ERRATA

Page v, paragraph 1, line 12: "*FLER* is not allelic" for "*FLER* is not be allelic"

Page v, paragraph 1 line 13: "*FLC*" not "FLC"

Page v, paragraph 2 line 14: Insert a comma after "However"

Page v, paragraph 2 line 15: "extended periods of cold" for "extended periods of a cold"

Page v, paragraph 2 line 17: Insert a comma after "process"

Early-flowering mutants of a late-flowering ecotype of *Arabidopsis thaliana*.

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ABSTRACT

Early-flowering mutant lines of the late-flowering Pitztal ecotype of *Arabidopsis* were identified in this study. Genetic analysis has indicated that all these mutant lines are disrupted in a single gene, the *FLER* (*F*loral *L*ate *E*cotype *R*epressor) locus. Several experiments were performed to determine if the *fler* mutation represented a lesion in either the dominant *FRI*, or semi-dominant *FLC* loci, as these genes are believed to be the major loci responsible for conferring the large delay in the flowering times of ecotypes such as Pitztal. Crosses of the *fler* lines with early- and late-flowering genetic backgrounds suggested that the *FLER* locus is not alllelic to the *FLC* locus. Physiological analysis of the mutant lines, and mapping of the mutation within these plants via microsatellite marker analysis, supports the hypothesis that *FLER* may be a *FRI* mutation however the presence of a late-flowering ecotype version of the *FRI* gene in the mutant background does not complement the mutant phenotype. It is therefore proposed that *FLER* is not be allelic to *FRI* or FLC, but rather represents a novel gene involved in the repression of flowering in late-flowering ecotypes. Several other recessive genes are also thought to be segregating within the *fler* lines that have minor though noticeable effects on the flowering times of these lines.

In order to idenify genes whose expression was disrupted in the mutant lines a cDNA subtraction technique was employed to isolate differentially expressed sequences between the mutant lines and wild-type Pitztal. This technique resulted in the isolation of many different types of gene sequences, with the majority encoding for light regulated genes. Northern blot analysis revealed that the expression of the majority of these transcripts was only slightly altered in the mutant lines compared to wild-type plants. The expression of one of the transcript sequences isolated from the subtraction was however dramatically altered in the mutant plants. No detectable expression of this transcript was observed in any of the early-flowering mutant lines or ecotypes analysed, while strong and relatively constant expression of this gene sequence was seen in all the late-ecotypes analysed. Further analysis showed that the lack of expression of this gene was also tightly correlated with the early-flowering phenotypes of the mutant lines. These results strongly suggest that this gene is involved with the repression of floral induction and the gene was subsequently termed *FRP33-4* (*F*loral *Rep*ressor 33-4). However the expression of this gene is elevated in wild-type plants that are exposed to extended periods of a cold or to long day photoperiods, conditions that promote floral induction. A model of the proposed mode of action for *FRP33-4* in a *FRI/FLER/FLC* mediated floral repression process, has been formulated.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other Degree or Diploma in any university or institute. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

Dale Wilson

Chapter 1

Literature Review
Floral induction in *Arabidopsis*

1.1 Introduction

Floral induction in plants is the process causing the transition from vegetative to reproductive growth. In many angiosperms floral induction is influenced by environmental conditions, as well as the physiological age of the plant (reviewed in Bernier, 1988 and Bernier *et al.*, 1993). The influence that these environmental and developmental factors have on the floral induction process varies amongst angiosperms. Autonomous flowering plants undergo the vegetative-to-floral transition largely independently of their environment, as developmental age is the most important factor in determining the competence of these plants to flower. Plants that require specific environmental conditions to become florally induced are referred to as obligate angiosperms. Facultative plants have no obligate requirement, however the transition to flowering can be influenced by environmental conditions such as day-length (Bernier *et al.*, 1993; Peeters and Koornneef, 1996; Martinez-Zapater *et al.*, 1994). *Arabidopsis* is a member of the globally distributed Brassicacea family and is considered to be a facultative long day requiring plant (Hempel, 1996). The availability of many well characterised metabolic and flowering time mutants, as well as the large variation in flowering time found amongst naturally occurring ecotypes of *Arabidopsis*, has meant that this species is often used as a model to study floral induction (Bernier *et al.*, 1993).

Morphological changes associated with floral induction in Arabidopsis

The vegetative and reproductive phases of *Arabidopsis* are temporally separated, and the transition between them involves the transformation of the shoot apical meristem (Martinez-Zapater *et al.*, 1994). The apical meristem contains groups of undifferentiated progenitor cells, or primordia, which grow from the flanks of the meristem and can differentiate into various vegetative (cotyledons or leaves) or floral organs (Ma, 1997). The fate of these primordia is controlled by an array of signals from the whole plant that are triggered by both environmental and developmental cues (Horsch 1990; Bernier *et al.*, 1993). It is still unclear however, whether these signals are received by a central zone of the apical meristem and subsequently transduced to the primordia, or if such signals act directly on the primordia themselves (Hempel, 1996).

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Before the apical meristem can become competent to receive any such floral induction signals the plant must pass through a vegetative, juvenile phase (Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Amasino, 1996a). It has therefore been suggested that this competence to receive floral stimulus is dependent upon the presence of enough vegetative tissue to produce the required signals (Hempel, 1996). This competence has been shown to be controlled by both genetic and environmental factors. Naturally occurring ecotypes of *Arabidopsis* that normally require five leaves before acquiring competence have for example, been shown to respond after the formation of only one leaf in conditions of nutrient stress (Martinez-Zapater *et al.*, 1994; Hempel, 1996)

The vegetative phases of *Arabidopsis* development are characterised by the shoot apical meristem reiteratively producing rosette leaves with no internode elongation (Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Hempel 1996). The first leaves of the juvenile phase of vegetative production are small, round, and entire and show an opposite phyllotaxy. Following the physiological change from juvenile to adult vegetative development, the rosette leaves become larger, spatulate, serrated, and exhibit a spiral phyllotaxy. This change in leaf morphology also marks the point at which the plant has become competent to receive the floral stimulus (Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995). Following the perception by the apical meristem of florally inductive conditions there is an increase in apical mitotic activity and the previously flat meristem changes into a dome-shaped structure. This physical transformation characterises the production of floral primordia rather than leaf primordia (Koornneef *et al.*, 1991; Martinez-Zapater *et al.*, 1994; Hempel, 1996). Often this transition can be very rapid, with pre-existing vegetative primordia undergoing a partial conversion that results in the production of chimæric organs showing both leaf and flower characteristics (Amasino, 1996a; Hempel, 1996; Ma, 1997; Pineiro and Coupland 1998).

After the development of the first floral primordia, there is an almost immediate elongation (bolting) of the internodes of the primary shoot to form an indeterminate inflorescence meristem (Martinez-Zapater *et al.*, 1994; Haughn *et al.* 1995). This primary inflorescence (bolt) has both early developing, rosette proximal nodes, and late developing, rosette distal nodes. Early inflorescence nodes bear cauline leaves that subtend auxiliary inflorescence meristems or coflorescences. These coflorescences are very similar in morphology to the primary indeterminate inflorescence. In contrast, late inflorescence nodes bear no leaves and produce only lateral branches that bear a determinate floral meristem (Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Hempel, 1996). After bolting, the primary inflorescence initiates the production of flowers followed by a downward activation of flowering in the coflorescences (Hempel, 1996). Secondary lateral meristems can also develop in the axils of the rosettes leaves and give rise to determinate floral meristems with no associated cauline

leaves. Secondary lateral meristems do not show internode elongation until the bolting of the primary inflorescence meristem has occurred (Haughn *et al.*, 1995). The flower inflorescences of *Arabidopsis* are open raceme and produce crucifer flowers displaying a whorled phyllotaxy of floral organs (reviewed in Martinez-Zapater *et al.*, 1994).

Pathways to floral induction in Arabidopsis

The morphological, physiological and genetic analysis of *Arabidopsis* flowering time mutants and variants has produced the framework for many models of floral induction (reviewed in Martinez-Zapater *et al.*, 1994). One of the main points described in these models is that flowering in *Arabidopsis* is an autonomous process, which can be promoted or inhibited but never completely stopped. The promotion and inhibition of this process is thought to be via the action of numerous flowering time genes, involved in several different pathways, whose activity is influenced by the environment as well as the developmental state of the plant (Koornneef *et al*, 1991; Peeters and Koornneef, 1996). The presence of more than one pathway to flowering is supported by several lines of evidence; firstly, no non-flowering mutants of *Arabidopsis* have been isolated (Burn *et al.*, 1994; Koornneef *et al.*, 1991) and secondly, there are no environmental conditions that will stop flowering in this species (Koornneef *et al.*, 1991). It is also believed that flowering time pathways are redundant to some extent, as even plants containing mutations in more than one pathway will eventually flower (Koornneef *et al.*, 1991; Pineiro and Coupland, 1998). The multiple flowering pathways, as well as their redundancy, are thought to allow sufficient plasticity for *Arabidopsis* to successfully reproduce in a diverse range of environmental conditions (Amasino, 1996a).

1.2 Environmental factors affecting flowering time in *Arabidopsis*

The sessile nature of plants means that their reproductive success is tightly linked to the correct timing of floral transition (Amasino, 1996b; Pineiro and Coupland, 1998). Many plants, including *Arabidopsis* therefore utilise environmental cues such as photoperiod, light intensity, temperature, and water availability to regulate flowering time and to ensure optimal seed production and germinating conditions. Environmental factors such as these, which exhibit predictable seasonal fluctuations, not only allow plants to flower in favourable conditions, they also ensure that most individuals of a species flower synchronously - a condition necessary for successful outcrossing (Bernier *et al.*, 1993; Martinez-Zapater *et al.*, 1994). There also appears to be some redundancy in the effects that different environmental factors can have in influencing flowering time. One example is the exposure of plants to extended periods of cold, termed vernalisation, that can substitute for the required increase in far red light to promote flowering in some late-flowering mutants and ecotypes of

Arabidopsis (Bernier *et al.*, 1993). The fact that the sensitivity to some environmental factors increases with age in *Arabidopsis*, suggests that these environmental cues also act in conjunction with endogenous signals to influence flowering time (Coupland, 1995a).

Arabidopsis exhibits variation in flowering time between naturally occurring populations, a phenomenon linked to the differing sensitivity to environmental factors amongst ecotypes. Karlsson *et al.* (1993) analysed the flowering times of 32 *Arabidopsis* ecotypes in standard growth conditions, and examined their responses to short days and vernalisation treatments. All but four ecotypes showed a decrease in leaf number and flowering time in long day conditions, while only 16 showed a response to a 24-day vernalisation treatment (Karlsson *et al.*, 1993). Ecotypes that show a strong vernalisation response are generally late-flowering ones that originated from high latitudes. It is thought that the requirement for exposure to an extended period of low temperature prevents flowering in these ecotypes until the favourable conditions of spring (Koornneef *et al.*, 1991; Burn *et al.*, 1994).

1.2.1 Vernalisation

Vernalisation is the exposure of plants to extended periods of cold. In some plants, it is required for flowering, whereas in others such as *Arabidopsis*, vernalisation only promotes the transition to flowering. Temperatures used for vernalisation of *Arabidopsis* range between 1°C and 7°C for a duration of three to twelve weeks, and generally, the longer the duration of vernalisation the greater the promotion of flowering (Dennis *et al.*, 1996). In the early-flowering ecotypes, vernalisation is not believed to be essential for early-flowering unless one or more of the other proposed flowering pathways are blocked or repressed, as thought to be the case in late-flowering ecotypes (Burn *et al.*, 1993a; Finnegan *et al.*, 1998). In ecotypes that flower late, it is thought that vernalisation and long day photoperiods may act additively to promote flowering. This was proposed following the observation that vernalisation is often not sufficient to overcome the delay in flowering observed in plants grown under short days (Amasino, 1996a).

The vernalisation signal is thought to be perceived directly by the shoot apical meristem where floral structures will ultimately form. This hypothesis is supported by the observations that vernalisation treatments that included the shoot apex of the plants resulted in the promotion of flowering, while vernalisation of only leaf tissue had no floral promotory effects (Wellensick, 1964; Burn *et al.*, 1994). The promotion of flowering by vernalisation has been proposed to occur by either increasing the levels of floral stimulating signals produced by the plants, or by increasing the sensitivity of the apical meristem to such factors (Bagnall, 1992; Amasino, 1996a).

In late-flowering ecotypes of *Arabidopsis*, low temperature treatments are effective in decreasing the flowering time of actively growing plants, although the magnitude of the response is greater when imbibed seed or very young plants are treated (Martinez-Zapater *et al.*, 1994). No such promotion of flowering is observed for plants grown from vernalised dry seed (Burn *et al.*, 1994). Vernalisation has also been shown to induce floral budding on mitotically active leaf or root explants from several species, whereas leaf explants from fully differentiated tissue show little response to such treatments. It is therefore proposed that vernalisation is only effective on tissues actively undergoing mitotic cell division (Burn *et al.*, 1993a; Burn *et al.*, 1994). The cold-induced state is stably inherited in the mitotic descendants of exposed apical cells, however the requirement for cold treatments to promote flowering is reset in each sexual generation (Burn *et al.*, 1994; Amasino, 1996a; Dennis *et al.*, 1996).

Methylation and Vernalisation

The epigenetic properties of vernalisation, such as the exclusively mitotic inheritance of the response, show similarities to the control of gene expression by methylation (Burn *et al.*, 1994). Methylation of residues within the promoter region, or even the coding sequence, can inhibit the transcription of a gene by preventing binding of transcription factors to the DNA (Burn *et al.*, 1993; Burn *et al.*, 1994; Finnegan *et al.*, 1996; Finnegan *et al.*, 2000). In plants, methylation is often associated with cytosine residues, and the methylation pattern of a DNA sequence is initiated and maintained during mitosis via methyltransferase proteins (Dennis *et al.*, 1996).

It has been proposed that genes critical for reproductive development may be methylated, and that cold treatment can cause demethylation and subsequent expression of these genes, leading to a promotion of flowering in vernalised plants (Burn *et al.*, 1993a, Burn *et al.*, 1994; Finnegan *et al.*, 1998). It is assumed that such demethylated genes are subsequently protected from re-methylation by bound transcription factors, or an altered chromatin structure (Finnegan *et al.*, 1998). Cold-induced demethylation of genes may occur during cell division by the uncoupling of DNA replication and maintenance methylation. The phenomenon of the effectiveness of vernalisation increasing over time may therefore be due to the larger numbers of cells adopting the demethylated vernalised state in successive generations (Dennis *et al.*, 1996; Finnegan *et al.*, 1998). It has further been postulated that the resetting of methylation patterns during gametogenesis or embryo development, may explain the requirement for subsequent cold treatments to promote flowering in the progeny of treated plants (Dennis *et al.*, 1996; Finnegan *et al.*, 1998).

Several lines of evidence support the involvement of methylation in the vernalisation response. Firstly, it has been observed from the restriction of DNA by methylation-sensitive enzymes that methylation levels decrease in vernalised plants compared to controls. In addition, methylation levels have been shown to increase in vernalised plants if they are transferred to higher than normal growing temperatures (Dennis *et al.*, 1996). Secondly, treatment of *Arabidopsis* plants with the demethylating agent 5-aza-cytidine, resulted in the promotion of flowering in two late-flowering, vernalisation-responsive, *Arabidopsis* ecotypes (Burn *et al.*, 1993a). This flowering time response however, was significantly less than that shown by plants given a vernalisation treatment of 21 days at 4°C. It has also been demonstrated that late-flowering mutants of early-flowering ecotypes that show a vernalisation response also exhibit a decrease in their flowering time following exposure to 5-aza-cytidine. No such response was observed in vernalisation-unresponsive mutants, suggesting that demethylation is specifically involved in cold-induced flowering (Burn *et al.*, 1993a; Dennis *et al.*, 1996, Finnegan *et al.*, 1998). The fact that 5-aza-cytidine and vernalisation both require cell division to be effective, and the responses to them are both mitotically but not meiotically inherited, generates further support for a role of methylation in vernalisation (Burn *et al.*, 1993a).

In an attempt to establish a definite causal link between demethylation and the promotion of flowering by vernalisation, plants transformed with an antisense methyltransferase sequence and DNA methylation mutants have been examined (Finnegan *et al.*, 1998). The antisense work utilised plants of the C24 ecotype of *Arabidopsis* that contained a 2.8 kb antisense fragment of the *MET1* (*Methyltransferase 1*) gene. This fragment spanned the entire coding region of the gene and part of the 3′ untranslated region. In short day conditions, which normally delay flowering, the antisense plants showed an early-flowering phenotype without the need for cold treatment. Furthermore, it was observed that the promotion of flowering seen in three transgenic lines grown under short day conditions, positively correlated with lower levels of DNA methylation (Finnegan *et al.*, 1998). Even when a 90% reduction in methylation was observed in transgenic plants however, the promotion of flowering was still only 70% of that observed in plants given a 4°C vernalisation treatment for 23 days (Dennis *et al.*, 1996; Finnegan *et al.*, 1998).

While this analysis strongly suggested that DNA methylation is integral to a vernalisation response, several aspects demonstrated that the cold-induced promotion of flowering was not solely due to decreased levels of the *MET1* gene (Finnegan *et al.*, 1998). Early-flowering antisense plants for example, still displayed a response to vernalisation, suggesting that demethylation does not fully compensate for vernalisation treatments (Dennis *et al.*, 1996; Finnegan *et al.*, 1998). Furthermore, the presence of an antisense *MET1* transcript in the progeny of antisense lines did not change the requirement of these plants for

cold treatment to induce earlier flowering in short days (Finnegan *et al.*, 1998). In addition, some antisense lines exhibited homeotic transformation of floral organs, ectopic expression of other floral genes, smaller rounded leaves, reduced fertility, a decrease in apical dominance, and increased branching in the root systems. The severity of these traits increased with lower levels of methylation. Such abnormalities have never been observed following vernalisation treatments (Burn *et al.*, 1993a; Finnegan *et al.*, 1996).

Arabidopsis ddm1 (*decreased DNA methylation*) mutants, which flower earlier in short days without vernalisation, have also been used to study links between methylation and vernalisation (Finnegan *et al.*, 1998). The *DDM1* gene maps close to the flowering time gene *FWA* which is situated in a hypomethylated five Mbp region of chromosome one (Kakutani *et al.*, 1995; Finnegan *et al.*, 1998). Plants homozygous for *ddm1* mutant alleles in an early-flowering Columbia background, exhibit a 30% decrease in cytosine methylation of both CG and CNG nucleotide sites (Kakutani *et al.*, 1995). The *DDM1* gene product has since been cloned and found to encode a member of a family of chromatin remodelling proteins and act as a component of the machinery that directs the localisation of DNA methyltransferases (Kakutani, 1997; Jeddeloh *et al.*, 1999; Finnegan *et al.*, 2000). In a similar manner to the *MET1* antisense plants, *ddm1* mutants flower earlier than wild-type plants in short days, however when grown for several generations in long days these lines flower successively later than wild-type plants. This latter phenotype is thought to be due to the segregation of different demethylated sites in the progeny (Finnegan *et al.*, 1998). The *ddm1* mutants also exhibit several pleiotrophic morphological abnormalities, such as rounded rosette leaves and an increased number of cauline leaves, that progressively increase in severity over several generations (Kakutani, 1997). These phenotypes, like those displayed by *MET1* antisense lines, are not seen in vernalised plants, and are probably due to a general demethylation of genes in *ddm1* mutants and *MET1* antisense lines (Finnegan *et al.*, 1998).

Gibberellins and Vernalisation

It has been demonstrated that gibberellic acid (GA) can promote flowering in several late-flowering mutants of *Arabidopsis* when grown under non-inductive conditions. These mutants show a positive correlation between flowering time response and either vernalisation or exogenous GA application. In addition, inhibitors of GA can block floral initiation even after cold induction, suggesting an important role for GA in vernalisation. Therefore it has been proposed that vernalisation may act by removing a methylation block in the GA biosynthetic pathway and that this results in a GA-mediated promotion of flowering (Dennis *et al.*, 1996). One such gene suggested to be demethylated and hence activated via cold treatment encodes kaurenoic acid hydroxlase (KAH) which is the rate-limiting enzyme in

gibberellin biosynthesis. Support for this theory comes from the observations that vernalised *Thalaspi arvesi* plants, a close relative of *Arabidopsis*, show an increase in the metabolism and turnover of kaurenoic acid in shoot tips. In addition, an increase in KAH activity and a corresponding decrease in kaurenoic acid levels has been observed in shoot tips but not in leaves, of vernalised *Arabidopsis* plants (Burn *et al.*, 1993a; Haughn *et al.*, 1995; Dennis *et al.*, 1996).

