A genetic and molecular analysis of two genes involved in flowering initiation of Arabidopsis

Een genetische en moleculaire analyse van twee genen die betrokken zijn bij de bloei initiatie van Arabidopsis

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A genetic and molecular analysis of two genes involved in flowering initiation of Arabidopsis

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Stellingen

1. De aanwezigheid van pleiotrope eigenschappen in een bloeimutant hoeft een verdere analyse niet in de weg te staan.
2. De kwaliteit van een mapping populatie is bepalend voor het succes van een map based cloning.
3. Het *FWA* gen kan gebruikt worden om zowel vroeg als laat bloeiende transgene planten te produceren.
4. Het is mogelijk dat organismen zich door middel van veranderingen in hun DNA-methyleringsniveau kunnen aanpassen aan plotselinge wijzigingen die zich voordoen in de omgeving.
5. Identificatie en isolatie van een "silencing element" in de buurt van het *FWA* gen kan tot toepassingen leiden bij het voorkomen van "silencing" in transgene planten.
6. Een probleem bij "in-silico" analyse van genen en genfamilies is de onnauwkeurigheid in de voorspelling van introns en exons op basis van de genomische DNA sequentie.
7. Het is slechts een kwestie van tijd voordat men binnen de biologische landbouw zal terugkomen op de afwijzing van GMO's.
8. De veelvuldige naamwisselingen en fusies van onderzoeksinstellingen binnen Wageningen leiden tot verwarring naar binnen toe en onherkenbaarheid naar buiten.
9. Het principe van gelijk inkomen voor gelijk werk gaat niet op voor personen die in tijdelijk dienstverband binnen de universiteit werken; hier bestaat onder meer een correlatie tussen land van herkomst en inkomen.
10. Optimisme leidt tot teleurstellingen.
11. Al te ludieke stellingen doen afbreuk aan de inhoud van een proefschrift.

Stellingen behorende bij het proefschrift getiteld "A genetic and molecular analysis of two genes involved in flowering initiation of Arabidopsis" door Wim Soppe, te verdedigen op 10 oktober 2000 te Wageningen.

Contents

Aan mijn ouders

Chapter 1

General introduction

Genetic control of flowering time in Arabidopsis

Maarten Koornneef, Carlos Alonso-Blanco, Anton J. M. Peeters and Wim Soppe

Summary

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

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Introduction

In order to achieve successful sexual reproduction, plants must be able to flower under favourable environmental conditions, and the proper timing of flowering is, therefore, supposed to have an important adaptive value for plants. The transition from vegetative to reproductive development is controlled by both environmental and endogenous factors. Plant physiologists have studied this important process by changing environmental factors and analysing the subsequent morphological, physiological and biochemical consequences of these treatments. More recently, genetics has been used to study the mechanism of flowering initiation by analysis of genetic variation in species, such as pea and Arabidopsis. Especially in Arabidopsis, the possibility to pursue the genetic analysis down to the molecular level is attractive and has generated the first positive results. This topic or aspects of it have been reviewed (Martínez-Zapater *et al.*, 1994; Okada and Shimura, 1994; Coupland, 1995; Haughn *et al.*, 1995; Weigel, 1995; Amasino, 1996; Koornneef and Peeters, 1997; Levy and Dean, 1998; Simpson *et al.*, 1999; Reeves and Coupland, 2000). In this chapter, we summarise the current progress made in the analysis of the transition to flowering using the genetic and molecular approaches as they have been applied to Arabidopsis.

The transition to flowering - meristem fate changes

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

A bi-directional development has been shown in this transition, with flowers being initiated acropetally. After floral initiation and following a basipetal direction, the axillary buds of the leaf primordia mostly develop into a secondary shoot (or paraclades or coflorescences) (Hempel and Feldman, 1994). In specific genotypes, they replicate the fate of the initial meristem by forming axillary rosettes. Following the fate change of these lateral meristems, internode elongation takes place (bolting). The elongated stem or inflorescence bears cauline leaves and flowers that are not subtended by leaves at higher internodes. The part of the inflorescence with leaves, which was called early inflorescence by Haughn *et al.* (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. Although the

appearance of flowers is the final and most dramatic result of the change to the reproductive phase, other changes occur earlier. These changes are characteristic for the transition from the juvenile vegetative phase, in which plants are not able to respond to factors inducing flowering, to the adult vegetative phase, in which plants are able to do so (Poethig, 1990). The changes are somewhat gradual and can be observed in leaf morphology (Martínez-Zapater *et al.*, 1995) and in the gradual appearance of trichomes at the abaxial side of the leaves and their gradual disappearance at the adaxial side (Chien and Sussex, 1996; Telfer *et al.*, 1997; Kerstetter and Poethig, 1998). It has been proposed that phase changes involve a decrease of a floral repressor (Sung *et al.*, 1992), called a *c*ontroller *o*f *p*hase *s*witching (COPS), which at critical low levels leads to the activation of the *fl*oral *i*nitiation *p*rocess (FLIP) (Schultz and Haughn, 1993). The latter is controlled by the so-called Floral Meristem Identity or FLIP genes, such as *LEAFY* (*LFY*), *APETALA1* and *2* (*AP1*, *AP2*), *CAULIFLOWER*, (*CAL*) and *UNUSUAL FLORAL ORGANS* (*UFO*) (Haughn *et al.*, 1995).

Environmental and endogenous control of flowering.

Arabidopsis is a facultative long-day (LD) plant, which means that plants flower earlier under LDs than under short days (SDs), but a LD treatment is not an absolute requirement for flowering. When plants of the common early laboratory genotypes are of sufficient age, indicating a certain competence for flowering, one LD is sufficient to induce flowering (Mozley and Thomas, 1995; 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.

The photoperiodic control of flowering is thought to be mediated by the interaction of photoreceptors, such as phytochrome and cryptochrome, and a clock mechanism or circadian rhythm. Photoreceptors play a role to set the phase of the circadian rhythm, but they can also affect flowering directly, thereby involving light quality in the control of this process. Blue (B) light and far-red (FR) light are known to be more effective to promote flowering than red (R) light (Brown and Klein, 1971; Eskins, 1992). Besides, the sensitivity of plants to light quality itself depends on a circadian rhythm (Carré, 1996). The importance of light quality in flowering is determined by the mechanism of light perception, since the ratio red : far-red (R/FR) determines the phytochrome status in the plant. Nevertheless, light is not a prerequisite for flowering, since flowering occurs rapidly in complete darkness when sufficient carbohydrates are provided to the growing shoot meristem (Rédei *et al.*, 1974; Madueño *et al.*, 1996; Roldán *et*

al., 1999). A higher light intensity also promotes flowering probably by its effect on carbohydrate supply (Bagnall, 1992; King and Bagnall, 1996).

Another important treatment promoting flowering is vernalisation, which is a transient exposure to low temperatures. The effectiveness of vernalisation depends on the stage of the plant, the length of the treatment and the temperature employed (Napp-Zinn, 1957; 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 differences in temperature effects on growth.

In Arabidopsis the effect of (sensitivity for) the environmental factors strongly depends on the genotype (see later). These environmental factors are thought to modulate certain endogenous components, thus affecting and controlling flowering. Many chemical treatments have been shown to promote flowering (Martínez-Zapater *et al.*, 1994) of which the application of gibberellins (GAs) (Bagnall, 1992; Wilson *et al.*, 1992) and base analogues (Rédei, 1970; Martínez-Zapater *et al.*, 1994) has attracted most attention, because of their relatively large effects.

Genes affecting flowering time

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

Natural variation

Genetic variation for flowering time has been described within and among Arabidopsis natural populations (accessions) since the earliest researchers (Laibach, 1951; Napp-Zinn, 1969; Rédei, 1970; Lawrence, 1976). Arabidopsis has a wide range of distribution along the Northern hemisphere (Rédei, 1970) and the differences found when growing different accessions under the same laboratory conditions are supposed to reflect particular adaptations

to different natural environments. To illustrate this genetic variation, Karlsson *et al.* (1993) analysed 32 accessions under SD and LD light conditions, with and without a vernalisation treatment. Interactions between the three parameters - accession, photoperiod and vernalisation - were found. The first genetic analyses of Arabidopsis flowering time made use of this natural variation to establish the minimum number of genes involved in particular crosses. These early studies often showed the segregation of one or two major genes (Van der Veen, 1965; Napp-Zinn, 1969; Karlovska, 1974). However, because different parental combinations were analysed it is not clear whether the same genes were segregating in those populations. Furthermore, segregation of genes with relatively small effects (minor genes) escaped to detection in such studies. Napp-Zinn (1957; 1969) studied in detail the flowering

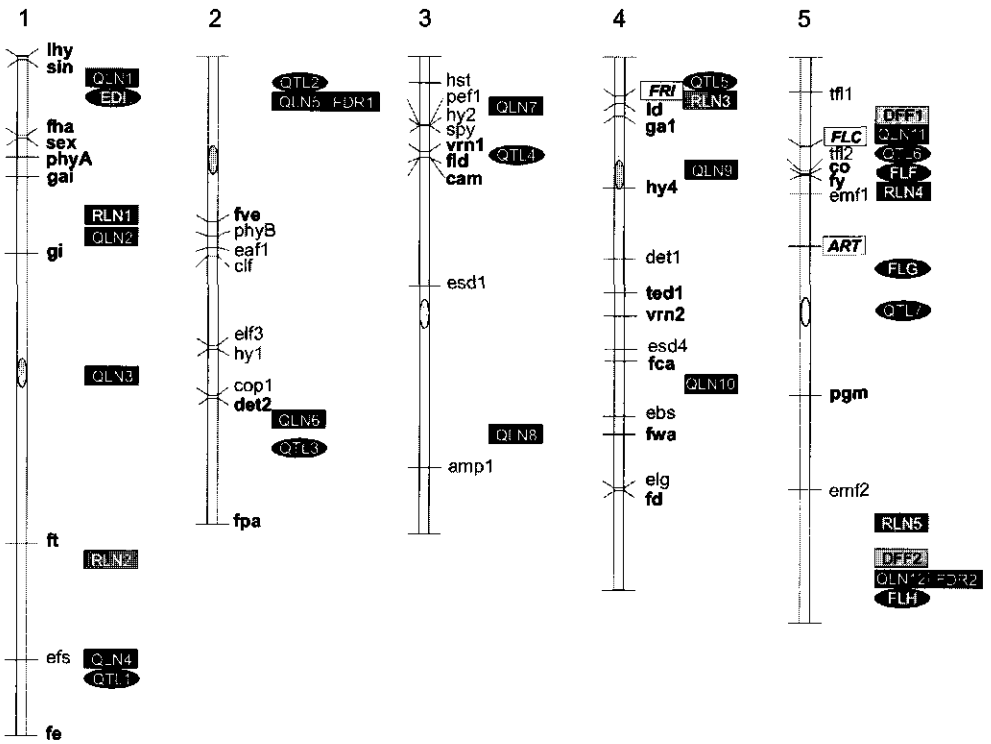

Figure 1.1. Arabidopsis genetic map showing the mutant loci and polymorphic QTLs identified affecting flowering time. Loci in bold correspond to genes with late flowering mutant phenotype, otherwise the mutant is early. *FLC, FRI* and *ART* loci, identified from natural populations, are indicated with white boxes. Black and grey boxes correspond to the approximate position of putative QTLs identified in different crosses; DFF1-2, QTLs in a Hannover/Münden F2 population (73); RLN1-5, QTLs in a *Ler* x H51 F2/F3 population (31); QLN1-12 in *Ler* x Col RIL population (60); FDR1-2 in the same *Ler* x Col RIL population (97); QTL1-7 in a backcross to Limburg-5, with selective genotyping , from F1 Limburg-5 x Naantali (74); EDI, FLF, FLG and FLH in a *Ler* x Cape Verde Island RIL population (3).

time differences and vernalisation requirement between the late accession Stockholm and the early Limburg-5 and isolated genotypes with single major flowering time gene differences. This analysis showed that at least four genes were involved and that alleles at the loci with larger effect were more or less epistatic to the alleles with smaller effect. At the locus *FRIGIDA* (*FRI*), the dominant allele produced a large delay in flowering time, and at the *KRYOPHILA* (*KRY*) and the *JUVENALIS* (*JUV*) loci the recessive alleles did so with a smaller effect. Vernalisation reverted most of the effect of these late alleles (Napp-Zinn, 1962).

The advent of molecular markers and the development of genetic maps have facilitated the localisation in the genome and the characterisation of some of the major loci controlling flowering time differences between very late and very early accessions. Napp-Zinn's *FRI* gene has been mapped on top of chromosome 4 (Clarke and Dean, 1994). It has been shown that the extreme lateness present in several ecotypes is due to dominant alleles at a locus mapping at a similar position, which is probably *FRI* (Napp-Zinn, 1987; Burn *et al.*, 1993b; Lee *et al.*, 1993; Grbic and Bleecker, 1996; Sanda *et al.*, 1997). The late flowering phenotype of *FRI* is very much suppressed under long-day light conditions by the Landsberg *erecta* (L*er*) allele at locus *Flowering Locus C* (*FLC*) mapping on top of chromosome 5 (Koornneef *et al.*, 1994; Lee *et al.*, 1994b), likely at a different position than any of the known flowering mutant loci (Figure 1.1). Therefore, the flowering time differences between late and early accessions are largely determined by these two loci, each one by itself having a small effect and requiring dominant alleles at both to produce extreme lateness. So far, only the laboratory strains L*er* and C24 (Koornneef *et al.*, 1994; Sanda and Amasino, 1995) have been found to contain early *FLC* alleles. The late flowering phenotype of *FRI* and *FLC*, present under both LD and SD conditions, is reduced by FR-enriched light and eliminated by vernalisation; saturation of vernalisation abolishes a further effect of FR light (Lee and Amasino, 1995). The L*er* early *FLC* alleles also suppress the lateness of mutant alleles at several loci (see later) such as *ld* (Koornneef *et al.*, 1994; Lee *et al.*, 1994b) and *fld* (Sanda and Amasino, 1996a), which were isolated in Columbia (Col) background but not in L*er*. The molecular analysis of *FLC* supported these observations; *FLC* was cloned and encodes a MADS domain protein which represses flowering. The levels of *FLC* mRNA are positively regulated by *FRI* and negatively by *ld* and vernalisation (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). Unexpectedly, the L*er* and C24 alleles of *FLC* do not show any differences in their coding sequence as

compared to the Col allele. Therefore, it is likely that they differ in some aspect of their regulation of *FLC* (Sheldon *et al.*, 2000).

A third locus, *A*erial *R*oset*T*e (*ART*), located on chromosome 5, has been identified by analysing another very late accession, Skye (Grbic and Bleecker, 1996). The dominant *ART* allele in combination with dominant alleles at another gene located on chromosome 4, probably *FRI*, delays the transition from vegetative to reproductive in the axillary meristems, giving rise to aerial rosettes under LDs. *ART* alone seemed to produce lateness, but taking into account the close location to *FLC*, it is unclear how much of the *ART* late phenotype comes from *FLC* and whether late *FLC* alleles are also necessary to produce the aerial phenotype. Epistatic analysis shows that aerial rosettes are produced by combining *ART* not only with *FRI* but also with the late flowering mutants *fca, fve, fpa, ld, fwa, co* and *gi* (see below) (Grbic and Gray, 1997). Thus, *ART* might act downstream in the flowering pathways, and in a late flowering background it would produce a prolonged insensitivity to the floral evocation signals in the axillary meristems.

To find other natural alleles of smaller effect has required the combination of molecular genetic maps with statistical methods to map quantitative trait loci (QTLs) (Jansen, 1996). QTL analyses have been performed using crosses between late and early accessions (Clarke *et al.*, 1995; Kuittinen *et al.*, 1997) and between early ones (Kowalski *et al.*, 1994; Jansen *et al.*, 1995; Mitchell-Olds, 1996; Alonso-Blanco *et al.*, 1998) (Figure 1.1). Multiple QTLs have been found in all the crosses and therefore differences in behaviour of flowering mutant alleles in different genetic backgrounds cannot be directly attributed to a single gene differing between accessions. Further analyses are needed to detect the interacting genes in each particular case. Such an analysis was done for four QTLs, derived from a cross between the accessions L*er* and Cape Verde Islands (Cvi). Different responses to vernalisation and photoperiod length changes and interactions between the QTLs were found (Alonso-Blanco *et al.*, 1998). Furthermore, introgression of the dominant Cvi allele of the QTL *EARLY DAYLENGTH INSENSITIVE* (*EDI*) into L*er* caused early flowering and almost daylength insensitive plants (Alonso-Blanco *et al.*, 1998).

The spectrum of natural variation is different from the spectrum of flowering-time variants obtained by mutational analyses. This is at least due to the limitations of the reduced number of accessions used to generate mutants, and to the possible deleterious pleiotropic effects of some of the induced mutations. For example, no mutant allele has been identified for the *FRI* locus. Some dominant late flowering mutants such as McKelvie's *florens* (*F*)

mutant (McKelvie, 1962) and the M73, L4, L5 and L6 mutants (Vetrilova, 1973) were reported allelic to *FRI*, but it was unclear whether they were mutants or contaminant natural variants (Koornneef *et al.*, 1994). Some of the putative QTLs locate at mutant gene positions and therefore it is expected that part of the natural variants will correspond to alleles of mutant flowering genes. However, there are known mutant flowering genes scattered all over the genome (Figure 1.1) and complex situations such as very closely linked QTLs might be expected. As an example, several analyses have detected QTLs on top of chromosome 5, a region enriched for mutant flowering genes, and at least some of these QTLs likely correspond to a different locus than *FLC* (Kuittinen *et al.*, 1997). Therefore, the identification of the individual alleles controlling this variation is necessary.

Late flowering mutants

Late flowering mutants with a strong effect but with no other obvious pleiotropic effects were described for the first time by Rédei (1962). He isolated the *constans (co)*, *gigantea (gi)* and *luminidependens (ld)* mutants in Col background. Later on more mutant alleles at these and nine other loci in L*er* were isolated and described by Koornneef *et al.* (1991) and in Wassilewskija (Ws) by Lee *et al.* (1994a). Thus, the loci *LD*, *CO*, *GI*, *FE*, *FT*, *FD*, *FY*, *FCA*, *FHA*, *FPA*, *FVE*, and *FWA* have been considered the classical late flowering genes (Figure 1.1). They have been physiologically characterised, and epistatic relationships have been examined in relation to early, late, and meristem identity genes (Halliday *et al.*, 1994; Ruiz-García *et al.*, 1997; Koornneef *et al.*, 1998a; Page *et al.*, 1999). Koornneef *et al* (1998a) constructed forty-two double mutants among ten of these loci. The epistatic interactions proved to be complex, but groups of loci similar to the ones established on the basis of their physiological behaviour were identified. A major epistatic group could be identified corresponding to the group of mutants *co*, *fd*, *fe*, *fha*, *ft*, *fwa* and *gi*. These mutants are late mainly under LD conditions, i.e. they show little or no response to daylength, and they have a low response to FR supplementary light and to vernalisation treatments. In contrast, the epistatic behaviour of the mutants that are much more responsive to these environmental factors (*fca, fpa, ld, fve* and *fy*) is more complex. Combining the *FLC*-Col allele with late flowering mutants in L*er* background, Sanda and Amasino (1996b) showed that the mutants *fca, fpa* and *fve,* of the same group, all have very enhanced late phenotypes like those of *ld, FRI* and *fld. Flowering locus D (fld)* is another late flowering mutant without apparent pleiotropic effects (Sanda and Amasino, 1996a). This mutant retains its response to photoperiod, and its flowering time can be reduced by cold treatment and

low R/FR light. A strong mutant allele of *FLD*, that produced aerial rosettes like *ART*, was obtained in Col by Chou and Yang (1998).

Six of these late flowering genes, *LD*, *FCA*, *CO*, *GI*, *FT* and *FHA* have been cloned:

- *LD* was the first flowering time gene to be cloned and encodes a glutamine-rich nuclear protein containing a possible homeodomain (Lee *et al.*, 1993). It is primarily expressed in apical proliferative regions of the shoot and root (Aukerman *et al.*, 1999).
- *FCA* encodes a protein containing two RNA-binding domains and a WW protein interaction domain, suggesting that it is functioning in the posttranscriptional regulation of transcripts involved in flowering (MacKnight *et al.*, 1997). An interesting characteristic of this gene is that the transcript is alternatively spliced; four different *FCA* transcripts have been found, the full-length transcript being only one third of the total amount. The WW domain, which is only present in the full-length transcript, seems essential for the flowering time effect.
- The *CO* gene was found to encode a protein with similarity to GATA-1 type transcription factors. (Putterill *et al.*, 1995). Constitutive expression of *CO* leads to earliness (Simon *et al.*, 1996), thereby confirming that this gene has flowering promoting properties. Besides, transgenic plants with extra copies of *CO* flower earlier than wild-type, suggesting that *CO* activity is limiting flowering time (Putterill *et al.*, 1995). The *CO* mRNA appears more abundant in plants grown under LDs than under SDs, in agreement with the role of this gene in promotion of flowering under LDs. It is interesting to note that two homologues of the *CO* gene, *CONSTANS LIKE 1 (COL1)* and *COL2*, have been described (Ledger *et al.*, 1996; Putterill *et al.*, 1997), and although quite similar in structure, their role in flowering has not yet been demonstrated.
-The *GI* gene was isolated during the past year. The presence of several membrane-spanning domains in its protein predicts that it is located in the plasma membrane. The amount of *GI* transcript is regulated by the circadian clock and there are indications for a role of GI in the control of expression of circadian-clock regulated genes in response to light (Fowler *et al.*, 1999; Park *et al.*, 1999).
- Cloning of *FT* showed that this gene has strong homology with the *TERMINAL FLOWER 1* (*TFL1*) gene which encodes a putative phosphatidylethanolamine-binding and nucleotide-binding protein that shares sequence similarity with membrane-associated mammalian proteins (Bradley *et al.*, 1997; Ohshima *et al.*, 1997). Furthermore, expression of *FT* is positively regulated by *CO* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999).
- The *FHA* gene encodes the CRY2 protein (Lin *et al.*, 1996; Guo *et al.*, 1998) and is thought to be involved in blue-light perception.

Besides these "classical" late flowering mutants, also other late mutants have been identified. Some of these are involved in light perception or transduction. The mutants *long hypocotyl 4 (hy4)* and *phytochrome A (phyA)* correspond to the blue light photoreceptor CRY1 (Ahmad and Cashmore, 1993) and phytochrome A (phyA) (Whitelam and Harberd, 1994), respectively. An elongated hypocotyl is also shown by the dominant, gain of function, *late elongated hypocotyl* (*lhy*) mutant (Simon and Coupland, 1996). This mutant is daylength insensitive and lacks circadian rhythms for leaf movement. It is suggested that this Myb-like transcription factor might be a component of the circadian clock (Schaffer *et al.*, 1998).

Mutants deficient in gibberellin biosynthesis, like *ga1*, or action, *gibberellin insensitive (gai)* show a late phenotype under short day conditions (Wilson *et al.*, 1992).

Late flowering mutants have also been identified as defective in starch metabolism, such as *phosphoglucomutase* (*pgm*) (Caspar *et al.*, 1985) and *ADP glucose pyrophosphorylase 1* (*adg1*) (Lin *et al.*, 1988), which lack leaf starch and flower late, mainly under SD conditions. In contrast, *starch excess 1* (*sex1*) (Caspar *et al.*, 1991) and *carbohydrate accumulation mutant 1* (*cam1*) (Eimert *et al.*, 1995), which also flower late, have increased starch content in leaves. This characteristic was also observed in the late mutant *gi* (Araki and Komeda, 1993; Eimert *et al.*, 1995). In the *pgm* and *sex1* mutants the late flowering phenotype could be suppressed by a vernalisation treatment (Bernier *et al.*, 1993). The late flowering phenotype observed in these mutants is not due to the defect in starch accumulation and the slow growth, but more to the inability to mobilise the stored carbohydrates (Bernier *et al.*, 1993; Eimert *et al.*, 1995). Nevertheless, it remains unclear how carbohydrate metabolism affects flowering time in Arabidopsis.

Additional mutants that show lateness either under specific conditions and/or with more pronounced pleiotropic effects are: *de-etiolated 2* (*det2*), *ted1* (a suppressor of *det1*) (Pepper and Chory, 1997), *ethylene insensitive* (*ein*) (Ecker, 1995), *ethylene responsive* (*etr1*) (Bleecker *et al.*, 1988), *short integument* (*sin*) (Ray *et al.*, 1996), and *vernalisation* (*vrn*) (Chandler *et al.*, 1996). Several of these genes have been cloned and are known to encode steps in brassinosteroid biosynthesis (*DET2*) (Li *et al.*, 1996) and ethylene action (*EIN, ETR1*) (Ecker, 1995).

Early flowering mutants

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

The early flowering mutants with the most dramatic phenotypes are *embryonic flower 1* and *2* (*emf1* and *emf2*). The *emf* mutants do not produce a normal rosette after germination, but they make only a few cauline leaves followed by floral buds. In addition, their flowers are usually abnormal and incomplete (Sung *et al.*, 1992). The phenotype indicates that most of the normal vegetative phase is bypassed, and *EMF* genes are therefore likely to play a central role in the COPS mechanism (Haughn *et al.*, 1995; Yang *et al.*, 1995). Double mutant analyses indicated that *emf* is epistatic to both early- and late-flowering mutants (Yang *et al.*, 1995), although differences have been found among double mutants of *emf* with several late flowering mutants (Haung and Yang, 1998). Interactions between *EMF* and genes regulating inflorescence meristem development and floral organ identity were revealed in the analysis of double mutants between *emf* and *tfl* and *agamous* (*ag*). It has been proposed that the *EMF* genes play a role during the different phase transitions of the plant by a gradual reduction in its activities (Yang *et al.*, 1995).

Several early flowering mutants are involved in light perception and light signal transduction pathways. Among these, *long hypocotyl 1* and *2* (*hy1* and *hy2*), which are defective in phytochrome chromophore biosynthesis (Parks and Quail, 1991), and *phytochrome B* (*hy3* = *phyB*), deficient in phytochrome B (Somers *et al.*, 1991), are daylength sensitive (Goto *et al.*, 1991). Overexpression of phytochrome B also leads to early flowering (Bagnall *et al.*, 1995), suggesting that the balance between different phytochromes is important for the proper timing of transition to flowering. Furthermore, phytochrome A and B are not the only phytochromes influencing this transition because *phyA phyB* double mutants still respond to increases in the proportion of FR light, by flowering early (Devlin *et al.*, 1996).

The *phytochrome-signaling early-flowering* (*pef1*) mutant shows a similar phenotype to *hy1* and *hy2* but cannot be rescued by the chromophore precursor biliverdin. It has been suggested that *pef1* has a mutation in a signaling intermediate, interacting with all the phytochrome family members (Ahmad and Cashmore, 1996). The *pef2* and *pef3* mutants more closely resemble *phyB* mutants. Therefore they may have lesions early in the signaling pathway primarily mediated by phyB and/or some of the other phytochrome gene family members (phyC, D, E) (Ahmad and Cashmore, 1996).

The *sucrose-uncoupled 2* (*sun2*) mutant has an early flowering phenotype, at least under LD conditions, and shows a long hypocotyl and reduced fertility (Dijkwel *et al.*, 1997). This mutant was initially isolated as showing reduced repression by sucrose of a transgenic

plastocyanin promoter. These phenotypes suggest an interaction between carbohydrate metabolism repression and light signaling in the flowering process.

Some of the mutants influence the circadian rhythm. The *early-flowering 3* (*elf3*) mutant lacks rhythmicity in circadian-regulated processes under constant light conditions (Hicks *et al.*, 1996), while *cop1* and *det1* show shorter circadian period lengths in constant darkness (Millar *et al.*, 1995). The short-period *toc1* mutant has a severely reduced daylength sensitivity and flowers equally early in LDs and SDs (Somers *et al.*, 1998b). The *elf3* mutant shows a similar photoperiod insensitivity, and has a long hypocotyl (most noticeably in blue and green light). Double mutant analysis with *hy4* and *hy2* indicates that *ELF3* is involved in blue light-regulated photomorphogenesis (Zagotta *et al.*, 1996). In contrast, the *cop1* and *det1* mutants are early flowering in SDs and also have a constitutive photomorphogenic phenotype. *DET1* encodes for a novel nuclear-localised protein, suggesting that it controls cell type-specific expression of light-regulated promoters (Pepper *et al.*, 1994). *COP1* encodes a protein with both a zinc-binding motif and a G_β homologous domain (Deng *et al.*, 1992). Double mutant analysis with *hy1* and *hy4* suggests that *COP1*, together with other *COP* and *DET* genes, acts downstream of phytochrome and the blue-light photoreceptor (Chory, 1992; Kwok *et al.*, 1996). The DET/COP protein complex formed in darkness negatively regulates transcription of certain genes involved in photomorphogenesis (Von Arnim *et al.*, 1997). It is thought that light signals mediated by multiple photoreceptors can be transduced to inactivate the pleiotropic COP/DET regulators and thus release the repression of seedling photomorphogenesis. Nevertheless, since the *cop/det* mutants also have a clear phenotype in light-grown plants, these genes may also function in other pathways that are not directly related to photomorphogenesis (Mayer *et al.*, 1996).