Vernalisation Mutants

In 1996, Chandler *et al.* identified five recessive mutations in the late-flowering, vernalisation-responsive *fca* mutant background that conferred a reduced vernalisation response. The mutations fell into three complementation groups defining three loci, *VRN1*, *VRN2*, and *VRN3*. The *vrn2-1* mutation resulted in the complete loss of any vernalisation response in the *fca* background. This mutation also caused delayed flowering of non-vernalised *fca* mutants however, suggesting a general role for *VRN2* in promoting flowering. The *vrn1-1* mutation reduced the vernalisation responsiveness of plants containing *fca* mutations by only 42%, and did not dramatically influence the flowering time of unvernalised *fca* plants. The *vrn1-1* mutation has also been introgressed into other late-flowering, vernalisation-sensitive mutants, such as the *fve*, *ld*, *fwa*, *fe*, *fpa*. and *ft* lines. This introgression again resulted in the partial loss of vernalisation responsiveness in all lines. It is not clear whether this incomplete suppression of the vernalisation response is due to the *vrn1-1* allele not representing a null mutant, or that the *VRN1* gene has a partially redundant function within a vernalisation-dependent floral pathway (Chandler *et al.*, 1996; Wilson and Dean, 1996).

The *vrn1-1* mutation has also been shown to decrease the vernalisation responsiveness of wild-type Landsberg *erecta* plants grown in short day photoperiods. The vernalisation-dependent flowering pathway, utilised for early-flowering in short days by early-flowering ecotypes, has therefore been suggested to be similar to that uncovered in the vernalisation responsive late-flowering mutants (Chandler *et al.*, 1996; Wilson and Dean, 1996). Interestingly, *vrn1-1* mutants and wild-type L. *erecta* plants grown in short days both displayed a similar acceleration in their flowering time in response to exogenous gibberellin application. This implies that gibberellins and vernalisation may act in separate pathways or alternatively, that gibberellin-mediated action is downstream of the *vrn* mutation in the same pathway. The latter hypothesis is supported by the fact that *gal-3* mutants which are defective in the gene involved in the first committed step of gibberellin biosynthesis, display no response to vernalisation when grown in short days (Chandler *et al.*, 1996; Wilson and Dean, 1996).

1.2.2 Photoperiod, light quality and intensity

For photoautotrophic organisms like plants, light has two major roles. Firstly, it provides a source of energy for the production of sugars via photosynthesis. Secondly, the intensity, quality and duration of light modulates a wide range of light-induced developmental processes, that are collectively known as photomorphogenesis (Deng *et al.*, 1992; Araki and Komeda, 1993a; Elich and Chory, 1994; Whitelam and Devlin, 1997). Responses controlled by light in plants include seedling germination and de-etiolation, the regulation of later vegetative development, and the onset of flowering (Whitelam *et al.*, 1998; Whitelam and Devlin, 1997). Indeed, day-length is thought to be one of the most influential environmental factors affecting flowering time in many species (Mozley and Thomas, 1995).

The majority of physiological analyses of the flowering time responses to day-length have been performed using photoperiod responsive species that can be induced to flower following exposure to a single day of an inductive photoperiod. These species include the short-day plant *Pharbitis nil* and long-day plants such as *Sinapis alba* and *Lolium temulentum* (reviewed in Bernier *et al.*, 1993). These studies have indicated that light quality, intensity, and duration is most likely to be perceived in the leaves, and depending on the light detected, floral promotory and inhibitory signals are transported to the apex of plants via the phloem (Bernier *et al.*, 1993; Martinez-Zapater *et al.*, 1994; Amasino, 1996a). The biochemical nature of such signals remains unclear (Bernier *et al.*, 1993). Plants at the appropriate developmental age may then initiate flower development within a few hours of exposure to inductive conditions. This rapid response suggests that signals produced in the leaves act to stimulate the pre-existing primordia at the apex to form floral structures (Corbeseir *et al.*, 1998; Onouchi and Coupland, 1998).

The influence of day-length on flowering time

Short-day plants will initiate flowering when the dark phase of the photoperiodic cycle is greater than a critical length. Photoperiodic regulation is more complex in long-day plants that flower when the light period is greater than a particular length. As mentioned previously, most ecotypes of *Arabidopsis* behave as facultative long-day plants, initiating flowering earlier in long days (14-to-16 hours) or continuous light, than in short days (8-to-10 hours) (Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Onouchi and Coupland, 1998). The L. *erecta* ecotype for example, flowers after the production of four rosette leaves if grown in a 16 hours light/8 hours dark cycle, and after 19 leaves have formed when grown in a 10 hours light/14 hours dark cycle (Onouchi and Coupland, 1998). All ecotypes of *Arabidopsis* will eventually flower however, even if grown in a minimum day-length of 4-to-5 hours (Martinez-Zapater *et al.*, 1994).

While all ecotypes of *Arabidopsis* show a promotion of flowering by long days, their requirement for such conditions can differ. In general, early-flowering natural populations from subtropical regions show less inhibition of flowering by short days than later flowering ecotypes from higher latitudes (Martinez-Zapater *et al.*, 1994). It has also been observed however, that the L. *erecta* line, itself derived from the early-flowering Columbia ecotype, is much less sensitive to photoperiod than wild-type Columbia (Corbeseir *et al.*, 1998). In addition, when Kakutani *et al.* (1995) examined the rosette leaf number at the time of flowering of 32 different ecotypes in response to different photoperiods, all ecotypes examined showed a long day promotion of flowering, however two ecotypes, TUL-O and SY-O, did not flower even after 110 days in long days (Kakutani *et al.*, 1995). This may suggest that these ecotypes have a decreased sensitivity to, or perception of, long day inductive conditions than other ecotypes.

In 1995, Mozely and Thomas performed a more comprehensive study of the effects of different photoperiods on the flowering time of L. *erecta* plants. A critical day-length of 10 hours was initially established as the requirement for early flowering, as plants subjected to 10-to-12 hours light still flower earlier than plants subjected to 8-to-10 hours light. The researchers also examined the flowering times and leaf numbers at flowering of plants switched from short days to long days at varying time intervals. As plants grown in short or long photoperiods for the first two days post-germination and subsequently transferred to the long days, flower at the same time, the researchers concluded that the presence of leaf tissue is required before a response to day-length can be observed. They also noted that short day exposure for 4-to-5 days post-germination delays seedling development, as such plants displayed delayed flowering but no increase in rosette leaf number at the time of flowering. It was further established that the 8-to-12 day post-germination period may represent a transitional interval where responsiveness to long days is progressively lost. Transfer of plants to long days after 55 days in short days has little effect on the flowering time of L. *erecta* plants (Mozely and Thomas, 1995). The authors have also established that the minimum number of long days needed to fully induce flowering in plants previously grown in short days, decreases as the age of the plants increases. In addition, it has been observed that 30-day old L. *erecta* plants grown in short days will initiate floral, instead of leaf primordia, within a few hours of being shifted to long days. Furthermore, even a transient exposure to long days irreversibly commits these plants to flower (Onouchi and Coupland, 1998; Pineiro and Coupland, 1998). In combination, these results confirm that there is a developmental 'window' in which plants that are vegetative in short days can be induced to flower by exposure to long days, prior to a time when flowering becomes autonomously induced (Bagnall, 1992; Mozely and Thomas, 1995). In 1996, Corbesier *et al.* also established that 45-to-60-day old Columbia plants, that were given a six week vernalisation treatment and grown in short days with low irradiance, can be induced to flower following

exposure to a single long day. This precise system of induction for *Arabidopsis*, in which non-induced plants stay vegetative for as long as possible, allows the examination of the expression of genes or proteins induced following exposure to inductive photoperiods and before flowers are initiated (Corbesier *et al.*, 1996).

In the physiological studies as described above, it is important to reduce the effect that sugar availability may play in controlling flowering time of plants grown in short days as opposed to long days (King and Bagnall, 1996). Researchers have therefore employed several methods to maintain a constant level of photosynthesis in different growth conditions. These have included growing plants in media containing sucrose, and altering the spectral quality and quantity of light during extensions of the short day photoperiod (Bagnall *et al.*, 1995; King and Bagnall, 1996).

Influences of the spectral quality of light and light intensity on flowering time in Arabidopsis

While photoperiod is probably the most influential light-related factor controlling flowering time in *Arabidopsis*, the spectral quality of the light received during these photoperiods is also important (Martinez-Zapater *et al.*, 1994). Several ecotypes of *Arabidopsis* have been shown to flower earlier when given long days of monochromatic far-red light rather than white light, while those given only red light flower later. This suggests that red light is inhibitory to flowering while far-red light appears to be promotive, although this response can vary depending on the developmental age of the plants (Eskin, 1992; Martinez-Zapater *et al.*, 1994; Mozely and Thomas, 1995; Amasino, 1996a). In addition, several researchers have shown that plants grown in continuous blue light flower earlier than those grown in continuous red light (Mozely and Thomas, 1995; Amasino, 1996a; King and Bagnall, 1996; Guo *et al.*, 1998). It has also been demonstrated that plants grown in long days and given pulses of blue or far-red light during the night periods, flower earlier than those given pulses of red light during the dark cycle (Martinez-Zapater *et al.*, 1994). The relative levels of wavelengths of light received by a plant throughout the day are also thought to be important in controlling flowering time. Several *Arabidopsis* ecotypes for example, flower earlier in response to low red to far-red ratios across a wide range of different light intensities (Johnson *et al.*, 1994; Martinez-Zapater *et al.*, 1994). Columbia and L. *erecta* plants grown in low irradiance conditions both display delayed flowering times, with Columbia plants being more sensitive. This indicates that the light intensity during inductive photoperiods is also influential in controlling the timing of floral induction (Corbesier *et al.*, 1996).

Perception of light via phytochrome

A number of different photoreceptors in *Arabidopsis* act to translate light into biochemical signals, that are subsequently transduced through several pathways, to in turn modulate growth and development. The main photoreceptor in *Arabidopsis*, phytochrome, is thought to be the primary photoreceptor involved in the perception of inductive dark periods and mediates blue, red, and far-red light responses (Coupland, 1995a; Amasino, 1996a; King and Bagnall, 1996; Whitelam and Devlin, 1997; Casal *et al.*, 1998).

Phytochromes exist as dimers of two identical 124 kDa polypeptides and are localised in the cytosol. Each of the subunits contains a covalently linked tetrapyrrole chromophore, phytochromobilin, attached to a conserved cysteine residue in the N-terminal domain of the phytochrome protein (Quail *et al.*, 1995; Whitelam *et al.*, 1998). These phytochrome molecules can exist in two photoreversible forms; a far-red light absorbing form (P_{fr}), with an absorption maximum at 730nm, and a red light absorbing form (P_r), with an absorption maximum at 660nm. There is a large overlap however, in the absorption spectra of P_{fr} and P_r in the visible spectrum (Amasino, 1996a; Whitelam *et al.*, 1998). Conformational interconversion between the P_r and P_{fr} forms is thought to involve isomerisation of the tetrapyrrole chromophore (Guo *et al.*, 1995). It is believed that phytochrome is initially synthesised in the red-light absorbing form. The photosensory function of phytochrome may involve the reversible conversion between this inactive P_r form to the biologically active P_{fr} form, following the absorption of red light (Casal *et al.*, 1998). Any phytochrome-mediated physiological responses initiated by red light exposure can be nullified by subsequent exposure of the plants to far-red light, which causes an almost complete reversion of P_{fr} into P_r (Amasino, 1996a; Chory *et al.*, 1996). The red to far-red light ratio to which plants are exposed can therefore determine the relative amounts of phytochromes existing in the active P_{fr} form and the types of responses initiated (King and Bagnall, 1996).

Arabidopsis has multiple discreet species of phytochrome apoproteins (A-E) which are encoded by a small family of five genes (*PHYA-E*), all of which have now been cloned (Amasino, 1996a; King and Bagnall, 1996; Whitelam and Devlin, 1997; Whitelam *et al.*, 1998). While all of these phytochromes are present in plants grown in both light and dark conditions, the various apoproteins can be differentially expressed (King and Bagnall, 1996; Whitelam and Devlin, 1997). The *PHYA* gene encodes a type I phytochrome protein that is light-labile, and is the most abundant phytochrome found in dark-grown etiolated seedlings. The other four genes (*PHYB-E*) encode type II phytochromes, which are light stable, and are the predominant forms detected in illuminated plants. The most abundant of these type II phytochromes is the PHYB apoprotein (Guo *et al.*, 1995; Amasino, 1996a; Whitelam and Devlin, 1997; Casal *et al.*, 1998; Whitelam *et al.*, 1998). Domain-swapping experiments

have also established that, at least for the PHYA and PHYB proteins, the carboxy terminus contains regulatory elements that are functionally similar. Residues within the amino terminus however, have been identified as specifying the functional differences between the two types (I and II) of phytochrome (Quail *et al.*, 1995; Guo *et al.*, 1995; Neff and Chory, 1998; Whitelam *et al.*, 1998).

The roles of the various phytochromes and components of their downstream signal transduction pathways, during flowering, have been extensively studied via mutant analysis and overexpression studies. Plants containing mutations within phytochrome genes were originally identified by examining the hypocotyl phenotype of mutated seedlings grown in white light. When grown in the dark, *Arabidopsis* seedlings normally display a light-foraging phenotype that involves an elongation of the hypocotyls, as well as an arrest in the developments of leaves and chloroplasts. When the seedlings are exposed to light, hypocotyl elongation ceases (de-etiolation) and the induction of leaf and chloroplast development begins (Pepper and Chory, 1997; Devlin *et al.*, 1998; Whitelam *et al.*, 1998). Phytochrome mutants were found to show an elongated hypocotyl phenotype in the light, and these mutants were collectively termed *hy* mutants. The specific phytochrome deficiencies associated with each *hy* mutant have since been determined. The *hy1* and *hy2* mutants are disrupted in the synthesis of the tetrapyrrole chromophore common to all phytochromes, and are therefore deficient in all forms of photoconvertable phytochrome (Whitelam *et al.*, 1998). *hy3* mutants appear to be specifically disrupted in the production of the PHYB apoprotein, while the *hy8* mutants have been shown to be deficient in the PHYA apoprotein (Mozely and Thomas, 1995). The *hy4* mutation is thought to cause disruption to the production of a blue light receptor (Johnson *et al.*, 1994). Plants containing mutations in the *HY5* gene, which encodes a basic leucine zipper transcription factor, are deficient in all red, far-red and blue light responses, suggesting that the mutated gene may act downstream of the photoreceptor molecules (Pepper and Chory, 1997). Many of these *hy* mutants, as well as transgenic plants overexpressing different phytochrome apoproteins, also exhibit altered flowering times when exposed to different spectral qualities of light. The pleiotrophic morphological and physiological alterations displayed by such plants however, may suggest that the flowering time effects seen in these plants are not always directly related to altered phytochrome levels (King and Bagnall, 1996; Whitelam and Devlin, 1997).

Analyses of plants defective in the *PHYA* gene have revealed that far-red light perception appears to be a major role of this locus, as the far-red light-induced early-flowering response is absent in *phyA* mutants (Chory *et al.*, 1996; Whitelam *et al.*, 1998). In support of this hypothesis, *phyA* mutants also show a germination deficiency when given both brief and prolonged exposure to far-red light, and show little or no de-etiolation in far-red light (Johnson *et al.*, 1994; Reed *et al.*, 1994; Whitelam and Devlin, 1997; Casal *et al.*, 1998;

Devlin *et al.*, 1998). It is also believed that PHYA acts to promote flowering by the detection of far-red light in photoperiod extensions, as *phyA* mutants flowered up to three weeks later than controls when grown in short days with end-of-day extensions (Johnson *et al.*, 1994; Chory *et al.*, 1996; Whitelam and Devlin, 1997; Whitelam *et al.*, 1998). The role of *PHYA* in the perception of inductive day length extensions is further confirmed from the observations that transgenic plants over-expressing *PHYA* show accelerated flowering and day-length neutrality (King and Bagnall, 1996; Whitelam *et al.*, 1998). *phyA* mutants display only a slight increase in flowering times compared to controls in either continuous light, long days, or short days photoperiods, indicating that the perception of day-length *per se* is not dependent on PHYA. Therefore, while *PHYA* may be important in the detection of far-red enriched end-of-day extensions, it may only play an important but minor role in the detection of high fluence long days (Johnson *et al.*, 1994; Reed *et al.*, 1994; Whitelam *et al.*, 1998).

phyB mutants are early-flowering in both short and long days, and continuous light. This early-flowering phenotype is believed to be partially due to the constitutive expression of a shade-avoidance response (Whitelam *et al.*, 1998). The shade-avoidance response of wild type *Arabidopsis* is associated with an elongation of internodes and petioles, a reduction in leaf growth, an increase in apical dominance, and an acceleration of flowering; responses all designed to increase the height of the plant (Casal *et al.*, 1998; Devlin *et al.*, 1998; Whitelam *et al.*, 1998). Light reflected from vegetation is richer in far-red light due to the absorbance of red light by chlorophyll, resulting in a high ratio of far-red to red light in shaded areas. In wild type plants, this higher ratio is thought to be important in altering the relative levels of PHYB (P_{fr}) and PHYB (P_r) within the plant, which in turn may mediate the initiation of the shade avoidance response. A disruption in the *PHYB* gene may therefore be related to the continual expression of a shade-avoidance response observed in *phyB* mutants, even in normal fluences of white light (Halliday *et al.*, 1994; Whitelam and Devlin, 1997; Devlin *et al.*, 1998; Whitelam *et al.*, 1998).

The early-flowering phenotype of *phyB* mutants suggests that the wild-type PHYB (P_{fr}) protein may, in contrast to PHYA (P_{fr}), act to inhibit flowering, unless high shade conditions are detected (Johnson *et al.*, 1994; Reed *et al.*, 1994; Devlin *et al.*, 1998). In addition, *PHYB* is thought to play a role in the perception of red rather than far-red light, as *phyB* mutants appear insensitive to brief or prolonged exposure to red-light and respond relatively normally to far-red and blue light (Casal *et al.*, 1998; Devlin *et al.*, 1998; Whitelam *et al.*, 1998). As *phyB* mutants do exhibit a day-length response, *PHYB*, like *PHYA*, may have a limited role in photoperiod detection (Johnson *et al.*, 1994; Whitelam and Devlin, 1997; Whitelam *et al.*, 1998). One mode of action for PHYB suggests that its P_{fr} form may antagonise the transition to flowering by promoting vegetative development. Genetic and

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environmental conditions that decrease *PHYB* levels therefore lead to early flowering without affecting the photoperiod response of plants (Mozely and Thomas, 1995).

Overexpression of *PHYB* in transformed plants causes an enhancement of the promotion of flowering caused by red-light enriched end-of-day treatments. It was shown however, that this response is limited to transformants exhibiting a 10 times or greater increase in the level of *PHYB* expression. Furthermore plants with greater phytochrome contents flowered earlier in white light and all overexpression lines flowered later than controls when given low fluence end-of-day extensions. In support of these observations Wester *et al.* (1994) reported that when wild-type *Arabidopsis* were transformed with a complete *PHYB* cDNA sequence, fused to 2.3 kb of upstream promoter region, the responsiveness of transformed plants to light intensities is positively correlated with *PHYB* copy number (Wester *et al.*, 1994; King and Bagnall, 1996). These results indicate a role for *PHYB* in regulating flowering in response to different intensities of light in end-of-day extensions, rather than to light received throughout the normal light cycle (King and Bagnall, 1996).