Cytokinins, applied to wild-type plants, result in a phenocopy of *det1* mutants (Chory *et al.*, 1994). Consistent with this the *altered meristem program 1* (*amp1* = *pt* = *hpt* = *cop2*) mutant, which has high levels of cytokinin, shows a constitutive photomorphogenic phenotype, flowers early, and is daylength insensitive, like the *det1* mutant (Chaudhury *et al.*, 1993) This suggests a role for cytokinins in the light signal transduction. Nevertheless, this mutant shows a strongly altered growth and leaf formation rate rather than altered flowering time. Other mutants like *spindly* (*spy*) and *early flowering 1* (*eaf1*) show the role of gibberellins in the transition to flowering. The *spy* mutant has the phenotype of wild-type plants treated with GAs and is therefore early flowering. The *SPY* gene is probably involved in the GA signal transduction pathway (Jacobsen and Olszewski, 1993). The *eaf1* mutant

flowers early under both LDs and SDs and germination of its seeds shows an increased resistance to the GA biosynthesis inhibitor paclobutrazol. This suggests an altered GA metabolism and/or response in the mutant (Scott *et al.*, 1999).

The early flowering *elongated* (*elg)* mutant shows a pleiotropic phenotype that suggests a disruption of phytochrome and/or GA function. However, it has been shown that *ELG* acts independently of phytochrome and GA action (Halliday *et al.*, 1996).

Another group of mutants involves genes whose function in the transition to flowering has not yet been determined. Two of these mutants, *early flowering 1* and *2* (*elf1* and *elf2)*, do not show clear pleiotropic phenotypes and have a daylength response (Zagotta *et al.*, 1992). In contrast, *early in short days 1* (*esd1*) (J.M. Martínez-Zapater, C. Gómez-Mena, L. Ruiz-García and J. Salinas, personal communication) and *4* (*esd4)* (Simon and Coupland, 1996; G. Murtas, P. Reeves, G. Coupland, personal communication) *early bolting in short days* (*ebs* = *speedy)* (J.M. Martínez-Zapater, C. Gómez-Mena, M. Pineiro and G. Coupland, personal communication) and *early flowering in short days* (*efs*) (Chapter 2) have a reduced daylength response and show pleiotropic phenotypes such as reduced fertility and/or plant size. Double mutant analysis indicated that these mutants interact with some of the late flowering mutants (Simon and Coupland, 1996; Chapter 2). The *ESD4* gene has been cloned but did not show homology to other genes of known function, although related sequences were found in a range of other organisms (Reeves *et al.*, 1997).

In a screen for mutations that accelerate the transition from the juvenile vegetative phase to the adult vegetative phase, the early flowering daylength sensitive *hasty* (*hst*) mutant was identified. *HASTY* is suggested to promote a juvenile pattern of vegetative development and to inhibit flowering by reducing the competence of the shoot to respond to *LFY* and *AP1* (Telfer and Poethig, 1998).

A number of early flowering mutants is involved in the later stages of floral transition. These genes are regulating the expression of floral meristem(-organ) identity genes like *AP1*, *LFY* and *AG*. Mutations in *TFL1* result in early flowering, replacement of coflorescences by flowers, and determinated growth of the apical meristem, which develops into a flower (Shannon and Meeks-Wagner, 1991). The *tfl* mutation shows ectopic expression of *LFY* and *AP1* in the apical meristem (Gustafson-Brown *et al.*, 1994; Blázquez *et al.*, 1997) agreeing with overexpression in transgenic plants of *LFY* and *AP1* giving a phenotype reminiscent of *tfl1* (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Blázquez *et al.*, 1997). Therefore, it appears that the *tfl1* mutant fails in negatively regulating *LFY* and *AP1*, thereby

promoting early flowering and the formation of a terminal flower (Shannon and Meeks-Wagner, 1991). The *TFL1* gene has been cloned and encodes a putative phosphatidylethanolamine-binding and nucleotide-binding protein (Bradley *et al.*, 1997; Ohshima *et al.*, 1997).

Mutations in the *curly leaf* (*CLF*) gene cause a very similar phenotype to the one conferred by constitutive expression of the meristem-organ identity gene *AG,* showing narrow and upwardly curled leaves as well as early flowering in short days (Mizukami and Ma, 1997). *CLF* has been cloned and encodes a protein with homology to polycomb-group genes. *CLF* is required to repress *AG* transcription in leaves, inflorescence stems and flowers (Goodrich *et al.*, 1997).

Flowering time genes identified by constitutive expression in transgenic plants

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

For a number of genes of unknown function transgenic plants suggested their role in promoting flowering, although no late mutants were available. The *FPF1* gene was cloned as a gene expressed immediately after photoperiodic induction. Constitutive expression of this gene leads to early flowering under LDs and SDs and to other associated changes that mimic the effect of GA applications (Kania *et al.*, 1997). In a search for genes whose products bind to the promoter of the meristem identity gene *AP1* (and its *Antirrhinum* ortholog *SQUAMOSA*), the *SPL3* gene was isolated. Its constitutive expression leads to earliness (Cardon *et al.*, 1997). Although overexpression phenotypes show the sufficiency of these genes to promote flowering, they do not prove that these genes are necessary for the timing of the transition. Therefore, late mutants at these loci may not be found. This is because the function of these genes may be redundant or they may be involved in other related processes. This is illustrated with the meristem identity genes *AP1* (Mandel and Yanofsky, 1995), *LFY* (Weigel and Nilsson, 1995) and the meristem-organ identity gene *AG* (Mizukami and Ma, 1997), for which mutants are available without an obvious flowering-time phenotype. However, transgenic plants expressing these genes constitutively do flower early.

Another way by which overexpression may indicate the function of a gene is by providing the endogenous gene with constitutive promoters or enhancers. A transposable element with

outward-directing 35S promoter has generated the dominant mutant *lhy* (Simon and Coupland, 1996; Coupland, 1997; Schaffer *et al.*, 1998), described above, which constitutively expresses this gene. A phenocopy of the *lhy* mutant was obtained in transgenic plants with constitutive expression of a related Myb-type gene called *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Wang *et al.*, 1997; Wang and Tobin, 1998). Expression of this gene is transiently induced by phytochrome and oscillates with a circadian rhythm, indicating a link with the phytochrome-related long hypocotyl phenotype (Wang and Tobin, 1998).

Methylation and epigenetics

During the past few years, it has become clear that DNA methylation plays an important role during development of eukaryotes. Its biological function, however, is less clear. DNA (de)methylation is involved in the control of gene expression during development and differentiation, by either negative or positive regulation (Bird, 1992; Martienssen and Richards, 1995). Furthermore, there is evidence that DNA methylation is one of the mechanisms to silence foreign DNA in eukaryotes (Matzke *et al.*, 1996).

In plants, like in mammals, methylation has dual functions in defence against invading DNA and transposable elements, and in gene regulation (Finnegan *et al.*, 1998b). The role of DNA methylation in some of these processes is becoming more clear. For instance in homology-dependent (trans)gene silencing in plants (Kooter *et al.*, 1999) and in the differential expression of maternal and paternal genes in endosperm of developing seeds (Finnegan *et al.*, 2000). For other processes, like flowering, the role of DNA methylation is not yet fully understood.

However, there are some indications that DNA methylation might be involved in the vernalisation response (Finnegan *et al.*, 2000). Arabidopsis plants either cold treated or treated with the demethylating compound 5-azacytidine show reduced amounts of 5-methylcytosine in their DNA. Among the late flowering mutants there are some, like *fca* and *fy*, responsive to vernalisation, and others, such as *gi*, *fd* and *ft*, that show little response to this treatment (Koornneef *et al.*, 1991). After treating these mutants with 5-azacytidine, earliness was observed in the responsive genotypes, but not in the nonresponsive ones, thus imitating the effect of vernalisation (Burn *et al.*, 1993a). Furthermore, transcription of the *FLC* gene, which plays a central role in the vernalisation response, is downregulated by both vernalisation and a decrease in genomic DNA methylation (Sheldon *et al.*, 1999). However, the role of methylation in vernalisation is still unclear, because substantial demethylation in antisense *METHYLTRANSFERASE1* plants did not prevent vernalisation from accelerating flowering in

these lines, nor did it prevent resetting of the vernalisation requirement in its progeny (Finnegan et al., 1998a).

DNA methylation has been reduced in transgenic plants. Transgenic C24 plants were constructed in which methylation was suppressed by the antisense methyltransferase cDNA *MET1* from *Arabidopsis thaliana* (Finnegan and Dennis, 1993; Dennis *et al.*, 1996; Finnegan *et al.*, 1996). This resulted in a reduction of total genomic cytosine methylation, which induced several developmental effects, and correlation was found between demethylation and reduction in flowering time. This was particularly clear under SDs where C24 shows a pronounced vernalisation response (Finnegan *et al.*, 1998a). Surprisingly, Ronemus *et al.* (1996) found late flowering transformants under LD conditions in Col genetic background, using the same antisense approach.

In addition to effects on flowering time, reduced methylation led to abnormal flowers due to an altered expression of genes such as *AG* and *APETALA 3* (*AP3*), probably caused by changes in chromatin structure (Finnegan *et al.*, 1996). These phenotypes are in some aspects similar to the phenotype of the early flowering mutant *clf*, defective in a gene encoding for a polycomb-like protein, which is known to affect chromatin structure (Goodrich *et al.*, 1997). Jacobsen and Meyerowitz (1997) showed that a *superman* (*sup*) mutant epi-allele found in antisense methyltransferase lines is due to highly localised hypermethylation in the *SUP* gene. Similar hypermethylation was found for the *ag* mutant epi-allele in the above mentioned hypomethylated background (Jacobsen *et al.*, 2000). The regulation of transcription of certain genes that are involved in the flowering initiation process is apparently either under control or may be influenced by DNA methylation as a component of cell memory.

A mutant, designated *ddm1* (*d*ecrease in *D*NA *m*ethylation) affected in DNA methylation but not exhibiting a flowering time phenotype, has been isolated in Arabidopsis (Vongs *et al.*, 1993). Cloning of *DDM1* revealed that it encodes a member of the SW12/SNF2 family of chromatin remodelling proteins (Jeddeloh *et al.*, 1999). The *ddm1* mutation causes hypomethylation up to 70% of the total genomic 5-methylcytosine levels, although these plants exhibit normal methyltransferase activity. The *ddm1* mutation induces other heritable mutations after repeated selfpollination (Kakutani *et al.*, 1996). Among them, there is a late flowering mutant designated *fts* mapped on chromosome 4 at a similar position as *fwa* (Kakutani, 1997). The latter late flowering mutant was described by Koornneef *et al* (1991) and both alleles, *fwa-1* and *fwa-2*, show strong hypomethylation in a 5 Mbase region were the gene has been mapped (Chapter 4).

It is evident that changes in the methylation level can affect the expression of genes, both under conditions generated in the laboratory (Finnegan *et al.*, 1996; Kakutani *et al.*, 1996; Ronemus *et al.*, 1996; Jacobsen and Meyerowitz, 1997; Jacobsen *et al.*, 2000), and under natural conditions (Cubas *et al.*, 1999). Strikingly, these changes often affect genes that are involved in flowering transition or flower morphology. However, it is not clear yet whether these changes in expression by altered methylation levels of the plant reflect an epigenetic regulation of gene expression or are merely caused by random changes in the methylation status of genes, due to an impaired methylation machinery.

Discussion: a working model for the control of flowering time

The complex multigenic control of flowering as revealed by genetic analysis in *Arabidopsis* (Martínez-Zapater *et al.*, 1994; Weigel, 1995; Peeters and Koornneef, 1996) and pea (Weller *et al.*, 1997b) indicates that the process is complex and influenced by many factors. This observation supports physiological evidence for a multifactorial control of the transition to flowering (Bernier, 1988). It has been proposed that the transition to flowering is the developmental default state (Rédei *et al.*, 1974; Sung *et al.*, 1992; Martínez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Weigel, 1995). This hypothesis is mainly based on two observations. First, Arabidopsis can flower with very few leaves in complete darkness when sufficient sucrose is provided to the shoot meristem (Rédei *et al.*, 1974; Madueño *et al.*, 1996). Under these conditions the late mutants, as far as tested, are as early as wild-type with the exception of *fwa* and *ft* (Roldán *et al.*, 1999). Second, no mutants without flower-like structures have been described, but in contrast, the *emf1* and *emf2* mutants with hardly any vegetative development have been isolated (Sung *et al.*, 1992). The *EMF* genes have been suggested to play a central role in the repression of flowering or promotion of vegetative development by being the final target for the flowering time genes (Sung *et al.*, 1992; Martínez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Weigel, 1995). However, although *emf1* and *emf2* are, respectively, epistatic to the late flowering mutants *gi* and *co* (Yang *et al.*, 1995), double mutants of the *emf* mutations with some of the other late flowering mutations flower with an intermediate number of leaves (Haung and Yang, 1998; Page *et al.*, 1999). This suggests that the wild-type products of these genes do not repress *EMF* function.

The genetical and physiological classification of several late mutants has led to group these genes into two different general modifying promotion pathways (Figure 1.2). The late flowering genes, *FCA*, *FY*, *FPA*, *FVE*, *LD* and *FLD*, are assumed to promote flowering autonomously, under LD and SD, and are therefore involved in the so-called autonomous promotion pathway. These mutants are highly daylength sensitive, presumably because when this pathway is defective the transition to flowering becomes very dependent on another pathway that is largely regulated by photoperiod. This second pathway has been called the LD promotion pathway, involving the late flowering genes, *CO*, *FD*, *FE*, *FHA*, *FT*, *FWA* and *GI*, which are believed to promote flowering mainly under photoperiodically inductive conditions, i.e., LDs. Nevertheless, since the mRNA level of *CO*, a gene that promotes flowering, is reduced in SD, the effect of LD might be the removal of a hypothetical SD repressor, and therefore this pathway could also be referred to as SD repression or, summarising, photoperiodic promotion pathway.

The reduced responsiveness to vernalisation of these photoperiodic promotion mutants does not imply that these genes are involved in sensing the cold signal, because long vernalisation treatments are effective in these mutants (Chandler and Dean, 1994) and the parental genotype L*er* also has a limited vernalisation responsiveness compared with mutants such as *fca*, even when it flowers late under SD (Chandler and Dean, 1994; Chandler *et al.*, 1996). Furthermore, double mutants involving representative genes of the two pathways are sensitive to vernalisation, although the absence of the LD promotion cannot be replaced by the vernalisation treatment (Koornneef *et al.*, 1998a). In contrast, the stronger vernalisation sensitivity of the autonomous promotion mutants suggests that this pathway and a third one, the vernalisation promotion pathway, might converge downstream and are able to replace each other. The candidate genes affecting the sensing or transduction of the cold signal are the *VRN* genes isolated on the basis of their lack of a vernalisation response in an *fca* mutant background (Chandler *et al.*, 1996). The *FLC* gene is probably functioning at the converging point of the two pathways. "Double mutant" analyses between the early *FLC-Ler* allele and the late mutations *fld*, *ld*, *fca*, *fve*, and *fpa* flower relatively early in comparison to the late flowering phenotype observed in these late mutants in a *FLC-Col* background (Sanda and Amasino, 1996b). This suggest that these late genes antagonise inhibitors. A vernalisation treatment might have the same effect. Analysis of *FLC* expression was in agreement with these interpretations; the level of *FLC* mRNA is downregulated by vernalisation and upregulated in late flowering mutants from the autonomous promotion pathway, whereas late flowering mutants from the

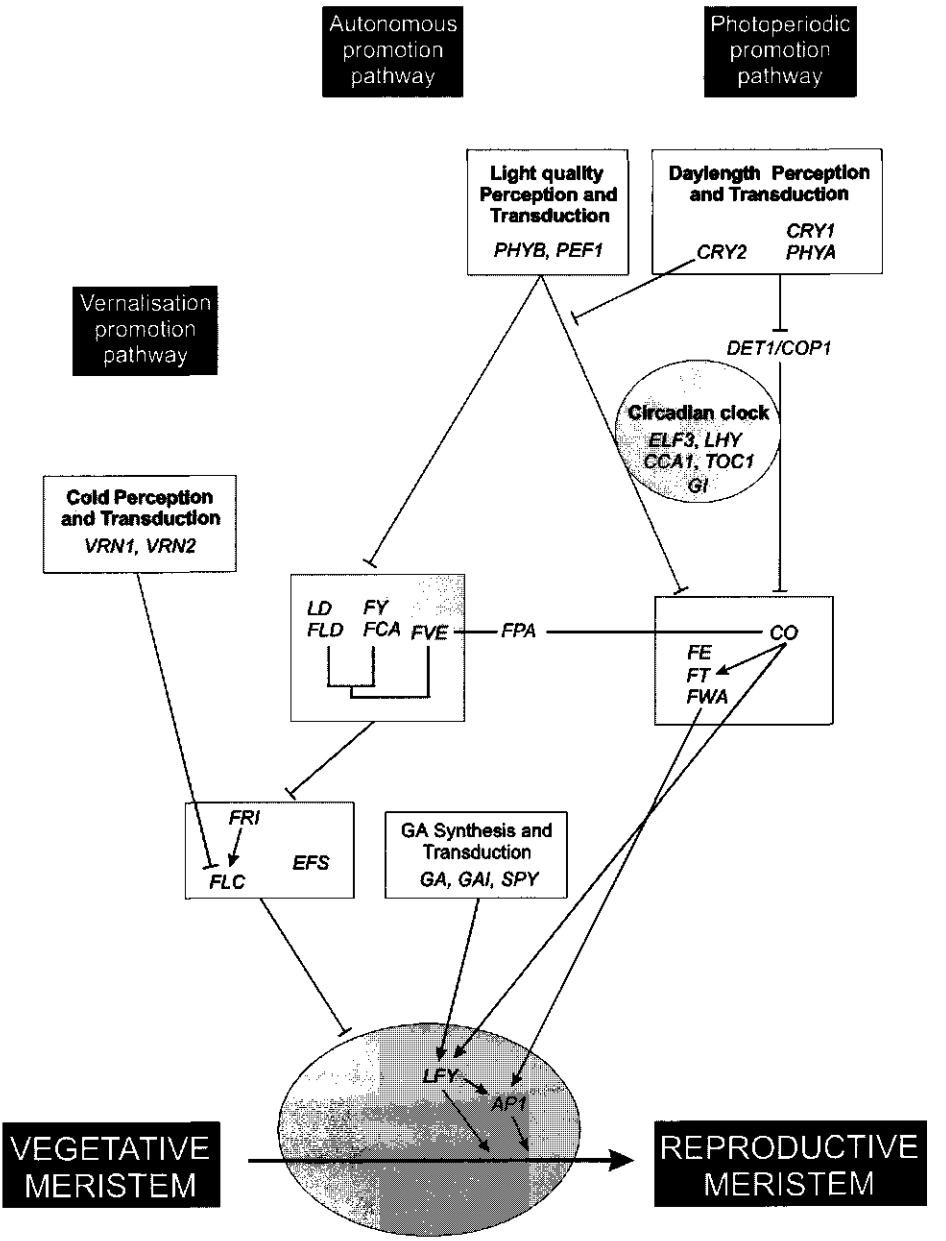

Figure 1.2. A model describing the interactions of flowering time genes in Arabidopsis. Different groups of genes, established according to their genetic and physiological behaviour, are shown in boxes. Lines within boxes indicate subgroups. The arrows represent a promotive effect, the "⊥" symbols represent a repressive effect.

photoperiodic promotion pathway have no effect on *FLC* expression (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999; Sheldon *et al.*, 2000).

Analysis of double mutants places the photoperiodic promotion mutations with similar phenotypes in the same epistatic group. This study also indicated that the situation for the autonomous promotion pathway mutants is more complex, suggesting parallel sub-pathways within this group. Particularly the *fpa* mutant shows a complex behaviour and might play a role in the two pathways (Koornneef *et al.*, 1998a).

To place other flowering genes, including those for which the recessive (probably loss of function) phenotype is earliness, in relation to these two general pathways can be attempted. However, since detailed genetic analyses of double mutants are lacking in most cases, this can only be done in a provisional manner. Furthermore, not knowing whether mutants are true null alleles complicates the interpretation of any double mutant analysis (Weigel, 1995; Koornneef *et al.*, 1998). In Chapter 2 it will be shown that the early flowering mutant *efs* can be placed in the autonomous promotion pathway by the analysis of double mutants with late flowering mutants.

It has been suggested that the outcome of the autonomous promotion pathway is similar to that of vernalisation and GAs. In agreement with this, a detailed morphogenetic analysis of *fve* mutants indicated that they show some symptoms of reduced GA levels or reduced GA action, although these are far less extreme than in *ga1* and *gai* mutants (Martínez-Zapater *et al.*, 1995). Besides, the implication of GA synthesis in vernalisation has been strongly suggested, not only by the work in *Thlaspi arvense* (Hazebroek *et al.*, 1993) but also in Arabidopsis by the finding that the *ga1-3* mutants do not respond to vernalisation in SDs. However, the observation that the *fca ga1-3* double mutant responds well to vernalisation under continuous light argues against the hypothesis that vernalisation acts through GA biosynthesis or through the *FCA* gene product (Chandler *et al.*, 1996; 2000). Furthermore, the *ga1-3* mutant also responds to vernalisation in a late flowering *FRI/FLC* background (Michaels and Amasino, 1999b). Nevertheless, GAs have been shown to be crucial for a number of processes associated with flowering, such as internode elongation and the suppression of adaxial trichomes, which indicates that there is a higher GA activity after the transition to flowering, which might be partially due to the promotive effect of LDs on the GA 20-oxidase encoded by the *GA5* locus (Xu *et al.*, 1997). It has also been shown that GAs stimulate flowering, both by activation of the *LFY* promoter and by control of the competence of the meristem to respond to *LFY* activity (Blázquez *et al.*, 1998). The actual sequence in the interaction among the autonomous promotion pathway, GAs and vernalisation remains to be solved and further research in this area is necessary. Besides, it has been suggested

that the vernalisation promotion involves modulation of gene expression through changes in methylation, which needs further confirmation by the study of the target genes. It is possible that GAs, vernalisation and the autonomous promotion pathway have a similar target that leads to floral induction. Therefore, their functions may overlap, and different environmental conditions may modulate the three pathways in a different way. A candidate target gene that probably is specific for GAs is *FPF1* (Kania *et al.*, 1997). *FPF1* is not responsible for the activation of *LFY* by GAs but probably acts to promote flowering in a parallel pathway to *LFY* (Melzer *et al.*, 1999).

The chromophore and *phyB* mutations cause early flowering, indicating that this phytochrome has an inhibitory role in flowering, which seems independent from the daylength sensing mechanism. The earliness conferred by the *hy* mutants to the *co, gi and fwa* mutant backgrounds under both LDs and SDs (Koornneef *et al.*, 1995) further indicates that the early flowering caused by the *hy* mutations does not act exclusively through these flowering time genes. However the *hy* mutants in the *fca* mutant background are late under SDs, suggesting that phyB, apparently, mainly represses the *FCA* gene pathway under SD conditions (Koornneef *et al.*, 1995). In contrast, under LDs, *hy* mutants in the *fca* background are early, suggesting that under these conditions another promotion pathway is repressed by phyB. Therefore, the phyB and other light-stable phytochromes might repress both the autonomous promotion and the photoperiodic promotion pathways. Reed *et al.* (1996) have shown that phyB decreases responsiveness to GAs, which suggests that this phytochrome might repress flowering through this mechanism.

The effect of the light labile phytochrome A is very different and more or less opposite to that of the light stable phytochromes. Phytochrome A promotes flowering, since overexpression of this gene leads to earliness (Bagnall *et al.*, 1995) and the mutant is late when SDs are extended by 8 hrs of light with a low R/FR ratio (Johnson *et al.*, 1994). Under LDs provided by "normal" fluorescent lamps, no lateness is observed, probably because other photoreceptors can compensate for the lack of phyA. In pea, PHYA deficient mutants have a much more pronounced late phenotype under LDs and are photoperiod insensitive (Weller *et al.*, 1997a). In this species, SDs lead to the production of a graft transmissible inhibitor which is under control of the pea genes *Sn*, *Dne* and *Ppp* . Based on grafting studies and the analysis of double mutants, it was concluded that phyA reduces the level of this inhibitor under LD conditions (Weller *et al.*, 1997a).

In addition to phytochromes, blue-light receptors, called cryptochromes, play a role in flowering. As in the case of phytochrome, the different members of this family of photoreceptor seem to have distinct roles in the transition to flowering. The promotive role of the cryptochrome I encoded by the *HY4* gene seems minor since the flowering time effect of this mutant is limited (Bagnall *et al.*, 1996). The effect of the cryptochrome II (CRY2) appears more important in LDs because these mutants (*fha*) are clearly late (Guo *et al.*, 1998). The similarity in phenotype of these mutants with the photoperiodic promotion pathway mutants strongly suggests that CRY2 and phyA are the photoreceptors for this pathway. However, *cry1/cry2* double mutant analysis revealed a redundant roles for CRY1 and CRY2 in blue light in the promotion of floral initiation (Mockler *et al.*, 1999). Furthermore, there are indications that phytochromes and cryptochromes interact with each other; CRY2 suppresses a blue-light inhibition of the phyB mediated red-light inhibition of floral initiation (Mockler *et al.*, 1999).

To measure the length of the photoperiod, apart from photoreceptors, a time measurement mechanism is required, which is probably provided by a circadian clock. Phytochromes and cryptochromes are important for the synchronisation of this clock. PHYB and CRY1 mediate signals for period length control under high fluence light whereas PHYA and CRY2 only seem to play a role under specialised conditions of low fluence light (Somers *et al.*, 1998a). The relation between daylength and a circadian rhythm mechanism affecting leaf movement and *CAB2* gene expression was studied in the Arabidopsis *elf3* mutant, which is early and daylength insensitive (Hicks *et al.*, 1996; Zagotta *et al.*, 1996). The *elf3* mutant lacks these circadian rhythms in continuous light but not in light/dark cycles and continuous darkness, suggesting that *ELF3* is involved in circadian regulation, especially in the transduction of light signals to a component of the clock. (Carré, 1996; Hicks *et al.*, 1996). Three other genes that may affect directly the clock and that show altered flowering time are *LHY*, *CCA1* and *TOC1*. The homologous genes *LHY* and *CCA1* are expressed rhythmically. When overexpressed, they cause late flowering and disrupted circadian rhythms (Schaffer *et al.*, 1998; Wang *et al.*, 1998). In the presence of the overexpressed copy of *LHY*, transcription from the endogenous *LHY* promoter is repressed, indicating that LHY is part of a transcriptional feed-back loop rhythmically (Schaffer *et al.*, 1998). Inactivation of *CCA1* also affects the circadian expression of clock-controlled genes, although no effect on flowering time was reported, possibly due to redundancy with *LHY* (Green and Tobin, 1999). The mRNA abundance of the recently cloned *GI* gene is also regulated by the circadian clock. In the *gi* mutant, not only the circadian expression pattern of *GI* was

altered, but also that of *LHY* and *CCA1*, suggesting that *GI* and *LHY/CCA1* affect each other's expression (Fowler *et al.*, 1999; Park *et al.*, 1999).

The early flowering phenotype under SD of mutants such as *det1* (Pepper and Chory, 1997) and *cop1* (Weigel, 1995), suggests that the DET1/COP1 proteins suppress flowering under SD, which might be done by repressing floral promoters such as *CO*. The simplest hypothesis to explain this SD inhibition would be through repression by DET1/COP1 in the absence of the LD signal, and this would predict that photoreceptor-deficient mutants, which would not be able to remove the suppression of flowering by DET1/COP1, should be late in LD. Although this might be the case for phyA and blue-light receptor mutants (Johnson *et al.*, 1994; Bagnall *et al.*, 1996), this is not the case for mutants affecting phyB (*phyB* = *hy3*) and the chromophore (*hy1* and *hy2*), which are relatively early in SD (Goto *et al.*, 1991) due to the inhibiting effect of phyB discussed above. Nevertheless, analyses of double mutants involving these genes are still needed in order to understand the role of *DET1/COP1* in this process.

Based on grafting studies, daylength is perceived by the leaves, and the signal is then transported to the apical meristem (Bernier, 1988). It is not clear whether the crucial target is the apical shoot meristem or the lateral leaf/flower primordia itself. The latter is suggested by the chimeric structures observed by Hempel and Feldman (1995) after the transfer of plants from SD to LD. In Arabidopsis, the shoot apical or inflorescence meristem remains undetermined, and to maintain this state the *TFL1* and *TFL2* genes are required. The *TFL1* gene is strongly expressed in a group of cells just below the apical dome of the inflorescence in accordance with a role in this meristem (Bradley *et al.*, 1997). Bradley *et al.* (1997) suggested that *TFL1* delays the commitment to flowering during the vegetative phase, where it is also weakly expressed. In contrast, its *Antirrhinum* ortholog *CEN* is not expressed during vegetative development, and *cen* mutants are not early (Bradley *et al.*, 1997). Double mutant analysis between *tfl* and the late flowering *fca*, *fpa*, *fve*, *fwa* and *co* indicates that to repress flowering initiation *TFL* requires the function of the late flowering loci tested (Ruiz-García *et al.*, 1997; Page *et al.*, 1999).

The floral meristem identity genes *LFY* and *AP1* are crucial early targets of the floral promotion process. *LFY* is the earliest acting and a direct upstream regulator of other meristem identity or meristem-organ identity genes (Parcy *et al.*, 1998), which has also been shown by its ability to induce transcription of *AP1* (Wagner *et al.*, 1999) and the presence of a LFY responsive enhancer in the second intron of the *AG* gene (Busch *et al*, 1999). Both LFY and AP1 can convert shoot meristems into floral meristems, as shown by the early flowering of transgenic plants that constitutively express these genes. However, expression of these genes

may only trigger floral development after the main shoot has acquired competence to respond to its activity, since constitutive expression of *LFY* still allows the formation of some leaves (Weigel and Nilsson, 1995). Elegant studies in which the *CO* function was regulated by the ligand-binding domain of the rat glucocorticoid receptor showed that *LFY* expression increased within 24 hours after the activation of *CO* (Simon *et al.*, 1996) and that *AP1* is expressed later. This sequence of gene expression was also observed in studies after the shift from SDs to LDs (Hempel *et al.*, 1997). The interaction of flowering time genes with *LFY* has shown the existence of two different classes. One class affects primarily the transcriptional induction of *LFY* and contains genes belonging to different flowering time pathways (*FCA*, *FVE*, *LD*, *CO*, *GI*, *GAI*) (Nilsson *et al.*, 1998; Aukerman *et al.*, 1999) . The other class of genes affects primarily the competence to respond to *LFY* expression and contains the genes *FT* and *FWA* (Nilsson *et al.*, 1998). Probably *FT* and *FWA* are involved in activation of *AP1* (see below).

Two more lines of evidence suggest that *FT* and *FWA* have effects in the floral induction process. Double mutants of *ft* and *fwa* with *lfy* virtually lack floral initiation and do not show *AP1* mRNA in the inflorescence apex, indicating the importance of these genes for the initiation of *AP1* expression (Ruiz-García *et al.*, 1997). Furthermore, in contrast to other late flowering mutants, *ft* and *fwa* are late in continuous darkness when sucrose was available at the aerial part of the plant (Madueño *et al.*, 1996; Roldán *et al.*, 1999). This indicates that their role is not restricted to modifying the level or effect of the light-induced floral repressor only, but instead these genes may work at the meristem level and may be required (also) for the flower initiation process itself. The normal flowers of these mutants show that genetic redundancy exists for the flower initiation program as well as for the control of flowering time (Ruiz-García *et al.*, 1997). The cloning of *FT* revealed strong homology with *TFL1*. Furthermore, the transcription of *FT* is positively regulated by CO whereas its expression is not affected by the *fwa* mutation, suggesting a role of FWA downstream or in parallel with FT (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). The opposite effect of mutations in the homologous genes *FT* and *TFL1* points to a different role, and the two genes might have in common their interaction with *LFY* and *AP1*.

In what way the promoting flowering environmental signals interact with the flowering genes, how these genes interact, and how they activate their targets is still mainly unknown. The phenotypic and epistatic analyses indicate a complex network and suggest various redundant pathways. Since some of the promotive flowering time genes may act as

transcription factors (*LD* and *CO*) or may affect RNA stability (*FCA*), a sequence of gene activation events is a likely mechanism. The combined genetic, physiological, and molecular analyses will provide answers to this just-started and evolving picture of the network.

Concluding remarks

Recent genetic, molecular, and physiological analysis of flowering initiation in Arabidopsis has led to the identification of components in this important developmental process. Molecular elements involved in some of the initial steps such as photoreceptors and components of the circadian clock, in intermediate steps such as some of the cloned flowering genes, and in the target genes of floral induction, are now known. However, many questions remain: how do these elements interact and transmit the signals? Intriguing questions are, for example, how light and clock signals are integrated and how these interact with the flowering genes. The effect of vernalisation at the molecular level is not yet understood although *FLC* has been identified as a crucial target. Furthermore, a role for GAs in flowering is strongly indicated but its function remains unclear, as does the role of other hormones such as cytokinins, and factors such a carbohydrates. Besides, the sequence of events and redundancy suggested by the genetics and physiology is not yet understood at the molecular level. However, the molecular and genetic tools are available in Arabidopsis and will further refine and modify the model presented in this review. It will be important to relate and complement these studies in Arabidopsis with those in other plants to identify both the differences and common aspects, as it has been done for flower development between *Antirrhinum* and Arabidopsis. For flowering timing, pea is particularly important because of its similarity with Arabidopsis in the physiological responses and its ability for grafting studies (Weller *et al.*, 1997b). This may aid in identifying the nature of the floral repressor, deduced thus far only from genetic and physiological studies, and in determining whether any of the flowering time genes encode the elusive graft-transmissible florigen.

Scope of this thesis

In contrast to most animals, plants are bound to a single location. In order to grow and reproduce most efficiently, they have developed good systems to perceive and react to the environmental conditions that they encounter. Understanding these systems is a major challenge in developmental biology. The transition of the plant from the vegetative to the generative phase is an excellent model to investigate such a system. Due to the advantageous properties of Arabidopsis, the study of flowering transition became concentrated on this plant. This introduction chapter showed the progress that has been made in the understanding of this process in Arabidopsis. In the following chapters, an additional stone will be added to this building in construction.

The thesis deals with one of the late flowering mutants, *fwa* that has been mentioned above. A particularly interesting feature of this mutant is its semi-dominant behaviour. In a mutagenesis experiment of *fwa*, a new early flowering mutant, *efs*, was obtained. Chapter 2 gives a genetical and physiological characterisation of this mutant. The remaining chapters of this thesis are focussed on the *FWA* gene itself. In Chapter 3, the map based cloning procedure is described, which led to the molecular identification of the *FWA* gene. Chapter 4 gives a description of the molecular properties of this gene. Here it is shown that the mutant phenotype of *fwa* is not caused by changes in the DNA sequence but by an altered methylation pattern. Finally, in Chapter 5 a summarising discussion of the work presented in this thesis will be given.

Chapter 2

The early flowering mutant *efs* is involved in the autonomous promotion pathway of *Arabidopsis thaliana*

Wim J.J. Soppe, Leónie Bentsink and Maarten Koornneef

Summary

The transition to flowering is a crucial moment in a plant's life cycle of which the mechanism has only been partly revealed. In a screen for early flowering, after mutagenesis of the late flowering *fwa* mutant of *Arabidopsis thaliana*, the *early flowering in short days* (*efs*) mutant was identified. Under long day light conditions the recessive monogenic *efs* mutant flowers at the same time as wild-type but under short day conditions the mutant flowers much earlier. In addition to its early flowering phenotype, *efs* has several pleiotropic effects such as a reduction in plant size, fertility and apical dominance. Double mutant analysis with several late flowering mutants from the autonomous promotion (*fca* and *fve*) and the photoperiod promotion (*co*, *fwa* and *gi*) pathways of flowering showed that *efs* reduces the flowering time of all these mutants. However, *efs* is completely epistatic to *fca* and *fve* but additive to *co*, *fwa* and *gi*, indicating that *EFS* is an inhibitor of flowering specifically involved in the autonomous promotion pathway. A vernalisation treatment does not further reduce the flowering time of the *efs* mutant, suggesting that vernalisation promotes flowering through *EFS*. By comparing the length of the juvenile and adult phases of vegetative growth for wild-type, *efs* and the double mutants it is apparent that *efs* mainly reduces the length of the adult phase.

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Introduction

A plant needs to flower at a suitable time of the year in order to produce a large number of viable seeds. Given its importance, it is not surprising that flower initiation is controlled by many environmental and endogenous factors. Physiological research in different plant species has identified numerous of these factors, leading to a multifactorial model of the control of flowering (Bernier, 1988). More recently, the combination of physiological and genetical research, especially in Arabidopsis (Koornneef *et al.*, 1991) and pea (Weller *et al.*, 1997b), has identified several of the genes that play a role in this process. In Arabidopsis, the interactions among these genes have been further studied by including molecular approaches (Levy and Dean, 1998). This has led to the identification of several pathways involved in the regulation of flowering time in this species. A model for flowering initiation has been established consisting of a photoperiodic promotion pathway that promotes flowering under long day (LD) light conditions, a vernalisation promotion pathway that promotes flowering at low temperatures and an autonomous promotion pathway that promotes flowering autonomously, independently of the promoting effects of the other two pathways. (Martinez-Zapater *et al.*, 1994; Koornneef *et al.*, 1998b; Levy and Dean, 1998). This flowering model constitutes an appropriate framework for the analysis of flowering but is still far from complete and many questions remain.

One way to refine the current model is to identify additional flowering time genes. The screening for late flowering mutants in Arabidopsis has been quite exhaustive. Few early flowering mutants were obtained initially because most screens were performed in early flowering ecotypes under LD conditions, which enhance flowering and do not allow a wide window for selection. However, some screens in which early flowering mutants were obtained have been described (Zagotta *et al.*, 1992; Ahmad and Cashmore, 1996). In addition, several early flowering mutants were identified on the basis of other pleiotropic phenotypes. For instance some phytochrome deficient mutants (Goto *et al.*, 1991) and mutants that are hypersensitive to gibberellins (Jacobsen *et al.*, 1996) or that overproduce cytokinins (Chaudhury *et al.*, 1993) also flower early. Recently, another class of early flowering mutants was obtained by overexpressing specific genes in transgenic plants (Cardon *et al.*, 1997; Kania *et al.*, 1997). How these early flowering mutants interact with the late flowering mutants of the photoperiodic promotion or the autonomous promotion pathways is very poorly understood, however.

Screens to obtain new early flowering mutants can be done more efficiently in genetic backgrounds or under environmental conditions where Arabidopsis flowers late. For example, vernalisation defective mutants have been obtained by mutagenising the late flowering and vernalisation responsive *fca* mutant (Chandler *et al.*, 1996).

Here, we describe a novel early flowering mutant, *early flowering in short days* (*efs*), which was obtained after mutagenesis of the late flowering *fwa-1* mutation in the Landsberg *erecta* (L*er*) background.

During the analysis of the *efs* mutant, special attention was given to the different developmental phases during the growth of the plant. The life cycle of most plants can be divided into three developmental phases: a juvenile vegetative phase in which plants cannot respond to factors inducing flowering, an adult vegetative phase in which plants are able to do so and a reproductive phase in which they flower (Poethig, 1990). The change from the juvenile to the adult phase has been extensively studied in woody plants where it is most obvious. However, it is also apparent in herbaceous plants. A way to distinguish between the phases is by monitoring changes in several diagnostic characteristics such as leaf shape, thorniness and branching pattern, most of these changes occurring gradually (Hacket, 1985; Zimmerman *et al.*, 1985; Poethig, 1990). In Arabidopsis the different developmental phases can be recognised by changes in leaf shape. However, the most useful trait to distinguish between the juvenile and adult phase of vegetative growth is the presence or absence of abaxial trichomes (Kerstetter and Poethig, 1998). Flowering time mutants affect the duration of these phases; for instance, most of the late flowering mutants enhance all the phases (Martinez-Zapater *et al.*, 1995; Telfer *et al.*, 1997). An early flowering mutant, *hasty*, specifically reduces the juvenile vegetative phase (Telfer and Poethig, 1998). In the present work, we found that in the *efs* mutant mainly the length of the adult vegetative phase is reduced.

Results

Isolation and mapping of the efs mutant

The *fwa* mutant flowers later than wild-type plants, both under LD and SD light conditions (Koornneef *et al.*, 1991). After γ-irradiation of seeds from a *cer2 ga5 fwa abi1* marker line, five plants that flowered early under LDs were obtained in the M_2 generation. These plants were crossed with the L*er* wild-type to separate them from the mutations of the marker line.

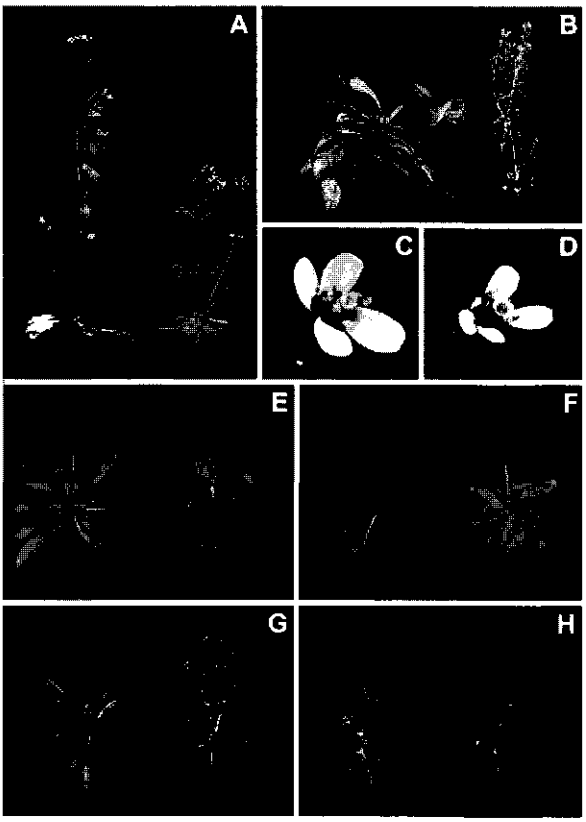

Figure 2.1. The phenotypes of L*er*, *efs* and double mutants with *efs*.

A. Wild-type L*er* (left) and *efs* mutant (right) plants, five weeks old and grown under LD conditions.
B. Wild-type L*er* (left) and *efs* mutant (right) plants, seven weeks old and grown under SD conditions.
C. Wild-type L*er* flower.
D. *efs* mutant flower.
E. *co* mutant (left) and *efs co* double mutant (right) plants, five weeks old and grown under LD conditions.
F. *fwa* mutant (left) and *efs fwa* double mutant (right) plants, five weeks old and grown under LD conditions.
G. *fca* mutant (left) and *efs fca* double mutant (right) plants, five weeks old and grown under LD conditions.
H. *tfl1* mutant (left) and *efs tfl1* double mutant (right) plants, five weeks old and grown under LD conditions.

For three of the plants the earliness could not be separated from the markers; these will be described in Chapter 4. Two other plants, derived from different M_1 batches, had similar pleiotropic phenotypes of which the bushy habit and small flowers were most obvious (Figure 2.1A). F_1 plants from a cross between these two mutants did not show complementation for both flowering time and pleiotropic traits, indicating that they are allelic. The mutant was named *efs* (*early flowering in short days*, see hereafter) and one of the two alleles was taken for further analysis. The phenotype of F_1 plants from a cross between *efs* and wild-type L*er* is indistinguishable from wild-type plants, indicating that *efs* is a recessive mutant.

The *EFS* locus is located 7.2 cM (± 2.1) below the microsatellite nga111 and 3.4 cM (± 1.8) below the CAPS marker ADH at the bottom of chromosome 1. To perform this mapping, F_3 lines were analysed from 91 F_2 plants, 12 of these were homozygous *efs*, 45 were heterozygous and 34 were homozygous wild-type. A similar deviation from the expected ratio has been observed before in this region for crosses between L*er* and Col (Lister and Dean, 1993) and is probably caused by gene(s), closely linked to *EFS*.

The efs phenotype

The efs mutation reduces flowering time under SD but not under LD conditions

The *efs* mutant was identified on the basis of its ability to reduce the flowering time of the late flowering mutant *fwa* under LD conditions. However, in a wild-type L*er* background *efs* does not reduce the flowering time under LDs. Flowering time, measured as number of days until first flower buds are visible and as total number of leaves, was similar for L*er* and *efs* (Figures 2.1A, 2.2A,B). Both L*er* and *efs* plants flower later under SDs than under LDs. However, *efs* is less sensitive to daylength and flowers much earlier than L*er* plants under SD conditions (Figures 2.1B, 2.2A,B).

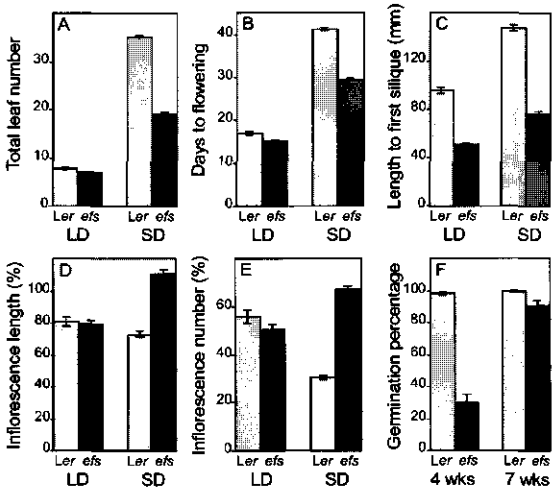

Figure 2.2. Comparative description of wild-type L*er* and the *efs* mutant.

Plants were grown under long (LD) or short day (SD) light conditions. The standard errors of the means are indicated on each bar.

A. The total leaf number of rosette and main shoot.
B. The number of days until the appearance of the first flower buds.
C. The length of the main stem from the rosette until the first silique.
D. The length of the longest secondary inflorescence as percentage of the length of the primary inflorescence.
E. The number of leaves with coflorescences or secondary inflorescences as percentage of the total number of leaves.
F. The mean percentage of germinating seeds at four and seven weeks after harvest.

To determine whether the difference in flowering time between *efs* and wild-type *Ler* is caused by a slower rate of leaf initiation, the rate of leaf initiation was measured for both *efs* and L*er* under SD conditions. As shown in Figure 2.3, the rate of leaf initiation in both genotypes is similar.

The efs mutation mainly reduces the adult vegetative phase

In the same experiment, *efs* mutant and wild-type L*er* were compared for the length of the three developmental phases: the juvenile vegetative phase characterised by leaves without abaxial trichomes, the adult vegetative phase characterised by leaves with abaxial trichomes and the reproductive phase characterised by an

inflorescence with cauline leaves (Telfer *et al.*, 1997; Lawson and Poethig., 1995). As shown in Figure 2.3, *efs* mainly reduces the number of leaves in the adult vegetative phase; the length of the juvenile vegetative phase and the reproductive phase were similar to those of wild-type L*er*.

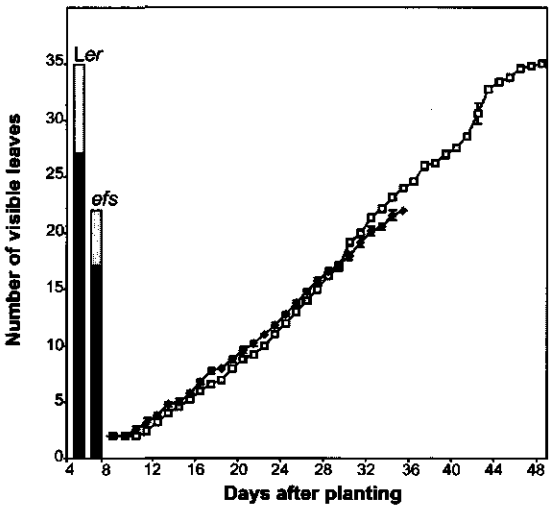

Figure 2.3. The rate of leaf initiation of L*er* and *efs* plants grown under short day conditions.

The data points represent the mean accumulated number of visible leaves, recorded till flowering, for five plants that made a total of 35 (L*er*, □) or 22 (*efs*, ♦) leaves. The standard error of the mean is indicated on each data point.

The average number of juvenile vegetative (■), adult vegetative (■), and reproductive leaves (■) for L*er* and *efs* is indicated in the two bars.

Pleiotropic traits of efs

In addition to its effect on flowering time in SDs, *efs* also has pleiotropic effects on several other traits (Figures 2.1, 2.2). In both LDs and SDs, *efs* is only half the size of L*er* as measured by the length of the stem from the rosette to the first silique (Figure 2.2C). The leaves, roots and flowers of *efs* mutants are reduced in size compared to wild-type plants. The secondary inflorescences of *efs* plants are relatively long, compared to the length of the primary inflorescence, indicating a reduced apical dominance (Figure 2.2D). This is also implied by the higher number of coflorescences and secondary inflorescences as compared to the total number of leaves in *efs* (Figure 2.2E). Although in this experiment the reduced apical dominance is only visible under SDs, *efs* plants that were grown in LDs in the greenhouse (as shown in Figure 2.1A) also showed a reduced apical dominance. This difference is probably due to the specific conditions since LDs were provided by supplementing the light period with incandescent bulbs.

Although at the macroscopic level there are several aberrations visible in the *efs* mutant, microsections through stem, hypocotyl, root and flower buds did not show any major structural differences. Representative photos of these microsections are shown in Figure 2.4.

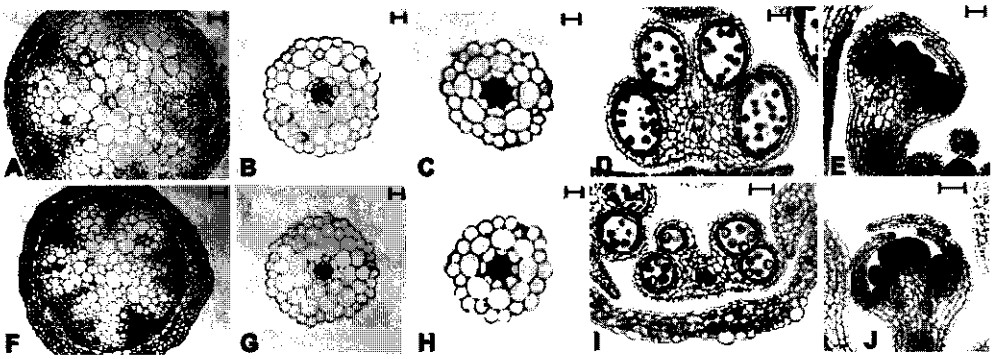

Figure 2.4. Microscopic sections from different tissues of wild-type L*er* and *efs*.
(A-E) Wild-type plants; (F-J) *efs* mutant plants. (A,F) Transverse section of the main stem (just above the first internode); (B,G) a transverse section of the hypocotyl; (C,H) a transverse section of the root; (D,I) a transverse section of an anther; (E,J) a longitudinal section of a flower meristem. Scale bar, 1 mm in A and F; 500 μm in B,D,E,G,I,J and 200μm in C and H.

The section through the main stem, taken just above the first internode (Figure 2.4A,F), also shows a reduced diameter of the *efs* stem which is mainly caused by a reduction in cell size.

Additional pleiotropic traits of the *efs* mutant are as follows.

(1) Incomplete development of first flowers. The first flowers that appear on the main shoot never open completely and wither, which causes a delay of the moment that the first open flower is visible. For this reason, the opening of the first flower was not taken as a measurement of flowering time in *efs*. Later flowers develop normally, although they are smaller than wild-type flowers (Figure 2.1C,D).

(2) Reduced fertility. The seed set of *efs* is lower than that of L*er*. Reciprocal crosses using *efs* either as female or as male parent gave a low seed set, indicating that both female and male fertility are reduced. As shown in Figure 2.1C,D, the anthers of *efs* flowers release less pollen than anthers of wild-type flowers. Microsections through the anther show that the *efs* anther is shrunken and deformed, and contains less pollen than wild-type anthers (Figure 2.4D,I). This could explain the reduced male fertility of *efs*.

(3) Increased dormancy and reduced germination. L*er* seeds that have been stored during four weeks germinate nearly 100% whereas *efs* seeds germinate 30%. Also, even after seven weeks of dry storage, germination of *efs* seeds does not reach 100% but remains at 90% (Figure 2.2F). Similar germination percentages were observed for *efs* seeds after more than one year of storage, indicating a reduced viability of the seeds.

(4) Reduced root growth. The root length of 16 L*er* and 19 *efs* plants, grown on MS 10 medium in vertically placed Petri dishes, was measured. After ten days, the lengths of L*er* and *efs* roots were respectively 32.6 mm (± 9.1) and 14.0 mm (± 6.7).

Is efs a specific flowering time mutant?

The pleiotropic traits of *efs* suggest that the reduction of flowering time in SDs is not caused by a specific defect in one of the flowering pathways but by a more general defect in plant growth, which simultaneously leads to early flowering. If this is the case, *efs* could not be placed in one of the flowering pathways and therefore should not be called a flowering time mutant. To determine whether *efs* specifically affects one of the pathways, double mutants were made with several late flowering mutants.

Double mutants of *efs* with three mutants, *fwa*, *constans* (*co*) and *gigantea* (*gi*) of the photoperiodic promotion pathway and two mutants, *fca* and *fve*, of the autonomous promotion pathway were constructed. The vernalisation response of *efs* and its double mutants was tested in order to see whether *EFS* might be involved in the vernalisation promotion pathway.

Furthermore, double mutants of *efs* with one other mutant involved in flowering, *terminal flower1* (*tfl1*) were made. *TFL1* promotes inflorescence meristem identity and the *tfl1* mutant shows an early flowering phenotype (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992).

Analysis of double mutants under LD conditions

Figure 2.5A shows the leaf number of the different mutants and double mutants with *efs*, grown under LD conditions. As already shown in Figure 2.2A, the leaf number of *efs* in these conditions is identical to that of wild-type L*er*. The late flowering mutants all have a higher leaf number than L*er*, but the double mutants with *efs* can be divided into two groups. One group, consisting of late flowering mutants belonging to the photoperiod promotion pathway (*gi*, *co*, *fwa-1* and *fwa-2*), has double mutants with a leaf number intermediate between the late flowering mutants and *efs* (Figures 2.1E,F, 2.5A). This indicates that *efs* behaves additively to these mutants. The other group consists of *fca* and *fve*, both belonging to the autonomous promotion pathway, and has double mutants with a similar leaf number as the *efs* mutant (Figures 2.1G, 2.5A). Therefore, *efs* is epistatic to these late flowering mutants. The double mutant with *tfl1* does not have a significantly different leaf number than the single mutants (Figures 2.1H, 2.5A).

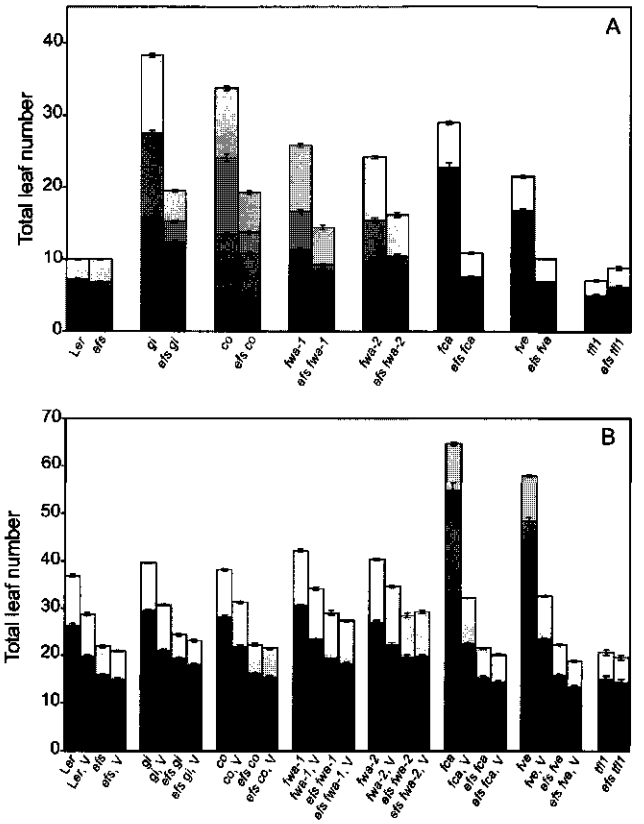

Figure 2.5. The number of juvenile, adult and reproductive leaves in different single mutants and double mutants with *efs*. (A) Leaf number under LD conditions; (B) leaf number under SD conditions, with (V) and without vernalisation. Juvenile vegetative (■), adult vegetative (■), and reproductive leaves (■) are shown. The standard error of the mean is indicated on each bar.