It has also been postulated that neither *PHYA* nor *PHYB* alone regulates light-induced processes such as flowering, and that the regulation of such responses is instead dependent on altered levels of the biologically active P_{fr} form of any phytochrome (Wester *et al.*, 1994, King and Bagnall, 1996). In support of this theory the *hy1* and *hy2* mutants, which are deficient in all forms of phytochrome, are early-flowering and show no end-of-day treatment response (Halliday *et al.*, 1994; Kim *et al.*, 1996; King and Bagnall, 1996). In addition, *phyA phyB* double mutants still show a promotion of flowering in response to low red to far-red ratios, or end-of-day far-red pulses, a phenotype that can be reversed by subsequent exposure to red light. This indicates that the actions of other phytochromes are controlling these responses in these lines (Whitelam and Devlin 1997).

The *PHYD* and *PHYE* genes encoding PHYD and PHYE apoproteins, have recently been isolated and may mediate the phytochrome responses seen in *phyA phyB* double mutants (Devlin *et al.*, 1996; Whitelam and Devlin 1997; Whitelam *et al.*, 1998). *PHYD* is believed to have a similar role to *PHYB* in initiating shade avoidance responses as *phyB phyD* double mutants show an additive phenotype (Devlin *et al.*, 1999). The presence of the *PHYE* gene was suggested following observations that *phyA phyB phyD* triple mutants still exhibit early-flowering in response to far-red light enriched end-of-day extensions (Devlin *et al.*, 1998; Whitelam *et al.*, 1998). It was also noted that *phyE* single mutants and *phyA phyE* double mutants had similar phenotypes to wild-type plants and *phyA* single mutants respectively, whereas *phyE phyB* double mutants flower even earlier than *phyB* single mutants. Therefore, similarly to *phyD*, the effects of the *phyE* mutation are only seen in the *phyB* background. As the *phyD* and *phyE* mutations do not cause earlier flowering unless in

combination with *phyB*, a major inhibitory role in floral induction has been suggested for *PHYB*. The relatively minor roles of *PHYE* and *PHYD* in repressing flowering then become more important in the absence of *PHYB*. The high degree of sequence similarity between *PHYB*, *PHYD*, and *PHYE* supports the theory that these genes may have partially redundant functions in controlling flowering time in *Arabidopsis* (Devlin *et al.*, 1998; Whitelam *et al.*, 1998).

The role of downstream signalling components of phytochrome in controlling flowering time

Although little is known about the types of signalling molecules that directly interact with phytochrome in *Arabidopsis*, signalling mechanisms of phytochromes are thought to involve phosphorylation of downstream molecules. This has been suggested as the C-terminus of phytochrome shows sequence similarity to histidine kinase catalytic domains, and contains an ATP binding site (Elich and Chory, 1994; Chory *et al.*, 1996). Several mutants containing defects in genes known to act one or two steps downstream of phytochrome have been characterised however, and several of these lines have been shown to have altered flowering times (Elich and Chory, 1994).

The *DEETIOLATED (DET)*, *CONSTITUTIVE PHOTOMORPHOGENIC (COP)* and *FUSCA (FUS)* genes are widely studied phytochrome signal transduction mutants (Chory *et al.*, 1989; Deng *et al.*, 1991; Castle and Meinke, 1994). Currently, sixteen mutant alleles of this group of genes have been identified. Mutations within these loci cause plants to exhibit the developmental characteristics of light-grown plants when grown in complete darkness, such as flowering. Plants homozygous for ten of these 16 mutant alleles (*det1*, *cop1*, *cop9*, *fus4*, *fus-5*, *fus6*, *fus8*, *fus9*, *fus11*, and *fus12*) display seedling lethality, suggesting that the genes affected are essential for the development of plants in both light or dark conditions (Chory *et al.*, 1996). Mutations in two other genes, *DWARFISM* (*cpd* mutants) and *EMBRYO DEFECTIVE* (*emb* mutants), cause similar phenotypes to the *det*, *cop*, *fus* mutants, and the gene products of these five loci are thought to act in common signal transduction pathways (Chory *et al.*, 1996).

The wild-type functions of all of these loci are thought to involve either the suppression of light-regulated genes in the dark, including those promoting or repressing flowering in response to various light conditions, or the promotion of genes required for dark development (Chory *et al.*, 1989; Deng *et al* 1991; Quail *et al.*, 1995; Chory *et al.*, 1996; Pepper and Chory, 1997). In support of the former hypothesis *det1-1* mutants are insensitive to day-length, flowering at the ten leaf stage in both long and short days, suggesting that flowering is actively repressed in short days to some extent by the *DET1* locus (Chory *et al.*, 1989; Pepper and Chory, 1997). It is also believed that light-regulated

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genes can be de-repressed when plants are again exposed to light, via the activation of other genes in phytochrome signalling pathways such as the *FHY1*, *FHY3*, *PEF* and *RED1* loci (Halliday *et al.*, 1994; Johnson *et al.*, 1994; Ahmed and Cashmore, 1996; Casal *et al.*, 1998; Whitelam *et al.*, 1998).

Other genes that may also affect the transcription of light-regulated genes, including those affecting flowering, have been identified following a screen for mutants that rescue some of the phenotypes of phytochrome, or phytochrome signal transduction mutants. The *ted1* (for reversal of the *det* phenotype) mutations for example, fully restore the etiolation response of *det-1* seedlings grown in the dark, and partially restore a day-length-sensitive flowering response of these mutants (Pepper and Chory, 1997). This suggests a role for the *TED1* locus in the promotion of processes *DET1* may inhibit, such as the repression of light-regulated flowering time genes. In addition, *shy1-1* mutants in the *SHY1* (*SUPRESSOR OF HY*) locus can suppress the elongated hypocotyl and early-flowering phenotypes of the *hy2* mutants. As treatment of *shy* mutants with a precursor of the phytochrome chromophore, bilidervin, has no effect on the mutant phenotype such mutations are thought to act not by restoring the level of the phytochrome chromophore, but rather downstream of the chromophore synthesis in a signal transduction pathway (Kim *et al.*, 1996). One mode of action for the *SHY* loci has since been proposed to involve the inhibition of the perception, or transduction, of a positive signal for photomorphogenesis produced via the P_{fr} form of phytochrome in the absence of light (Kim *et al.*, 1996; Kim *et al.*, 1998).

Perception of light via cryptochrome

Another role of phytochrome is believed to be in the perception of blue light, which also promotes flowering in *Arabidopsis* (Haughn *et al.*, 1995). As with many other plant species *Arabidopsis* also possesses specific blue light receptors that may act synergistically or additively with phytochrome (Coupland, 1995b; Bagnall *et al.*, 1996; Cashmore, 1998; Whitelam *et al.*, 1998). Blue light receptors are collectively termed cryptochromes and in *Arabidopsis* are encoded by two genes, *CRY1* and *CRY2*. *Arabidopsis* cryptochromes proteins exhibit sequence similarity to a group of flavoproteins, known as photolyases, which mediate repair of pyrimidine dimers generated by UV-B light. This repair involves a redox reaction between pyrimidine and $FADH_2$ that is mediated by the photolyases only in response to light (Bagnall *et al.*, 1996; King and Bagnall, 1996; Cashmore, 1998). CRY1 and CRY2 are not classed as a photolyases however, as they lack DNA repair activity. Unlike photolyases they contain unique and gene specific C-terminal extensions important for light detection (Cashmore, 1998). Chimæric proteins of CRY1 and CRY2 have also demonstrated that the domains of the two proteins are functionally interchangeable, suggesting they have similar modes of action (Cashmore, 1998; Guo *et al.*, 1998).

The *hy4* mutant is now called *cry1* as it appears deficient in the CRY1 protein and shows an acceleration in flowering only in response to night breaks of red or white-light, and not blue light (Bagnall *et al.*, 1996). When this mutation is expressed in the *L. erecta* background there is little difference in the flowering time of the mutants compared to wild-type controls, except in conditions in which a blue light receptor response is required. When the mutation is introgressed into the Columbia ecotype however, the flowering time of mutants increases compared to controls if day-length extensions are given using far-red light deficient lamps. The difference in the expression of the mutant phenotype in L. *erecta* and Columbia may be attributable to the differential expression of the flowering time gene, *FLC*, in these ecotypes (Bagnall *et al.*, 1996; Cashmore, 1998).

The *CRY2* gene is also postulated to play a role in floral induction, as *cry2* mutants flower later than wild-type plants in long days, and slightly earlier than controls in short days. This suggests that these mutants are partially disrupted in processes influencing the photoperiodic regulation of flowering (Guo *et al.*, 1998). Interestingly, overexpression of *CRY2* causes a slight decrease in the flowering time of transgenic plants grown in short days, and results in little difference in the flowering time of such plants grown in long days (Guo *et al.*, 1998). Plants heterozygous for the *cry2* mutant allele, while still exhibiting a decrease in *CRY2* mRNA levels, show little if any difference in their flowering time compared to controls. This suggests only the complete loss of *CRY2* activity affects the timing of reproductive development (Guo *et al.*, 1998). As *cry2* mutants were only found to flower later in white light, or in a combination of blue and red light, but not in continuous blue light, it was hypothesised that cryptochrome may affect a blue-light dependent inhibition of phytochrome, which itself causes a red light mediated inhibition of flowering (Guo *et al.*, 1998). Hence, only in the absence of *CRY2* expression will plants flower late when exposed to red light (Cashmore, 1998; Guo *et al.*, 1998). Further support for this proposed inhibitory hierarchy comes from the observation that the *phyB* mutant background suppresses the *cry2* phenotype in blue and red light, however *cry2* cannot repress the early-flowering phenotype of *phyB* mutants in red light (Guo *et al.*, 1998).

The *CRY2* gene and the flowering time gene, *FHA*, map to the same region of chromosome one. In addition, late-flowering *fha* mutants have now been shown to contain a missense mutation within the *CRY2* locus (Guo *et al.*, 1998). Characterisation of the *fha* mutants has shown that the *CRY2* locus may be involved in a long day floral promotory pathway. In support of this proposal, mRNA levels of the flowering time gene *CO*, whose stronger expression in long days is thought to promote flowering, are decreased in *cry2* mutants grown in long days but not in those grown in short days. The decreased expression of CO in these mutants may also explain the late flowering phenotype exhibited by such plants in long days. Furthermore, it has been shown that overexpression of *CRY2* in transgenic

plants results in an increase in the levels of *CO* detected in short-day grown plants, and it is thought that this may contribute to their slightly earlier flowering phenotype (Guo *et al.*, 1998). It has therefore been suggested that *CRY2* is a positive regulator of *CO* in a long day dependent promotion of flowering (Guo *et al.*, 1998). Interestingly, the *hy1* mutant which is deficient in all forms of phytochrome, shows a two-fold increase in the levels of *CO* mRNA, suggesting an inhibitory role of phytochrome on *CO* expression. The antagonistic roles of cryptochrome, and some species of phytochrome, in influencing flowering times may therefore be at least partially explained via their contradictory regulation of the flowering time gene *CO* (Guo *et al.*, 1998).

The effect of interactions between circadian rhythms and the perception of photoperiod on flowering time

It has been shown via several physiological experiments that the interaction of light signals and the circadian clock provides a timing mechanism for plants to detect long and short days, with inductive photoperiods promoting flowering only when given at the correct phase of the circadian cycle (Hamner and Bonner, 1983; reviewed in King, 1984; Lumsden, 1991; Coupland, 1997). In order to measure the length of the dark period of the cycle, endogenous rhythms must be phased from the end of the light period, suggesting a role for phytochrome in this process (Coupland, 1995a; Coupland, 1997). Little is known about the biochemical nature of the endogenous circadian rhythm in plants, how it is affected by light receptor input, or how circadian rhythm clock outputs affect flowering. A clock is thought to be synthesised daily however, as rhythms persist in constant light and temperature with a periodicity of almost 24 hours.

One gene that is thought to be important in clock oscillation is the *TOC1* locus (*TIMING OF CAB EXPRESSION*). Semi-dominant *toc1-1* mutants were observed to have a circadian cycle two-to-three hours shorter than wild-type controls with respect to the cycling of a CAB luciferase reporter gene construct, the cycling of two RNA binding proteins, as well as in the movements of expanding cotyledon and leaves (Kreps and Simon, 1997; Somers *et al.*, 1998). The *toc1-1* mutant also causes slightly earlier flowering in plants grown in short days and slightly later flowering in those grown in long days, resulting in an almost day neutral phenotype in the C24 background. These phenotypes support the hypothesis that disruptions in the circadian rhythms of a plant can affect the perception of photoperiod in *Arabidopsis*. In addition, these observations show that only a change of a few hours in the phase length of the circadian cycle can dramatically affect the correct processing of day-length signals (Somers *et al.*, 1998). Interestingly, when the *toc1-1* is introgressed into the L. *erecta* background, mutants flower much earlier than wild-type plants in short days. These plants exhibit little difference however, in their flowering time in long days compared

to controls. Ecotype differences such as these have been suggested to be due to the interactions of *toc1-1* with the L. *erecta* allele of the *FLC* gene (Somers *et al.*, 1998).

Support for a connection between circadian rhythm and the control of flowering time was also provided from analyses of the *elf3* (*early flowering 3*) mutant (Somers *et al.*, 1998). The *elf3* mutant is a day-length insensitive early-flowering mutant which shows arrhythmic expression of the *CAB* genes when grown in both light and dark cycle regimes, and in continuous light but not in continuous dark (Onouchi and Coupland, 1998). It has since been proposed that the *ELF3* gene may act in the input pathway leading from phytochrome to the central oscillator of the circadian clock (Onouchi and Coupland, 1998). *elf3* mutants also display less inhibition of hypocotyl elongation than wild-type plants when grown in light across the visible spectrum. This difference is most evident when plants are exposed to monochromatic blue and green light. This suggests that such mutants may also be impaired in some aspects of photoreception (Zagotta *et al.*, 1996; Onouchi and Coupland, 1998).

Analysis of the *lhy* (*late elongated hypocotyl*) mutants has also suggested a link between the response of plants to different photoperiods and the circadian clock. These plants display a disruption in their circadian rhythms and also exhibit a constitutive increase in the expression of the flowering time gene *CO* which is involved in the long day dependent floral promotory pathway. The *lhy* mutant phenotype has since been shown to be caused by overexpression of the *LHY* gene which encodes a MYB DNA binding transcription factor. The constitutive expression of this gene is believed to be the cause of the disrupted circadian responses of the mutants, and implies that the *LHY* gene is closely associated with the circadian oscillator of the clock (Schaffer *et al.*, 1998). Overexpression, or the absence of expression, of a transcription factor *CCA1* (*CIRCADIAN CLOCK ASSOCIATED-1*), which shows homology to *LHY*, also results in plants exhibiting disruptions in the circadian oscillations of several genes. In addition, constitutive expression of this gene causes plants to flower later in long day conditions (Wang and Tobin, 1998; Green and Tobin, 1999). Such observations again implicate circadian rhythms in the control of flowering time of *Arabidopsis* in response to photoperiod (Onouchi and Coupland, 1998).

Recently another gene, *FKF1* has also been cloned which is also thought to provide a link between flowering time and the endogenous circadian clock in *Arabidopsis*. *fkf1* mutants are late flowering and deletion of *FKF1* alters the rhythmic expression of two circadian regulated genes (Nelson *et al.*, 2000). This gene contains a domain similar to the flavin-binding domain of various photoreceptors (Nelson *et al.*, 2000). This implies that *FKF1* may function in the input pathway of the circadian clock.

Flowering in darkness

Wild-type *Arabidopsis* seedlings do not develop past the cotyledon stage in darkness, unless supplied with a source of carbohydrates such as sucrose. It has been demonstrated that for floral differentiation to occur in plants following a shift from dark to light conditions, a transferral from liquid cultures to solid media is required (Araki and Komeda, 1993a). If this transfer occured within ten days of dark growth, wild type Columbia were found to flower within approximately 18 days, while the late-flowering *gi-2 (gigantea-2)* mutants took approximately 40 days. If the transfer occurred after this ten day period, the flowering times of the *gi-2* and *co* mutants were not significantly different from those of wild-type plants treated similarly. This suggests that flowering in the dark, which is presumably controlled mainly by photosynthetic input in the form of sucrose, may utilise a different mechanism than the more complex light-induction of flowering (Araki and Komeda, 1993a). Unlike the Columbia ecotype, L. *erecta* plants rarely formed flowers in the dark when grown in media supplemented with sucrose. This indicates that genes controlling the differences in flowering time between *Arabidopsis* ecotypes may interact with processes affecting flowering in the dark. Overall, these results demonstrate that dark flowering conditions can effectively replace light signals in the photoperiodic induction of flowering. In addition, the dark-to-light transfer experiments lend support to the proposal that light signals may normally act to delay the action of a default flowering pathway until a suitable developmental stage of growth is reached (Araki and Komeda, 1993a).

1.3 Endogenous factors affecting flowering time in *Arabidopsis*

All aspects of plant development, including flowering, are influenced by the interaction of signals perceived and transduced from the environment with downstream endogenous factors within the plant, that act to cause a specific physiological action (Li *et al.*, 1996). It has been established for example, that various phytohormones can induce responses similar to those initiated by molecules involved with the perception of environmental stimuli such as phytochrome (Chory *et al.*, 1996). Increased levels of the phytohormone gibberellic acid, have been associated with the induction of flowering in long days (Blazquez *et al.*, 1998). Other compounds involved in floral induction include phytohormones such as brassinosteroids or cytokinins, as well as carbohydrates such as sucrose (Eimart *et al.*, 1995; Dennis *et al.*, 1996; Kauschmann *et al.*, 1996; Thomas *et al.*, 1997)

1.3.1 The role of carbohydrates in floral induction

Analysis of several flowering time mutants of *Arabidopsis* has supported the important role that sucrose plays in floral induction. It has been demonstrated that when sucrose is supplied to the apices of several late-flowering mutants, they flower early without the need for vernalisation. The supply of photosynthate to the apex is therefore implicated in a vernalisation-independent floral-promotory pathway in *Arabidopsis* (Dennis *et al.*, 1996). Physiological studies utilising the recently established system of floral induction of *Arabidopsis* by a single long day or displaced short day (a short day given at an unusual time in the 24 hour cycle) have also demonstrated that there is a two to three fold higher level of carbohydrates in the apex of induced plants. This elevation is believed to be due to an increased mobilisation of sucrose from the leaves, which occurs rapidly and transiently following induction, before any mitotic activity is observed at the apex. It is also believed that the increase in leaf export of sugars is not simply due to increased leaf carbohydrate availability, because there exists a sensitive system of synthesis and degradation of sugars within the plant that allows regulation of export into the phloem sap (Eimart *et al.*, 1995, Corbesier *et al.*, 1998).

The *adg-1* and *pgm* starch biosynthesis mutants of *Arabidopsis* are defective in two of the main enzymes in the starch biosynthesis pathway, ADP-glucose phosphorylase (ADGase) and phosphoglucomutase (PGM) respectively. The growth rate and net photosynthetic ability of these mutants are indistinguishable from those of wild-type plants, however, their flowering times are severely delayed providing further evidence for the role of sucrose in floral induction (Eimart *et al.*, 1995). In 1998, Corbesier *et al.* demonstrated that when the *pgm* mutants were florally induced by one long day there was a similar increase in sucrose levels to those observed in wild-type Columbia controls, although only a small decrease in their flowering time. If these mutants are florally induced by a single displaced short day, no increase in sucrose is observed and little if any reduction in flowering time is noted. If plants induced by displaced short days are subsequently treated with sucrose however, floral induction increases from 25% to 60% of individuals. The lack of full floral induction by one long day in *pgm* mutants suggests that floral signals other than sucrose, which are induced by long days, may be deficient in these mutants. The lack of floral induction of *pgm* mutants in displaced short day is thought to be due to their severe deficiency of starch reserves (Corbesier *et al.*, 1998). Such analyses indicate that while a fully functional starch metabolic pathway is not essential for floral induction, a disruption in it may alter the regulation of induction (Eimart *et al.*, 1995). This hypothesis is further supported following an examination of the starch degradation *sex-1* (*s*tarch in *ex*cess) mutant of the early-flowering Columbia ecotype. The *sex-1* mutant was found to be vernalisation-responsive and late-flowering when grown in short days, and displays little if any mutant phenotype in

continuous light. *sex-1* plants have a very low flux of carbohydrates through the starch pool, suggesting that it is not the levels of starch within the plant *per se*, but rather the problem of mobilisation of this starch to the apex, that causes a delay in floral induction in these lines. Such observations have lead to the proposal that the accumulation of metabolites in the chloroplasts of these mutants, may be the cause of their altered floral responses (Eimart *et al.*, 1995, Corbesier *et al.*, 1998).