The total number of leaves was subdivided into the number of juvenile vegetative, adult vegetative and reproductive leaves (Figure 2.5A). In the late flowering mutants the number of leaves in all three phases is increased compared to the wild-type. For the double mutants of *efs* with those, the leaf number in all the phases is reduced compared to the late flowering mutants. However, the main reduction in leaf number is in the adult vegetative phase. As shown in Figure 2.6A, for the late flowering mutants, an increasing number of juvenile vegetative leaves correlates with an increasing number of adult vegetative leaves. However, in double mutants with *efs* an increasing number of juvenile vegetative leaves does not lead to an increase of the number of adult vegetative leaves.

Analysis of double mutants under SD conditions

In Figure 2.5B the leaf number of the different mutants and double mutants with *efs*, grown under SD conditions is shown. As described above and shown in Figure 2.3, *efs* has a reduced number of leaves in these conditions compared to the wild-type *Ler*. The late flowering

mutants can be divided into three groups. The first group, consisting of *gi* and *co*, has a similar number of leaves as L*er*. The double mutants *efs gi* and *efs co* have the same number of leaves as *efs* . The second group, with *fwa-1* and *fwa-2*, flowers with a few more leaves than L*er* and the *efs fwa-1* and *efs fwa-2* double mutants flower with a leaf number between *fwa* and *efs*. The flowering time of the last group with mutants of the autonomous promotion pathway (*fca* and *fve*), is strongly delayed under these conditions. The double mutants *efs fca* and *efs fve* have a leaf number similar to *efs*. So, like under LD conditions *efs* is additive to *fwa-1* and *fwa-2* and epistatic to *fca* and *fve*. The *efs tfl1* double mutant has a leaf number similar to the single *efs* and *tfl1* mutants.

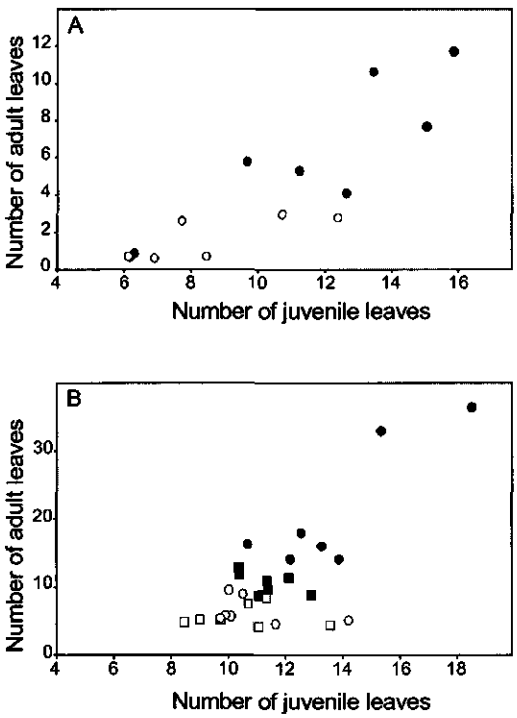

Figure 2.6. The relation between juvenile and adult leaves for the late flowering mutants and the double mutants with *efs*.
(A) Plants grown under LD conditions. ●, wild-type L*er* and late flowering mutants; O, *efs* and the double mutants of *efs* with the late flowering mutants. (B) Plants grown under SD conditions. ●, wild-type and late flowering mutants without vernalisation; ■, wild-type and late flowering mutants with vernalisation; O, *efs* and the double mutants of *efs* with the late flowering mutants without vernalisation; □, *efs* and the double mutants of *efs* with the late flowering mutants with vernalisation.

A comparison of the leaf numbers of the different phases for the wild-type and the late flowering mutants in LDs with those in SDs shows that the higher leaf number in SDs is mainly caused by an increase in leaf number of the adult vegetative phase (Figure 2.5). As in LD conditions, *efs* decreases the leaf number in the double mutants mainly by reducing the adult vegetative phase (Figure 2.5B). This is especially striking for the *efs fca* and *efs fve* double mutants. An increasing number of juvenile vegetative leaves correlates with an increasing number of adult vegetative leaves in late flowering mutants but not in an *efs* genetic background (Figure 2.6B).

Analysis of double mutants after vernalisation

The behaviour of late flowering mutants and the corresponding doubles with *efs* in SD conditions after a vernalisation treatment is shown in Figure 2.5B. As previously described, L*er*, *gi*, *co*, *fwa-1* and *fwa-2* react little to vernalisation by flowering with a slightly reduced leaf number whereas *fca* and *fve* react strongly. The monogenic *efs* and double mutants hardly respond to a vernalisation treatment.

The reduction in leaf number produced by a vernalisation treatment for L*er* and the late flowering mutants is mostly caused by a decrease in the number of adult vegetative leaves (Figure 2.5B), although the number of juvenile vegetative leaves is slightly reduced in the *fca* and *fve* mutants. The effect of the vernalisation treatment and the *efs* mutation on the ratio adult vegetative leaves to juvenile vegetative leaves is similar. However, the *efs* mutation is able to even further reduce the number of adult vegetative leaves in vernalised plants (Figure 2.6B).

Pleiotropic effects of double mutants

The double mutants of *efs* with the late flowering mutants showed the same pleiotropic phenotypes as *efs*: they are smaller in size, have less apical dominance and are less fertile than wild-type plants. Therefore *efs* does not need the function of any of the late flowering genes in its role on these other traits.

The double mutant of *efs* with the meristem identity mutant *apetala1* showed additivity of the two phenotypes, indicating that *efs* does not affect meristem identity as no novel synergistic interactions were observed (data not shown).

Discussion

EFS is a novel flowering time locus

The early flowering mutant under SDs, *efs*, identifies a novel flowering time locus, controlling an inhibitor of flowering as shown by its novel phenotype and its location at a map position where no other flowering locus has previously been mapped.

Although *efs* has several pleiotropic traits it can be considered as a flowering time mutant according to the double mutant analysis. Several other early flowering mutants with pleiotropic traits have been obtained in Arabidopsis (Levy and Dean, 1998; Koornneef *et al.*, 1998b). The early flowering phenotype of *phyB* for instance is caused by a defect in

phytochrome B which also causes abnormal elongation of hypocotyls, stems, petioles and root hairs (Reed *et al.*, 1993). The *spindly* (*spy*) mutant is not only early flowering but also has pale green foliage, partial male sterility and parthenocarpic fruit development which is caused by a constitutive activation of *GA* signal transduction (Jacobsen *et al.*, 1996). The pleiotropic effects of phytochrome and gibberellin mutants indicate that these factors control many aspects of plant development of which flowering time is one. A similar situation cannot be excluded for *efs*.

The nature of the relationship between pleiotropic traits and flowering behaviour of *efs* is difficult to assay and might need a molecular identification of the locus. The observation that the *efs* mutant influences plant size, germination and fertility but does not change the overall structure of the tissues and cells (Figure 2.4) suggests that the locus may be involved in cell expansion. In this aspect, its effects are opposite to those of the early flowering phytochrome and *spy* mutants in which mutations result in more elongated cells in specific tissues (Reed *et al.*, 1993; Jacobsen *et al.*, 1996). Since most of the dwarf mutants in Arabidopsis do not have an early flowering phenotype, it is likely that the cell elongation defects of *efs*, leading to the pleiotropic phenotype, are probably independent of its early flowering effect. The interpretation of the dormancy phenotype of *efs* in relation to its flowering behaviour has similar complexities as the interpretation of the plant size.

Efs reduces the adult vegetative phase

Phase changes in Arabidopsis are affected by both environmental and genetical factors. Application of gibberellins for instance accelerates the onset of abaxial trichomes and therefore shortens the juvenile phase (Chien and Sussex, 1996). Furthermore, all three growth phases are decreased in LDs, compared to SDs (Chien and Sussex, 1996; Telfer *et al.*, 1997; present work). Genetic factors that influence phase changes are noticed in the late flowering mutants that cause a delay of phase changes (Telfer *et al.*, 1997). This has been demonstrated in detail for the *fve* mutant where trichome distribution was studied in combination with leaf shape changes (Martinez-Zapater *et al.*, 1995). In agreement, under LDs *gi*, *co*, *fwa*, *fca* and *fve* mutants are all delayed in their phase changes. However, under SDs only the mutants belonging to the autonomous pathway, *fca* and *fve*, show this delay in accordance with the reduced function of the photoperiodic promotion pathway under these conditions.

Besides the above mentioned factors that have a similar influence on the length of the different growth phases, there are also some factors that mainly affect one of the growth phases. The *efs* mutant is one of these because the reduction in flowering time is primarily

caused by a reduction of the adult vegetative phase (Figure 2.3). Apart from the *efs* mutation, another genetic factor that has been reported to reduce mainly the adult phase is the constitutive expression of the *LFY* gene by a *35S::LFY* construct (Weigel and Nilsson, 1995). This implies that *LFY* only influences the meristem after the adult vegetative phase has started, i.e. after the plant gained the ability for flower initiation. One other early flowering mutant with a specific reduction of one of the growth phases is *hasty* which hardly affects the adult vegetative phase but has a reduced juvenile vegetative phase. The *hasty* mutant appears to affect flowering time primarily by an acceleration of the transition to a reproductively competent phase (Telfer and Poethig, 1998). Vernalisation mainly affects the length of the adult vegetative phase (Figures 2.5B, 2.6B). This supports the view that plants can only respond to factors inducing flowering when they are in the adult vegetative phase (Poethig, 1990). However, vernalisation occurs in seedlings whereas its effect is seen later, indicating that the plant must store this signal.

Despite the lack of a flowering phenotype under LDs, *EFS* is needed under these conditions in a L*er* wild-type genetic background as indicated by its effect in the double mutants. The *efs* mutant does not flower early in LDs because wild-type L*er* plants have hardly any adult vegetative leaves in these conditions, therefore the reducing effect of *efs* on the adult phase has no effect on flowering time. The *fca* and *fve* mutants have a longer juvenile vegetative phase than the other late flowering mutants. The doubles of *efs* with *fca* and *fve* also show a considerable reduction of the juvenile vegetative phase (Figure 2.5). This points to some influence of *EFS* in the juvenile vegetative phase as well. It could be that this effect is normally completely suppressed by *FCA* and *FVE* wild-type alleles and only becomes apparent when one of these genes is mutated. Probably *EFS* influences the length of all developmental phases although its effect on the adult vegetative phase is most obvious.

EFS interacts with the autonomous promotion pathway

The interaction of *efs* with the late flowering mutants is similar under LD and SD conditions (Figure 2.5). The only exception is for *efs co* and *efs gi*, in SDs these double mutants flower only slightly later than the single *efs* mutant. This is probably a result of the low expression and flowering promotion of *CO* and *GI* under these conditions. In agreement with this, it has been shown that the expression of *CO* in SDs is reduced as compared to the expression in LDs (Putterill *et al.*, 1995).

The double mutant analysis indicates that *EFS* represses flowering in the autonomous promotion pathway and that *FCA* and *FVE* require the function of *EFS* to promote flowering;

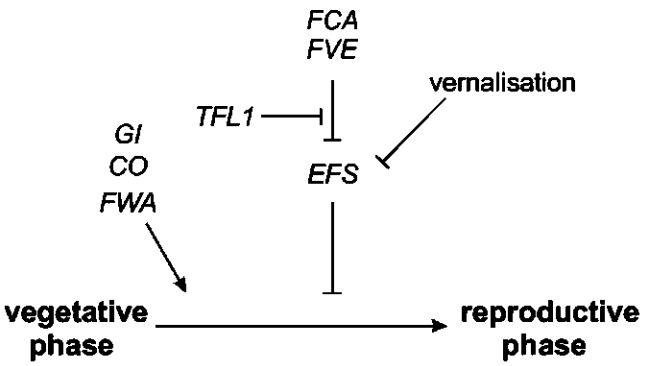

Figure 2.7. A model of the possible role of *EFS* in floral transition.
The genes from the LD promotion pathway (*CO*, *GI* and *FWA*) promote flowering independently of *EFS*. *FCA*, *FVE* and a vernalisation treatment promote flowering by decreasing the repressing effect of *EFS* on flowering. The role of *TFL1* could be a repression of the function of *FCA* and *FVE*. The horizontal arrow symbolises the transition from the vegetative to the reproductive meristem. The other arrow represents an enhancing influence, the "⊥" symbols represent a reducing effect.

in other words, *FCA* and *FVE* counteract the inhibitory function of *EFS* on flowering transition (Figure 2.7). A similar repression role, and interactions with *FCA* and *FVE*, has been considered for two other genes, *FRI* and *FLC* (Sanda and Amasino, 1996b; Michaels and Amasino, 1999a).

Another gene that may negatively regulate flowering through this pathway is *TFL1*. Under both LD and SD conditions the double mutant of *efs* with *tfl1* does not flower significantly earlier than the single mutants (Figure 2.5B). *TFL1* is involved in the promotion of inflorescence meristem identity and has a role in the autonomous promotion pathway because it delays flowering by repressing the promotive function of *FVE* (Ruiz-Garcia *et al*, 1997) and *FCA* (Page *et al*., 1999). This means that the *efs fve* and *efs fca* double mutants behave opposite to the *tfl1 fve* and *tfl1 fca* double mutants. Whereas *TFL1* reduces the promotive function of *FVE* and *FCA* on floral transition, *FVE* and *FCA* reduce the repressive function of *EFS* on floral transition (Figure 2.7).

Two arguments indicate an epistatic relationship between the *efs* mutant and vernalisation. Firstly, a vernalisation treatment has hardly any effect on the flowering time of *efs* and the double mutants with *efs* in SDs (Figure 2.5B). Secondly, the effect of vernalisation on the relative length of the different growth phases of Arabidopsis is comparable to the effect of the *efs* mutation on these, but smaller (Figure 2.5B). A longer vernalisation treatment could give a stronger effect. Therefore, one can conclude that vernalisation acts on the *EFS* gene product or on its downstream components. However, the possibility that vernalisation does not have an effect on the *efs* mutant because the plants flower so early that vernalisation cannot further promote flowering under short days cannot be ruled out. The function of *EFS*

might be to prevent meristems that are in the adult vegetative phase, so meristems that have obtained the competence to flower, from flowering. Flowering stimuli like vernalisation but also the wild-type *FCA* and *FVE* gene products from the autonomous promotion pathway might reduce this function of *EFS* (Figure 2.7).

The interaction of *EFS* with vernalisation is again rather similar to that of the other repressors of the autonomous promotion pathway, *FRI* and *FLC*, (Michaels and Amasino, 1999a) suggesting that these repressors work at the same point. However, where *FCA* and *FVE* seem to function completely through *EFS*, they are suggested to work only partially through *FLC*. Further investigation of the interaction from the *efs* mutation with *FRI* and *FLC* and the role of vernalisation in this pathway will help to elucidate the role of these genes and stimuli on the promotion of flowering.

Materials and methods

Plant material

The mutant lines that were used are all in Landsberg *erecta* (L*er*) genetic background. The monogenic mutants *co-3*, *fca-1*, *fve-1*, *fwa-1*, *fwa-2* and *gi-3* were described by Koornneef *et al.* (1991) and *tfl1-2* by Alvarez *et al.* (1992). The L*er* marker line containing the mutations *cer2-1 ga5-1 fwa-1 abi1-1* was constructed by M. Koornneef and A.J.M. Peeters for the fine mapping of *FWA*. Seeds of the Columbia wild-type were obtained from C. Somerville (Carnegie Institute of Washington, Stanford, USA).

The *efs* mutant was obtained by γ irradiation of the *cer2-1 ga5-1 fwa-1 abi1-1* multiple marker line with 400 Gy. Out of 5000 M_2 plants, grown under LD light conditions, early flowering plants were selected and crossed with L*er*. In the F_2 generation, derived from these crosses, putative mutant plants that did not contain the marker genes were isolated for further analysis.

Double mutants were constructed by crossing the monogenic *efs* mutant with lines carrying the mutations *co-3*, *gi-3*, *fwa-1*, *fwa-2*, *fca-1*, *fve-1* and *tfl1-2*. Double mutants were selected from the selfed progeny of F_2 plants that either showed the *efs* phenotype or were late flowering and which segregated for the second mutation. The genotype of *efs fca* and *efs fve* double mutants was confirmed by crosses with monogenic *fca* and *fve* respectively, which resulted in late F_1 plants.

Growth conditions and physiological characterisation

Seeds were sown on wet filter paper (no 595, Schleicher and Schuell, Germany) in plastic Petri dishes. Thereafter, they were transferred to a climate room (25°C, 16 hours light for LD experiments; 24°C, 8 hours light for short day (SD) experiments) and incubated for three days. Germinated seeds were

planted on potting compost in pots and grown in a greenhouse with long day light regime (at least 14 hours daylight) or in a climate chamber with SD light conditions (24°C, 8 hours light per day). For the physiological comparison of the monogenic *efs* mutant with wild-type, both LD and SD experiments were carried out in similar growth chambers whereby long day conditions were created by additional supply of 8 hours of incandescent light, as described in Koornneef *et al.* (1995).

Seeds used in the vernalisation experiments were surface sterilised with 20% bleach in 96% ethanol and rinsed twice with ethanol. After drying they were sown on Murashige and Skoog plates containing 1% sucrose and stored in dark at 4°C during four weeks before planting.

For the physiological characterisation shown in Figure 2.2, flowering time was recorded as the number of days from the time that the seedlings were planted till the visualisation of the first flower buds by naked eye. In the rest of the experiments the total number of leaves is taken as a measurement of flowering time. The number of leaves within the rosette was counted shortly after the plant bolted. A magnifying glass was used to detect the presence of abaxial trichomes. The number of juvenile vegetative leaves was determined as rosette leaves without abaxial trichomes and the number of adult vegetative leaves as rosette leaves with abaxial trichomes. At the time of first flower opening the number of leaves on the main stem was counted, representing leaves from the reproductive phase.

For the germination assay, mature seeds were harvested from ripe siliques. After storage at room temperature, the seeds were sown on water-saturated filter paper in plastic Petri dishes and incubated at 25°C under 16 hours white light. The seeds were scored for germination after 1 week.

Mapping

A cross was made between *efs* and the Columbia ecotype. The F_2 and F_3 populations derived from this cross were scored for the *efs* phenotype. DNA was isolated from 91 F_2 lines and analysed using CAPS (Konieczny and Ausubel, 1993) and microsatellite (Bell and Ecker, 1994) markers. To estimate the recombination fractions, the RECF2 program, which produces maximum likelihood estimates with their standard errors, was used (Koornneef and Stam, 1992). For the construction of the linkage map, the JOINMAP program (Stam, 1993) was used applying the Kosambi function to convert recombination fractions into genetic distances.

Microscopical characterisation

For the preparation of microscopic sections, plant material was immersed in 4% formaldehyde for at least 16 hours at 4°C and subsequently dehydrated in a graded series of acetone. Thereafter, acetone was replaced by resin (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim, Germany). After polymerisation of the resin the blocks were cut with a microtome (Leitz Wetzlar, Germany) and sections were stained with 0.05% (w/v) toluidine blue in water.

Acknowledgements

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Chapter 3

Map based cloning of the *FWA* gene

Wim J.J. Soppe, Carlos Alonso-Blanco, Anton J.M. Peeters and Maarten Koornneef

Summary

The *fwa* mutant was identified in Arabidopsis by its late flowering phenotype. With detailed genetic and physical mapping, the *FWA* locus could be located in a region of 60 Kb. Transformation with two overlapping cosmids, obtained from *fwa-1* mutant DNA, converted late flowering to L*er* wild-type plants. This indicated that *fwa* is located in the overlap between both cosmids and that the mutant allele behaves as a gain of function allele, suppressing flowering. The overlap contains only one complete predicted gene that encodes a homeodomain containing transcription factor.

Introduction

The *FWA* locus was identified in *Arabidopsis thaliana* by its mutant phenotype. In comparison to the wild-type, the *fwa* mutant is delayed in the transition from the vegetative to the generative phase (Chapter 4). There are two mutant alleles available; *fwa-1* was identified after treatment with ethyl methanesulphonate and *fwa-2* after fast neutron irradiation (Koornneef *et al.*, 1991). The *FWA* locus has been mapped to chromosome 4. However, nothing is known about the molecular function of *FWA*. To understand this function and the role of the *FWA* gene in the flowering process, an effort is made to clone this gene.

There are several strategies to clone a gene, depending on the available molecular information (Gibson and Somerville, 1993). When the function of a gene is known, it is possible to isolate the gene by its ability to complement mutations in bacteria or yeast (Minet *et al.*, 1992). A gene with a characterised pattern of expression can be cloned by differential screening (Park *et al.*, 1998). In the case that only the mutant phenotype of a gene is known, other strategies have to be used. When a T-DNA or transposon is inserted in the gene, it may cause a mutant phenotype. In such a tagged mutant, the gene can be very effectively cloned by isolation of DNA fragments flanking the insertion (Aarts *et al.*, 1995; Schaffer *et al.*, 1998). If the mutant phenotype is caused by a deletion, the gene can be cloned by subtractive hybridisation. However, cloning by subtractive hybridisation in Arabidopsis is complicated and has only been proven successful for two loci (Sun *et al.*, 1992; Silverstone *et al.*, 1998). If none of the above mentioned methods is applicable, a gene can be cloned using a map-based approach (Putterill *et al.*, 1995; Macknight *et al.*, 1997).

The gene product and function of *FWA* are unknown and no tagged alleles are available which excludes the first methods, mentioned above, as cloning strategy. Preliminary attempts to clone the *FWA* locus by subtractive hybridisation remained unsuccessful (Peeters, personal communication). Map-based cloning is the best way for cloning a gene like *FWA* of which only the mutant phenotype and genetic map position are known. Map-based cloning in Arabidopsis is facilitated by the fact that it has one of the smallest genomes among higher plants with very low levels of repetitive DNA. Furthermore, there are many genetic loci identified by mutations, it has a dense molecular marker map and at the time that the cloning was started, there was almost a complete yeast artificial chromosome (YAC) coverage (Dean and Schmidt,1995). In addition, the complete DNA sequence of chromosome 4 has recently

been published (Mayer *et al.*, 1999) and soon the DNA sequence of the complete genome will be available.

The first step in map-based cloning is to locate genetically the locus of interest as accurate as possible with the help of linked markers, either morphological or molecular. The most closely linked molecular markers can be used to isolate clones that contain the region of the genome covering the locus. Thereafter, the gene will be identified by complementation of the mutant phenotype in transgenic plants containing the candidate gene. DNA sequencing of wild-type and mutant alleles will reveal the nature of the mutation and a comparison with sequences in the databases can indicate the putative molecular function of the gene.

This chapter deals with the map-based cloning of the *FWA* gene. First the segregating population that was used for the fine mapping of *FWA* is described, followed by the YAC and cosmid contigs that were constructed. Finally the plant transformation experiments that gave complementation will be discussed.

Results and Discussion

Genetic mapping

The mapping population

For the genetic mapping of *FWA* both morphological and molecular markers have been employed. Morphological markers are based on differences in phenotype while molecular markers detect polymorphisms at the DNA level. The latter implies that a mapping population should be derived from a cross between two plants that do not only differ for the locus of interest but also for their DNA sequence. Such DNA polymorphisms can be found between accessions in Arabidopsis. For the mapping of *FWA*, the accessions Landsberg *erecta* (L*er*) and Columbia (Col) were used.

A complication of the use of accessions is that they may differ in loci affecting the trait of interest, in this case flowering time. An analysis of recombinant inbred lines derived from a cross between L*er* and Col revealed genetic variation for flowering time at twelve different loci (Jansen *et al.*, 1995). In this population, the effects of individual loci were relatively small, in contrast with the finding of large gene effects in the progeny of the cross between the early flowering accessions L*er* and Cape Verde Islands (Alonso-Blanco *et al.*, 1998). However, the accumulation of either many early or many late alleles in specific progeny plants

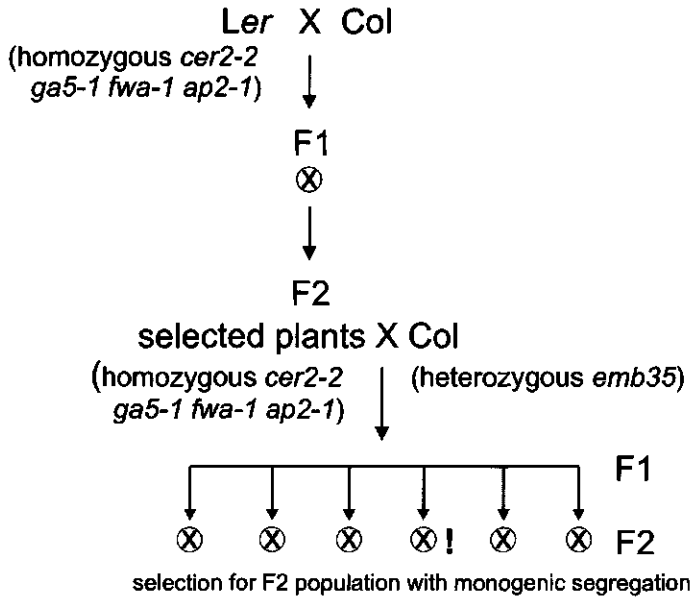

Figure 3.1. Schematic presentation of the pedigree from the mapping population.

from a L*er* x Col cross may affect the phenotype for flowering time of these plants in such a way that their classification for the *FWA* gene can not be done unambiguously.

To solve this problem a mapping population was constructed with a more uniform genetic background (Figure 3.1). First a cross was made between L*er* and Col whereby L*er* was homozygous for *fwa* and for the recessive morphological markers *cer2*, *ga5* and *ap2*. Late plants, homozygous for *cer2*, *ga5*, *fwa* and *ap2* were selected in the F2 generation. This selection ensured that in the *FWA* region of chromosome 4 these plants were homozygous L*er*, and thus *fwa* mutants, whereas the rest of the genome contains both L*er* and Col DNA. Some of these F2 individuals were crossed with Col plants, heterozygous for the *emb35* mutation that is linked to *FWA*. This cross increased the contribution of Col in the genome of the resulting F2 plants, leading to less variation in flowering time. From three different F2 populations, variation in flowering time and segregation for the *emb35* mutation was analysed. The use of a lethal marker linked in repulsion to the semi-dominant late flowering mutant *fwa* implies that hardly any early plants are expected, unless unlinked flowering time modifiers segregate. This allowed a clear distinction between F2 populations where such modifiers did segregate and those where this was not the case. One F2 population with a clear monogenic

segregation for flowering time and segregating *emb35* was selected as mapping population and further investigated.

Figure 3.2 shows the segregation of flowering time in this mapping population. The plants were grown under long day light conditions in a greenhouse. In these conditions Col flowered between 27 and 31 days, whereas the progeny of the parental *fwa* mutant line that was selected for the cross flowered between 42 and 51 days. As *fwa* is a semi-dominant mutant, one quarter of the mapping population should flower as early as Col. However, this fraction does not exist in the population because, due to linkage, these plants are homozygous for *emb35* and therefore embryo lethal. The few early plants found in the population must be the result of a cross-over between *fwa* and *emb35*. Therefore, the overall shape of the flowering time frequency distribution with two major peaks of different size can be explained because approximately 2/3 of these plants will be heterozygous for *fwa* (the heterozygous

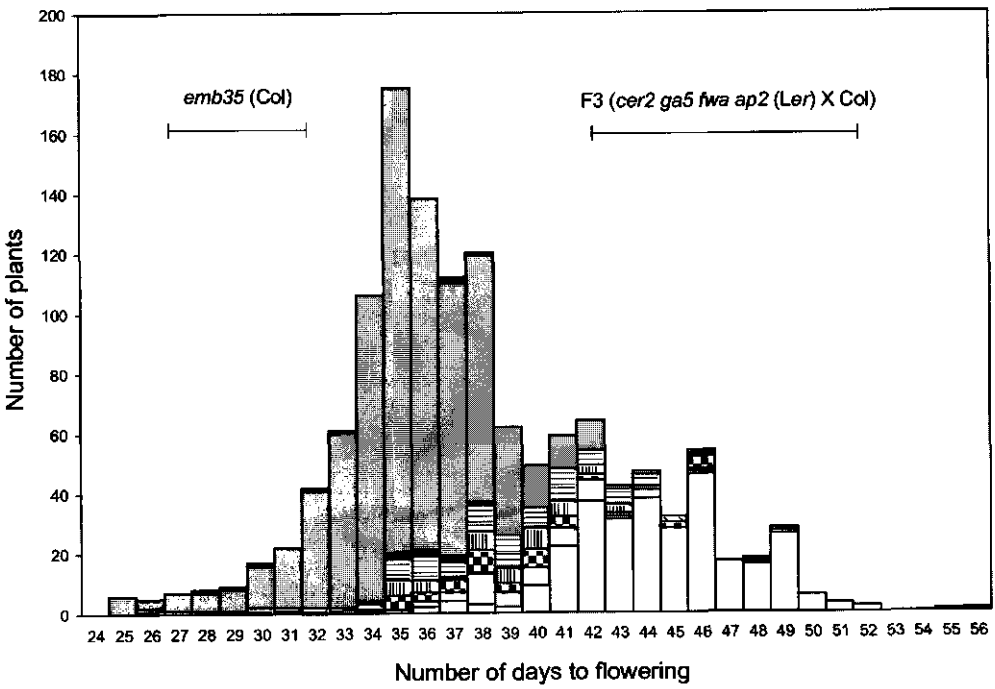

Figure 3.2. Segregation of flowering time in the mapping population. The different fractions of recombinant and parental phenotypes are shown: ■ = wild-type; ▨ = heterozygous *emb35*; ■ = homozygous *cer2*; ■ = homozygous *cer2*, heterozygous *emb35*; ■ = homozygous *ga5*, heterozygous *emb35*; ▤ = heterozygous *emb35*, homozygous *ap2*; ▥ = homozygous *ap2*; ▩ = homozygous *cer2*, *ga5*, heteozygous *emb35*; ▨ = homozygous *cer2*, *ga5*; □ = homozygous *cer2*, *ga5*, *ap2*. Flowering time of the Col parent and progeny of the *cer2 ga5 fwa-1 ap2* parent is indicated in the top (⊢—⊣).

FWA fwa plant flowers earlier than the homozygous *fwa fwa* plant). The flowering time of most of the plants of the mapping population is between the values of the parental lines, although a very small fraction of transgressive phenotypes might be present due to the segregation of some other flowering loci of minor effect differing between L*er* and Col. Figure 3.2 shows all recombinants that were obtained between the different morphological markers, classified according to their flowering time. From this mapping population the recombinants between *ga5* and *emb35* were selected for the fine mapping of *FWA*, using molecular markers.

Mapping with morphological markers

The different classes of recombinant F2 plants, segregating in the mapping population, were used to estimate the genetic distances between the morphological markers *cer2*, *ga5*, *emb35* and *ap2* (Table 3.1). Because of the variation in flowering time, it was not possible to score the *FWA* phenotype of the F2 plants unambiguously. Therefore, the *FWA* genotype of F2 plants was determined by analysing the flowering time of the F3 lines, derived from recombinant plants. These lines were only grown from the 120 recombinants between *GA5* and *EMB35*. F3 lines from homozygous *fwa* mutant F2 plants were completely late flowering; F3 lines from heterozygous *FWA fwa* F2 plants segregated flowering time while F3 lines from homozygous wild-type *FWA* F2 plants were early flowering. Out of these 120 recombinants, only two were recombinant between *GA5* and *FWA* and 118 had undergone recombination between *FWA* and *EMB35*. This means that *FWA* maps 0.1 cM below *GA5*.

These calculated distances are generally in agreement with the ones in the classical genetic map (Table 3.1; http://www.arabidopsis.org/cgi-bin/maps/Genintromap) apart from the distance between *GA5* and *FWA*. In the classical map this distance is 1 cM. This is probably due to the relatively small mapping population previously used and the fact that the

Table 3.1. Genetic distances between the morphological markers.

Morphological markers	Accumulative genetic distance (cM) according to mapping population	Map position on Meinke's classical genetic map
cer2	0	52
ga5	0.6	53
fwa	0.7	54
emb35	6.8	58
ap2	16.4	68

distance in this map is based on an integration of distances from different mapping populations.

Fine mapping with molecular markers

The genetic fine mapping of *FWA* was performed with molecular markers; 15 different restriction fragment length polymorphism (RFLP) markers and one codominant cleaved amplified polymorphism (CAPS) marker were used). DNA isolated from F3 lines derived from the 120 F2 plants, showing recombination between *GA5* and *EMB35*, was analysed with polymorphic molecular markers. From this analysis the location of the *FWA* locus could be limited to a region of 0.7 cM, between the morphological marker *ga5*, and the molecular marker pcr23 (Figure 3.3). For *ga5* also a molecular marker was available (Xu *et al.*, 1995), which was used as an extra control for the scoring of this morphological marker, revealing that for one recombinant the *ga5* phenotype was scored wrong. Further fine mapping within this region narrowed the location of *FWA* down to a small region between the markers CC128 and pcr28. There was only one recombinant with CC128 and there were two recombinants with pcr28. Recombinants between these flanking markers and the *FWA* locus were also recombinant between both molecular markers.

For the majority of the used molecular markers, RFLP's could be found between L*er* and Col. However, for three markers (cos20, pcr34 and pcr23) such RFLP's were not found but instead, RFLP's were detected between wild-type *FWA* and mutant *fwa* DNA. Further research indicated that these RFLP's were not caused by differences in DNA sequence but by stable differences in methylation level (Chapter 4).

Physical mapping

The YAC contig

Once *FWA* was located within a small region between CC128 and pcr28, a YAC contig was constructed in order to locate *FWA* within a YAC. For this purpose nine YAC's were selected from the published YAC contig of chromosome 4 (Schmidt *et al.*, 1995). The relative positions of these YAC's were refined by hybridisation with all molecular markers in this region that were used for the mapping. The relative position of a YAC was deduced, according to whether a marker hybridised completely, partially or not at all with the YAC. The resulting YAC contig is shown in Figure 3.3.

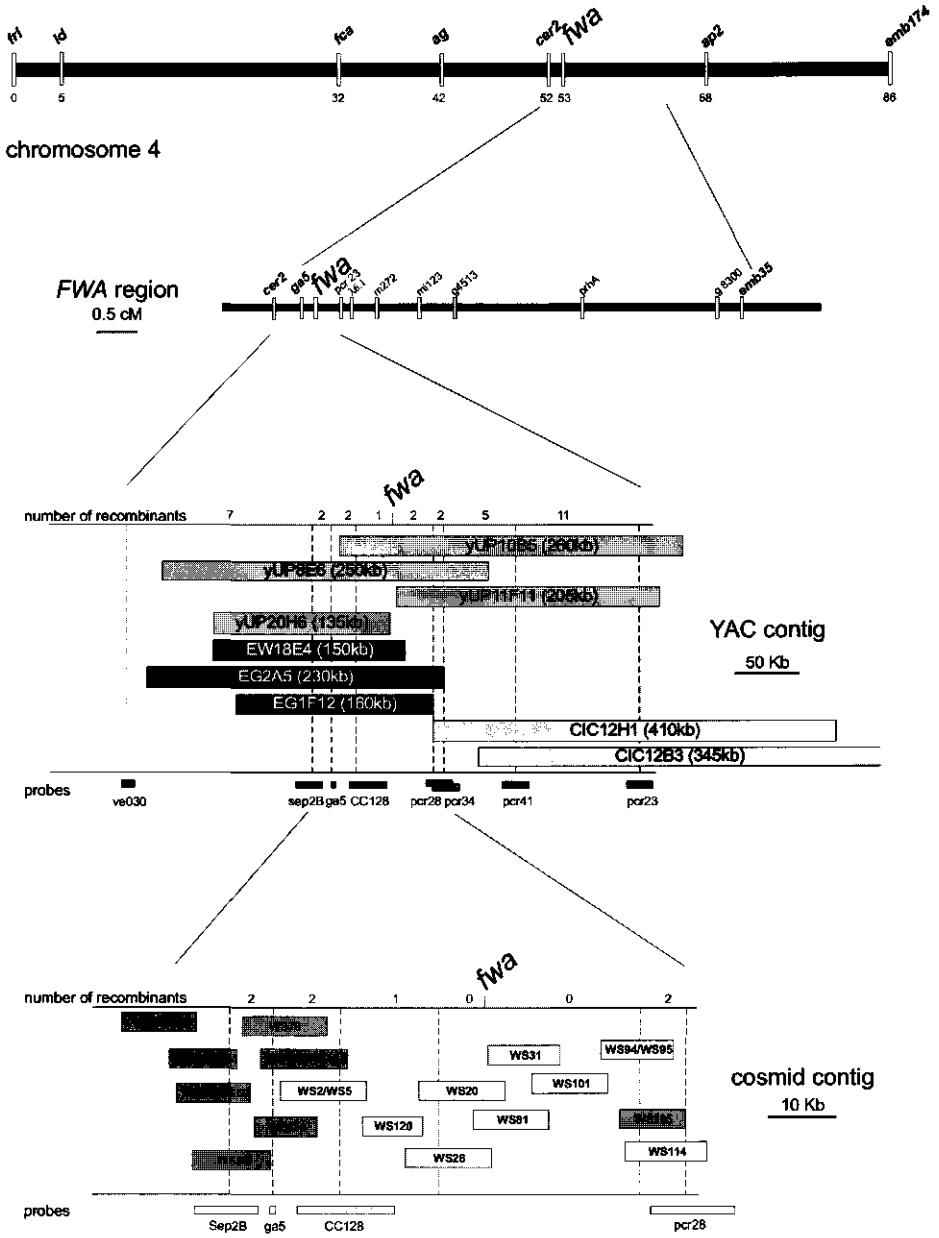

Figure 3.3. Position of the *fwa* locus on chromosome 4. The upper part of the figure shows the whole chromosome with several morphological markers. Below this the *fwa* region is shown with morphological and molecular markers that were used for the mapping of the *fwa* locus. The middle part of the figure shows the YAC contig from a small part of this region, together with the probes that were used to construct this contig. The number of recombinants between every marker and *FWA* is indicated. The cosmid contig that was generated after screening of the *fwa-1* cosmid library with YAC EG1F12 is shown in the bottom of the figure. Cosmids in white were used for plant transformation experiments.

A comparison of this YAC contig with the contig from Schmidt *et al.* (1995) did not show any significant differences in the positions of YAC's. However, the use of more markers for the construction of the YAC contig for *FWA* improved its accuracy. For instance, EG2A5 and CIC12H1 are not overlapping in Schmidt's contig but they are in the *FWA* contig, because in the first contig no probe, located in the overlapping part, was used.

Since the molecular sizes of the YAC's could be estimated, it was possible to compare genetic and physical distances in the *FWA* region. The genetic distance of 0.7 cM between ga5 and pcr23 corresponds to a physical distance between 200 and 250 Kb. This means that the ratio of physical to genetic distance in this region of chromosome 4 is about 300 Kb/cM. The average ratio for this chromosome is 175 Kb/cM, varying from 30 Kb/cM to more than 550 Kb/cM (Schmidt *et al.*, 1995). Therefore the ratio in the *FWA* region is higher than average, which is not favourable for map-based cloning.

The cosmid contig

The *fwa*-mutant is semi-dominant and probably a gain of function mutation (Chapter 4). This raises the possibility that complementation of a mutant plant with the wild-type gene might not be possible. Therefore the complementation experiment should be done by transforming a wild-type plant with the *fwa* mutation. In this case a complementing transformant should confer later flowering to wild-type plants.

To achieve this, a genomic library was made from *fwa-1* mutant DNA. This library was constructed in a cosmid binary vector because of the relatively large insert size and the advantage of being able to use the clones directly for plant transformation. The resulting cosmid library consisted of 27.264 clones with an average insert size of 16 Kb. Therefore, in theory, the library should contain four genome equivalents. However, due to cosmids without a good insert, the library probably contains between two and four genome equivalents.

YAC EG1F12 was selected from the YAC contig to screen the cosmid library (Figure 3.3). This YAC completely covered the genomic region that contains *FWA* because it contains both markers, CC128 and pcr28, that flank the *FWA* locus. Hybridisation of this YAC with the library yielded 22 positive cosmids. Five pairs of these clones were identical, which means that the screen yielded in total 17 different cosmids. After hybridisation of these cosmids with each other and with several YAC's and molecular markers in this region, they could be arranged into a contig (Figure 3.3). The overlaps between the different cosmids were at least

five Kb, apart from the overlap between cosmids WS2/WS5 and WS120, which was only a few Kb. Ten of the cosmids covered the region between the markers CC128 and pcr28.

Some cosmids were used to find RFLP's that cosegregated with *FWA*. Indeed such RFLP's were found for both WS20 and WS94. There were no recombinants left between these polymorphisms and *FWA*. However, it was not possible to further genetically limit the region where *FWA* is located. The recombination events between *FWA* and pcr28, detected in two recombinants, occurred between the left end of WS94 and the right end of pcr28. The recombination between *FWA* and CC128 occurred between the left end of CC128 and the right end of WS20.

Using the molecular sizes of the cosmids, the region in which *FWA* is located was estimated to be 60 Kb.

Plant transformation and complementation

Nine cosmids were selected for the plant transformation experiment (indicated in white in Figure 3.3). These cosmids span the complete region where *FWA* is located, ranging from the left end of CC128 to the right end of pcr28. All these cosmids were introduced into wild-type L*er* plants. The number of T1 transformants from every cosmid that was checked for flowering time and the flowering time behaviour of these transformants are shown in Table 3.2.

Table 3.2. Numbers of obtained T1 Transformants.

Genotype of transformed plant	Cosmid	Total number of T1 transformants	Number of late flowering transformants	Number of wild-type flowering transformants
L*er* wild-type	WS2	47	0	47
L*er* wild-type	WS120	48	0	48
L*er* wild-type	WS28	48	14	34
L*er* wild-type	WS20	47	5	42
L*er* wild-type	WS31	40	0	40
L*er* wild-type	WS81	22	0	22
L*er* wild-type	WS101	41	0	41
L*er* wild-type	WS94	34	0	34
L*er* wild-type	WS114	16	0	16
fwa-1	WS28	44	27	17
fwa-1	WS20	23	19	4
fwa-1	WS31	48	48	0

Several T1 transformants of WS20 and WS28 were clearly flowering later than the wild-type. However, most of the transformants with these two *fwa* cosmids did not show complementation because they flowered as early as the wild-type. For both cosmids it was shown by PCR analysis that all the late flowering T1 transformants contained the insert but several of the early flowering transformants did not.

Complementation of the phenotype by transformation is never achieved in 100% of the T1 transformants because several causes might impede the right expression of inserted genes, leading to gene silencing. These causes can range from transgene copy number and arrangement to nuclear architecture and chromosomal location (Gallie, 1998). However, in the case of *fwa*, the frequency of complementing transformants was rather low compared to literature data for other flowering genes. For instance, the two cosmids that complemented the flowering time mutant *constans* showed complementation in respectively 6 out of 9 and 12 of 13 transformants (Putterill *et al.*, 1995). This suggest that there might be specific reasons for *fwa*'s low complementation rate.

Cosmids WS20, WS28 and WS31 were not only transformed into L*er* wild-type plants but also into late flowering *fwa-1* mutant plants. Transformation with WS20 and WS28 yielded several wild-type flowering T1 plants (Table 3.2). This means that these two cosmids are not only able to confer late flowering to wild-type plants but also to suppress the late flowering phenotype of *fwa-1* mutant plants. This dual behaviour of WS 20 and WS28 will be further discussed in Chapter 4.

The FWA gene

The above mentioned complementation analysis indicated that the *FWA* locus must be located in the overlap of cosmids WS20 and WS28. The DNA sequence and predicted genes of this region could be obtained from the Arabidopis Genome Initiative (AGI) in the Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/agi.html). This revealed the presence of only one complete predicted gene in the overlap of the two cosmids, encoding a homeodomain containing transcription factor, which will be further described in Chapter 4.

Materials and methods

Plant material

Seeds of the Col accession and Col containing the *emb35* mutation were obtained from David Meinke (Oklahoma State University, Stillwater, OK, USA). The L*er* marker line, containing the mutations *cer2-2*, *ga5-1*, *fwa-1* and *ap2-1* was generated before in our laboratory. These morphological markers are listed in Table 3.3.

Seeds were sown in plastic Petri dishes on a filter paper soaked with water and incubated in a cold room (4°C) for three days. After this they were transferred to a climate room (25°C, 16 hours light per day) and incubated for two days. Germinated seeds were planted on potting compost in individual clay pots and grown in a greenhouse with long day light conditions (at least 14 hours daylight).

Table 3.3. Morphological markers, used for the genetic mapping of the *FWA* locus.

Symbol	Locus name	Phenotype	Reference
ap2-1	apetala-2	homeotic transformation of sepals to leaves and petals to stamens or stamenoid petals	Jofuku *et al.*, 1994
cer2-2	eceriferum-2	very bright green stems and siliques due to altered wax composition	Negruk *et al.*, 1996
emb35	embryo defective-35	embryo lethal	Franzmann *et al.*, 1995
fwa-1	late flowering	late flowering	Koornneef *et al.*, 1991
ga5-1	gibberellin deficient-5	dwarf	Koornneef *et al.*, 1980

Genetic analysis

To estimate the recombination fraction in the mapping population the RECF2 program, which produces maximum likelihood estimates and standard errors, was used (Koornneef and Stam, 1992). For the construction of the linkage map the JOINMAP program (Stam, 1993; Stam and van Ooijen, 1995) was used, applying the Kosambi function (Kosambi, 1944) to convert recombination fractions into genetic distances.

DNA isolation

DNA was isolated from plants grown in the greenhouse, following basically the protocol of Bernatzky and Tanksley (1986). Approximately 4 g of fresh leaf material was ground in a mortar filled with liquid nitrogen. The powder was transferred to a tube containing 20 ml extraction buffer (0.1 M Tris pH7.5, 0.35 M Sorbitol, 5 mM EDTA). After centrifuging at 4000rpm for 30 min, the supernatant was discarded and 1.25 ml extraction buffer, 1.75 ml nuclei lysis buffer (0.2 M Tris pH7.5, 50 mM EDTA, 2M NaCl, 2% CTAB) and 300 µl 10% sarkosyl were added, mixed with the pellet and incubated at 65°C for 30 min. Then 7.5 ml chloroform/isoamylalcohol (24:1) was added and the tube was rotated for 15 min at room temperature (RT). After centrifuging at 4000 rpm for 30 min, 1 volume of isopropanol was added to the upperphase to precipitate the DNA. The tube was centrifuged again for 30 min; the pellet was dried and dissolved in 400 µl sterile milli-Q water (mQ). RNAase A was added to an end concentration of 10 µg/ml and the tube was incubated at 37°C for 30 min. The solution was extracted twice with respectively phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol. Thereafter the DNA was precipitated with 0.1 volume of 3 M NaAc (pH 5.2) and 2.5 volumes of 96% ethanol, washed with 70% ethanol and dissolved in an appropriate volume of sterile mQ. DNA concentrations were measured with a TKO 100 fluorimeter (Hoefer Scientific Instruments, San Francisco, CA, USA).

Plasmid and cosmid DNA was isolated, following the "small-scale preparations of plasmid DNA" protocol of Sambrook *et al.* (1989). When the DNA was used as a probe it was purified with Qiagen-tip 20 columns (Qiagen, Chatsworth, CA, USA), following the manufacturers instructions.

Phage DNA was isolated, following the "rapid analysis of bacteriophage λ isolates, plate lysate method" protocol of Sambrook *et al.* (1989).

Total genomic YAC DNA was isolated from a 5 ml culture of yeast, which was grown in YPD medium (10 g yeast extract, 20 g peptone and 20 g dextrose per liter) at 30°C. After centrifuging the culture at 4K for 5 min, the pellet was washed in 5 ml of 50 mM EDTA, then washed in 20 mM EDTA, 1 M sorbitol; after this it was resuspended in 150 µl of 20mM EDTA, 1M sorbitol. Thereafter, 35 µl lyticase (5U/µl) and 11.5 µl β-mercaptoethanol was added and the solution was incubated for 2 hours at 37°C. After centrifuging at 1200 g for 5 min, the pellet was dissolved in 0.5 ml of 0.1 M EDTA, 0.15 M NaCl, then 25 µl of 20% SDS was added and the solution was incubated at 65°C for 20 min. Next, 200 µl of 5 M KAc was added and the tube was left on ice for 30 min after which it was centrifuged for 3 min. The supernatant was poured in a 1.5 ml Eppendorf tube that was filled with 96% ethanol and then centrifuged for 10 min at RT. The pellet was resuspended in 250 µl of mQ, after which an equal volume of 4.4 M LiCl was added and the tube was left on ice for 30 min. After centrifuging for 5 min the supernatant was taken and the DNA was precipitated with 96% ethanol and washed twice with 70% ethanol. Finally the DNA was dissolved in 50 µl mQ.

Complete YAC's were isolated from 100 ml cultures of yeast. Cells were pelleted and washed as described above. After washing, the pellet was warmed to 38°C and 14 µl lyticase (5U/µl), 4.6 µl β-mercaptoethanol and 180 µl low melting agarose was added (amounts should be adapted, according to the volume of the pellet). After mixing quickly, the solution was transferred to a mould to cast plugs. The plugs were transferred to a small volume of LET (0.5 M EDTA, 10 mM Tris pH8.0) with 7.5 µl β-mercaptoethanol and 0.1 mg/ml RNAaseA and incubated overnight at 37°C. Hereafter, they were washed three times in NDS buffer (0.5 M EDTA, 10 mM Tris pH8.0, 1% sodium N-Lauroylsarcosine) for 15 min. Then they were transferred to NDS with 2 mg/ml proteinase K and incubated overnight at 50°C. Finally, they were washed in 50 mM EDTA pH8.0 for 15 min, left overnight in fresh 50 mM EDTA and washed again. The plugs were stored at 4°C in 50 mM EDTA pH 8.0. To separate complete YAC's, the plugs were cast in a 1% agarose gel, which was run by pulsed field gel electrophoresis in a CHEF-DR™II (Bio-Rad, Hercules, CA, USA) apparatus.

Preparation of probes

The molecular probes that were used for the fine-mapping of *FWA* are listed in Table 3.4. The insert of a cosmid or plasmid was released by digestion with the appropriate restriction enzymes. The resulting fragments were separated by gel electrophoresis and the fragment(s) corresponding to the insert were cut out of the gel. YAC's were released as described above.

The DNA was liberated from these agarose blocks by electro-elution. An electro-elution device (Harvard Bio Labs Machineshop, Cambridge, MA, USA) was filled with elution buffer (10mM Tris pH 7.5, 5mM NaCl and 1mM EDTA) and 70µl of 20% NaAc was added to the salt bridge. Two µl of loading buffer was added to the agarose blocks and these were put in the reservoir. Electrophoresis lasted 45 minutes at 80V after which the DNA was pipetted out of the salt trap (two times 175µl). The DNA was first extracted with phenol/chloroform/IAA (25:24:1), then with chloroform/IAA (24:1) and finally precipitated with 2.5 volumes of absolute ethanol overnight at –20°C. The precipitate was washed with 70% ethanol and dissolved in mQ water.

Southern blotting and hybridisation

Three µg of genomic plant DNA was cut with the appropriate restriction enzymes and the DNA fragments were separated by agarose gel electrophoresis. Thereafter, they were transferred to a Hybond-N nylon membrane (Amersham Pharmacia, Uppsala, Sweden) by vacuum blotting, following the procedures recommended by the manufacturer (Pharmacia LKB-VacuGeneXL, Amersham Pharmacia, Uppsala, Sweden). The time periods for depurination, denaturation, neutralisation and transfer were respectively 10 min, 10 min, 10 min and 2 hours. After blotting, the blot was soaked in 2

Table 3.4. DNA probes, used to detect molecular polymorphisms between the accessions L*er* and Col or between wild-type (*FWA*) and mutant (*fwa*) DNA.

Name	Description	Enzymes that give RFLP's	Obtained from
ve030	plasmid (cDNA)	*Cfol* (Ler-Col)	D. Bouchez[a]
sep2B	phage	*MspI, HpaII* (Ler-Col)	C. Dean[b]
ga5	plasmid	*DraI* (Ler-Col)	J. Zeevaart[c]
(pA17-4S2)	(contains both introns of GA5)		
CC128	cosmid	*MspI* (Ler-Col)	C. Dean[b]
cos20	cosmid	*HhaI* (*FWA-fwa*)	cosmid contig
cos94	cosmid	*HhaI* (Ler-Col)	cosmid contig
pcr28	plasmid (left end of YAC EW3H7)	*HpaII* (Ler-Col)	J. Leung/J. Giraudat[d]
pcr34	plasmid (right end of YAC EG2A5)	*HhaI, ClaI* (*FWA-fwa*)	J. Leung/J. Giraudat[d]
pcr41	plasmid (right end of YAC EW18C4)	*EcoRI, HhaI, HpaII* (Ler-Col)	J. Leung/J. Giraudat[d]
pcr23	plasmid (right end of YAC EW3H7)	*HpaII* (*FWA-fwa*)	J. Leung/J. Giraudat[d]
Lambda 6.1	phage	*EcoRI* (Ler-Col)	J. Leung/J. Giraudat[d]
m272	phage	*ClaI, PstI* (Ler-Col)	ABRC[e]
mi123	plasmid	*EcoRI* (Ler-Col)	C. Dean[b]
g4513	cosmid	*HindIII, ClaI* (Ler-Col)	ABRC[e]
prhA	CAPS marker	*DdeI* (Ler-Col)	TAIR[f]
g8300	cosmid	*EcoRI* (Ler-Col)	ABRC[e]

[a] INRA, Versailles, France
[b] JIC, Norwich, UK
[c] Michigan State University, East Lansing, MI, USA
[d] CNRS, Gif-sur-Yvette, France
[e] ABRC = Arabidopsis Biological Resource Center, Ohio, USA
[f] TAIR = The Arabidopsis Information Resource (www.arabidopsis.org)

x SSC for 1 min, UV irradiated in an ultraviolet crosslinker (Ultra Lum, Paramount, CA, USA) with 120,000 μJ/cm^2 and baked at 80°C for 2 hours.

Hybridisations were performed in a Hybaid oven (Hybaid, Teddington, UK). A blot was prehybridised with 10 ml of hybridisation solution (5 x SSC, 5 x Denhardt's solution and 0.5% SDS) for 4 hours at 65°C. [^{32}P] Random prime labelled DNA fragments were used as probe for hybridisation overnight. Blots were washed at 65°C in 0.1% SDS and respectively 5 x SSC, 3 x SSC and 1 x SSC (every wash step took half an hour). The activity of a blot was visualised with a phosphor imager.

Cosmid library

The T-DNA cosmid vector 04541 was used to prepare the genomic library. This vector was derived from SLJ1711 (Jones *et al.*, 1992) by the insertion of a fragment containing a cos site between the *BglII* sites. SLJ1711 was derived from pRK290 (Ditta *et al.*, 1980). The vector contains the kanamycin resistance gene (NPTII), a cos site and a polylinker, with blue/white selection, between T-DNA borders. Furthermore, it carries a SURE™ bacterial tetracycline resistance gene.