To further examine the roles of starch and sucrose availability in controlling flowering time in *Arabidopsis*, Eimart *et al.* (1995) examined two late flowering mutants, the *gi* and *cam-1* (*carbohydrate accumulation-1*) mutants. The EMS-induced, day-neutral *cam-1* mutant exhibits elevated levels of starch in its photosynthetic tissues prior to the onset of flowering. The levels of several starch metabolism enzymes are also higher in *cam-1* plants, indicating that the accumulation of starch observed in these mutants may be due to an increase in starch synthesis rather than a lack of degradation. The *gi* mutant is the only late-flowering mutant previously characterised which shows elevated levels of starch that co-segregate with the late-flowering phenotype. In contrast to *cam1* mutants, the increase in starch levels in the *gi* mutants occurs only at early stages of growth. Eimart *et al.*, (1995) observed that *gi pgm* and *cam-1 pgm* double mutants were all late-flowering, although they displayed no further increases in starch levels. This indicated that the elevated starch levels observed in the *gi* and *cam-1* mutants are not responsible for the late-flowering phenotypes of these lines. It was therefore suggested that the flowering phenotype of these mutants may instead be due to changes in intermediate metabolites. An alternative explanation is that the *GI* and *CAM-1* loci are directly involved in a pathway regulating both starch metabolism and floral initiation, with mutations in these genes resulting in alterations in both processes (Eimart *et al.*, 1995). Researchers have concluded that while increases in starch levels are not the cause of the late-flowering and vice versa, common regulatory pathways of starch metabolism and floral initiation may exist (Eimart *et al.*, 1995, Corbesier *et al.*, 1998).

1.3.2 The role of gibberellins in floral induction

Gibberellins are tetracyclic diterpenoid carboxcylic acid growth factors that have diverse effects on plant growth and development. Gibberellins have been associated with floral induction in many species (Phillips *et al.*, 1995; Peng *et al.*, 1997; Cowling *et al.*, 1998; Evans, 1999). It has been demonstrated that GA can substitute for a vernalisation treatment or an inductive photoperiod in a number of long day and cold requiring plants, provided that the plants are old enough to be competent to flower (Wilson *et al.*, 1992; Martinez-Zapater *et al.*, 1994; Evans, 1999). In *Arabidopsis*, gibberellins have been implicated in a slow autonomous age-dependent flowering pathway (Langridge, 1957; Blazquez *et al.*, 1998; Koornneef *et al.*, 1995).

Active gibberellins such as GA_1 and GA_4 are synthesised from the gibberellin skeleton following several oxidation and hydroxylation steps catalysed by GA-oxidases and 3β-hydroxylase enzymes (Phillips *et al.*, 1995, Cowling *et al.*, 1998). The GA-oxidases, in particular the GA 20-oxidases, which act to oxidise C-20 containing gibberellins, are also thought to be involved in a control point in the gibberellin pathways by which it can regulate its own production (Phillips *et al.*, 1995, Wu *et al.*, 1996). GA 20-oxidase genes have been cloned from several species such as spinach (Wu *et al.*, 1996), pumpkin (Lange *et al.*, 1994; Phillips *et al.*, 1995), peas and beans (Garcia-Martinez *et al.*, 1997). The activity of these enzymes has been implicated in the photoperiodic control of flowering. In the long day plant spinach it has been observed that the inactive GA_{20} is metabolised more quickly in plants grown in long days. In addition, the conversions of GA_{53} to GA_{44} and GA_{19} to GA_{20} catalysed by GA 20-oxidases, are increased in spinach plants shifted from short days to long days. Furthermore, levels of GA_{20} are successively decreased in this species when these plants are shifted from long days, to short days, and then to darkness (Wu *et al.*, 1996). Three different GA 20-oxidases have been cloned from *Arabidopsis* by using degenerate primers based on the pumpkin GA 20-oxidase sequence. Different spatial and developmental expression patterns were detected for all three transcripts suggesting specific *Arabidopsis* GA 20-oxidases may be important in several aspects of plant development (Phillips *et al.*, 1995).

While the exogenous application of gibberellins has been shown to promote flowering in many angiosperms, the doses required and the choice of gibberellin varies (Bagnall, 1992; Evans *et al.*, 1990, Evans *et al.*, 1994a, Evans *et al.*, 1994b, Evans, 1999), which can affect different developmental processes such as stem elongation and flowering (Ross. 1994; Evans, 1999). Indeed, while the bolting and flowering processes are very tightly linked in rosette plants like *Arabidopsis*. it has been demonstrated that they are separate developmental mechanisms stimulated by distinct gibberellins (Evans, 1999). The considerable diversity of gibberellins found in *Arabidopsis*. and their involvement in three separate gibberellin biosynthetic pathways, has also lead to uncertainty as to which gibberellin is the most biologically active in this species (Ross, 1994; Evans, 1999). It has been observed that *Arabidopsis* contains higher levels of GA_4 than GA_1, both of which are believed to be biologically active in that species, although GA_1 is implicated as the major active gibberellin in maize, pea and rice (Ross, 1994, Evans, 1999). This hypothesis is supported by the fact that GA_4 is generally converted into GA_1 in feeding experiments, and that GA_1 levels correlate better than GA_4 levels with shoot height in both wild-type plants and gibberellin biosynthesis mutants (Ross, 1994, Evans, 1999).

Further information on the roles of gibberellin in floral induction has been elucidated from an analysis of gibberellin mutants of *Arabidopsis*. These mutants can be classified into two

main groups: the dwarf gibberellin biosynthetic mutants and the gibberellin response, or signal transduction mutants. This latter group can be further sub-classified into gibberellin response dwarf mutants and elongated gibberellin overexpression mutants (Wilson *et al.*, 1992; Peng and Harberd, 1997). The dwarf gibberellin biosynthetic mutants *ga1*, *ga2*, and *ga3* are disrupted in the *GA1*, *GA2*, and *GA3* genes which are involved the early steps of the gibberellin biosynthetic pathway. The two other biosynthetic mutants, *ga4* and *ga5*, carry mutations in the *GA4* and *GA5* genes that code for a gibberellin 3β-hydroxylase or GA 20-oxidase respectively, enzymes that act later in gibberellin biosynthetic pathway (Phillips *et al.*, 1995; Xu *et al.*, 1995; Peng and Harberd, 1997; Evans, 1999). These gibberellin biosynthesis mutants all have a characteristic late-flowering, dark-green, dwarf phenotype, and show reduced apical dominance, germinal frequency and fertility. The majority of these mutants show a complete reversion to a wild-type phenotype when treated with exogenous gibberellin (Peng and Harberd, 1997; Cowling *et al.*, 1998).

The *GA1* gene controls the first committed step of the gibberellin biosynthesis pathway, which is the formation of *ent*-kaurene, and the gene was found to encode copalyl diphosphate synthetase which is localised in the chloroplast (Wilson *et al.*, 1992; Silverstone *et al.*, 1997; Mander *et al.*, 1998). *ga1* mutants exhibit a decrease in gibberellin levels, although they do not show a complete lack of gibberellin, making them similar to other gibberellin biosynthesis mutants (Wilson *et al.*, 1992). *ga1-3* mutants cannot flower in short day conditions without the addition of exogenous gibberellin, and these mutant plants undergo senescence after five to six months in short days without ever flowering. In addition, microscopic signs of flower formation (floral primordia) were never seen in excised apices of *ga1-3* plants grown in short days. Furthermore, cold treatments of short day-grown *ga1-3* plants had little, if any, effect on their flowering times (Wilson *et al.*, 1992; Peng *et al.*, 1997). *ga1-3* mutants will flower readily however, in long days or continuous light conditions. Plants carrying weak mutant alleles of *GA1* such as the *ga1-6* mutants will eventually flower in short days albeit much later than wild-type plants (Wilson *et al.*, 1992). In conjunction, these results suggest that a certain level of gibberellin is required for a plant to flower, and that these levels are more limiting in short day conditions (Wilson *et al.*, 1992). Furthermore, a connection between vernalisation and the production of gibberellins is supported, as these observations demonstrate a minimum requirement of endogenous level of gibberellin before vernalisation can be effective in *Arabidopsis* (Wilson *et al.*, 1992; Blazquez *et al.*, 1998; Evans, 1999). These results also agree with the hypothesis that gibberellins act via the slow autonomous flowering pathways in this species, whuch is the pathway proposed to be responsible for flowering of *Arabidopsis* in short days. In addition, these observations support the theory that the predicted long-day dependent, promotory flowering pathway is only minimally affected by altered gibberellin levels, although not completely gibberellin-independent (Wilson *et al.*, 1992; Evans, 1999).

In 1998, Helliwell *et al.* reported the map-based cloning of the *GA3* gene which encoded a cytochrome P450 enzyme that is involved in converting *ent*-kaurene to GA_{53}. This gibberellin intermediate is then further metabolised into biologically active GAs by dioxgenase enzymes. As *ga3-1* mutants of *Arabidopsis* are deficient in *ent*-kaurene oxidase, and therefore accumulate *ent*-kaurene, it is believed that this cytochrome P450 enzyme is *ent*-kaurene oxidase. It has since been shown that the two mutant alleles of *GA3* contain single base changes that introduce stop codons into the ORF of the gene. Unlike the GA oxidases that catalyse latter steps of the GA biosynthesis pathway, there is believed to be only one *ent*-kaurene oxidase gene in the genome. The *GA3* transcript levels are high in dividing and expanding cells, elongating stems and inflorescences, and developing seeds. This expression pattern supports the important role GA biosynthesis plays in processes such as germination, stem growth, and flowering (Helliwell *et al.*, 1998).

Expression of the GA 20-oxidase enzyme encoded by the *GA5* gene appears to correlate with early-flowering and stem elongation, as *ga5* mutants are late-flowering and dwarfed. Mutations in the *GA5* locus also result in decreased levels of both bioactive gibberellins and those involved in the later steps of the 13-hydroxylation pathway (Talon *et al.*, 1990a; Evans, 1999). As mentioned above, GA 20-oxidases are thought to be involved in the feedback-regulation of gibberellin biosynthesis, an hypothesis confirmed by the observation that the expression of this gene is upregulated by approximately 40% in plants shifted from short days to long days, and decreased by treatment with an active gibberellin (Xu *et al.*, 1995). Therefore, mutants defective in this GA biosynthesis gene presumably lack any ability to increase production of gibberellins in response to photoperiodic conditions, a factor that may be important in their late-flowering phenotype observed in long days (Xu *et al.*, 1997). Sponsel *et al.* (1997) isolated two new lines defective in gibberellin biosynthesis, and termed them *ga6* mutants. It is believed that the *GA6* locus also encodes for a GA 20-oxidase enzyme that is expressed in the inflorescences and siliques of *Arabidopsis* (Sponsel *et al.*, 1997)

Further information of the regulation of gibberellin-mediated growth responses, such as flowering, has come from an analysis of the *gai* gibberellin response mutants. These semi-dominant dwarf mutants are later flowering in short days than wild-type plants, and show several other phenotypes characteristic of gibberellin biosynthesis mutants. Unlike those mutants however, neither exogenous gibberellin treatment nor vernalisation can accelerate the flowering of *gai* mutant plants in short days. *gai* mutants also contain high endogenous gibberellin levels and accumulate bioactive 3β-hydroxylated gibberellins (Talon *et al.*, 1990b). The late-flowering of the *gai* mutant in short days only, again suggests that some important changes other than a rise in gibberellin levels promotes flowering *Arabidopsis* plants grown in long days (Wilson *et al.*, 1992; Evan, 1999).

The *GAI* gene has been cloned and the mutant protein was found to contain a 17 amino acid deletion in a region of the protein potentially involved in gibberellin interactions (Peng *et al.*, 1997). The GAI protein also contains features such as leucine heptad repeats, LXXLL motifs and nuclear localising signals common to several regulatory proteins involved in modifying transcription of nuclear genes (Peng *et al.*, 1997; Silverstone *et al.*, 1998). These observations have lead to the proposal that *GAI* may be involved in the perception of gibberellin, or in a gibberellin signal transduction pathway (Jacobsen *et al.*, 1996; Peng *et al.*, 1997; Peng and Harberd, 1997; Blazquez *et al.*, 1998; Cowling *et al.*, 1998; Silverstone *et al.*, 1998). In 1998, Harberd *et al.* further proposed that the GAI protein acts as a repressor of gibberellin-mediated responses in wild-type plants, and that gibberellins may mediate their affects on growth via a repression of GAI activity. In the GA-deficient mutants, this level of control is removed and gibberellin-mediated processes, such as rapid flowering in short days, are continually repressed by the activity of proteins such as GAI. Harberd *et al.* (1998) also postulated that the *gai* mutants produce a defective form of the GAI protein that can no longer be repressed by endogenous gibberellins. These lines therefore display a lack of gibberellin-mediated growth responses which is a phenotypes that cannot be rescued by exogenous gibberellin application (Harberd *et al.*, 1998).

The proteins encoded by two other loci, *SPY (SPINDLY)*, and *RGA (REPRESSOR OF GAI-3)* have since been suggested to have a similar role to GAI (Jacobsen and Olszewski, 1993; Silverstone *et al.*, 1997; Silverstone *et al.*, 1998; Evans, 1999). *spy* mutants are pale green in colour, display early-flowering, show partial male sterility, are resistant to the gibberellin biosynthesis inhibitor paclobutrazol, and have elongated hypocotyls and stems. These phenotypes are similar to those observed when wild-type plants are treated with exogenous gibberellins. This observation is in agreement with the idea that *SPY*, like *GAI*, may function as a repressor of gibberellin-mediated interactions, except in the presence of gibberellin (Jacobsen *et al.*, 1996). As both strong and weak mutant alleles of *SPY* are completely epistatic to the *gai* mutation, the activity of SPY is postulated to be downstream of the GAI protein in a process repressing a gibberellin signalling pathway (Jacobsen *et al.*, 1996; Peng *et al.*, 1997; Peng and Harberd, 1997; Silverstone *et al.*, 1998). In support of the hypothesis that the *SPY* gene may have a regulatory role similar to *GAI*, the product of the *SPY* gene shows similarities to a ser(thr)-O-linked N-acetyl glucoisomerase transferase, which is a protein that plays an important role in regulating the activity of various nuclear and cytosolic proteins (Silverstone *et al.*, 1998).

The pale green recessive *rga* mutants were originally isolated as mutants that could reverse the late-flowering and dwarf phenotypes of the *gal-3* mutants, suggesting that in a similar manner to that predicted for the *SPY* locus, the wild-type *RGA* gene product can act as a negative regulator of gibberellin responses. It is believed that the *RGA* and *SPY* genes may

act to control separate branches of the gibberellin signal transduction pathway however, as *rga* and *spy* mutations have additive effects in the *gal-3* background (Silverstone *et al.*, 1997; Silverstone *et al.*, 1998). The *RGA* locus has since been cloned, and like the *GAI* gene, was found to be a member of a family of putative transcription factors that are characterised by the presence of a VHIID domain. At present, the function of this domain is unclear. Differences in the sequence of the N-terminal region of the GAI and RGA proteins may reflect their overlapping, although not completely redundant, functions (Silverstone *et al.*, 1998).

A relationship between gibberellin levels and the photoperiod response of *Arabidopsis* has been suggested following the observation that plants overexpressing phytochromes, often exhibit a dark-green dwarf phenotype and may have low levels of endogenous gibberellins. In addition, plants displaying phytochrome deficiencies showed similar phenotypes to the *spy* mutants that constitutively display gibberellin-induced responses (Peng and Harberd, 1997). To examine the interactions between phytochrome and gibberellin levels further, Peng and Harberd (1997) examined the phenotypes of *gal-3, gai, hyl-1* and *phyB-1* double mutants. The authors demonstrated that the gibberellin-deficient mutants were epistatic to the effects of the *hyl-1* or *phyB-1* mutations, with respect to seed germination, mature plant height, and floral fertility. The effects of the mutations were additive however, in relation to hypocotyl elongation, as the *gai hyl-1* and *gai phyB* double mutants showed intermediate hypocotyl heights. The authors concluded that a functional gibberellin system is needed before the full effects of phytochrome deficiencies can be seen (Peng and Harberd, 1997). These observations support the hypothesis that gibberellin activity is downstream of the perception of light by phytochrome (Koornneef *et al.*, 1995).

1.3.3 The role of other plant hormones in floral induction

Brassinosteroids

Brassinosteroids were first identified from pollen extracts of *Brassica napus* and found to be structurally similar to animal steroid hormones. They are distributed throughout the plant kingdom and to date approximately 30 have been identified, all of which have unique biological activity when applied to plants (Kauschmann *et al.*, 1996; Li *et al.*, 1996). General effects of brassinosteroids have been suggested and include a stimulation of growth through enhanced cell division and cell elongation. These observations have lead to the proposal that brassinosteroids may act synergistically with auxins and additively with gibberellins (Kauschmann *et al.*, 1996).

Analysis of the *det2* mutants of *Arabidopsis* has also implicated brassinosteroids in light-regulated developmental processes. When grown in the dark, these mutants display short, thick hypocotyls, accumulate anthocyanins, show expansion of their cotyledons, develop primary leaves, and show decreased expression of several light-regulated genes (Chory *et al.*, 1989). If these plants are grown in the light however, they appear dwarfed, darker green in colour, exhibit a decrease in cell size, apical dominance, male fertility, and delay their flowering such that they are no longer photoperiod-responsive (Chory *et al.*, 1989). Light-grown *det2* plants also show a shortening of the circadian cycle of *CAB* gene expression, and a delay in leaf and chlorophyll senescence (Li *et al.*, 1996). The DET2 protein has since been isolated and found to show similarity to a mammalian steroid 5α-reductase, which catalyses nicotinamide adenine dinucleotide phosphate (NADPH) dependent conversion of testosterone to dihydro testosterone. A missense mutation in the *DET2* gene completely abolishes *DET2* function, and it is believed that the *det2* phenotype is caused by a decrease in brassinosteroid biosynthesis due to a block in the step involving the reduction of the intermediate campesterol to campestenol. This theory was supported by the observation that treatment of the *det2* mutants with brassinolide which is the end product of the brassinosteroid biosynthesis pathway, completely rescues the *det2* phenotype, however has little effect on wild-type and *det1* control plants (Li *et al.*, 1996). Analyses of the *det2* mutants has therefore lead to the proposal that light may act in part, to regulate processes such as the expression of photosynthesis genes, leaf and chlorophyll senescence, and the promotion of flowering, by modulating the brassinosteroid signals in plants (Li *et al.*, 1996; Kauschmann *et al.*, 1996). The altered skotomorphogenic (development in dark) and photomorphogenic (development in light) responses of several other mutants deficient in the biosynthesis or perception of brassinosteroids, such as the *dwarf* (*dwf*), *diminuto* (*dim-1*), and *cabbage-3* (*cbb-3*) mutants, supports the hypothesis that brassinosteroids may play a role in light-regulated processes (Kauschmann *et al.*, 1996; Choe *et al.*, 1999).

Cytokinins and Auxins

The effects of floral induction on the levels of the phytohormones cytokinin and auxin in *Arabidopsis*, and a related mustard plant *Sinapis alba*, has been examined (reviewed in Bernier *et al.*, 1993). It was noted that induction of flowering in *S. alba* plants by a single long day resulted in rapid changes in the cytokinin flux throughout the plants, with levels of the zeatin riboside increasing early and transiently in response to the long day signal. This increase is thought to be due to increased cytokinin export from the roots, rather than an increase in cytokinin production, as the root exudate of plants shows lower levels of cytokinin following induction (reviewed in Bernier *et al.*, 1993; Dennis *et al.*, 1996). In addition, it is currently believed that the root-to-shoot flux of cytokinins is essential for flowering, as plants whose xylem flow is inhibited via growth in conditions of 100%

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humidity, fail to flower following induction (Bernier *et al.*, 1993). Exogenous application of cytokinin to *Arabidopsis* plants can also decrease their flowering time, although this response is dependent upon growth in low irradiances (Dennis *et al.*, 1996).

Further support for the role of cytokinin in flowering has come from an analysis of the *Arabidopsis* mutant *amp-1*. This mutant has seven times higher cytokinin levels than wild-type plants and flowers early in both long and short days (Chaudhury *et al.*, 1993). Interestingly, when the *amp-1* mutation was introgressed into the late-flowering *gi* mutant background, the double mutants flowered early. Double mutants of *amp-1* and the vernalisation-dependent late-flowering *fca* mutants flowered as late as *fca* single mutants however, indicating that the early-flowering phenotype of the *amp1* mutant requires a vernalisation-independent *FCA* pathway to be functional (Dennis *et al.*, 1996).

Long day induction of flowering in *Arabidopsis* has also been shown to be associated with a decrease in the auxin levels of the apical buds of induced plants. This decrease consequently alters the developmentally important endogenous auxin/cytokinin ratio within the plants and the resulting higher relative levels of cytokinin within the plants are thought to contribute to the promotion of flowering by long days (Bernier *et al.*, 1993).

Many other compounds have also been found to affect flowering time possibly via interactions with cytokinin. *S. alba* plants treated with the polyamine biosynthesis inhibitor, DL-α-difluromethylornithine (DFMO) show a decrease in their flowering response to long days (Havelange *et al.*, 1996). It is further believed that polyamines may promote flowering via their interaction with cytokinins following induction by long days. Such interactions have been reported to be involved in several different developmental processes within the plant, including cell division (Bernier *et al.*, 1993). Early and transient increases in calcium levels in the roots of *Arabidopsis* and *S. alba* plants have also been observed following induction via one long day or one displaced short day, indicating that calcium may act as a second messenger for cytokinins in their effects on floral induction (Bernier *et al.*, 1993).

Other compounds or factors which have non-specific effects on plant growth and metabolism may also indirectly affect the timing of floral induction, and include base-analogues, the organic molecule isoprene, and mineral nutrition (Martinez-Zapater *et al.*, 1994; Terry *et al.*, 1995). Like the control of flowering time by environmental factors, there appears to be a multifactorial control of flowering time in *Arabidopsis* by numerous endogenous compounds with no compound being singly important (Bernier, 1988; Bernier *et al.*, 1993).

1.4 Mutations affecting the flowering process in *Arabidopsis*

The flowers of *Arabidopsis* contain four types of organs; sepals, petals, stamens, and carpels arranged in four concentric whorls. The maintenance of this structure is controlled by three classes of homeotic genes A, B or C, that all act in two adjacent whorls in the flower (Bowman *et al.*, 1991). When class A genes act alone in the first whorl, sepals are produced, when A and B genes operate together in the second whorl, petals are formed, the action of the B and C class genes in the third whorl cause stamens to be formed, while the action of C class genes alone in the fourth whorl confers carpel formation. Inactivation, or ectopic expression of these genes has shown that one of their functions is a mutual inhibition of one another in adjacent whorls. Mutations in one or more of these genes result in the wrong type of organ being formed in the wrong whorl. The homeotic genes responsible for these functions include the class A, *APETELA2* (*AP2*) gene, the class B genes *APETELA3* (*AP3*) and *PISTILLATA* (*PI*), and the class C *AGAMOUS* (*AG*) gene (Weigel and Meyerowitz, 1993a; Weigel and Meyerowitz, 1993b; Mandueno *et al.*, 1996; Simon *et al.*, 1996; Goodrich *et al.*, 1997; Jurgens, 1997; Pineiro and Coupland, 1998).

Cadastral genes are thought to activate the expression of these homeotic genes, as the inflorescences of plants containing mutations in these cadastral genes often show homeotic transformations (Shultz and Haughn, 1993; Weigel and Meyerowitz, 1993a; Weigel and Meyerowitz, 1993b). The coordinated action of these cadastral genes, or Floral Meristem Identity (FMI) genes, confers floral identity on developing primordia at the shoot apical meristem following floral induction. FMI genes include the *LEAFY* (*LFY*), *APETELA1* (*AP1*), *APETELA2* (*AP2*), *CAULIFLOWER* (*CAL*), *TERMINAL FLOWER1*, *TERMINAL FLOWER2* (*TFL1* and *TFL2*), and *UNUSUAL FLORAL ORGANS* (*UFO*) genes. Mutations in the FMI genes result in the generation of inflorescences, or modified flowers with inflorescence characteristics, in positions where flowers would normally form (Mandueno *et al.*, 1996). Several of these genes, such as *LFY*, *AP1*, and *AP2* are thought to have partially redundant functions in initiating the floral program, as inactivation of one of these genes only causes a delay, rather than the complete absence of the transition of coflorescences into flowers (Shultz and Haughn, 1993).

The *LEAFY* gene is thought to be one of the most important loci necessary for the initiation of flowers in coflorescences. While overexpression and reporter gene studies have shown that the *LFY* gene is extensively expressed during vegetative development, *LFY* expression is upregulated more rapidly in long days than in short days. Such changes in *LFY* expression prior to the transition to flowering suggest that floral induction is partially controlled by levels of the *LFY* gene product (Blazquez *et al.*, 1997). In support of this theory, plants overexpressing *LFY* produce flowers earlier, while the loss of *LFY* function

causes the production of leaves, or secondary inflorescences subtended by leaves, where flowers would normally form. Later developing flowers are not as affected by low *LFY* levels, possibly due to the activation of other FMI genes (Coupland, 1995b; Coupland, 1995c; Blazquez *et al.*, 1997). *LFY* and another FMI gene, *AP1*, are thought to reinforce the activity of each other, as overexpression of either can accelerate flowering time and causes the development of flowers in almost every available shoot meristem. Ectopic expression of these genes does not cause the production of flowers after germination however, indicating that a period of vegetative growth is required for the plant to become competent to receive *LFY* signals (Coupland, 1995c; Mandel and Yanofsky, 1995; Mandueno *et al.*, 1996; Blazquez *et al.*, 1997).

It has also been suggested that the plant hormone gibberellin may act to promote floral induction through a pathway that controls both *LEAFY* promoter activity, and the ability of the plants to respond to this activation. This hypothesis is supported by the observations that *LEAFY* promoter activity is delayed and reduced in gibberellin biosynthetic mutants, and an increase in the expression of the *LEAFY* gene can induce *ga1-3* mutants to flower in short days. In addition, *spy* mutants, which show constitutive expression of gibberellin-mediated responses, also show elevated levels of *LEAFY* (Blazquez *et al.*, 1998).

Alvarez *et al.* (1992) identified the FMI gene, *TERMINAL FLOWER1* (*TFL1*) following the isolation of eight recessive *tfl1* mutants. In these lines the normally indeterminate inflorescence became determinate, forming a terminal flower after the formation of several normal flowers. These plants exhibit a reduction in bolt height, produce numerous rosette inflorescences, and flower early. The *TFL1* gene has been cloned and encodes a membrane-associated protein (Alvarez *et al.*, 1992; Bradley *et al.*, 1997). The *TFL1* gene was found to be expressed in a group of cells just below the apical meristem, and could be detected two days post germination (Amasino, 1996a; Bradley *et al.*, 1997). This expression pattern, and the *tfl1* mutant phenotype, suggests that the primary role of this gene is to repress the expression of other FMI identity genes, such as *LFY* and *AP1* thereby maintaining the indeterminate nature of the inflorescence (Alvarez *et al.*, 1992; Shannon and Meeks-Wagner, 1993; Hicks *et al.*, 1996a; Simon and Coupland, 1996; Simon *et al.*, 1996).

Larsson *et al.* (1998) reported the identification of a new gene, *TFL2*, mutations in which result in plants that flower early and produce a terminated flower structure. These mutants also display several other pleiotrophic effects, such as a decrease in photoperiod sensitivity, and more variability in the terminal flower structure. These phenotypes suggest a more general regulatory role for the *TFL2* gene compared to that of the *TFL1* gene. In addition, double mutants containing mutations in both the *TFL1* and *TFL2* genes show an enhanced terminal flower phenotype, suggesting these genes may act in separate pathways

(Hicks *et al.*, 1996a; Larsson *et al.*, 1998). *tfl2 apl* and *tfl2 lfy* double mutants have also demonstrated that *TFL2* functions may influence processes controlled by *AP1* but not by *LFY* (Larsson *et al.*, 1998).

Mutations in the *AP1* FMI gene cause the production of secondary inflorescences that are subtended by leaves instead of basal flowers or primary inflorescences. In addition, plants homozygous for the *apl* allele flower slightly earlier in constant light or long days (Shultz and Haughn, 1993; Page *et al.*, 1999). A role for *AP1* in the control of homeotic gene expression is suggested by the flower phenotype of *apl* mutants that maintain a whorled phyllotaxy, but form ectopic secondary flowers in the axils of the first whorl floral organs (Huala and Sussex, 1992; Weigel *et al.*, 1992; Mandeuno *et al.*, 1996; Ruiz-Garcia *et al.*, 1997).

Mutations within the *SHORT INTEGUMENT (SIN1)* FMI gene result in female sterile mutants that display abnormal ovule development. *sin1* mutants also have an increased number of vegetative leaves and are therefore late-flowering. It has been demonstrated that this late-flowering was not the result of a defect in day-length perception or gibberellin metabolism, and furthermore, vernalisation had no effect on the flowering time of these mutants (Ray *et al.*, 1996). These phenotypes, and the map location of *SIN1*, indicate that the *sin1* mutation is not an allele of any known late-flowering mutation that has been mapped so far (Ray *et al.*, 1996). The *SIN1* gene has instead been suggested to be an FMI gene that affects both the vegetative-to-inflorescence transition, and the inflorescence-to-floral transition. In addition, the phenotype of *sin tfl1* double mutants indicates *SIN* activity is required for the early transition to flowering found in *tfl1* mutants (Ray *et al.*, 1996).

In 1998, Tefler and Poethig identified nine mutant alleles of the *HASTY* gene, which accelerated the production of leaves with abaxial trichomes which are an indicator of the adult vegetative phase. These mutations also hasten the loss of adaxial trichomes on leaves and decreases the total leaf number of the plant (Tefler and Poethig, 1998). Double mutant analysis between *hasty* and the *ga-1* and *gai* mutants suggests that the increase in reproductive competence of the shoots in these mutants does not require gibberellin production or response. The phenotype of the *hasty* mutant alleles is also not suppressed by the *lfy*, *ag*, or *apl* mutations. The predicted role of the *HASTY* gene is therefore thought to be to inhibit flowering by decreasing the competence of the shoot to respond to the *LFY* or *AP1* gene products (Tefler and Poethig, 1998).

As the expression of several of the FMI genes alters in response to environmental conditions, such as day-length, it is believed that the expression of these genes may be influenced by upstream 'flowering time' genes that control the switch to reproductive development within *Arabidopsis*. As *LFY* has characteristics of a gene affecting flowering

time and an FMI gene, it is believed that *LFY* may act as a direct target for the protein products of flowering time genes. This theory is supported by the observations that upregulation of *LFY* is detected in both future and previously initiated primordia following the growth of plants in long days (Simon and Coupland, 1996; Blazquez *et al.*, 1997; Pineiro and Coupland, 1998). Further evidence that *LFY* may act as a link between the floral induction process and the activation of floral meristems is provided by the observation that the early-flowering associated with *phyB* mutations also correlates with an increase in *LFY* expression (Blazquez and Weigel, 1999). This increase in *LFY* expression is proposed to result from increased GA levels, as there is no *PHYB*-mediated repression of GA biosynthesis in such lines (Blazquez and Weigel, 1999). It is not thought that the role of flowering time genes is to simply activate *LFY* however, as plants overexpressing *LFY* still produce a vegetative phase before flowering. Therefore it is believed that the co-operative action of several flowering time genes may help to stimulate *LFY* activity at the time when the plant becomes competent to flower, thus resulting in a cascade of *LFY*-mediated expression of FMI and homeotic genes to produce floral meristems and flowers (Ma, 1997; Pineiro and Coupland, 1998). *tfl* mutants have also been found to be early-flowering in long and short days while maintaining a short day response. This observation suggests that like *LFY*, *TFL* may also provide a connection between flowering time and FMI genes (Hicks *et al.*, 1996a; Pineiro and Coupland, 1998).

The recently cloned *AGL20* (Borner *et al.*, 2000) which is also known as the *SOC1* (Samach *et al.*, 2000) locus has also been suggested to act in parallel or upstream of *LFY* as a target gene for several flowering time genes such as *CO* and *GI* (Borner *et al.*, 2000; Lee *et al.*, 2000; Samach *et al.*, 2000). This gene was found to be activated in the shoot apical meristems during the transition to flowering. Plants overexpressing *AGL20* flower early, while *agl20* mutants show delayed flowering (Borner *et al.*, 2000; Lee *et al.*, 2000). The expression of this gene is also of interest since it is upregulated not only in long days and down-regulated in *co* and *gi* mutants, however the expression also appears to be regulated by genes within the autonomous flowering pathway. In addition, *AGL20* expression increases following exogenous application of GA, which indicates that *AGL20* may play a role in converging the signals from different flowering pathways to promote the transition to flowering (Borner *et al.*, 2000).

Flowering time genes have been discovered and isolated following the production of mutants in *Arabidopsis* that have altered flowering times and leaf number either in response to, or independently of, various environmental conditions. Two main classes of mutants have been identified; those that delay, and those that accelerate flowering time. The existence of both late- and early-flowering mutants confirms the existence of at least two antagonistic flowering pathways that either promote or inhibit flowering (Coupland *et al.*, 1993; Aukerman and Amasino, 1996). Flowering is thought to be primarily controlled by a central

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autonomous flowering pathway which requires a gradual increase in levels of a floral promotory substance. Other promotory and inhibitory pathways may act in conjunction however, to decrease or promote the levels of this substance in response to various environmental conditions (Haughn et al., 1995; Mandueno et al., 1996; Koornneef and Peeters, 1997).

1.4.1 Mutations conferring a late-flowering phenotype in *Arabidopsis*

Approximately twenty mutations have been identified that cause a delay in flowering and an extension of vegetative growth (Haughn et al., 1995; reviewed in Koornneef, 1997). In 1962, Redei described four recessive late-flowering 'supervital' mutants in the early-flowering Columbia ecotype. These were the *ld* (*luminidependens*), *gi-1* and *gi-2* (*gigantea-1 and gigantea-2*), and the *co* (*constans*) mutants (Redei, 1962). In 1973, Vetrilova also reported the isolation of four late-flowering mutants in the early-flowering Dijon-G ecotype, following treatment of the plants with N-methyl-N-nitrosource (MNH). These semi-dominant mutants were termed *L4*, *L5*, *L6*, and *L7* and exhibited some response to vernalisation (Vetrilova, 1973). It has since been suggested that *L4* and *L5* may be alleles of the flowering time locus *FRIGIDA* (Burn et al., 1994). The most comprehensive collection of mutants was isolated by Koornneef et al. (1991), following EMS treatment of the L. *erecta* ecotype of *Arabidopsis*. 42 mutants were originally isolated that flower approximately one to two weeks later than wild-type L. *erecta* in long days. Three of these mutants (*fg*, *fb-1* and *fb-2*) were found to be allelic however, to the previously isolated Columbia mutants *co*, *gi-1*, and *gi-2*. The remaining 39 mutants represented alleles at nine other loci (*FCA*, *FPA*, *FVE*, *FY*, *FE*, *FT*, *FD*, *FWA*, *FHA*). These 11 loci were distributed amongst all chromosomes except number three (Koornneef et al., 1991; Bernier et al., 1993; Burn et al., 1994). Further mutations resulting in late-flowering plants include the late-flowering *F2* mutant isolated by McKelvie (1962) that has been found to represent an allele of the *FHA* gene (reviewed in Martinez-Zapater et al., 1994). It is assumed that all of these late-flowering mutants interfere in some way with the synthesis or action of floral promoters, or alternatively, that these mutations may act to enhance the production of substances that are inhibitory to flowering (Koornneef et al., 1991; Burn et al., 1994).

Several of the late-flowering mutants have since been classified into groups based on their flowering time responses to both vernalisation and photoperiod. The classification of these mutants was confirmed via double mutant analysis which demonstrated that mutants in separate classes gave an additive late-flowering phenotype, while double mutants of lines in the same group flowered no later than the latest parental mutant line (Koornneef et al., 1991; Burn et al., 1994; Koornneef et al., 1998; Pinciro and Coupland, 1998).

One group, represented by the *fca, fpa, fve, fy*, and *ld* mutants, flowered earlier in response to a vernalisation treatment and later in short day conditions compared to long days. A second group of mutants, *fe, ft, fd*. and *fwa*. showed little response to vernalisation and exhibited only a small delay in flowering time in short day conditions. A third grouping included the *co, gi*, and *fha* mutants, which showed a minimal response to vernalisation, and flowered as late in long days as in short days (Koornneef *et al.*, 1991). This classification of responsiveness to vernalisation was based on the observation that mutants in classes two and three required extended treatments to show any phenotypic change, and that this response was never as great as that seen for the mutants in class one (Koornneef *et al.*, 1991; reviewed in Martinez-Zapater *et al.*, 1994; Dennis *et al.*, 1996; Koornneef, 1997). In addition, the photoperiod response shown by group two mutants is far less than that shown for mutants classified in group one (Amasino, 1996a). A more generalised grouping therefore suggests that mutations in the first class are defective in genes involved with a constitutive or autonomous flowering pathway that promotes flowering independently of the environmental conditions. The mutations causing plants to exhibit a decreased response to short day photoperiods (group 2 and 3 mutants) are suggested to affect genes involved in a pathway that promotes flowering primarily in long day conditions. Some partial redundancy however, is believed to exist between these pathways (Koornneef *et al.*, 1991; Okada and Shimura, 1994; Koornneef *et al.*, 1998; Pineiro and Coupland, 1998).

In early-flowering ecotypes, vernalisation has been shown to only partially overcome the flowering time delay caused by short days, therefore it is believed that vernalisation may need to act in conjunction with long days to saturate the early-flowering response. The inability of a vernalisation treatment to promote flowering in group two and three mutants may therefore also reflect their decreased flowering response to inductive long day conditions (Amasino, 1996a).

1.4.2 Group one late-flowering mutants; *fca, fpa, fve, fy, fld*, and *ld*

As mentioned above, group one late-flowering mutants show a further delay in their flowering time in response to short days and a long vernalisation treatment can completely rescue their late-flowering phenotype (Koornneef *et al.*, 1991; Lee *et al.*, 1994a). This latter phenotype indicates that the physiological outcome of a vernalisation treatment is the same as that from the constitutive flowering pathway. This vernalisation response is only shown when these mutants are grown in fluorescent light however, and not in combinations of fluorescent and incandescent lighting, or natural sunlight. In addition, these mutants were found to be more responsive to low red to far-red ratios than wild-type plants and other late-flowering mutants (Bagnall, 1992; Martinez-Zapater *et al.*, 1994).

The LUMINIDEPENDENS (LD) gene

One of the first late-flowering mutants to be characterised was the *ld-1* mutant that is defective in the *LD* gene. In 1994, Lee *et al.* described the isolation of a new recessive T-DNA tagged mutant allele, *ld-3*, in the Wassilewskija ecotype that failed to complement the late-flowering phenotype of the original Redei mutant (Redei, 1962). *ld-3* is likely to represent a null mutant allele as the *LD* transcript could only be detected when highly polyA+ enriched RNA samples from mutant plants were examined. The *ld-1* mutant contains a missense mutation in the splice donor site that results in the production of a truncated protein product in the mutant plants. Another mutant allele of this gene, *ld-2*, has also been isolated and found to contain an in-frame nine base pair deletion in the *LD* gene (Aukerman and Amasino, 1996). *ld-3* plants flower later than wild-type plants in long and short days and at the same time as the *ld-1* and *ld-2* mutants. Vernalisation could partially overcome the flowering time phenotype in all *ld* lines, and mutant plants could maintain some photoperiod responsiveness when grown under high light intensities (Lee *et al.*, 1994a). If *ld* mutant plants were given day-length extensions of low intensity light with high red to far-red ratios however, mutants no longer flowered earlier in long days. These results therefore predict that the role of the *LD* gene in the constitutive flowering pathway may be one involving the perception of both light quantity and quality (Lee *et al.*, 1994a; Coupland, 1995a). The *LD* gene has been mapped to the top of chromosome four, and encodes a protein containing two consensus bipartite nuclear localisation signals and a glutamine-rich domain in the carboxy terminus, that shows similarities to those found in several transcription factors (Lee *et al.*, 1994a; Aukerman and Amasino, 1996). In addition, the N-terminal domain of LD was found to contain several of the conserved amino acids found in homeodomain DNA-binding motifs from various yeast or plant proteins. A transcriptional regulatory role has therefore been suggested for the *LD* gene (Lee *et al.*, 1994a; Aukerman and Amasino, 1996; Pineiro and Coupland, 1998).