To prepare the library, genomic DNA of the *fwa*-1 mutant was partially digested with the restriction enzyme *Sau3AI*, treated with calf intestinal phosphatase and size fractionated over a sucrose gradient to obtain fragments in between 15 and 25 Kb. These fragments were ligated into the *BamHI* site of the cosmid vector. After that the DNA was packaged with Gigapack II packaging extract (Stratagene, La Jolla, CA, USA), mixed with SURE™ cells (Stratagene, La Jolla, CA, USA) and plated out on LB (10 g peptone, 5 g yeast extract and 5 g NaCl per liter) plates with tetracycline (10µg/ml), 0.004% Xgal and 0.2 mM IPTG for blue/white selection. Single white colonies were picked and put into wells of high density (384 wells) microtiter plates (Genetix, Dorset, UK) that were filled with freezing medium (LB, containing 36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM Na citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$ and 4.4% glycerol). In total, 71 high density plates were filled and stored at –80°C. To prepare library filters, cells were transferred from the microtiter plates to agar plates with a replicator and grown overnight. Hybond-N filters (Amersham Pharmacia, Uppsala, Sweden) were placed on the plates with colonies for 1 minute, denatured and neutralised in trays containing these solutions and baked at 80°C for 2 hours. Hybridisation of the filters was similar as mentioned above (southerns, blotting and hybridisation), but the filters were hybridised in trays instead of bottles.

Electroporation of Agrobacterium tumefaciens

Cosmids that were selected for plant transformation were transferred from *Escherichia coli* cells (SURE™) to *Agrobacterium tumefaciens* (AGLO strain; Lazo *et al.*, 1991) by electroporation. To prepare competent cells a 50 ml liquid culture of LB with selective antibiotics was inoculated with *A. tumefaciens* and grown overnight at 28°C. The next day a 500 ml liquid culture (LB without salts) was inoculated with 25 ml of the overnight culture. Cells were harvested at an OD600 of 0.6 by centrifugation (5K, 5min, 4°C) and gently resuspended in 250 ml of ice-cold mQ water. Thereafter, cells were centrifuged again and resuspended in 100 ml of ice-cold mQ water. Finally, the cells were resuspended in 10 ml of ice-cold 15% glycerol in mQ water, aliquoted in 100 µl portions and stored at –80°C.

For electroporation, an aliquot was thawed and 1 to 5 µl of cosmid DNA was added. The mixture was transferred to a cuvet, which was placed in the cuvetchamber of an electroporator set at 2.2 kV (*E. coli* pulser from Bio-Rad, Hercules, CA, USA), after which a pulse was given. Immediately after the pulse one ml of SOC (2% bactotrypton, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. The SOC medium with cells was transferred to a sterile tube and incubated for 1 to 2 hours (225 rpm, 28°C). Subsequently, the cells were plated on LB plates with selective antibiotics and grown for two days at 28°C.

Transformation of Arabidopsis plants

For transformation of Arabidopsis the protocol of Bechtold *et al.* (1993) was adapted. *A. tumefaciens* cells of the strain AGLO (Lazo *et al.*, 1991), with the appropriate cosmid, were grown in 15 ml liquid culture (LB with 50 µg/ml kanamycin and 50 µg/ml rifampicin) at 28°C during 48 hours. One day before transformation, 4 flasks with 0.5 liter of liquid medium were inoculated with 0.5 ml of the 15 ml culture and grown overnight. The cells were harvested at an OD600 of 0.8 by centrifugation (5K, 15min, RT) after which the pellet was gently resuspended in 0.5 liter of infiltration medium, pH 5.8 (0.5 X Murashige & Skoog salts, 5% sucrose, 0.05% MES, 0.02% Silwet L-77 (Lehle seeds, Round Rock, TX, USA). The infiltration medium with *A. tumefaciens* was put in two jars on top of which the pots with Arabidopsis were placed upside down with the flowering shoots completely submerged in the medium. Thereafter, the jars with pots were placed in vacuum for five minutes. Finally, the pots with Arabidopsis were transferred to the greenhouse.

The seeds that were harvested from these plants were sterilised for 15 minutes with 20% bleach in absolute ethanol solution, after which they were rinsed two times in absolute ethanol and dried overnight in a flow cabinet. Seeds were sown on plates with selective medium (1 X Murashige & Skoog salts, 1% sucrose, 40µg/ml kanamycin, 0.8% agar, pH 5.8). The plates were kept in the cold room (4°C) for 4 days and then transferred to the growth room (16 hours light, 25°C). After 10 days, transformed seedlings were visible as green plants with several green leaves and a root, whereas untransformed seedlings were yellow and did not develop any true leaves.

PCR analysis

DNA was isolated from a few leaves of a T1 transformant and amplified through 35 cycles (10 sec 94°C, 30 sec 54°C and 2 min 72°C) in standard PCR conditions. Presence of the cos20 insert in the plant was confirmed by appearance of a 1.1 Kb band after amplification with the T3 primer, (5'-AATTAACCCTCACTAAAGGG-3') and the primer HD8F (5'-GCTTCGGAACTAAGGAACCC AAGC-3'). For cos28 a 0.8 band was amplified, using the T3 primer and the primer HD1F (5'-GAGTCTTGCTTTATGCCAAGCCGC-3').

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Chapter 4

The late flowering phenotype of *fwa* mutants is caused by gain of function epigenetic alleles of a homeodomain gene

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Summary

The transition to flowering in *Arabidopsis thaliana* is delayed in *fwa* mutant plants. *FWA* was identified by loss of function mutations in normally-flowering revertants of the *fwa* mutant, and encodes a homeodomain containing transcription factor. The DNA sequence of wild-type and *fwa* mutant alleles was identical in the genomic region of *FWA*. Furthermore, the *FWA* gene is ectopically expressed in *fwa* mutants and silenced in mature wild-type plants. This silencing is associated with extensive methylation of two direct repeats in the 5' region of the gene. The late flowering phenotype, ectopic *FWA* expression, and hypomethylation of the repeats were also induced in the *ddm1* hypomethylated background. Mechanisms for establishment and maintenance of the epigenetic mark on *FWA* are discussed.

This chapter will be published in *Molecular Cell* with minor modifications

Introduction

Induction of flowering at the appropriate moment is essential for many plant species to reproduce successfully. The fine-tuning of the transition from the vegetative to the reproductive phase is believed to be under control of multiple factors. These are both endogenous, such as gibberellins and carbohydrate metabolites, and environmental, like daylength, temperature and light quality. To understand this process, a genetic approach is underway in Arabidopsis in which a multitude of mutants influencing the timing of flowering are being studied. The combination of physiological, genetic and molecular approaches using these mutants has led to a model of floral induction that consists of a photoperiodic promotion pathway, a vernalisation promotion pathway and an autonomous promotion pathway (Koornneef *et al.*, 1998b; Levy and Dean, 1998; Simpson *et al.*, 1999). The cloning and molecular characterisation of several of the involved genes are allowing a molecular interpretation of these pathways. However, the available information is fragmented and many aspects of this developmental process remain poorly understood.

One of the factors thought to play a role in the regulation of gene expression affecting flowering transition is DNA methylation (Finnegan *et al.*, 2000). The actual significance of DNA methylation for gene regulation in plant development remains unknown. An overall reduction in total genomic cytosine methylation of up to 70% has been found in transgenic plants with reduced amounts of DNA methyltransferase (Finnegan *et al.*, 1996; Ronemus *et al.*, 1996) and in *d*ecrease in *D*NA *m*ethylation (*ddm1*) mutant plants that are defective in a protein that is likely to be involved in chromatin remodelling (Jeddeloh *et al.*, 1999). Such plants develop a number of phenotypic abnormalities (Vongs *et al.*, 1993; Finnegan *et al.*, 1996; Kakutani *et al.*, 1996; Ronemus *et al.*, 1996). Furthermore, it has been observed that stable enhancement of the methylation level in specific genes can suppress expression of these genes, leading to mutant phenotypes (Jacobsen and Meyerowitz, 1997; Cubas *et al.*, 1999; Jacobsen *et al.*, 2000).

In relation to flowering, experimental arguments supporting a role for DNA methylation are largely correlative (Finnegan *et al.*, 1998b). For instance, Arabidopsis plants that are exposed to low temperatures during a prolonged period (vernalisation), and plants that are treated with the DNA demethylating agent 5-azacytidine show reduced levels of 5-methylcytosine and early flowering as compared to untreated plants (Burn *et al.*, 1993a). Thus, it has been hypothesised that vernalisation promotes flowering through demethylation of the genome. Recently, it has been shown that the expression level of the flowering

repressing gene *FLC* is downregulated by a decrease in genomic DNA methylation suggesting that either *FLC* or a regulator of *FLC* is directly controlled by the DNA methylation status (Sheldon *et al.*, 1999, 2000). Apart from early flowering plants, late flowering plants were derived from the hypomethylated backgrounds of antisense DNA methyltransferase (as-*MET1*) (Ronemus *et al.*, 1996) and *ddm1* (Kakutani *et al.*, 1996). Therefore, contrasting phenotypes have been caused by altering methylation, suggesting that multiple genes with opposite effects might be involved in the epigenetic regulation of flowering. Nevertheless, to prove and understand the involvement of such mechanisms awaits the identification of target genes that are affected directly by methylation.

The late flowering trait induced by *ddm1* hypomethylation background were genetically mapped to the chromosomal region containing *FWA* (Kakutani, 1997), a well-characterised flowering time gene. The *fwa* mutant is delayed in the transition to flowering and is semi-dominant, unlike most flowering time mutants (Koornneef *et al.*, 1991). Based on double mutant genetic and physiological analyses, *FWA* is presumed to affect flowering through the speculated photoperiodic promotion pathway in the current model for the control of flowering initiation (Koornneef *et al.*, 1998b). In addition to its function in the transition from the vegetative to the reproductive meristem, several observations indicate that *FWA*, together with the recently cloned flowering promoting gene *FT*, also plays a role in the control of flower meristem identity (Ruiz-Garcia *et al.*, 1997; Nilsson *et al* 1998; Roldán *et al.*, 1999; Onouchi *et al.*, 2000). It has been suggested that *FWA* and *FT* affect meristem identity in a pathway that operates parallel to that of the well-characterised *LFY* gene (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999).

In this work, we describe the molecular identification of the *FWA* gene and show that the late flowering phenotype of *fwa* mutants is caused by gain of function epi-alleles that lack methylation in two repeated sequences located in the 5' region of *FWA*.

Results

Characterisation of FWA mutants

Two different *fwa* mutant alleles have previously been described (Koornneef *et al.*, 1991); *fwa-1* was induced by ethyl methanesulphonate (EMS) and *fwa-2* by fast neutrons. Plants carrying these *fwa* mutations flower later than Landsberg *erecta* (L*er*) wild-type plants (Figure 4.1). This delay in flowering is relatively stronger under long day (LD) light

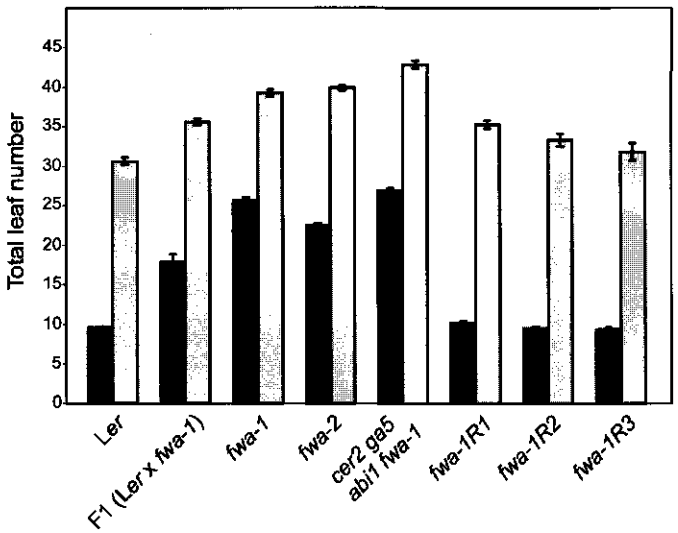

Figure 4.1. Flowering time of the different *FWA* alleles.

Mean flowering time (measured as the total number of leaves produced by the plant before flowering) of 10-15 plants grown under LD conditions (■) or under SD conditions (▒) is shown. The standard error of the mean is indicated on each bar.

conditions than under short day (SD) light conditions. Plants heterozygous for the *fwa* mutation flower intermediate between wild-type and the homozygous *fwa* mutant plants, indicating that *fwa* alleles are semi-dominant (Figure 4.1).

To determine if the dominance of *fwa* mutations is due to gain of function of these alleles, we attempted to obtain intragenic suppressor mutations of *fwa* that show a wild-type-like phenotype. Seeds of the *fwa-1* marker line carrying the mutations *cer2 ga5 fwa-1 abi1* were γ irradiated and approximately 5000 M2 plants were screened under LD conditions for altered flowering time. Five early flowering plants were obtained and crossed with the L*er* wild-type to try to separate the new mutation causing the early flowering from the mutations of the marker line. Two of the early mutants produced F_1 hybrids that flowered late, indicating that the *fwa-1* mutation was present together with a recessive extragenic *fwa* suppressor mutation that produced early flowering. Both mutations were allelic and have been further characterised (Soppe *et al.*, 1999; Chapter 2). The three other revertant plants were named *fwa-1R1*, *fwa-1R2* and *fwa-1R3* and gave rise to F_1 hybrids that flowered early. In addition, no late flowering plants were observed in F2 progenies of 356 plants and therefore these revertants are likely to carry intragenic suppressor mutations in the *fwa* mutant gene. Figure 4.1 shows the flowering time, under LD and SD conditions, of the revertants and the marker

line from which they were derived. These results strongly suggest that *fwa* mutants carry gain of function alleles of the *FWA* gene, while the second site mutations *fwa-1R1*, *fwa-1R2* and *fwa-1R3* result in loss of function alleles of *FWA*.

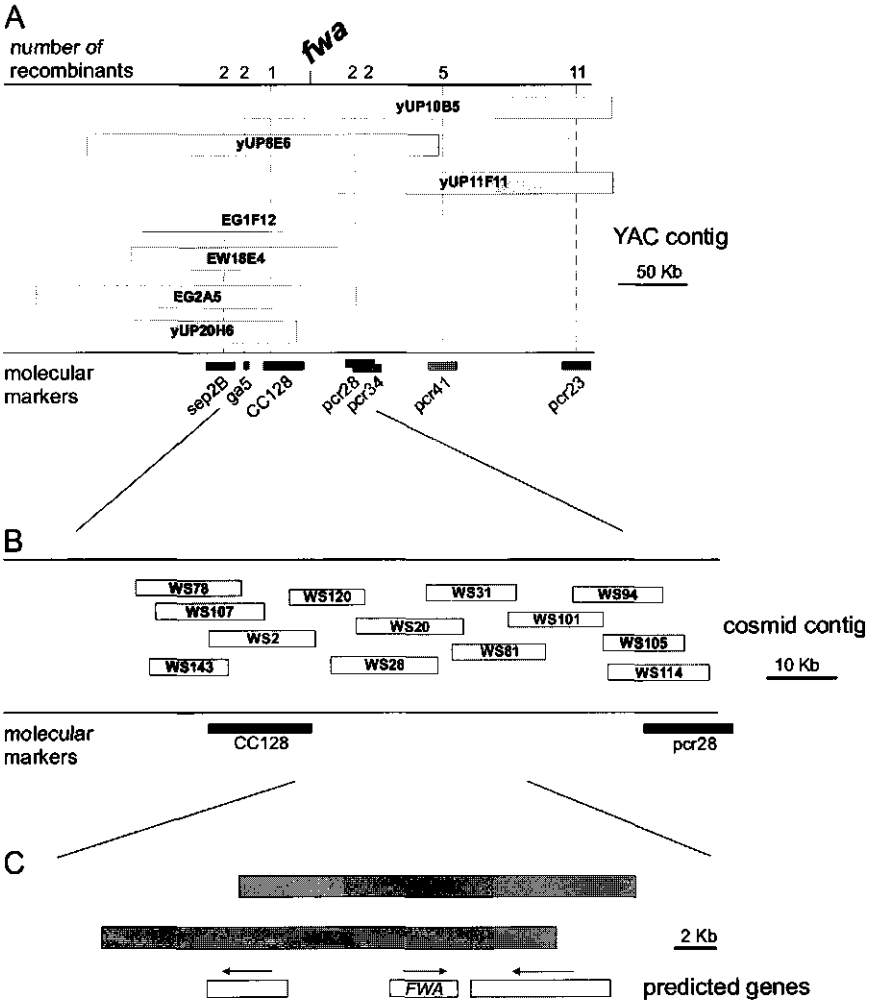

Figure 4.2. Molecular markers, YACs and cosmids in the *FWA* region.

A. The YAC contig and molecular markers used to locate the *FWA* locus. The number of recombinants between every molecular marker and *FWA* is indicated in the top.

B. The cosmid contig; white coloured cosmids were used for plant transformation experiments.

C. Predicted genes in the overlap of cosmids WS20 and WS28. Arrows above the genes show the direction of transcription.

Positional cloning of FWA

FWA is located on chromosome 4 between the two morphological markers *ga5* and *emb35*, which are 6.2 cM apart. From a mapping population of 1306 plants, 120 recombinants were identified between these two markers. Two of them had cross-overs between *GA5* and *FWA*, indicating that *FWA* maps only 0.1cM from *GA5*, while 118 had cross-overs between *FWA* and *EMB*35. The location of *FWA* was further refined with molecular markers located within this region. The markers CC128 and pcr28 flanked the locus and had respectively one and two cross-overs remaining (Figure 4.2A). Several YAC's were selected from the published YAC contig of chromosome 4 (Schmidt *et al.*, 1995) and their relative positions in relation to the molecular markers were further refined. Thus, *FWA* could be located in a region of about 60 Kb between markers CC128 and pcr28. A genomic library was made from *fwa-1* in a binary cosmid vector that was screened with the YAC clone EG1F12 containing both markers CC128 and pcr28. The positive cosmid clones from this screen were arranged into a contig (Figure 4.2B) and used as markers in RFLP analysis which indicated that clones WS20 and WS94 did not have any cross-overs left with the *FWA* locus.

Nine overlapping cosmids (see Figure 4.2B), spanning the region between CC128 and pcr28, were used to transform wild-type plants and between 16 and 48 transformed plants were generated per cosmid. Late flowering was only observed in transformants with the two overlapping cosmids WS20 and WS28. Respectively 5 out of 47 and 14 out of 48 plants transformed with WS20 and WS28 flowered significantly later than L*er*, indicating that *FWA* is on the overlap of these two cosmids. The DNA sequence of this region for the Columbia

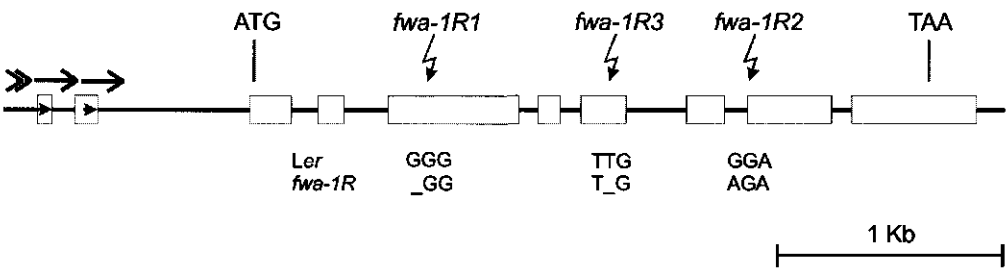

Figure 4.3. Schematic representation of the *FWA* gene.

Open boxes represent exons. The start codon (ATG), stop codon (TAA) and the position and nature of the mutations in the three revertants are indicated. The arrows above the 5' region mark the two direct repeated sequences, while arrows within the first two exons show the position of the direct repeat in the untranslated leader of the mRNA. The sequence of *FWA* is available through the GenBank database (genomic DNA: accession number AF178688, cDNA: accession number AF243535)

(Col) accession was obtained from the *Arabidopsis thaliana* database and showed that the overlap of these two cosmids contains only one complete predicted gene which encodes a homeodomain (HD) transcription factor (Figures 4.2C, 4.3 and 4.4).

A 5.3 Kb region corresponding to this HD gene was sequenced in the L*er* wild-type, the two *fwa* and three revertant alleles to look for mutations. The three revertant alleles all contained different mutations within the open reading frame of this gene; *fwa-1R1* and *fwa-1R3* both have a single base pair deletion causing a premature stop of translation and *fwa-1R2* has a single base pair change resulting in a glycine to arginine transition (Figure 4.3). However, the sequences of wild-type and both mutant alleles were identical, indicating that the cause of the *fwa* mutant phenotype cannot be due to mutations in the *FWA* gene itself.

Analysis of expression of this HD gene in *fwa* mutants showed that this is altered in both *fwa* mutant alleles as compared to L*er* wild-type plants. However, expression of other genes in the region did not show differences between *fwa* and wild-type plants (data not shown). Therefore, we conclude that the late flowering of *fwa* mutants is due to a direct regulation of this HD gene, which is considered to be the *FWA* gene.

To explain the upregulation of *FWA* in *fwa* mutants we further analysed the structure and expression of this gene. The complete cDNA of *FWA* was obtained by RACE-PCR from total RNA of the *fwa-1* mutant because we could not detect any cDNA for this gene in wild-type cDNA libraries, nor in EST databases. Comparison of the cDNA with the genomic sequence showed that *FWA* contains 10 exons (Figure 4.3). The predicted translation start is in the third exon, the first two exons being located 700 base pairs upstream of this start. The cDNA encodes a predicted protein of 686 amino acids. A database search with this putative FWA protein sequence revealed strong homology with proteins belonging to the subclass of plant HD-ZIP homeodomain proteins, named HD-GL2 (homeodomain Glabra2) (Rerie *et al.*, 1994; Lu *et al.*, 1996). The highest homology of FWA was found with ANTHOCYANINLESS2 (ANL2) (Kubo *et al.*, 1999). FWA showed all the characteristics of HD-GL2 transcription factors; the presence of a homeodomain in the N terminal part followed by a leucine zipper (Di Cristina *et al.*, 1996), and a StAR-related lipid-transfer (START) domain (Ponting and Aravind, 1999). As shown in Figure 4.4, comparison of the putative FWA protein with ANL2 and two other members of the HD-GL2 class revealed amino acid conservation throughout the whole protein. Homology was especially strong in the regions of the homeodomain and the START domain but weaker at the amino terminus.

An interesting feature of the *FWA* genomic sequence was the presence of two direct repeats in the 5' region, one of 38 base pairs with 100% homology and one of 198 base pairs

with 94% homology (Figure 4.3). The small repeat was located in the promoter region of *FWA* while the larger one covered the first two exons and part of the first two introns. Consequently, the cDNA contains a direct repeat of 56 base pairs (with 91% homology) in the 5' untranslated leader. The two repeats appear to be unique in the Arabidopsis genome because homologous sequences could not be found in the databases.

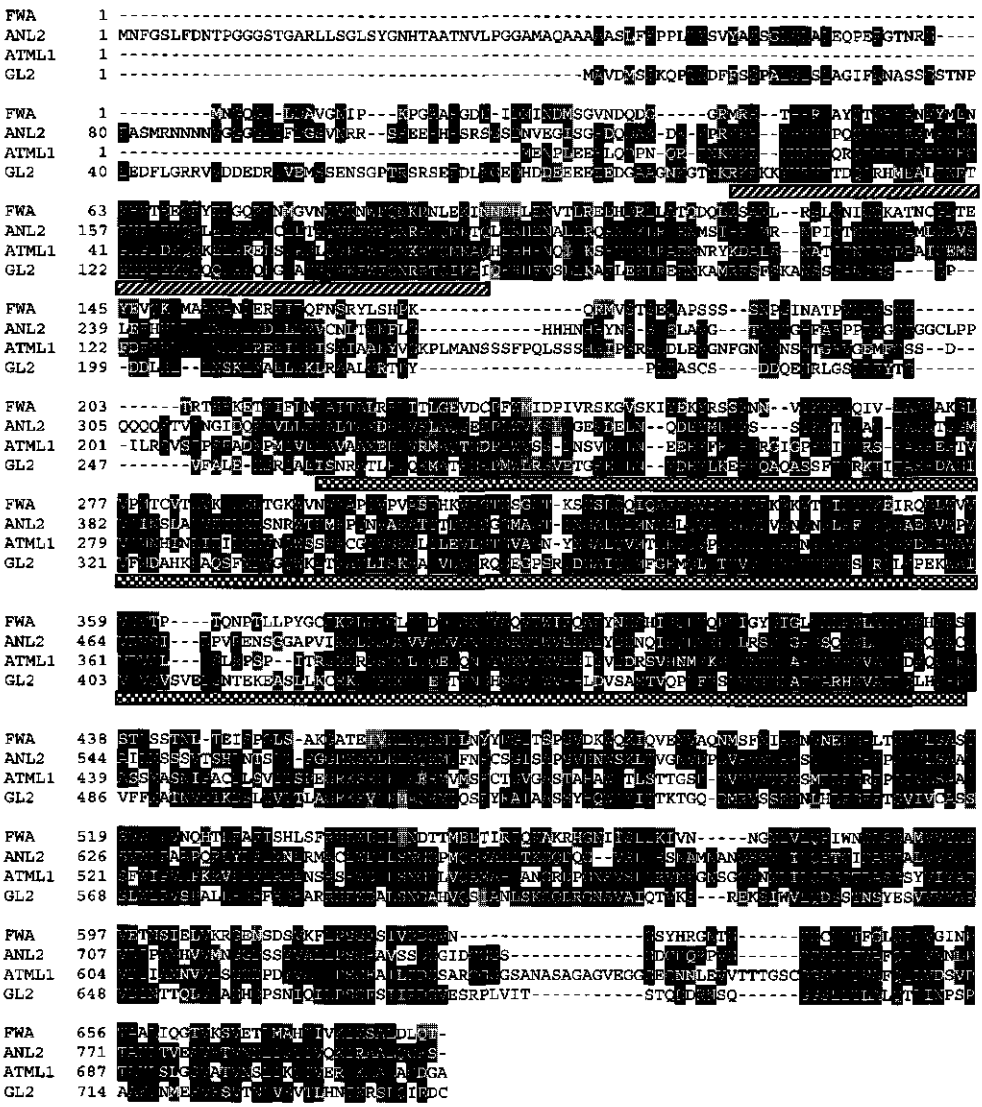

Figure 4.4. The FWA protein.

The deduced amino acid sequence of the FWA protein compared with ANL2 (GenBank accession number AF077335), ATML1 (U37589) and GL2 (L32873). Identical amino acids are shaded in black and conservative changes are shaded in grey. The homeodomain is underlined with a hatched box and the START domain with a blocked box.

Expression of the FWA gene

The expression of *FWA* was analysed in different *FWA* genetic backgrounds by Northern blot hybridisation. RNA was extracted from whole plants of various ages, which were grown under LD and SD light conditions. The two *fwa* mutant alleles showed a similar expression pattern of *FWA*. Transcripts were present through the full life cycle of the plant and in different plant organs, including flowers (Figure 4.5A). In contrast, no expression could be detected in wild-type and revertant alleles.

To detect whether the transcript might be present at a very low level in wild-type plants, RT-PCR was used. The transcript could not be detected in RNA isolated from whole plants at

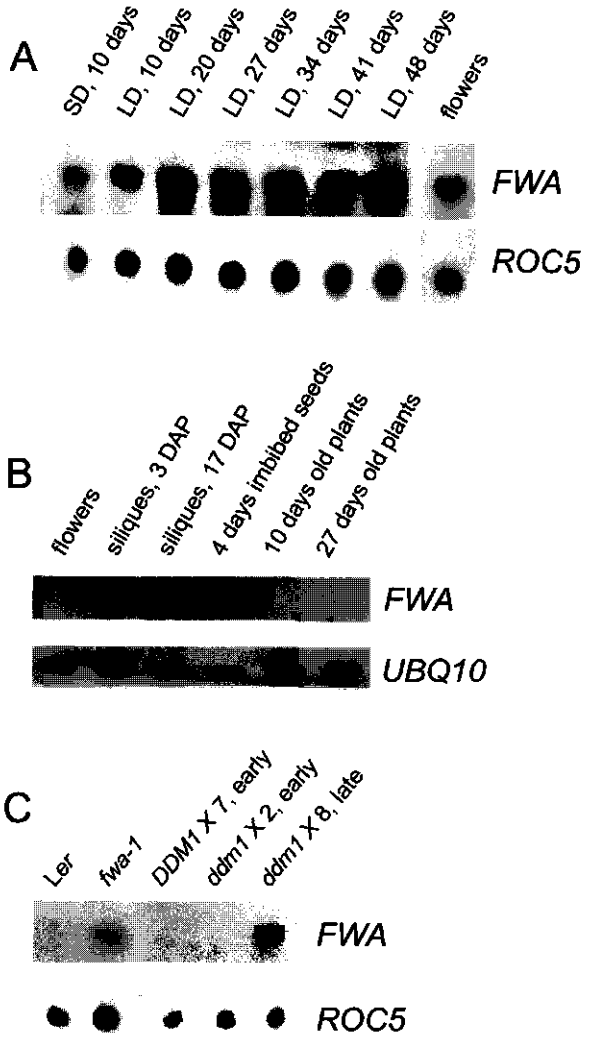

Figure 4.5. Expression of *FWA*.