Northern and Western blot analysis has revealed that the *LD* gene and its protein product are primarily expressed in regions of the root and shoot apex that contain dividing cells such as the apical meristem, with stronger expression detected in the shoots. Localisation of the LD protein within plants has also been examined following a fusion of the *LD* promoter and half of the *LD* coding region to the *E. coli* β-glucoronidase (GUS) gene. As expected, GUS activity in these transgenic plants was localised to the nucleus of cells and was again found to be strongest in rapidly growing meristematic regions of the plant. Newly emerging leaves also showed GUS activity which decreased in intensity as these tissues expanded. (Aukerman and Amasino, 1996; Aukerman *et al.*, 1999).

Floral induction models predict that the production of a floral stimulus in the leaves of plants in response to inductive light conditions, precedes its transport to the apex. The higher expression levels of the LD protein in the shoot apical meristem suggests a role for *LD* in the generation of an apex that is competent to receive such signals. The presence of *LD* expression in the root meristems however, indicates that the role of *LD* is not specific to reproductive development. A more general role of *LD* may entail making any meristem within the plant competent to receive and/or respond to appropriate signals (Aukerman and Amasino, 1996).

The FCA gene

The EMS-induced *fca-1* mutant was isolated by Koornneef *et al.* (1991) in the L. *erecta* ecotype, and is one of the latest flowering mutants of early-flowering ecotypes ever discovered, flowering after the production of approximately 30 rosette leaves. This mutant is also one of the most responsive to vernalisation (Martinez-Zapater and Somerville, 1990; Macknight *et al.*, 1997). While wild-type plants only remain fully responsive to vernalisation treatments if given within two days after germination, *fca* mutants have been shown to flower earlier in response to an eight week vernalisation treatment even if given up to 25 days post germination. These vernalisation responses remain similar whether mutant plants were grown in short days or continuous light conditions, and are enhanced in continuous dark conditions. (Chandler and Dean, 1994).

Chandler *et al.* (2000) investigated the role of gibberellins, phytochrome, and ABA in the vernalisation response, by combining *fca-1* mutant alleles with mutations affecting the biosynthesis or response to gibberellins, ABA, or phytochrome. The lack of a vernalisation response by *gal-3* gibberellin biosynthetic mutants has suggested that gibberellins do play a role in mediating the vernalisation response in *Arabidopsis*. Phytochrome B has also been implicated in this process as the late-flowering mutants showing the strongest response to vernalisation, such as *fca*. also display the greatest decrease in their flowering times in response to far-red enriched light, a process mediated by *PHYB*. As *PHYB* has also been shown to decrease gibberellin biosynthesis and the responsiveness of a plant to GA, phytochrome may mediate its effects in a vernalisation response by altering gibberellin biosynthesis, or sensitivity of the plant (Martinez-Zapater and Somerville, 1990; Bagnall *et al.*, 1995). The role of ABA in vernalisation was also investigated as it had been shown previously that ABA can often act as an antagonist to GA (Jacobsen *et al.*, 1996). Prior to the analysis by Chandler *et al.* (2000) it had been speculated that vernalisation and gibberellins may act via a common process to promote flowering (Dennis *et al.*, 1996). In support of this hypothesis, it has been observed that the gibberellin responsiveness of the *fca* mutant does not appear to be impaired (Bagnall, 1992). In addition, the gibberellins GA_1,

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GA_4, GA_5, and GA_9 were able to promote flowering in all *fca* mutants tested, and GA_3 was also able to partially substitute for a vernalisation treatment decreasing the flowering time of the *fca-1* mutant (Chandler and Dean, 1994).

Contradicting previous hypotheses however, Chandler *et al.* (2000) found that the *fca-1 gal-3* double mutants flowered much later than either single mutant, suggesting that *GA1* and *FCA* promote flowering via independent pathways. In addition, it was also observed that gibberellins were not required for a vernalisation response, as *fca-1 gal-3* lines still responded strongly to vernalisation with a reduction in flowering time. Both the *phyB*, and ABA insensitive mutations, *abi1-1* and *abi2-1*, accelerated flowering of *fca-1* mutants, suggesting that phytochrome and ABA may repress flowering even in the absence of the *FCA* gene product. Furthermore, as neither of these types of mutations altered the responsiveness of *fca-1* mutations to vernalisation, it was also suggested that like gibberellins, neither phytochrome nor ABA plays a major role in the vernalisation response (Chandler *et al.*, 2000).

The molecular mechanism of *FCA* action remains obscure, however, it is known that the FCA protein contains two RNA recognition motifs (RRM) that show similarity to those in the protein encoded by the *ELAV*-like genes of *Drosophila*. These ELAV-like proteins have been shown to function in a variety of processes, including splicing of transcripts, nuclear export, polyadenylation, and maintenance of RNA stability (Macknight *et al.*, 1997; reviewed in Koornneef, 1997). The FCA protein also contains a 26 amino acid region that is identified as a WW, or WWP, protein-protein interaction domain. The presence of this region does suggest that FCA may interact with at least one other protein in plant cells (Macknight *et al.*, 1997).

Four transcripts, α, β, γ, and δ are produced from the alternate splicing of the *FCA* mRNA (Macknight *et al.*, 1997; reviewed in Koornneef, 1997; Pineiro and Coupland, 1998). Only the γ transcript however, is believed to produce a functional FCA protein that contains both the RRM and WW domains. This was suggested as the overexpression of the β transcript alone does not alter the flowering phenotype although plants overexpressing the *FCA* gene flower early, accumulate very high levels of β transcripts, and only slightly higher levels of the γ and δ transcripts. The production of the other *FCA* transcripts has been suggested to be a mechanism by which *FCA* can post-transcriptionally regulate itself (Macknight *et al.*, 1997; reviewed in Koornneef, 1997). The late-flowering *fca 1* mutant accumulates only δ-like transcripts that contain the RRM domain and lack the WW domain, due to the introduction of an in-frame stop codon within the *FCA* gene (Burn *et al.*, 1994; Macknight *et al.*, 1997; Pineiro and Coupland, 1998). This suggests that it is the WW domain of the FCA protein that is responsible for influencing flowering time. In addition, the relative level

of each type of *FCA* transcript remains the same throughout development, in all tissues tested, indicating a continual requirement for *FCA* within the plant (Macknight *et al.*, 1997). In 1999, Page *et al.* further investigated the interaction of the *FCA* gene with the meristem identity genes *LFY*, *TFL*, and *AP1*. It was observed that mutations in the *FCA* gene delayed the formation of the terminal flower in *tfl* mutants, and increased the number of coflorescences formed in *lfy* and *ap1-1* mutants. It was therefore concluded that the *FCA* gene product is required for the early transition of the apex into a floral meristem, and that *FCA* may be required for the function of genes that act in conjunction with *LFY* and *AP1* to initiate flowers (Page *et al.*, 1999). The different flowering time phenotypes in double mutants of *lfy* and *ap1* with strong (*fca-1* and *fca-9*) or weak (*fca-4*) mutant alleles of the *FCA* gene have also been examined (Page *et al.*, 1999). Combination with weaker mutant alleles of *fca* resulted in double mutant plants that flowered later than the original *fca* mutant line. Double mutants containing strong *fca* mutant alleles however, flowered only as late as the *fca* mutant parent. It was also observed that the late-flowering phenotype of the *fca-4* mutation was epistatic to the early-flowering phenotype of transgenic plants overexpressing the *LFY* gene. This led to the proposal that the *FCA* gene may act in two separate pathways. One pathway is postulated to act in parallel with *LFY* and *AP1* to promote flowering, and the other is proposed to involve the activation of *LFY* and *AP1* directly via *FCA* activity. The *fca-4* mutants are suggested to have lost activity in the parallel pathway only, while both pathways involving *FCA* may be defective in the *fca-1* and *fca-9* mutants. This may result in a low level of expression of the *LFY* and *AP1-1* genes within the *fca-4* lines only, and help to explain the slightly earlier flowering phenotype of these lines compared to the *fca-1* or *fca-9* mutants (Page *et al.*, 1999).

The FVE gene

The *fve* mutant was originally isolated by Hussein (1968), following a screen for mutants showing delayed flowering in long days. Two recessive mutant alleles, *fve-1* and *fve-2* of this locus have been isolated following EMS treatment of the L. *erecta* ecotype, with the *fve-2* allele conferring slightly later flowering on plants (Martinez-Zapater *et al.*, 1995). *fve* mutants show delayed flowering in both continuous light and short days, although they flower later in short days. Plants heterozygous for *fve* flower at the same time as wild-type plants in long days, however they also exhibit slightly later flowering times than wild-type plants in short days. This completely recessive phenotype in long days, and a semi-dominant phenotype in short days, may suggest that while the *FVE* gene is required for floral promotion in all photoperiods, the gene product may be more limiting in short days. Therefore the later flowering of *fve* heterozygous plants in short days may be due to the decreased expression of *FVE*, or of other loci that normally act together with *FVE* to promote flowering in short days (Martinez-Zapater *et al.*, 1995).

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fve mutants have been found to be delayed in flowering due to a lengthening of all stages of development (Martinez-Zapater *et al.*, 1995). *fve* mutants produce more rounded juvenile rosette leaves, more ovulate adult rosette leaves, and more cauline leaves than wild-type plants, especially in short day conditions. Vernalisation treatments decreased the time of flowering of these plants which is a phenotype indicated by a decrease in all leaf types in mutant plants. *fve* lines also exhibit extended growth of the inflorescence, and consequently produce more coflorescences than wild-type plants. *FVE* is therefore predicted to have a role in the promotion of both the vegetative-to-reproductive and inflorescence-to-floral transition (Martinez-Zapater *et al.*, 1995).

The FY gene

The EMS-induced *fy* mutants were isolated in the L. *erecta* background and are responsive to both short days and vernalisation. These *fy* mutants are only slightly late-flowering however, with the latest flowering wild-type plants flowering at the same time as the earliest flowering *fy* mutants (Koornneef *et al.*, 1991).

The FLD gene.

The *FLD* gene which maps to the top of chromosome three, was identified through the isolation of the *fld-1* mutant allele following EMS mutagenesis of Columbia ecotype plants (Sanda and Amasino, 1996a). The *fld-1* mutant is photoperiod-sensitive and shows an acceleration of flowering in response to vernalisation and far-red light. When this mutation is introgressed into the L. *erecta* background however, minimal alteration in flowering time is observed. This may be due to the requirement of the *fld-1* mutation for a non-L. *erecta* version of the flowering time gene, *FLC*, to express a late-flowering phenotype (Sanda and Amasino, 1996a, Chou and Yang, 1998).

In 1998, Chou and Yang reported the characterisation of a new *FLD* mutant allele, *fld-2*. This mutant has an extended rosette development, producing on average, 70 more rosette leaves than wild-type plants. *fld-2* mutants also exhibit an elongated inflorescence with many axillary inflorescences, suggesting a role for *FLD* in the conversion of inflorescences to floral meristems. The *fld-2* mutant also produces an excess of rosette inflorescences that arisie from the internodes between rosette leaves, which terminate in morphologically normal rosettes instead of flowers. Indeed, the majority of *fld-2* mutants never produce flowers even after 100 days of growth. In those plants that do flower, at least ten secondary branches arise before the flower is formed. These secondary branches can also form rosettes at their base, so that plants can have tertiary or even quaternary rosettes. The flowers that are observed in these mutants are male sterile due to the production of immature stamens. Long

vernalisation treatments were able to overcome all of these phenotypes. The demethylating agent, 5-aza-cytidine however, had no effect on the late-flowering phenotype of *fld-2* mutants. *fld-2* mutants were also not impaired in their responsiveness to gibberellin, showing both decreased flowering time and increased internode elongation in response to exogenous gibberellin application (Chou and Yang, 1998).

It is proposed that the *FLD* gene product may function as an inhibitory factor suppressing vegetative shoots, thus promoting inflorescence development, by decreasing the activity of a substance which promotes vegetative development (Chou and Yang, 1998). If no inhibitory FLD protein is detected, as in the *fld-2* mutant, the period of vegetative development lengthens. As the plant ages, and the substances actively promoting vegetative development naturally decrease, the shoot apical meristem may begin the production of inflorescences in the *fld-2* mutant, or more often, revert to the production of rosette development. Further reduction of the activity of this promoter of vegetative development may eventually allow the plant to flower (Chou and Yang, 1998).

The interactions of the *FLD* gene with FMI genes have also been investigated (Chou and Yang, 1998). Combinations of the *fld-2* mutant allele with weak or strong *lfy* alleles were found to result in intermediate flowering plants that exhibit more inflorescence defects than *lfy* single mutants. This enhancement of the *LFY* phenotype by the *fld-2* mutation suggests that *LFY* acts downstream of *FLD*, and that *FLD* promotes *LFY* activity. This may be either through a direct promotion of *LFY*, or via the suppression of a floral inhibitory substance (Chou and Yang, 1998). *fld tfl* double mutants display an additive phenotype, showing earlier flowering than the *fld* parental line and a less severe terminal flower structure. These phenotypes are similar to those seen in *tfl* single mutants grown in short days. This indicates that *TFL* and *FLD* act in different pathways to alter the activity of genes which promote floral development (Chou and Yang, 1998).

1.4.3 Group two late-flowering mutants; *fe*, *ft*, *fd* and *fwa*

The EMS-induced late-flowering phenotype of this group of mutants cannot be overcome with vernalisation. Their delay in flowering-time compared to wild-type plants grown in long days is also significantly more than the slight differences seen in short days. Therefore these mutants are believed to be altered in genes primarily affecting a flowering pathway that is dependent upon inductive long days for its function. All of these mutants are recessive, except the *fwa* mutant which is dominant (Martinez-Zapater *et al.*, 1994; Coupland, 1995a; Haughn *et al.*, 1995).

The FT and FWA genes

In 1998, Araki *et al.* proposed that the genes *FWA* and *FT* may compose a subgroup within this class of mutants. This is based on the observations of several unique phenotypic traits displayed by these two mutants. Firstly, unlike most other late-flowering mutants, *ft* and *fwa* mutations dramatically enhance the phenotype of plants mutated in the floral meristem identity genes, *LFY* and *AP1*. Secondly, weak *ft* alleles can partially suppress the early-flowering phenotype of *emf-1* mutants (discussed below in section 1.5), whereas *emf* alleles are completely epistatic to other 'late-flowering' genes (Araki *et al.*, 1998). Furthermore, unlike several late- (*co, gi*. and *ld*) and early-flowering (*hy2*) mutants whose flowering times are the same as wild-type plants when all are grown in the dark, the *ft* and *fwa* mutants flower late in these conditions. The addition of sucrose to the media also failed to rescue the late-flowering phenotype of such lines, suggesting that *FT* and *FWA* may function downstream of genes involved in sugar transport to the apex (Araki *et al.*, 1998).

It has further been suggested that the role in the floral induction process of the transcription factor FWA, which contains a homeodomain (Soppe *et al.*, 2000), is not to directly promote flowering, but rather to help counteract an inhibition of flowering. This is suggested since the two *fwa* alleles isolated, both cause dominant phenotypes, and have been suggested to be gain-of-function mutants due to the hypomethylation of a normally hypermethylated 5 Mbp region of chromosome 4 spanning the *FWA* locus (Araki *et al.*, 1998; Finnegan *et al.* 1998). In support of this, the sequences of the two *fwa* alleles are found to be identical to that of the wild-type sequence. In addition, the *FWA* gene is normally silenced in wild-type plants via methylation of the 5′ region, however this gene is ectopically expressed in the mutants (Soppe *et al.*, 2000). Further support is provided by the isolation of a dominant late-flowering trait following successive in-breeding of the *ddm1* mutants, which exhibit decreased levels of methylation (Finnegan *et al.* 1998). The *DDM1* locus maps near the *FWA* locus, however these genes are not thought to be identical, since *fwa* mutants do not respond to vernalisation in either long or short days. Vernalisation however can accelerate the flowering of *ddm1* mutants in non-inductive conditions. The *ddm1* mutation is believed to activate *FWA* by demethylation of the surrounding chromosomal region, resulting in a late-flowering phenotype. This is similar to the situation though to occur in the *fwa* gain-of-function mutants (Araki *et al.*, 1998; Finnegan *et al.* 1998).

The *FT* gene has recently been cloned following the isolation of a T-DNA tagged mutant line (Araki *et al.*, 1998). The FT protein shows significant homology to the *TFL-1* gene product which belongs to a protein family showing similarity to mammalian phosophatidylethanolamine-binding proteins (Araki *et al.*, 1998; Onouchi and Coupland, 1998; Pinciro and Coupland, 1998). The expression of the *FT* gene was found to be low

and is first seen in two day-old seedlings. Expression levels of the gene gradually increase with the age of the plant, although this occurs earlier than the increase in expression of other flowering time genes such as *CO, LFY,* and *TFL-1* (Araki *et al.,* 1998). It is now believed that *FT* may act downstream and antagonistically with *TFL,* as the *ft* and *tfl-1* mutations have opposite effects on flowering time. In addition, *tfl ft* double mutants show an additive phenotype, flowering slightly earlier than *ft* single mutants and forming terminal flowers later than *tfl* mutants. A similar phenotype is also observed in *fwa tfl-1* double mutants, whereas other late-flowering mutants are episatic to the *tfl* mutation. This supports the proposal that *FT* may have a role in the promotion of FMI genes, whereas the *TFL* gene has been proposed to inhibit the action of such genes (Araki *et al.,* 1998; Onouchi and Coupland, 1998). Araki *et al.* (1998) further postulated that *FT* may act at the shoot apical meristem to control floral evocation, rather than regulating photoperiodic induction in leaves, and that the activity of FT and FWA may then determine the competence of the apex to flower (Araki *et al.,* 1998; Onouchi and Coupland, 1998).

Further support for the hypothesis that the *FT* and *FWA* genes act directly at the apex to enhance the actions of FMI genes, in a similar manner as the *LFY* gene, comes from an analysis of the phenotypes of *ft lfy* or *fwa lfy* double mutants. These lines flower as late as the *fwa* or *ft* single mutants and show an extreme *LFY*-like phenotype, as they only form inflorescences that never produce flowers. This latter phenotype indicates a lack of function of floral development genes, such as *AP1* (Mandueno *et al.,* 1996; Ruiz-Garcia *et al.,* 1997; Pineiro and Coupland, 1998). In addition, combinations of *ap1* and the *ft* or *fwa* mutations also resulted in the production of an extreme *ap1*-like phenotype. Such extreme phenotypes were not observed in double mutants of *lfy* and *ap1,* or double mutants of *lfy* with other flowering time mutants such as the *fve* and *fpa* lines (Mandueno *et al.,* 1996; Ruiz-Garcia *et al.,* 1997; Pineiro and Coupland, 1998). These observations support the proposal that *FT* and *FWA* have an additional role to that of other flowering time genes, to act redundantly, or in parallel with, the *LFY* gene product to activate FMI genes such as *AP1.* It has been postulated further that other genes such as *FPA* and *FVE* may play a role in indirectly activating FMI genes via an interaction with the pathway involving *FT, FWA,* and *LFY* (Mandueno *et al.,* 1996; Ruiz-Garcia *et al.,* 1997; Araki *et al.,* 1998; Nilsson *et al.,* 1998; Page *et al.,* 1999).

1.4.4 Group three late-flowering mutants; *co, gi,* and *fha*

Mutations affecting genes in this group result in plants flowering late only in long day conditions that are completely insensitive to vernalisation. It is therefore thought that such genes act in a floral pathway that is only active in inductive long day conditions (Martinez-Zapater *et al.,* 1994; Coupland, 1995a; Haughn *et al.,* 1995; Simon and Coupland, 1996).

The CONSTANS (CO) gene

The late-flowering *co* mutants were first identified by Redei (1962) in the Columbia ecotype, and show a day-neutral flowering phenotype. This phenotype indicated that the CO protein is important in regulating flowering time in response to environmental conditions, particularly photoperiod (Putterill *et al.*, 1993; Putterill *et al.*, 1995). The *CO* gene has recently been cloned and the 373 amino acid CO protein was found to contain two putative zinc finger domains that are similar to those found in the GATA1 family of transcription factors from humans. This protein also contains a putative nuclear localisation domain in the carboxy terminus (Putterill *et al.*, 1995; Amasino, 1996a; Simon and Coupland, 1996; Onouchi and Coupland, 1998; Pineiro and Coupland, 1998).

All *co* mutant alleles contain deficiencies in either the putative zinc finger domains or in the carboxy terminus of the CO protein. The 9 bp deletion in the *co-1* mutant allele, and the G-to-A nucleotide transition found in the *co-2* allele for example, both occur in a region containing the putative zinc finger domains. A more recently isolated allele, *co-8*, has the entire DNA binding domain deleted and is predicted to be a null mutant. This mutant can still flower however, and like all *co* alleles, plants heterozygous for the *co-8* mutation flower at intermediate times. This latter phenotype suggests that the *co* mutation is semi-dominant and that the *CO* gene product may act to promote flowering in a dose-dependent manner (Putterill *et al.*, 1995; Simon and Coupland, 1996).

co mutant phenotypes have also suggested that the *CO* gene product is more limiting in short days than in long days. *CO* levels are low in long days and a further decrease in expression of this gene is observed in plants grown in short days (Putterill *et al.*, 1995; Onouchi and Coupland, 1998; Pineiro and Coupland, 1998). Furthermore, some *co-2* mutant plants transgenic for several copies of the *CO* gene, flowered earlier than wild-type plants in short days (Putterill *et al.*, 1995). It has also been proposed that the *phyB* mutation which causes early-flowering in short days, results in an increase in *CO* levels. *co phyB* double mutants are found to be later flowering than *phyB* single mutants, although they still flower earlier than wild-type plants in long and short days (Putterill *et al.*, 1995).

The *CO* gene has been found to be expressed in both shoots and leaves with the highest expression detected in meristematic tissues, inflorescences, and floral buds (Simon and Coupland, 1996). This expression pattern therefore predicts other roles for the *CO* gene besides its contribution to the facultative long day requirement of *Arabidopsis* for early-flowering. To further investigate the role of the *CO* gene in flowering, it has been placed under the control of the constitutive CaMV35S promoter and transformed into plants (Onouchi and Coupland, 1998). As expected, transformants flowered earlier than wild-type

plants in both long and short days. In addition, plants overexpressing both *CO* and *GI* flowered at the same time as those only overexpressing *CO*, which places *CO* downstream of the *GI* gene in the long day inductive pathway. Furthermore, when *fca* mutations were introgressed into plants overexpressing *CO*, transformants flowered later than 35S::*CO* plants but earlier that the *fca* mutants. Therefore, increases in the activity of the long day-dependent pathway can partially compensate for a defect in the constitutive flowering pathway (Simon and Coupland, 1996; Onouchi and Coupland, 1998).

Transformants have also been generated containing the CO protein fused to a hormone-inducible mammalian glucocorticoid receptor protein under the control of the CaMV35S promoter. Hormone-treated plants containing this construct flowered rapidly in both long and short days, and earlier application of the hormone produces a more dramatic phenotype. This suggests that plants can respond to altered *CO* levels, or activity, at any time throughout development (Pineiro and Coupland, 1998). A model to explain these observations has been proposed suggesting that the CO protein may act as a timer that responds to the environment. In long days, this timer may run fast, causing rapid flowering. In short days, the *CO*-induced timer runs too slow and flowering becomes dependent upon a different mechanism (Pineiro and Coupland, 1998).

Further genetic analysis of the role of *CO* in flowering has utilised double mutants. The introduction of the *co* mutant allele into lines containing defects in the *GAI* and *GAI* genes resulted in plants showing a severe delay in flowering in both long and short day conditions. In addition, several phenotypes associated with the *gai* mutation were enhanced in the *co gai* double mutants, suggesting redundant roles of the *CO* and *GAI* genes in a single pathway. Exogenous application of gibberellin had only a small effect on the phenotype of short day-grown *co-2* plants however, and no effect on the late-flowering of long day-grown plants. This indicates that the *CO* gene product is not required for gibberellin biosynthesis. Instead, *CO* may function in a similar manner to the products of gibberellin signal transduction pathways to promote flowering, perhaps by altering the transcription of genes involved in floral inhibition (Coupland *et al.*, 1993; Putterill *et al.*, 1995).

One of the most widely studied interactions between genes involved in flowering is that between the *CO* and *LFY* genes. As mentioned above the *LFY* gene affects the transition from inflorescence to floral meristems by activating genes controlling the floral transition. Several lines of evidence suggest a positive correlation between induction of *CO* activity and a subsequent upregulation of *LFY*. Plants transformed with the hormone-inducible *CO* overexpression construct for example, showed a co-induction of the *LFY* gene with hormone-induced *CO* activity in short days, or when such plants were shifted from short to long days. In addition, the phenotype of *lfy* mutants was enhanced when introgressed into

plants containing weak *co* mutant alleles. These results suggest that at least one function of *CO* may be to activate *LFY* and/or to enable the shoot apical meristem to respond rapidly to the upregulation of *LFY* (Putterill *et al.*, 1995; Simon *et al.*, 1996; Ma, 1997; Onouchi and Coupland, 1998; Pineiro and Coupland, 1998). It has been further postulated that the downstream activation of *LFY* by a long day-dependent floral induction pathway involving *CO* may be paralleled by one involving gibberellins. This is suggested following the observation that treatment of *co* mutants with the gibberellin biosynthesis inhibitor, paclobutrazol, prevents upregulation of *LFY* in such plants (Blazquez *et al.*, 1998)

It was also noted that both the *LFY* and *TFL* genes are expressed 24 hours after the hormone-mediated induction of *CO* in transgenic plants grown in both long and short days. This provides further evidence for a role of *CO* in promoting the activity of FMI genes. Expression of the *AP1* gene however, was only observed 72 hours after *CO* induction in plants grown in long days, and after 120 hours in plants grown in short day conditions. These results are consistent with the placement of *CO* in a long day-dependent pathway that activates both the *LFY* and *TFL* genes and indirectly the downstream *AP1* gene. The fact that *AP1* induction occurs much less rapidly in short days compared to long days, suggests that induction of this gene is dependent upon long day-induced factors other than the *CO* gene product (Simon *et al.*, 1996; Pineiro and Coupland, 1998).

Further target genes for the CO protein have been identified through the screening for mutants that suppress the phenotype of plants overexpressing *CO* (Onouchi *et al.*, 2000; Samach *et al.*, 2000). These screens have resulted in the isolation of novel genes, such as the *SOC1* (*AGL20*) gene discussed above, and alleles of the flowering time genes *FT* and *FWA*. This indicates that *FT* and *FWA* may act after *CO* in the hierarchy of flowering time genes. It was also noted that the *fwa* mutation also suppressed floral development after bolting had occurred in these plants, again indicating an alternate role for *FWA* other than promoting floral induction (Onouchi *et al.*, 2000)

The GIGANTEA (GI) gene

The first two mutant alleles (*gi-1* and *gi-2*) of the *GIGANTEA* locus, which maps to chromosome one, were described by Redei (1962) in the Columbia background. In 1991, Koornneef *et al.* reported the isolation of a further eight *gi* (originally termed *fb*) mutant alleles in the L. *erecta* background (Koornneef *et al.*, 1991; Araki and Komeda, 1993b). All of these mutations result in plants that are later flowering in long days, but show little difference in their flowering times in short days compared to the wild-type controls. In 1993, Araki and Komeda analysed in detail the responses of the two Columbia mutant alleles (*gi-1* and *gi-2*) and one L. *erecta gi* allele (*gi-3*). In short days, the *gi-1* and *gi-2* mutants

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were found to flower at the same time as wild-type, however the *gi-3* mutant was slightly later-flowering than wild-type in these conditions. While all these mutant alleles resulted in plants that flowered later than wild-type ecotypes in long days, the *gi-3* mutant showed a decrease in flowering time in long days compared to short days. The *gi-1* mutants also flowered earlier in long days than short days although this induction was markedly less than that observed for the *gi-3* mutant. In contrast, the *gi-2* mutation resulted in plants that were slightly delayed in their flowering time in long days. The differing responses of the *gi* alleles to short and long days are believed to be due to the extent of the lesion within each mutant allele, as the most severely altered allele, *gi-2*, is also the latest to flower. In addition, the Columbia background enhances the severity of the late-flowering phenotype of *gi* alleles, as alleles in the L. *erecta* background flowered earliest in long days. This has lead to the proposal that *GI* may be involved in regulating the conversion of a floral inhibitor produced under short days, into a less inhibitory form in long days. In the *gi* mutants the level of this inhibitor may remain unconverted in inductive conditions and therefore result in late-flowering plants in long days (Araki and Komeda, 1993b).

The *gi-2* mutants were also observed to display several phenotypic traits, such as an elongated hypocotyl, that co-segregate with the late-flowering phenotype. In addition, both the late-flowering and hypocotyl phenotype were found to segregate in a co-dominant manner. The long hypocotyl phenotype was found to be due to a decreased inhibition of cell elongation by light in these mutant lines, as no increase in cell number in the hypocotyls was observed. This supports the suggested role of *GI* in the regulation of light-controlled processes, particularly in long day conditions. The *gi-2* mutation was also observed to be temperature-sensitive, with mutants flowering twice as late at 21°C compared with 28°C. Lower temperatures also enhanced the elongated hypocotyl phenotype. Furthermore, temperature-shift experiments established that plants were committed to express the earlier-flowering phenotype at 28°C

Another pleiotrophic effect of the *gi* mutations is the accumulation of starch. It has therefore been predicted that the *gi* mutation may also have a role in carbohydrate transport to the apical meristem (Amasino, 1996a). It has recently been noted however, that the accumulation of starch in these mutants is not correlated with the late-flowering phenotype (Fowler *et al.*, 1999)

The role of the *GI* gene in the circadian clock has recently been investigated following the cloning of the *GI* gene from a T-DNA tagged mutant of the Wassilewskija ecotype (Richardson *et al.*, 1998; Fowler *et al.*, 1999). The predicted GI protein contains eleven possible transmembrane domains and has 71% homology to a rice EST that contains four such transmembrane domains in its N-terminus. Expression of *GI* mRNA was observed

throughout the development of the plant and found to cycle every 24 hours, with peak expression noted in long days, approximately 10 hours after dawn. This cycling pattern persists even when plants are grown in continuous light, strongly suggesting that *GI* expression is regulated by circadian rhythms. The peak of *GI* expression was also observed to be later and lower in long days compared with short days, however it takes longer for transcript levels to fall to trough levels in long days. This extended period of relatively high levels of GI expression in long days, compared with short days, may reflect the role of *GI* in a long day-mediated promotion of flowering (Fowler *et al.*, 1999).

Further support for the role of GI in processes mediated by circadian rhythms stem from observations that *elf3* mutants, which are disrupted in a gene known to be involved in the input domain of the circadian clock, have also been shown to have altered levels of *GI* expression. *GI* levels still oscillate with a periodicity of 24 hours in *elf3* mutants, however *GI* expression is elevated above the lowest levels detected in wild-type plants at all stages of this cycle. As the *elf3* mutation results in plants that flower early, it has been suggested that the elevated levels of *GI* in such plants may influence this flowering time phenotype. In addition, the abundance of the CCA1 and the LHY proteins which are also involved processe related to the circadian clock, were drastically decreased in *gi-3* mutant plants. A late-flowering phenotype similar to that observed in *gi* mutants however, has also been observed when these proteins are overexpressed in plants. Furthermore, levels of the LHY protein are found to be lower in *elf3* mutants than those observed in wild-type plants. Taken in conjunction, these observations suggest that arrhythmic expression of the CCA and LHY proteins, rather than their absolute levels, is the critical factor causing the altered day-length response observed in mutants such as *gi* and *elf3*. While *GI*, *CCA1*, *LHY*, and *ELF3* may affect the same light-regulated pathway, the complex relationships between these genes make it unlikely that these interactions act in a linear pathway (Fowler *et al.*, 1999). Instead, it has been suggested that *GI* can affect circadian rhythm-mediated processes in plants via a feedback loop (Park *et al.*, 1999)

The FHA gene

The leaky *fha* late-flowering mutant is also believed to be affected in a gene involved in the long day-induced floral promotory pathway. The *FHA* gene has now been shown to be allelic to the *CRY2* gene that encodes a flavin-containing blue light receptor. Characterisation of the *fha* mutant has therefore shown that blue light may act via a long day-induced pathway (Guo *et al.*, 1998). *FHA* is believed to act upstream of *CO* in such a pathway, as *fha* mutants show lower levels of *CO* than wild-type plants (Guo *et al.*, 1998; Onouchi and Coupland, 1998).

1.4.5 The *FPF* gene

In 1997, Kania *et al.* reported the use of a subtractive hybridisation technique to identify genes that respond to photoperiodic induction in the apical meristem of mustard, a close relative of *Arabidopsis*. From this procedure, the *FPF1 (FLOWERING PROMOTING FACTOR1)* gene was isolated which was specifically activated following the induction of flowering. An *Arabidopsis* homologue of *FPF1*, *(FPF1(At))*, has subsequently been cloned and found to be almost 93% homologous to the mustard gene (Kania *et al.*, 1997). This sequence mapped to chromosome five at a location distinct from those of any other flowering time genes also mapping to this linkage group. The mRNA levels of *FPF1(At)* were shown to increase in apical buds up to day seven following floral induction, and subsequently decrease at approximately ten days post-induction. As floral induction is associated with an increase in mitotic activity in the peripheral zone of the apical meristem, a region where *FPF1* is first upregulated, it is postulated that *FPF1* is involved in the early stages of the transition to flowering (Kania *et al.*, 1997)

When the *FPF1(At)* gene was overexpressed in plants they became early-flowering in both long and short days. Indeed, these transformants often flowered before the production of rosette leaves had stopped and hence produced several rosette leaves in the positions normally occupied by cauline leaves. Other pleiotrophic effects of *FPF1(At)* overexpression included earlier germination and the formation of longer hypocotyls and larger rosette leaves. These features are reminiscent of the phenotypes observed in wild-type plants treated with gibberellin. In addition, gibberellin treatment of *FPF1(At)* transformants gave an additive phenotype with respect to flowering time. Furthermore, treatment with the gibberellin inhibitor, paclobutrazol, caused a severe delay in the flowering time of transformed plants. These results indicate that gibberellin synthesis is a limiting factor for expression of the *FPF1(At)*-mediated flowering phenotype (Kania *et al.*, 1997).

To further investigate the interaction of *FPF1* and gibberellin, lines overexpressing *FPF1(At)* were introgressed into strong *(ga2-1)*, or weak *(ga4-1)*, gibberellin biosynthesis mutant backgrounds. It was observed that the *ga2-1 FPF1(At)* double mutant was as late-flowering as the *ga2-1* mutant, whereas *ga4-1 FPF1(At)* double mutants displayed intermediate flowering times. These observations again indicate that the function of the *FPF-1* gene is dependent upon, or responsive to, gibberellins. When levels of the gibberellins GA_4 and GA_{20} were examined in the transgenic lines, only a slight elevation in their levels was observed. It has therefore been postulated that the transgenic plants may be more responsive to gibberellins therefore requiring lower levels than wild type plants to initiate responses. This decreased requirement for gibberellin may alter the feedback regulation of gibberellin biosynthesis, decreasing the pools of free gibberellin in the plant (Kania *et al.*, 1997). The

increased gibberellin responsiveness of lines overexpressing *FPF-1(At)* may then lead to an activation of the gibberellin signalling pathway used to promote flowering (Kania *et al.*, 1997)

1.4.6 Interactions between late-flowering mutants

As mentioned above two floral promotory pathways are proposed to exist; a constitutive pathway, and a long day-dependent pathway, that are defined by the genes affected in late-flowering mutants. It has also been postulated that genes affected in both pathways act to promote flowering by an inhibition of a major floral repressor (Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Weigel and Nilsson, 1995). The observation that the *co*, *gi*, and *ld* mutants all flower at the same time as wild-type plants in complete darkness for example, suggests that the main role of the long day-dependent promotive pathway may be to counteract repression pathways (Martinez-Zapater *et al.*, 1994).

In addition to the long day-dependent and autonomous flowering pathways, there is also believed to be a vernalisation-dependent floral promotion pathway whose effects, like those of the constitutive pathway, may be dependent upon gibberellin biosynthesis (reviewed in Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995). The additive effect of gibberellin and vernalisation in promoting the flowering of the *fca* late-flowering mutants however, suggests that vernalisation and gibberellin synthesis may also act in at least partially independent pathways. Vernalisation may therefore act not by increasing gibberellin levels in the plant, but rather, by enhancing the responsiveness of the plant to gibberellin (Martinez-Zapater and Somerville, 1990; Chandler and Dean, 1994; Dennis *et al.*, 1996). Furthermore, it is thought that vernalisation has little effect on genes involved in the constitutive flowering pathway, as cold treatment of late-flowering mutants affected in genes in the long day-dependent pathway only, show little response to such treatment (Martinez-Zapater *et al.*, 1994).

When Koornneef *et al.* (1991) isolated twelve different late-flowering mutants in the L. *erecta* ecotype their placement into different pathways was dependent upon the results of double mutant analysis. The various physiological responses of these groups led to the proposal of a complicated system of promoters and inhibitors that control floral induction via both the long day-dependent and autonomous flowering pathways (Koornneef *et al.*, 1991). In 1998, Koornneef *et al.* performed a further comprehensive analysis of the *fca*, *fld*, *fpa*, *fv*, *fy*, and *ld* mutants which are affected in genes involved in an autonomous flowering pathway, and also studied the *co*, *gi*, *fd*, *fe*, *fha*, *ft*, and *fwa* mutants which are defective in genes involved in a long day-dependent floral promotion pathway. Double mutants between these lines were constructed in order to organise these mutants further into epistatic groups

and to confirm their roles in proposed flowering pathways. Due to linkage between the *co* and *fy* genes, or between the *fca* and *fwa* genes, double mutants could not be constructed. In addition, it was believed that the *fpa* and *fy* double mutants may not be viable as homozygotes could never be isolated (Koornneef *et al.*, 1998). Between the remaining mutant lines, three main types of double mutants were found; those in which the flowering time of the double mutants was the sum of those of the parental lines, those in which the double mutants flowered earlier than the sum of the parental lines (the most extreme cases flowering at the same time as a single parent), and those in which a synergistic effect was found, so that double mutants flowered later than the sum of the parental lines flowering times. If additive or synergistic effects on the flowering time phenotype were observed in the double mutants it was thought that the genes affected in the mutant parentals act in two separate parallel pathways that lead to the same end result. If an epistatic relationship is shown between the two parental mutant lines then the affected genes are thought to affect subsequent steps in the same developmental pathway (Koornneef *et al.*, 1998).

Amongst the mutants affected in the long day flowering pathway, the *gi* mutant was found to be epistatic to the *co, fwa, fha, fe.* and *ft* mutants, *co* mutations were found to be epistatic to *ft, fe,* and *fwa*, and *fwa* was shown to be epistatic to *ft.* Amongst the mutants affected in the constitutive flowering pathway the interactions were found to be more complex with the only clear epistatic relationship shown between *fca* and *fy.* and *fve* and *fpa.* When mutants from the two different flowering pathways were combined, the most common response was an additive delay in flowering times. Occasionally, a synergistic effect was observed however, with double mutants between the *fpa* mutant and other lines showing the most complex interactions (Koornneef *et al.*, 1998).