A. Northern blot analysis of *FWA* expression in *fwa-1* plants. Plants were grown under SD or LD conditions and total RNA was extracted from flowers and complete plants, 10, 20, 27, 34, 41 and 48 days after planting. The blot was probed with a fragment of the *ROC5* gene as a loading control.

B. Analysis of *FWA* expression in different tissues or complete plants of wild-type L*er* by RT-PCR with *FWA* gene specific primers. A fragment of the *UBIQUITIN10* gene was amplified as a control.

C. Northern blot analysis of *FWA* expression in different early and late flowering *DDM1* lines that were self-fertilised for two, seven or eight generations. Plants were grown under LD conditions and total RNA was extracted three weeks after planting. The blot was probed with a fragment of the *ROC5* gene as a loading control.

vegetative or reproductive phases. However, it could be detected in siliques of different ages, from 3 days after pollination (DAP) until maturity of the seeds and in germinating seeds (Figure 4.5B).

FWA expression was analysed by northern blot hybridisation in three week old plants of the late flowering mutants *fca-1*, *fve-1*, *co-3*, *gi-1*, *ft-1* and of the floral meristem identity mutant *lfy-6*. The *FWA* transcript could not be detected in any of these mutants (data not shown). Furthermore, *FWA* RNA could not be detected in three week old plants of different early flowering (Col and Wassilewskija), middle late flowering (Fukuyama and Llagostera) and late flowering (Canary Islands and Saint Feliu) accessions (data not shown).

DNA hypomethylation in the fwa mutants

During the map based cloning of *FWA* several RFLP's between wild-type and *fwa* mutant DNA were detected with methylation sensitive restriction enzymes. Southern hybridisation of genomic DNA cleaved with the isoschizomeric enzymes *HpaII* and *MspI* was used to examine whether there was a difference in DNA methylation level between *fwa* mutants and wild-type. Hypomethylation in the *fwa* mutant was found with 14 probes that were located in a region of 5 Mb, surrounding the *FWA* locus (an example of this hypomethylation is shown in Figure 4.6). Five single copy probes located elsewhere in the genome did not show a

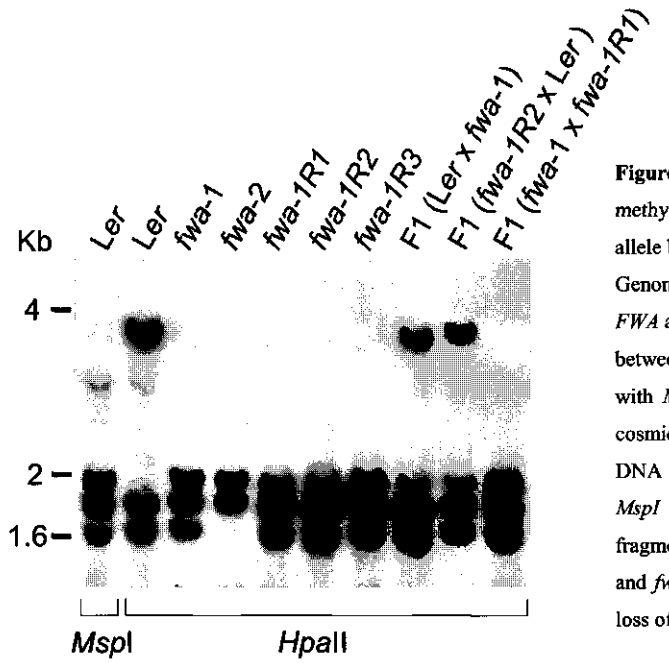

Figure 4.6. Southern analysis of methylation patterns in different *FWA* allele backgrounds.
Genomic DNA of plants with different *FWA* alleles and of F1 plants from crosses between different alleles was digested with *MspI* or *HpaII* and hybridised with cosmid WS31 as probe. In wild-type, a DNA fragment of about 4 Kb, is cut by *MspI* but not by *HpaII*. However, this fragment is cut by *HpaII* in *fwa* mutant and *fwa-1R* revertant alleles, indicating a loss of methylation.

difference in DNA methylation levels. Furthermore, *fwa-1* and *fwa-2* do not have an identical methylation pattern and the three revertants show the same methylation pattern as *fwa-1*, from which they derived. In addition, plants that are heterozygous for *fwa* show both the methylated and the unmethylated restriction sites, suggesting that the wild-type *FWA* allele is normally methylated and the mutant *fwa* allele is hypomethylated (Figure 4.6). The genomic DNA methylation status was also analysed in repeated sequence regions outside the *FWA* locus using probes for the 180bp centromere repeats (Martínez-Zapater *et al.*, 1986), rDNA (Ronemus *et al.*, 1996) and the retrotransposon Ta3 (Konieczny *et al.*, 1991; Kakutani *et al.*, 1999). In all cases the same methylation pattern was observed in *fwa* and wild-type DNA (data not shown). Therefore, the hypomethylation of *fwa* seems to be restricted to the region of the *FWA* locus. These observations prompted us to further investigate methylation as a possible cause for the upregulation of *FWA* expression in *fwa* mutants.

Inverted repeats and multiple-copy sequences have been shown to be more sensitive to methylation and gene silencing than single-copy sequences (Jacobsen, 1999). Therefore, we looked in detail at the cytosine methylation status of the repeated sequences located in the 5' region of the *FWA* gene (Figure 4.3). Using bisulfite sequencing (Jacobsen *et al.*, 2000) on DNA isolated from whole rosettes at the vegetative phase, we analysed a region of approximately 1.4 Kb containing the two direct repeats just upstream of the translation start site. In wild-type plants, methylation was restricted to the repeats only and found at all 20 CG sites. Analysis of 8 top strand and 10 bottom strand clones revealed that within the wild-type repeats, 89% of cytosines in symmetric CG sequences context are methylated (Figure 4.7). However, methylation is not restricted to these symmetric sites, cytosines in a non-symmetric context were also methylated 13% of the time (Figure 4.7). Furthermore, we found wide variation in cytosine methylation between individual clones. The pattern of non-CG methylation seems to be variable with little preference for sequence context. Analysis of five top and three bottom strand clones of *fwa-1* showed complete bisulfite conversion, indicating that no cytosine residues in this region were methylated in the mutant plants. The methylation of the repeats in the *fwa-2* mutant and three revertant alleles of *FWA* was also analysed and found to be completely absent, as in *fwa-1* (Table 4.1).

Methylation has been associated with repression of gene expression and gene silencing in Arabidopsis (Jacobsen and Meyerowitz, 1997; Jacobsen, 1999; Kooter *et al.*, 1999; Jacobsen *et al.*, 2000). Therefore, we conclude that *fwa* mutants carry epi-alleles of *FWA*, and that the dense CG methylation of the repeated sequences is associated with the prevention of *FWA* expression in wild-type plants.

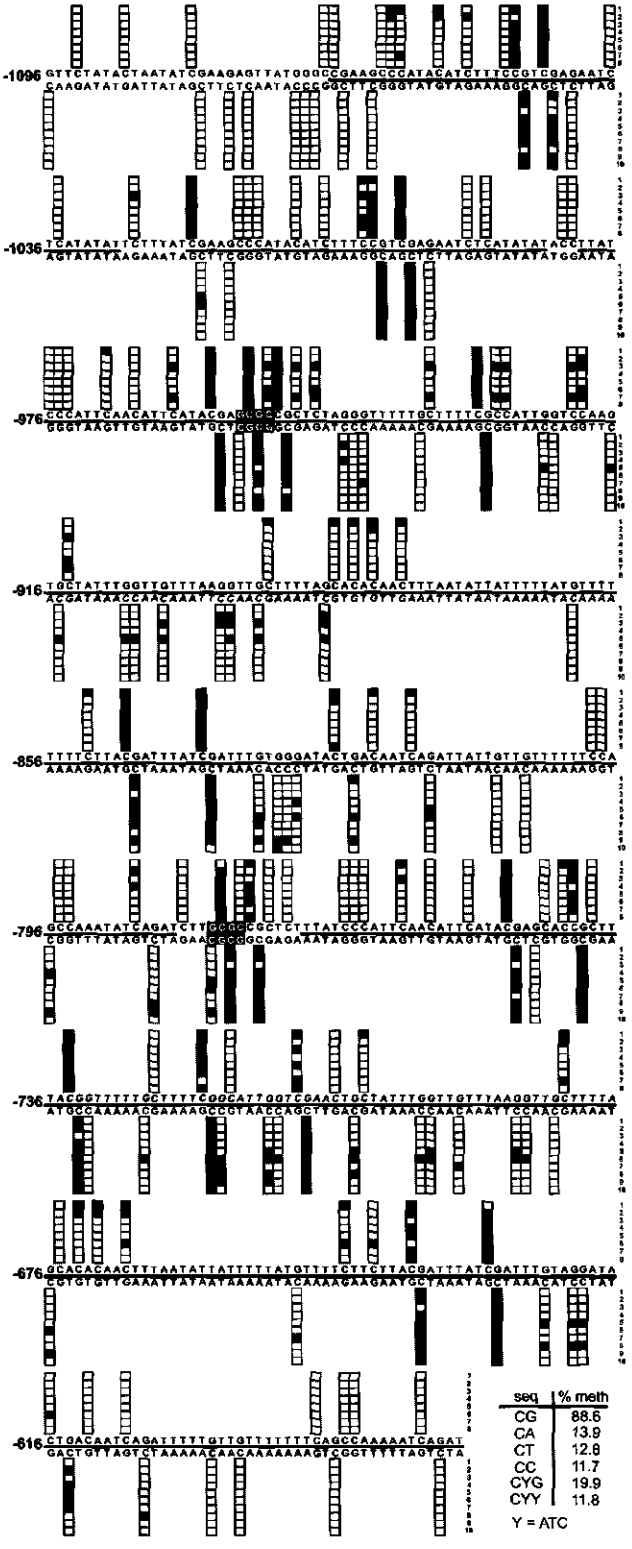

Figure 4.7. Methylation pattern of the *FWA* direct repeats in L*er* wild-type.

Ten bottom strands and eight top strand clones were sequenced. Rows represent methylation status of individual clones (clone numbers are noted to the right side of the sequence). Filled boxes indicate a 5-methylcytosine in the respective clone, while open boxes denote an unmethylated cytosine residue. The direct repeats are underlined and numbering of the sequence is relative to the translational start site. The grey shaded GCGC sequences indicate restriction sites for the *CfoI* restriction enzyme. The table shows percentages of methylated cytosines within different sequence contexts, calculated from the first methylated cytosine to the last.

A late flowering ddm1 line contains an FWA epi-mutation

DNA of the *ddm1* mutant was shown to be hypomethylated throughout the genome (Vongs *et al.*, 1993). In the progeny of this mutant, stable dominant late flowering lines were observed after several generations. These late flowering traits were genetically mapped to the same position as *FWA* and named *fts* (Kakutani, 1997). To find whether *FWA* might be the cause of the abnormal flowering in these lines, we studied the expression and methylation of *FWA* in early and late flowering *ddm1* lines. Northern blot hybridisation showed the presence of *FWA* expression in a late flowering *ddm1* line whereas no expression could be detected in early flowering lines (Figure 4.5C). The methylation level of the *FWA* repeated sequences in different early and late flowering *ddm1* lines was analysed by bisulfite sequencing. Because the *ddm1* mutant was obtained in the Col genetic background, this genotype was also analysed. The repeated sequences of Col wild-type were found to be as densely methylated as in L*er*. A similar level of methylation was found in early flowering *ddm1* lines (Table 4.1). However, in a late flowering *ddm1* line these sequences were not methylated, like in the *fwa* mutants. Therefore, we suggest that the late flowering of lines derived from the *ddm1* background is caused by an *FWA* epi-mutation.

Table 4.1. Flowering time and methylation of the *FWA* repeats in different wild-type and mutant plants.

Genotype	Accession	Flowering time	Methylation of repeats
L*er*, wild-type	L*er*	early	yes
Col, wild-type	Col	early	yes
fwa-1	L*er*	late	no
fwa-2	L*er*	late	no
fwa-1R1	L*er*	early	no
fwa-1R2	L*er*	early	no
fwa-1R3	L*er*	early	no
ddm1	Col	early	yes
ddm1	Col	late	no

The late flowering behaviour of plants transformed with FWA is unstable

The late flowering phenotype of the *fwa* mutants is very stable since a screen among 4000 plants of the *fwa-1* marker line for spontaneous early flowering plants did not yield any revertant. In contrast, the flowering behaviour of *FWA* transgenic plants was rather unstable. Transgenic L*er* wild-type plants transformed with either of the two cosmids WS20 and WS28, were analysed for their flowering behaviour through four subsequent generations. Only a small portion of these T1 plants showed a delay in flowering time. None flowered as late as the *fwa* mutant, probably because they are hemizygous for the insert (Figure 4.8). Late

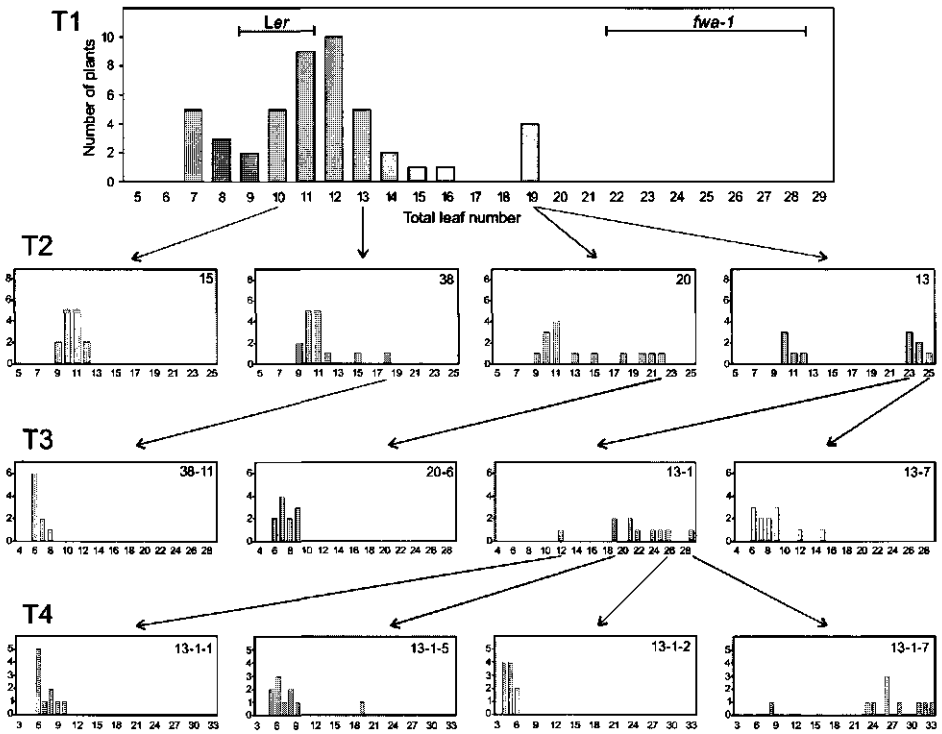

Figure 4.8. Flowering time of *Ler* plants transformed with the *FWA*-containing cosmid WS20.

Frequency distribution of the number of leaves in the T1 transformants obtained after transformation of *Ler* wild-type plants with cosmid WS20 and some of the subsequent T2, T3 and T4 populations. All the T1 plants contained the insert and all T2, T3 and T4 populations were either homozygous or segregating for the WS20 insertion. The Y-axis indicates the number of plants and the X-axis the total number of leaves produced by the plant. The ranges of variation for leaf number of *Ler* wild-type and *fwa-1* under the growth conditions of this experiment are indicated as horizontal bars.

flowering plants were never observed in the progeny of early transgenic plants. However, the progeny of late flowering T1 plants segregated for flowering time and included plants that flowered as late as the *fwa* mutant (Figure 4.8). In all cases tested, the segregation for flowering time did not fit Mendelian ratios for either one or multiple copies of the cosmid. An excess of early flowering plants was observed through T2, T3 and T4 generations. In contrast, Mendelian ratios were observed for the segregation of the cosmid insert in all tested families. This indicates that the distorted segregation for flowering time was not due to reduced transmission of chromosomes bearing the transgene.

We analysed the expression of *FWA* in different T2 populations. As shown in Figure 4.9A, the transcript could only be detected in the T2 populations that were segregating late

flowering plants and not in the T2 population that only contained early flowering plants. Thus, *FWA* expression correlated with the flowering phenotype.

In addition, *fwa* mutant plants were transformed with cosmids WS20 and WS28. Surprisingly, several T1 plants that flowered as early as L*er* wild-type were obtained (4 out of 23 plants for WS20 and 17 out of 44 plants for WS28). As shown in Figure 4.9B, expression of *FWA* was only detected in the late flowering T2 populations and not in the early flowering populations. This indicated that presence of the transgene induced silencing of the endogenous copy of *FWA*.

FWA transgenic plants were also obtained with smaller genomic inserts than those contained in cosmids WS20 and WS28. For that, two constructs were made; one containing a 6.1 Kb genomic fragment from the *fwa-1* mutant and another containing a 5.2 Kb fragment from the L*er* wild-type, both spanning the complete *FWA* gene and promoter. Transformation of these constructs into L*er* wild-type plants did not yield any late flowering T1 plants, and only a few plants were flowering slightly later than wild-type plants. However, *fwa-1* mutant plants transformed with the same constructs, produced mostly early flowering T1 plants (32 out of 38 for the 6.1 Kb construct and 43 out of 48 for the 5.2 Kb construct. Therefore, the smaller constructs are stronger inducers of silencing than the larger genomic inserts.

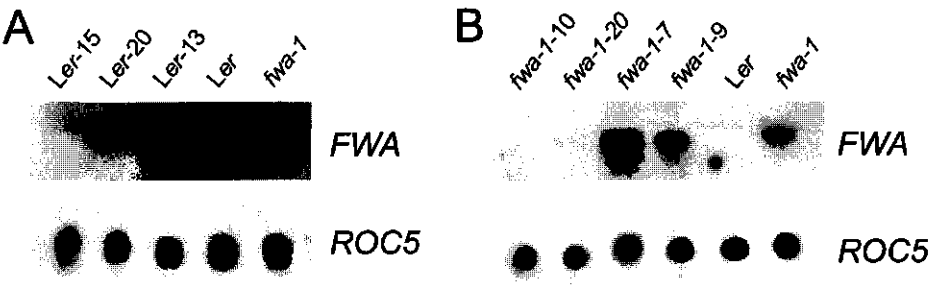

Figure 4.9. *FWA* expression of L*er* and *fwa-1* plants transformed with the *FWA*-containing cosmid WS20.

A. Northern blot analysis of *FWA* expression in different T2 bulked populations of L*er* plants, transformed with cosmid WS20. As shown in Figure 4.8, the T2 population L*er*-15 was early flowering and L*er*-20 and L*er*-13 contained both early and late flowering plants. Plants were grown under LD conditions and total RNA was extracted three weeks after planting. The blot was probed with a fragment of the *ROC5* gene as a control for loading.

B. Northern blot analysis of *FWA* expression in different T2 bulked populations of *fwa-1* plants, transformed with cosmid WS20. The T2 populations *fwa-1*-10 and *fwa-1*-20 were early flowering and *fwa-1*-7 and *fwa-1*-9 were late flowering. Plants were grown under LD conditions and total RNA was extracted three weeks after planting. The blot was probed with a fragment of the *ROC5* gene as a control for loading.

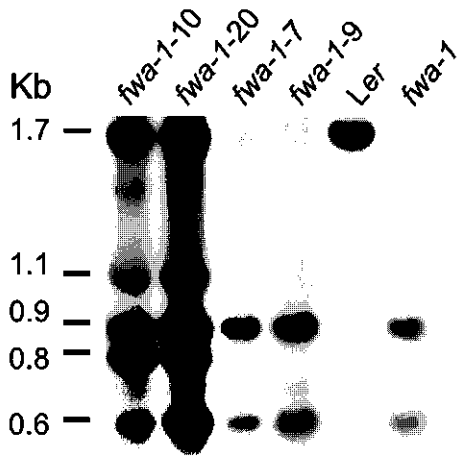

Figure 4.10. Methylation pattern of *fwa-1* plants transformed with the *FWA*-containing cosmid WS20.
Southern blot analysis of the methylation pattern for the same T2 populations analysed in Figure 4.9B. Genomic DNA was digested with *CfoI*. A 0.95 Kb probe that completely covered the genomic region, containing the two direct repeats, was used for hybridisation. Three DNA fragments of 0.9, 0.6 and 0.2 (not visible) Kb were present in the *fwa-1* mutant and one fragment of 1.7 Kb in L*er*.

To test whether *FWA* silencing and the loss of late flowering might be caused by de novo methylation of the *FWA* repeats, we looked at the methylation of several cytosines by southern blot analysis. This assay was used on *fwa-1* transformed plants because in this case *FWA* silencing was induced on both transgene and endogene copies. Genomic DNA was extracted from whole plants of T2 populations and digested with the restriction enzyme *CfoI* that cuts twice in the repeats and is sensitive for methylation (Figure 4.7). Late flowering T2 *fwa-1* populations show the same pattern as *fwa-1* itself (Figure 4.10). However, T2 populations derived from T1 plants flowering at the same time as wild-type L*er*, showed both the L*er* wild-type and *fwa* mutant fragments and two other additional fragments, presumably due to methylation of only one of the two *CfoI* sites. Therefore, silencing of the *FWA* gene correlates with the presence of methylation in the *FWA* repeated sequences.

Discussion

FWA encodes a homeodomain containing transcription factor

We have identified the *FWA* gene by positional cloning, revealing that it encodes a protein that belongs to the HD-GL2 family (Figure 4.4) which is a subclass of plant HD-ZIP homeodomain proteins. Several arguments indicate that *fwa* mutants carry gain of function alleles while *fwa-1R* revertants are loss of function mutants of this gene: i) In *fwa* mutants, the flowering delay correlated with overexpression of this gene, compared to wild-type plants. ii)

Similar correlation was found in transgenic plants that carry an additional copy of *FWA*. iii) Mutations in the *FWA* DNA sequence of *fwa-1R* revertants suppress the late flowering phenotype of the *fwa-1* mutant.

Homeodomain proteins are transcription factors that play an important role in the regulation of developmental decisions through cell fate specification in both animal and plant development. It has been shown that the homeodomain can bind to DNA in a sequence-specific manner and activates or represses the transcription of specific target genes. The leucine zipper can form a dimer that is required for this DNA binding. In addition, the START domain can bind to lipids, which suggests that HD-GL2 proteins function in a lipid-dependent manner (Ponting and Aravind, 1999). The only two genes of this family with a known function are *GLABRA2* (*GL2*), which plays a role in specification of trichome producing and root hair developing cells (Rerie *et al.*, 1994; Di Cristina *et al.*, 1996), and *ANL2* which is involved in anthocyanin distribution and root development (Kubo *et al.*, 1999). The *fwa* mutants are characterised by a delay in flowering initiation, and thus, HD-GL2 proteins also appear to be involved in cell fate changes that occur during transition from the vegetative to the reproductive meristem.

fwa is a gain of function epi-mutant

FWA overexpression in *fwa* mutants indicates that they are gain of function alleles of this gene. Although none of the *fwa* alleles have mutations in the DNA sequence of *FWA*, they show a complete absence of cytosine methylation in two direct repeated sequences located in its 5' promoter and coding regions. This is opposite to the strong methylation observed in wild-type *FWA*. Methylation of coding regions has been shown to lead to a reduction of gene activity in plant cells (Hohn *et al.*, 1996; Jacobsen *et al.*, 2000). Therefore, we concluded that *fwa* mutants are gain of function epi-alleles of the *FWA* gene in which hypomethylation activates expression and leads to late flowering. Interestingly, overexpression of the most homologous gene to *FWA* (*ANL2*) by activation tagging also resulted in a late flowering phenotype (Weigel *et al.*, 2000). This delay in flowering could be an indirect consequence produced by dominant negative interference with the function of flowering time genes.

In recent years, several loss of function epigenetic mutations have been found and studied in plants. As shown for *SUPERMAN* (*SUP*) and *AGAMOUS* (*AG*) epi-alleles in Arabidopsis (Jacobsen and Meyerowitz, 1997; Jacobsen *et al.*, 2000) and a naturally occurring epi-allele of the *Lcyc* gene in *Linaria vulgaris* (Cubas *et al.*, 1999), these mutations are characterised by extensive methylation of a gene, leading to silencing in the mutant. In contrast to these epi-

mutants where the wild-type allele is expressed, *fwa* mutants provide the first example of an epigenetic mechanism that leads to ectopic expression and gain of function of an otherwise silenced gene.

FWA messenger was only detected in developing and germinating seeds of wild-type plants indicating that *FWA* expression is regulated through development. The mechanism by which this occurs remains unknown. It is possible that changes in methylation of the repeated sequences in the 5' region of *FWA* are involved. These repeats contain both promoter and transcribed regions and two silencing mechanisms can be speculated: transcriptional gene silencing (TGS), characterised by methylation of promoter regions and post transcriptional gene silencing (PTGS), associated with methylation of transcribed regions (Kooter *et al.*, 1999). Typical for PTGS is reactivation of the silenced genes at the onset of each generation (Depicker and Van Montagu, 1997; Kooter *et al.*, 1999) which we observed for *FWA* expression. Furthermore, methylation at non-symmetrical sites as we found in the *FWA* repeats is characteristic for RNA-directed DNA methylation (RdDM) which can be part of PTGS (Pélissier *et al.*, 1999). In RdDM, RNA elements located in the coding region of a certain mRNA could induce heavy methylation of the corresponding genomic region (Jones *et al.*, 1999). The direct repeat present in the *FWA* mRNA is a candidate for such an RNA element. PTGS has only been observed in the silencing of transgenes thus far. However, it has been speculated to constitute a form of gene regulation that is important for plant growth and development (Depicker and Van Montagu, 1997). Another possibility is that methylation does not affect gene expression equally in all tissues throughout development. Perhaps methylation of the repeats cannot prevent expression of *FWA* during seed development and germination. Consistent with this, the *CfoI* sites in the *FWA* repeats were methylated in 4 days old imbibed seeds which showed expression of *FWA* (data not shown). In this respect it should be noted that plants with a *SUP* epi-mutation have a wild-type *SUP* phenotype and expression in ovules, even though the gene is silenced in other tissues (unpublished observations, Hajime Sakai, S.E.J. and Jean Finnegan).

fwa is locally defective in DNA methylation

The *fwa* mutants are characterised by stable hypomethylation of the *FWA* direct repeated sequences and surrounding sequences. This hypomethylation must have been caused during the mutagenesis experiments that yielded these mutants. Several hypotheses could explain this.

The *FWA* hypomethylation might have originated by a wide swath of demethylation of chromosome 4, as a direct consequence of the mutagenesis. In this light it is interesting to note that one of the *SUPERMAN* hypermethylated epi-alleles (*clk-1*) was found in the same plants that contained *fwa-1*. Therefore, this mutagenised plant might have shown disruptions in genomic methylation due to genomic shock caused by EMS.

Secondly, a *ddm1* or *ddm1*-like mutation might have occurred in the mutagenesis experiment, which induced hypomethylation of *FWA* repeats and late flowering. Hypomethylation in *ddm1* is spread over the whole genome, including the 180bp centromere repeats, rDNA and the retrotransposon TA3 (Kakutani *et al.*, 1999) which all have a wild-type methylation pattern in *fwa* mutants. During backcrosses with wild-type the original mutation and hypomethylation outside the *FWA* region could have been eliminated from *fwa* mutant plants. In agreement with this, hypomethylation of sequences that are segregated away from the *ddm1* mutation is very stable (Kakutani *et al.*, 1999).

Finally, a mutation in a region containing cis-acting local information for methylation might be closely linked to *fwa* and could have caused hypomethylation. In this respect, the characteristics of *fwa* are very similar to those of the human neurogenetic disorders called the Angelman and Prader-Wili syndromes. In patients with these syndromes, chromosome region 15q11-q13 shows abnormal DNA methylation and gene expression in about 2Mb. Deletions in a region that contains an imprinting centre or switch element have been suggested as the cause (Buiting *et al.*, 1995). It has also been shown that a fragment from this region can function as a silencer in transgenic flies, suggesting a link between genomic imprinting and an evolutionary conserved silencing mechanism (Lyko *et al*, 1998). It could be possible that an element, similar to an imprinting centre, is linked to *FWA* and essential for proper methylation in this region. A mutation in this centre could explain the local nature of the hypomethylation in *fwa*.

Unlike the *fwa* mutant alleles, *FWA* transgene expression and phenotype is not stably maintained. Interestingly, the late flowering trait is lost after a few generations (Figure 4.8). Furthermore, silencing of both the *FWA* trans- and endo-genes was observed in *fwa* plants that are transformed with constructs containing the *FWA* gene, as shown by the early flowering *fwa* transformants. These plants showed a correlation between silencing of *FWA* and methylation of the repeats (Figure 4.9B and 4.10). Therefore, we suggest that this is at least partly caused by a de novo methylation of the repeats. These findings are similar to those reported for the inverted repeats found in the *PAI1-PAI4* gene which triggers methylation of previously unmethylated *PAI* endogenes after introduction into the plant (Luff et. al., 1999). It

is likely that the presence of multiple copies (from both the endogene and the transgene) induces homology dependent gene silencing (HDGS) in the transformants (Kooter *et al.*, 1999). Since this silencing occurs in *fwa* transgenic plants, the transgene dependent silencing mechanism is able to overcome the factor that causes the *fwa* hypomethylation.

Smaller constructs, carrying the *FWA* gene showed a stronger induction of silencing in both L*er* wild-type plants and *fwa* mutants. The lack of late flowering phenotype in transformed wild-type plants might be caused by the absence of an upstream enhancer element. A stronger induction of silencing in both wild-type and *fwa* mutants could be explained by an easier concatenation of these smaller constructs, as compared to cosmids, which causes more copies of *FWA*

The transition from the vegetative to the reproductive phase is mediated by expression of FWA

Increased expression of *FWA* in the *fwa* mutants leads to late flowering. Therefore, *FWA* either represses flowering or promotes vegetative development in these mutants. The molecular mechanism through which this repression occurs is still unknown. 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). In addition, double mutants *fwa ap1* and *ft ap1* have a strongly delayed floral initiation while the double mutants *fwa lfy* and *ft lfy* completely lack flowerlike structures (Ruiz-Garcia *et al.*, 1997). Constitutive expression of *LFY* cannot substitute for the late flowering of *ft* and *fwa* and these mutants in their turn do not interfere with promoter activity of *LFY* as other late flowering mutants do (Nilsson *et al* 1998). Thus, it has been speculated that *FT* and *FWA* have similar roles. They control not only the transition to flowering but also floral meristem identity through a common pathway parallel to *LFY* action. 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). However, we could not detect altered expression of *FWA* in the *ft* mutant or in any of the other late flowering mutants. These results suggest that although the *FWA* and *FT* products might work in a common target, their expression is independent of each other.

The loss of function mutations of *FWA* (revertant alleles) did not show a flowering phenotype, which makes it unlikely that *FWA* has a function in flowering of wild-type plants. However, it is possible that *FWA* only functions under specific environmental conditions or

external stresses in which plants benefit from late flowering. Such conditions might induce hypomethylation of the repeats, enabling expression of *FWA*. In this way silenced genes may act as a reserve of activatable genes relevant for plant adaptation.

The availability of the *FWA* gene should improve our understanding of its true role in the control of flowering initiation and clarify the significance of the methylated repeats for gene regulation in the near future.

Experimental procedures

Plant material

Both *fwa* mutants are in a L*er* background; the *fwa-1* mutant was identified after treatment with EMS and the *fwa-2* mutant after fast neutron irradiation (Koornneef *et al.*, 1991). The L*er* marker line containing the mutations *cer2-1*, *ga5-1*, *fwa-1* and *abi1-1* was constructed by crossing lines carrying these mutations and selection in subsequent generations.

The *ddm1* mutant lines are in a Col background. The late lines obtained after repeated self-pollination of *ddm1* lines were described by Kakutani (1997).

Growth conditions and measurement of flowering time

Plants were grown either in a greenhouse with LD light regime (at least 14 hours daylength) or in a climate chamber with SD light conditions (8 hours light per day) as described in Koornneef *et al.* (1995).

Flowering time was measured by counting the total number of leaves, excluding the cotyledons, since there is a close correlation between leaf number and flowering time (Koornneef *et al.*, 1991).

Construction of the YAC and cosmid contigs

YAC clones were obtained from C. Dean (John Innes Centre, Norwich, UK) and analysed by hybridisation with RFLP markers sep2B, CC128 (from C. Dean), GA5 (from J. Zeevaart, Michigan State University, East Lansing, MI, USA), pcr28, pcr34, pcr41, pcr23 (from J. Giraudat and J. Leung, CNRS, Gif-sur-Yvette, France).

A genomic DNA *fwa-1* library of 27.262 clones with inserts of 15-20 Kb was constructed using the binary cosmid vector pCLD 04541, which carries the Agrobacterium LB, RB sequences, and a 35S-NPTII fusion (supplied by C. Dean and C. Lister). The YAC clone EG1F12 was gel purified and hybridised to filters of this library. A cosmid contig was constructed by hybridisation of the positive cosmids with YAC's EW18E4, yUP10B5 and yUP11F11 and by hybridising the cosmids with themselves.

Transformation of Arabidopsis

Selected cosmids for plant transformation were transferred from *Escherichia coli* to *Agrobacterium tumefaciens* (AGLO strain; Lazo *et al.*, 1991) by electroporation. Plants were transformed using the vacuum infiltration transformation procedure (Bechtold *et al.*, 1993). Seeds obtained after infiltration were sterilised for 15 minutes with 20% bleach in absolute ethanol solution after which they were rinsed two times in absolute ethanol and dried overnight in a flow cabinet. Seeds were sown on plates with selective medium (1 X Murashige & Skoog salts, 1% sucrose, 40µg/ml kanamycin, 0.8% agar, pH 5.8). The plates were kept in the cold room (4°C) for 4 days and then transferred to a growth room (16 hours light, 25°C). After 10 days, resistant seedlings were transferred to soil.

DNA and RNA detection by gel blot hybridisation

DNA was isolated from plants, grown in the greenhouse, following basically the protocol of Bernatzky and Tanksley (1986). RNA was isolated from plants grown in the greenhouse or climate chamber, following the protocol of Puissant and Houdebine (1990). Three µg of genomic DNA was used for Southern blot analysis and 25 µg of total RNA for Northern blot analysis. Southern and Northern blot analyses were performed, following the protocol supplied with the Hybond™-N nylon membranes (Amersham Pharmacia, Uppsala, Sweden). *FWA* expression was detected with a 1.1 Kb probe, corresponding to exons 4-8 of the *FWA* gene. A 0.57 Kb probe, corresponding to the constitutively expressed cyclophilin gene *ROC5* (Chou and Gasser, 1997) was used as a positive control on Northern blots.

Detection of mRNA by RT-PCR

RNA for RT-PCR was isolated with the Rneasy plant mini kit from Qiagen (Chatsworth, CA, USA). For first strand cDNA synthesis, 5-10 µg of total RNA was used and cDNA synthesis was primed by using the standard $dT_{12\text{-}18}$ adapter primer. The product of the first-strand synthesis reaction was then used for PCR with the primers FWA-E6/7 (5'-GCTCAC TCCAACAGATTCAAGCAG-3'), located at the junction of the sixth and seventh exon of the *FWA* gene, and FWA-R2 (5'-GTTGGTAGATGAAAGGGTCGAGAG-3'), located in the eighth exon, which yielded a 0.35 Kb fragment. For the control reaction, a fragment of the constitutively expressed *UBIQUITIN10* mRNA (Callis *et al.*, 1995) was amplified, using the primers UBQ10F1 (5'-GATCTTTGC CGGAAAACAATTGGAGGATGGT-3') and UBQ10R1 (5'-CGACTTGTCATTAGAAAGA AAGAGATAACAGG-3'), which yielded a 0.5 Kb fragment.

Isolation of complete FWA cDNA by 5' and 3' rapid amplification of cDNA ends (RACE)

10 µg of total RNA was used for first strand cDNA synthesis. The cDNA was amplified and sequenced in three parts. The 5' part after dCTP tailing with the primers anchor (5'-AAATGGATCCTTCTAGATGCGGGGGGGGGGGGGGGG-3'), adapter (5'-AAATGGATCCTT CTAGATGC-3') and the gene specific primers fwa-5-1 (5'-ATCTG TCATGCTCTTCTCTA-3') and fwa-5-2 (5'-TACATTCTCAAGGTGGTCAT-3'). The middle part was amplified with the primers FWA-F2 (5'-ACAGAGGTACGAGCTTGGACAAAG-3') and FWA-R2. The 3' part was amplified with the 3' RACE system kit from Gibco BRL (Rockville, MD, USA), using adapter and amplification primers from the kit and the gene specific primer fwa-3-1 (5'-ACTCTACCAGCCTTTGATTGGC-3').

Bisulfite Sequencing

Genomic DNA, isolated from vegetative plants of each genotype was cleaved with *DdeI* and *DraI* restriction enzymes. DNA was then treated with sodium bisulfite, amplified, cloned and sequenced as previously described (Jacobsen, 2000). Clones were derived from PCR products of bisulfite treated DNA using the Invitrogen Original TA Cloning Kit. Several sets of PCR primers were used to amplify the direct repeats and regions outside these repeats.

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Chapter 5

Summarising discussion

The transition from the vegetative to the reproductive phase (flowering initiation) in plants has a complex regulation which is affected by environmental and internal plant factors. The understanding of this process is not only of fundamental interest but could also lead to practical applications. Early investigations of this process identified the roles of a cold treatment (vernalisation) (Gassner, 1918) and daylength (Garner and Allard, 1920) in the flowering initiation of several plant species. Flowering research initially focussed on the identification of environmental and chemical factors regulating flowering time and the classification of plants according to their response to these factors. Of particular interest was the identification of a graft transmissible signal, called florigen, proposed to be critical for flowering initiation (Lang, 1965). However, the biochemical nature of this compound was never discovered. The observation that many factors influence flowering time led to a multifactorial model of flowering control (Bernier, 1988). In the meantime, a genetic approach was initiated in different plant species (Murfet, 1977). In Arabidopsis several late flowering mutants were isolated and analysed (Rédei, 1962; Hussein, 1968; Vetrilova, 1973) and classified into several groups based on genetic and physiological characterisations (Martínez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991). By this time, tools became available which made it possible to isolate and analyse these genes at the molecular level, which promised more insight into their cellular functions and molecular relationships with each other. Due to its genetic and molecular features Arabidopsis was the most suitable organism for this purpose (Meinke *et al.*, 1998) and the research on flowering initiation became focussed on this plant. More and more genes influencing flowering time were characterised and different, partially redundant and interacting pathways affecting flowering were identified (Martínez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Koornneef *et al.*, 1998b; Levy and Dean, 1998; Simpson *et al.*, 1999; Reeves and Coupland, 2000). The present state of knowledge about the genetic control of flowering time in Arabidopsis is described in Chapter 1. Although several genes that play important roles in flowering initiation have now been identified and an increasing amount of information about their mutual interactions has been obtained, the picture is still far from complete.

The aim of the work presented in this thesis is to increase our knowledge of flowering time regulation. It focussed on the genetic and molecular characterisation of the semi-dominant mutant *fwa* which flowers late under long daylength conditions and has been proposed to be part of the photoperiodic promotion pathway (Chapter 1). One approach sought to identify additional genes that affect flowering, by mutagenesis of the *fwa* mutant. In addition to three different intragenic revertants of *fwa*, this screen yielded a novel early

flowering mutant which is described in Chapter 2. Another approach consisted of the map based cloning and molecular characterisation of the *FWA* gene, presented in Chapters 3 and 4.

The novel early flowering mutant, obtained after mutagenesis of *fwa* was named *early flowering in short days* (*efs*). Its phenotypic characterisation has shown that the main role of the wild-type *EFS* gene is to delay flowering in plants that have entered the adult vegetative phase, which is considered to be the phase where plants are able to respond to environmental signals in order to flower (Poethig, 1990). Consistent with this, *efs* mutant plants do not show an early flowering phenotype when grown under environmental conditions that lead to a shortened adult vegetative phase such as long days and vernalisation. To learn more about the role of *EFS* in relation to other genes involved in flowering initiation, double mutants were isolated and characterised. This analysis showed that *efs* is epistatic to the late flowering mutants *fca* and *fve* which belong to the autonomous promotion pathway, indicating that *EFS* acts downstream of these genes in the same pathway. This result, together with the lack of a vernalisation response, suggests that *EFS* is likely to represent a new element acting at a point close to the convergence of signals from the autonomous promotion pathway and the vernalisation promotion pathway (Figure 1.2). A similar position has been proposed for the *FLC* gene (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999) and therefore, further study of the relationship between these two genes could provide new insights into this aspect of the flowering initiation process.

The main topic of this thesis concerns the map based cloning of the *FWA* gene. This cloning was started in 1991 and proved to be a laborious and time consuming exercise. Initial fine mapping of the *FWA* locus failed because many genes with minor effects on flowering segregated in the original mapping population. This caused misscoring for the *fwa* phenotype and, therefore, misclassification of recombinants. As shown in Chapter 3, this problem was solved by using a mapping population with a more uniform genetic background, by which the *FWA* locus could be located in a region of about 60 Kb. Plant transformation experiments with cosmids spanning this region showed that the gene is located in the overlap of two cosmids. This overlap contained only one complete gene that encodes a homeodomain containing transcription factor. The altered expression of this gene in *fwa* mutants together with DNA mutations in the intragenic revertants of *fwa-1* further proved that this gene is *FWA*.

Analysis of *FWA* revealed several interesting characteristics. Surprisingly, the mutant and wild-type alleles had an identical DNA sequence in the *FWA* region, excluding DNA mutations in the gene as a cause for the mutant phenotype. Furthermore, two direct repeated

sequences were found in the 5' genomic region of *FWA*. In wild-type plants these repeats were heavily methylated, whereas in the mutant alleles the repeats were completely un-methylated. In contrast to *fwa* mutant plants, which showed a high expression of *FWA* at all developmental stages, wild-type plants showed only a low expression of *FWA* in siliques and germinating seeds. Taken together, these findings suggest that loss of methylation of the *FWA* repeats in the *fwa* mutant causes a high level of expression of the gene, leading to a late flowering phenotype. A similar correlation of late flowering, *FWA* overexpression and hypomethylation of *FWA* repeats was found in late flowering plants which were derived from the *ddm1* hypomethylation mutant. The late flowering phenotype of these plants had previously been mapped to the *FWA* region (Kakutani, 1997). Nevertheless, the correlation between hypomethylation of the *FWA* repeats and *FWA* expression was not found in germinating seeds of wild-type plants which showed expression of *FWA* but methylation of the repeats. Although this expression might come from residual mRNA produced earlier in developing seeds, it is possible that methylation of the repeats does not always prevent expression of *FWA*. Perhaps a different epigenetic mechanism early in development can induce expression of methylated genes. In Chapter 4 three hypotheses are given as possible explanations for the local hypomethylation in *fwa* mutants: a genomic shock due to mutagenesis, a second site mutation that has been segregated away, and a closely linked mutation that still could be present. An indication that might support the last hypothesis came from wild-type plants that were transformed with a cosmid (WS31) carrying an *fwa-1* insert which is closely linked to the *FWA* locus. In T2 and later generations of these transformants late flowering and other mutant phenotypes were observed. This suggested that cosmid WS31 contains a mutation that causes hypomethylation in cis, thereby altering the expression of nearby genes. However, late flowering T3 transformants did not show expression of *FWA* nor hypomethylation of the *FWA* repeats, indicating that overexpression of the *FWA* gene was not the cause of the late flowering phenotype.

The correlation of *FWA* expression with late flowering indicates that *FWA* is a repressor of flowering. This repression phenotypically and genetically mimics loss of function mutations in the *FT* gene. However, *FT* expression is not altered in an *fwa* mutant background (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) suggesting that *FWA* might act downstream of *FT*. A genetic analysis by Ruiz-García *et al.* (1997) showed that the double mutants of *fwa* and *ft* with *leafy* (*lfy*) did not make any flower like structures. Also, the combination of *fwa* with a constitutively expressed flowering promoting gene (35S::*CO*) resulted in near-complete loss of floral organs (Onouchi *et al.*, 2000). This suggests a role for *FWA* as

meristem identity gene, redundant with *LFY*. However, we could not detect any expression of *FWA* in flowers or buds of wild-type plants indicating that *FWA* probably inhibits floral meristem identity only when ectopically expressed in the *fwa* mutants.

The cloning of *FWA* could not further clarify the role of this gene in the flowering initiation process. The loss of function alleles of *FWA* generated in the *fwa-1R* revertants did not show any obvious phenotype and no expression of *FWA* could be detected in other late flowering mutants, suggesting that these mutants do not flower late due to *FWA* expression. Possibly, *FWA* has no function in flowering initiation of wild-type plants. It might participate in a seed-specific process, as suggested by its expression in seeds. However, the lack of an obvious phenotype in the revertants suggests that this role is minor or redundant with other genes.

A role for methylation in the regulation of *FWA* expression can be proposed. It may be that *FWA* only has a function in wild-type plants under specific environmental conditions or external stresses in which plants benefit from flowering late. Such conditions might induce hypomethylation of the repeats, enabling expression of *FWA*. An environmental condition that influences flowering time and that has been speculated to be associated with changes in methylation level is vernalisation. It has been suggested that low temperature treatment reduces the methylation status of gene(s) that promote flowering (Burn *et al.*, 1993a; Dennis *et al.*, 1996). For the recently cloned flowering repressing *FLC* gene it has been shown that both vernalisation and a reduction of the methylation status downregulate the level of its mRNA (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999), although no direct effect on the methylation status of *FLC* or any other gene has yet been reported. However, demethylation of *FWA* does not cause early flowering but late flowering instead. Therefore, it is unlikely that *FWA* plays a central role in the vernalisation process itself, although it may be associated with a similar regulation mechanism.

The results discussed in this thesis have contributed to the existing knowledge of flowering initiation by the isolation of a mutant at a novel locus and the cloning of a previously known gene which are both involved in this process. In addition, the results indicate a possible role for DNA methylation in gene regulation of Arabidopsis.

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Samenvatting

Het is belangrijk voor een plant om op een gunstig tijdstip in het jaar tot bloei te komen teneinde een zo groot mogelijk aantal levensvatbare zaden te kunnen produceren. Deze bloei initiatie is complex gereguleerd. Hierbij spelen zowel omgevingsfactoren als interne plantfactoren een rol. Een goed begrip van dit proces is niet alleen van fundamenteel belang, maar zou ook tot praktische toepassingen kunnen leiden. Het onderzoek naar bloei initiatie heeft al een geschiedenis van meer dan 80 jaar. Aanvankelijk werd de nadruk gelegd op fysiologisch en biochemisch onderzoek dat leidde tot de identificatie van verschillende omgevings- en plantfactoren voor bloei. Sinds de jaren zestig kent dit onderzoek ook een genetische aanpak, waarbij gebruik gemaakt wordt van planten die mutaties hebben in genen die invloed hebben op het tijdstip van bloei. Vooral in Arabidopsis werd een groot aantal van dergelijke mutanten verkregen, die vervolgens genetisch en fysiologisch gekarakteriseerd zijn. Hieruit bleek dat de bloei initiatie in Arabidopsis via verschillende, met elkaar in verband staande routes gereguleerd is. Dit zijn onder andere de daglengte route, waarin bloei onder invloed van lange daglengtes gestimuleerd wordt, de vernalisatie route, waarin bloei gestimuleerd wordt na blootstelling aan lage temperaturen en de autonome route, waarin bloei onafhankelijk van de omgeving gestimuleerd wordt. Dankzij de gunstige eigenschappen van Arabidopsis concentreerde het onderzoek naar bloei initiatie zich vervolgens op deze plant. Sinds het begin van de jaren negentig zijn ook verschillende van de genen in deze reguleringsroutes gekloneerd. Dit gaf meer inzicht in de functie van deze genen in de cel en de relaties die ze hebben met elkaar. Hoewel door dit onderzoek nu al veel opgehelderd is, blijven er toch nog onduidelijkheden bestaan over de regulering van bloei initiatie.

Het in dit proefschrift gepresenteerde onderzoek heeft als doel een bijdrage te leveren aan de kennis over bloei initiatie. Hierbij zijn twee verschillende strategieën gevolgd, beide gebaseerd op de laat bloeiende mutant *fwa*. De *fwa* mutant is gedeeltelijk dominant en behoort tot de daglengte route. Eén strategie was om meer genen te verkrijgen welke invloed hebben op de bloei door middel van het mutageniseren van de *fwa* mutant. Hierbij werden niet alleen drie intragene revertanten van *fwa* gevonden, maar ook een nieuwe vroeg bloeiende mutant welke is beschreven in hoofdstuk 2. De tweede strategie bestond uit het kloneren en de verdere moleculaire analyse van het *FWA* gen, wat beschreven is in de hoofdstukken 3 en 4.

De nieuw verkregen vroeg bloeiende mutant werd *efs* genoemd (hetgeen staat voor early flowering in short days = vroege bloei in korte dag). Een fenotypische karakterisering van

deze mutant wees uit dat de belangrijkste rol van het normale (wildtype) *EFS* gen is het remmen van de bloei van planten in de volwassen vegetatieve fase. De volwassen vegetatieve fase wordt beschouwd als de fase waarin planten kunnen reageren op omgevingsfactoren om tot bloei te komen. In overeenstemming hiermee bleek ook dat mutante *efs* planten niet vroeger bloeiden als ze werden opgekweekt onder condities zoals lange dagen en vernalisatie die leiden tot een kortere volwassen vegetatieve fase. Uit een analyse van dubbelmutanten is gebleken dat het *EFS* gen een rol speelt bij de autonome regulering van bloei initiatie. Dit gegeven, samen met het gebrek aan een vernalisatie respons duiden erop dat *EFS* een rol speelt in het punt waar de autonome en de vernalisatie route samenkomen.

Het belangrijkste onderwerp van dit proefschrift betreft de klonering van het *FWA* gen. Met behulp van planten die een overkruising bezitten tussen *FWA* en omliggende markers kon het gebied waarin het *FWA* locus moet liggen verkleind worden tot 60 Kb. Plant transformatie experimenten met cosmiden, die dit gebied omvatten, lieten zien dat het *FWA* gen op de overlap van twee van deze cosmiden ligt. Deze overlap bevat slechts één compleet gen, dat voor een homeodomein bevattende transcriptie factor codeert. Een gewijzigde expressie van dit gen in *fwa* mutanten, samen met DNA mutaties in de intragene revertanten van de *fwa* mutant bewezen verder dat dit gen *FWA* is.

Analyse van het *FWA* gen onthulde verschillende interessante kenmerken. Het bleek dat de mutante en wildtype allelen een identieke DNA volgorde hebben in het gebied op het genoom waar het *FWA* gen ligt. Dit sluit uit dat een DNA mutatie in het gen verantwoordelijk is voor het mutante fenotype. In het 5' gebied van *FWA* werden twee repeterende DNA sequenties gevonden. Eén van deze is gelegen in de eerste twee exons van het gen, hetgeen ook een kleine repeterende sequentie in het onvertaalde 5' gedeelte van het mRNA tot gevolg heeft. Het DNA van deze repeterende sequenties is sterk gemethyleerd in wildtype planten, terwijl dit in de *fwa* mutanten in het geheel niet gemethyleerd is. In tegenstelling tot mutante *fwa* planten, waar voor alle ontwikkelingsstadia van de plant een hoge expressie van *FWA* gevonden werd, is in wildtype planten slechts een lage expressie gevonden in hauwtjes en kiemende zaden. Deze gegevens maken het zeer waarschijnlijk dat het verlies aan methylering van de repeterende sequenties in de *fwa* mutant een hoge expressie van het gen veroorzaakt, hetgeen leidt tot het late bloei fenotype. Een soortgelijke correlatie tussen late bloei, overexpressie van *FWA* en hypomethylering van de repeterende *FWA* sequenties werd ook gevonden in laat bloeiende planten die verkregen zijn uit de *ddm1* hypomethylerings mutant. Het laat bloeiende fenotype van deze planten was voorheen al genetisch gelokaliseerd in het *FWA* gebied. De correlatie tussen hypomethylering van de *FWA* repeterende sequenties en

FWA expressie werd echter niet gevonden in kiemende zaden van wildtype planten die *FWA* expressie vertoonden in aanwezigheid van gemethyleerde repeterende sequenties. Het is mogelijk dat deze expressie nog afkomstig is van eerder geproduceerd mRNA, maar het kan ook dat methylering niet altijd expressie van *FWA* kan voorkomen.

De correlatie tussen *FWA* expressie en late bloei geeft aan dat *FWA* de bloei onderdrukt. Eerder onderzoek had al aangetoond dat *FWA* niet alleen een rol speelt bij bloei initiatie, maar ook in het meristeem dat tot de vorming van bloemen leidt. In wildtype planten kon echter geen expressie van *FWA* worden aangetoond in bloemknoppen of bloemen en het zou kunnen dat het gen alleen dit proces beïnvloedt wanneer het een hoge expressie vertoont in de *fwa* mutant. Het is daarom mogelijk dat *FWA* geen rol speel bij het tot bloei komen van wildtype planten, maar een functie heeft in een zaad specifiek proces, gezien de gevonden expressie in zaden.

Uit de klonering van *FWA* bleek dat de afwezigheid van methylering van de repeterende sequenties in het 5' gebied van het *FWA* gen een verhoogde expressie geeft in de *fwa* mutant. Het is echter niet duidelijk geworden of deze correlatie direct of indirect is. Ook het belang van deze methylering in wildtype planten is nog onduidelijk. Het is mogelijk dat het een rol speelt in de expressie van het gen onder bepaalde omgevingsfactoren.

De resultaten die in dit proefschrift beschreven zijn, hebben een bijdrage geleverd aan de bestaande kennis over bloei initiatie, zowel door de isolatie van een mutant in een nieuw locus als door de klonering van een al eerder bekend gen, die beide bij dit proces betrokken zijn. Bovendien hebben de resultaten aanwijzingen gegeven voor een mogelijke rol van DNA methylering in genregulatie bij Arabidopsis.

Publications

Articles from this thesis:

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

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

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Additional articles:

Koornneef M., Bade, J., Hanhart, C., Horsman, K., Schel, J., **Soppe, W.**, Verkerk, R. and Zabel, P. (1993). Characterisation and mapping of a gene controlling shoot regeneration in tomato. *Plant J.* **3**, 131-141.

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Patent:

Soppe, W.J.J., Peeters, A.J.M. and Koornneef, M. Genetic control of flowering using the FWA gene. Patent Cooperation Treaty, International Application PCT/NL99/00414. 2 July 1999, Wageningen University.

Curriculum vitae

Wim Soppe werd op 8 juni 1967 in de Noordoostpolder geboren. Na het behalen van het VWO diploma in 1985 aan het Prof. ter Veenlyceum in Emmeloord, begon hij in hetzelfde jaar met de studie Plantenveredeling aan de Landbouwuniversiteit te Wageningen. Tijdens de doctoraalfase werden de afstudeervakken Plantenfysiologie, Plantenveredeling en Erfelijkheidsleer gevolgd, alsmede een stage Plantenveredeling bij het Scottish Crop Research Institute in Dundee (Schotland). Na het afstuderen in 1991 werkte hij in het kader van zijn vervangende dienstplicht van januari 1992 tot mei 1993 op de vakgroep Plantenveredeling van de Landbouwuniversiteit te Wageningen. Vervolgens was hij van juli 1993 tot september 2000 aangesteld bij het laboratorium voor Erfelijkheidsleer van dezelfde universiteit om onderzoek te verrichten aan bloei initiatie mutanten bij Arabidopsis. De resultaten hiervan staan beschreven in dit proefschrift. Met ingang van september 2000 werkt hij als postdoc aan de relatie tussen DNA organisatie en genactiviteit bij het Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben (Duitsland).

Nawoord

Hoewel het in dit proefschrift beschreven werk ook door anderen verricht had kunnen worden zonder mij, zou ik nooit in staat zijn geweest dit te doen zonder hulp van anderen. Hierbij wil ik graag al diegenen bedanken waarbij een aantal mensen in het bijzonder.

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