In most cases, the response to vernalisation was as expected for the double mutant lines, with double mutants of mutations affecting the same pathways retaining the vernalisation responsiveness or unresponsiveness of the parental mutants. Only the *fca* and *fpa* double mutant displayed a lesser response to vernalisation than expected. In addition, a combination of mutants from the different pathways resulted in double mutants showing intermediate vernalisation responses, except for the *fe* or *ft* double mutants which retained the responsiveness of the *fe* and *ft* lines (Koornneef *et al.*, 1998).

The majority of the observations confirmed the classification of the genes into the two pathways previously described. The synergistic flowering time phenotype of the *fpa* and *fe* or *ft* double mutants however, suggests that *FPA* may play an additional role, similar to that of the *FE* and *FT* genes, in the long day-dependent pathway. Thus when *FPA* and either *FE* or *FT* functions are removed, there is not only a block in both pathways that results in an additive late-flowering phenotype, there is also a loss of the floral promotory function

performed by both the *FPA* and *FE* or *FT* genes in the long day-dependent pathway. This results in a further delay in flowering. The observation that vernalisation did not alter the flowering times of mutants in the long day-dependent pathway in either single or double mutants supports the theory that vernalisation acts in a similar manner as the constitutive pathway to promote flowering. The common target of these pathways may be to alter gibberellin metabolism or sensitivity within the plant (Koornneef *et al.*, 1998).

1.4.7 Mutations conferring an early-flowering phenotype in *Arabidopsis*

There are at least 24 mutations in separate genes in *Arabidopsis* that result in an early-flowering phenotype. Several of these have been discussed above and were isolated based on other pleiotrophic characteristics. These include the elongated hypocotyl mutants (*hy1-8*) (Johnson *et al.*, 1994: Mozely and Thomas, 1995; Whitelam *et al.*, 1998), the constitutive photomorphogenic (*cop1*) mutant (Deng *et al.*, 1991) and the terminal flower mutant (*tfl1*) (Alvarez *et al.*, 1992). Other early-flowering mutants have been identified following screens of early-flowering ecotype plants grown in tissue culture or short days (Coupland *et al.*, 1993). All early-flowering mutants so far identified in these backgrounds are recessive or semi-dominant (Coupland, 1995a; Coupland, 1995b; Peeters and Koornneef, 1996). The genes altered in early-flowering mutants are believed to have a role in the repression of flowering (Martinez-Zapater *et al.*, 1994; Coupland, 1995b).

Early-flowering mutants have been classified into two main groups that may define two pathways; a constitutive floral repression pathway and a short day-dependent repression pathway (Martinez-Zapater *et al.*, 1994). Mutants affected in the constitutive floral repression pathway are earlier flowering than wild-type plants in both long and short days, while still displaying some delay in their flowering in response to short days. Mutants in this group include the *elg* (*elongated*), *elf1* and *elf2* (*early-flowering1* and 2), *svp* (*short vegetative phase*), *tfl* (*terminal flower-1*), *eafl* (*early flowering 1*), and *emf* (*embryonic flower*) mutants. The second group, defining a short day-mediated floral repression process, includes the *esd-2*, *esd-3*, *esd-4* (*early short days-2, -3, -4*), *efs* (*early-flowering in short days*), and the *elf3* (*early-flowering-3*) mutants, which are early flowering in long and short days and no longer display a delay in flowering in short day conditions (Zagotta *et al.*, 1992; Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Amasino, 1996b; Hicks *et al.*, 1996b; Peeters and Koornneef, 1996; Scott *et al.*, 1999; Hartman *et al.*, 2000). It is unclear whether a short day repression pathway exists, as it has been suggested that genes repressing flowering in short days may do so by removing the inhibition of these genes, which is normally caused by the products of the long day-dependent floral promotory pathway (Martinez-Zapater *et al.*, 1994). Alternatively, genes involved with a short day-

dependent repression of flowering may exert their effects via a repression of genes involved within a long day-dependent pathway.

1.4.8 Genes involved in a putative short day repression pathway

The EARLY-FLOWERING 3 (ELF 3) gene

Zagotta *et al.* (1992) isolated three classes of early-flowering mutants following a screen of EMS-mutagenised Columbia plants. One of these groups was defined by the previously characterised *tfl* mutant (Shannon and Ry Meeks-Wagner, 1993; Alvarez *et al.*, 1992). Class two was described by two recessive mutants, *elf1* and *elf2*, that were earlier flowering in long and short days, while still showing a photoperiod response. The third class was defined by the *elf3* mutant, which showed the same early phenotype when grown in 20, 16, or 10 hours light. *elf3* mutants have since been studied in detail, and the *ELF3* gene has been mapped to the central region of chromosome two. Six *elf3* alleles have now been isolated in two different ecotypes (Zagotta *et al.*, 1996; Hicks *et al.*, 1996a). These *elf3* mutants also have elongated hypocotyls and their rosette leaves are pale green due to decreased levels of chlorophyll (Zagotta *et al.*, 1992; Martinez-Zapater *et al.*, 1994; Zagotta *et al.*, 1996). The elongated hypocotyl phenotype of these mutants initially suggested a possible light signalling defect in these lines, however *elf3* plants failed to complement any of the *cry* or *hy* mutants isolated (Zagotta *et al.*, 1996; Onouchi and Coupland, 1998).

Further evidence has been presented that *elf3* may be involved in the promotion of flowering by influencing a light-mediated signal transduction pathway related to photoreceptors other than phytochrome or cryptochrome (Zagotta *et al.*, 1996). *elf3 hy2* double mutants for example, were found to flower very early, suggesting that phytochrome-mediated floral induction pathways remain intact in *elf3* mutants. In addition, *elf3* mutants were less sensitive to blue-green light than controls, a phenotype similar to *hy4* mutants. Unlike these mutants however, *elf3* plants retained normal red light responses. Furthermore, the *elf3* mutation is epistatic to *hy4* with respect to flowering time, but not hypocotyl length, indicating that *elf3* is not simply downstream of *hy4* in a signal transduction pathway (Hicks *et al.*, 1996a; Zagotta *et al.*, 1996; Onouchi and Coupland, 1998).

It is now believed that *ELF3* may play a pivotal role in plant development via regulation or interactions with circadian rhythms (Zagotta *et al.*, 1996; Koornneef and Peeters, 1997). This theory is based on several observations. Firstly, leaf movements of *elf3* plants are abolished when they are grown in constant light, suggesting an impaired circadian response. Secondly, when the *elf3* mutation was introduced into transgenic plants containing the luciferase reporter gene under the control of the *CAB* gene promoter, the rhythmic activity of

this gene was three- to four-fold less than in controls. *elf3* mutants retained normal circadian rhythm functions however, when grown in continuous dark (Hicks *et al.*, 1996b). It is proposed that *ELF3* may therefore function in a light input pathway to the circadian oscillator, and that an aberration in the coordination of light and circadian rhythm pathways contributes to the phenotype of the *elf3* mutants (Amasino, 1996a; Hicks *et al.*, 1996a; Hicks *et al.*, 1996b; Onouchi and Coupland, 1998; Pineiro and Coupland, 1998).

One of the main downstream effects of genes such as *ELF3* is postulated to be in the repression of FMI genes such as *LFY*, as overexpression of *LFY* results in an early-flowering phenotype (Zagotta *et al.*, 1996). The positioning of FMI genes, such as the *TFL1* gene downstream of *ELF3* is also suggested by the fact that the *tfl1 elf3* double mutants show a phenotype similar to *tfl* single mutants (Martinez-Zapater *et al.*, 1994; Zagotta *et al.*, 1996).

The EARLY SHORT DAYS (ESD) genes

Five different early-flowering mutants within this group have been identified, and these mutants define four genes (*ESD1, ESD2, ESD3* and *ESD4*), in the *L. erecta* ecotype. The *esd1* mutant is recessive and is day-length sensitive. *esd2* and *esd3* are also recessive mutants, which flower at the same time as wild-type in long days, but earlier in short days. The *esd4* mutant shows an intermediate early-flowering phenotype in heterozygotes and contains a lesion in a gene mapping to chromosome three. This mutant has the most extreme phenotype of the *esd* mutants, flowering after the production of only two leaves in long days and four leaves in short days. This mutant is also partially epistatic to several late-flowering mutants (Coupland *et al.*, 1993; Martinez-Zapater *et al.*, 1994; Amasino, 1996a; Hicks *et al.*, 1996b).

The CURLY LEAF (CLF) gene

Recessive mutations (*clf-1, clf-2, clf-7,* and *clf-19*) at the *CLF* locus, which maps to chromosome two, confer pleiotrophic effects on leaf and flower development, as well as early-flowering (Coupland *et al.*, 1993; Jurgens, 1997; Goodrich *et al.*, 1997). The leaves of *clf* mutants are narrow and curled around the midrib, with later formed leaves showing more severe curling. The inflorescence and floral bud development of *clf* mutants are also disrupted. *clf* mutants are reported to flower two days earlier in long days and three weeks earlier in short days than controls. The expression of the *clf* mutant phenotype is also temperature sensitive, as these plants are almost indistinguishable from wild-types when grown at 16°C (Coupland *et al.*, 1993; Goodrich *et al.*, 1997; Pineiro and Coupland, 1998). The *clf* mutant phenotype is also similar to those seen in plants overexpressing the homeotic

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gene, *AG. clf* mutants have subsequently been found to show ectopic expression of *AG*, suggesting a role for the *CLF* gene in repressing *AG* transcription in the leaves, inflorescences, stems and flowers (Coupland *et al.*, 1993; Goodrich *et al.*, 1997; Pineiro and Coupland, 1998).

The *CLF* has been cloned and found to encode a protein containing two conserved regions with homology to the *Drosophila* gene, *ENHANCER* of *ZEST* (*E(Z)*), as well as a nuclear localisation signal (Goodrich *et al.*, 1997; Pineiro and Coupland, 1998). The E(Z) molecule belongs to a group of polycomb proteins that alter chromatin structure and are believed to be involved in the heritable maintenance of homeotic gene transcription. The similarity of *CLF* to a polycomb gene further supports the hypothesis that the main role of *CLF* is to maintain the correct repression patterns of homeotic genes such as *AG* and *AP3*, particularly in the later stages of *Arabidopsis* floral development (Simon and Coupland, 1996; Goodrich *et al.*, 1997; Pineiro and Coupland, 1998).

The *EARLY-FLOWERING IN SHORT DAYS* (*EFS*) gene

Recently, researchers have been trying to isolate further early-flowering mutants of *Arabidopsis* by screening for such mutants in initially late-flowering genetic backgrounds. In 1999, Soppe *et al.* reported the isolation of two *early-flowering in short days* (*efs*) mutant alleles of the *EFS* gene that maps to chromosome one, following irradiation of the *fwa-1* mutants in the L. *erecta* background. In the *fwa* mutant background *efs* mutations result in a decrease in the flowering time of plants grown in long day conditions. When *efs* alleles are introduced into wild-type L. *erecta* plants however, there was no reduction in flowering time observed. While both wild-type L. *erecta* and *efs* mutants flower later in short days than in long days, the *efs* mutants flower significantly earlier than wild-type plants in these conditions (Soppe *et al.*, 1999). Measurements of the appearance of adaxial trichomes on rosette leaves indicate that the early-flowering phenotype of the *efs* mutants is due to a reduction in the adult vegetative phase of plants carrying this mutation (Soppe *et al.*, 1999).

Along with their early-flowering phenotype, *efs* mutants also show several pleiotrophic phenotypes. These include a reduction in the size of the leaves, roots and flowers, decreased apical dominance, incomplete development of the first flowers, and decreased germination and fertility (Soppe *et al.*, 1999). This suggests that the influence of *EFS* in affecting flowering time is only one function of this gene, and that *EFS* may also have other roles particularly in cell expansion processes (Soppe *et al.*, 1999). To determine where *EFS* acts in the proposed flowering time pathways, double mutants were created between *efs* and the late-flowering *co, gi, fwa, fca,* and *fve* mutants. For combinations of *efs* and the late-flowering mutants altered in a photoperiod responsive pathway (*co, gi,* and *fwa*), an

intermediate flowering time phenotype was observed in plants grown in both long and short days. The *efs* phenotype was found to be epistatic however, to the late-flowering phenotype of mutants altered in the constitutive flowering pathway (*fca* and *fve*) in all photoperiods tested. In addition, double mutants of the *efs* and *fca* or *fve* mutants no longer displayed the strong vernalisation response of the monogenic late-flowering mutants, but rather the weak response shown by *efs* single mutants. This double mutant analysis indicates that *EFS* may act by repressing the promotion of flowering by the autonomous flowering pathway. Furthermore, it has been postulated that *FVE* and *FCA* act to promote flowering via their inhibition of the floral inhibitory effects of the *EFS* gene. It is also believed that vernalisation may interact with the *EFS* gene product, or those genes downstream of *EFS*, as a vernalisation treatment had little effect on the flowering time of *efs* single or double mutants in short days (Soppe *et al.*, 1999).

1.4.9 Genes involved in the constitutive floral repression pathway

The EMBRYONIC FLOWER (EMF)gene

The *emf* (*embryonic flower*) mutants display an extreme early-flowering phenotype, in which all vegetative development is bypassed. As a consequence, these mutants also undergo senescence faster than wild-type plants (Bai and Sung, 1995). The flowering time of these mutants does not alter in response to any changes in environmental conditions. This not only suggests that *EMF* is a key gene required for vegetative development and the repression of reproductive development, but that flowering is the default state for plants (Martinez-Zapater *et al.*, 1994; Bai and Sung, 1995; Haughn *et al.*, 1995; Yang *et al.*, 1995a; Amasino, 1996a; Pineiro and Coupland, 1998; Koornneef and Peeters, 1997).

The first *emf* mutants were isolated by Sung *et al.* (1992) who identified four recessive *emf* alleles in the L. *erecta* ecotype. The only leaves formed in these mutants are sessile, with no petioles, and mutant callus tissue of these lines is unable to ever produce rosette leaves (Sung *et al.*, 1992; Yang *et al.*, 1995a). The most severe *emf* allele, *emf1-2*, only ever produced carpelloid structures following germination (Haughn *et al.*, 1995; Yang *et al.*, 1995a; Pineiro and Coupland, 1998). Multiple floral buds containing flowers that were abnormal and produced no viable seed, are also found in several of these *emf1* mutant lines. The cotyledons of these mutants lack petioles and are oval-shaped, which are features characteristic of cauline leaves. The root growth of *emf1* mutants however, is not phenotypically different from that of wild-type plants. Surgical experiments to reveal the site of action of the *EMF* gene, suggests that the phenotype of these mutants is independent of the presence of cotyledons and that *EMF* acts at the shoot apex (Sung *et al.*, 1992; Yang *et al.*, 1995a).

The embryonic flowering phenotype of *emf* mutants was examined in further detail by Bai and Sung in 1995. They concluded that *emf* mutants show immediate flowering after germination since the mutant shoot apical meristems enlarge and become dome-shaped, which are characteristics seen in a florally induced apex, as early as five days after germination. The authors also demonstrated that the precocious flowering phenotype of *emf* mutants cannot be suppressed by varying their day-length or temperature regimes. The defect in these mutants is not thought to be in their light signalling responses however, as the hypocotyls of *emf* mutants respond normally to light. In addition, the normal root growth of the mutant lines suggests that the role of *EMF* is not in cellular growth and differentiation. Gibberellin treatment of *emf1-2* mutant seedlings results in elongated and branched shoots similar to those seen in gibberellin-treated wild-type plants, indicating that the potential to form an inflorescence is also unaffected in these mutants. *emf* mutants differ from wild-type plants however, in the development of their shoot apical meristem. The mutant apex is not flat and lacks the single epidermal, and two hypodermal, cell layers seen in wild-type plants undergoing vegetative development. These three cell layers are formed at the heart stage of embryo development, suggesting that the earliest time of action of *EMF* occurs at this stage or earlier (Bai and Sung, 1995; Amasino, 1996a). The inability of *EMF* mutant callus to form vegetative shoots indicates that *emf* mutants are not simply defective in an embryo-specific gene required for shoot organogenesis, but rather that *EMF* acts to specify vegetative fate (Bai and Sung, 1995).

A role for the *EMF* gene in the transition from inflorescence to floral meristems has also been proposed. This theory is based on the observations that the number and structure of the floral organs of *emf* mutant flowers are often altered, although the severity of this phenotype varies between the *emf* alleles (Bai and Sung, 1995). The effects of the *emf* mutation on several FMI genes have since been investigated via double mutant analysis. The *emf1* mutation was found to be epistatic to the *lfy-1* mutation, indicating that *LFY* action is after *EMF1*. *LFY* is also believed to reciprocally antagonise *EMF* activity, as constitutive *LFY* expression in weak *emf1* mutants enhances the *emf1* phenotype (Yang *et al.*, 1995a; Chen *et al.*, 1997). Such analysis also suggested that *EMF1* and *TFL1* genes appear to act synergistically in their effects on flowering time, as an enhancement of the *emf1* phenotype was observed in the *tfl1-1* mutant background. This further indicates that *TFL1* may act to promote *EMF1*. As *EMF1* suppresses *LFY* activity, the *TFL1* promotion of *EMF1* has also been suggested to be a route by which *TFL1* can alter *LFY* expression (Martinez-Zapater *et al.*, 1994; Yang *et al.*, 1995a; Chen *et al.*, 1997). Both the *AP1* and *AG* genes were found to be ectopically expressed in the shoot meristem, leaves, hypocotyls, and cotyledons of *emf1* plants. *EMF1* is therefore believed to be required to negatively regulate expression of these loci (Pineiro and Coupland, 1998). Furthermore, *emf* seedlings show phenotypes similar to *ap2* mutants. It is therefore suggested that the negative regulation of the *AG* and

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AP1 genes is via the promotion of the *AP2* gene product or vice versa (Yang *et al.*, 1995a; Hicks *et al.*, 1996a).

Following these analyses, a model for the role of *EMF* throughout development has been postulated suggesting that the levels of *EMF* activity are highest during embryonic growth, and gradually decrease throughout development. As the plant ages the activity of *EMF* decreases and therefore allows both the transitions from rosette to inflorescence and inflorescence to floral development to occur. Furthermore, it is postulated that the levels of *EMF* may be promoted and inhibited throughout the life cycle of the plant by the products of several flowering time and FMI genes (Yang *et al.*, 1995a). The proposal that *EMF* levels, rather than an absolute presence or absence, is important in mediating various phase transitions. Heterozygotes of *emf1-2* alleles show an intermediate phenotype, and *emf-1 emf-2* double mutants display a more severe *emf* phenotype, supporting this hypothesis (Yang *et al.*, 1995a).

The ELONGATED (ELG) gene

Halliday *et al.* (1996) identified a novel elongated mutant following EMS mutagenesis of *ga-4* mutants. This *elg* mutant, which is defective in the *ELG* locus that maps to chromosome four, shows elongation of its hypocotyls and petioles, narrow leaves, epinastic growth and early-flowering. In addition, *elg* mutants exhibit a full range of photomorphogenic and gibberellin-induced responses, suggesting the *ELG* gene product acts independently of phytochrome and gibberellin action (Halliday *et al.*, 1996).

The SHORT VEGETATIVE PHASE (SVP) gene

Recently, Hartmann *et al.* (2000) reported the cloning of a floral repressor gene, *SHORT VEGETATIVE PHASE* (*SVP*), from transposon-tagged early-flowering mutants of the Columbia ecotype. These *svp* mutants flower earlier than wild-type plants in both long and short days while retaining a short day response. When grown in short day conditions these lines also respond to a vernalisation treatment and flower earlier. The *SVP* gene maps to chromosome two, near the other flowering time genes, *ELF3* and *EAF1*. *svp* mutants do not display the photoperiod insensitivity of *elf3* mutants however, nor the elongated petiole and light green colour of the *eaf1* mutants (Hartmann *et al.*, 2000).

The expression of the two SVP transcripts that have been detected remains relatively constant during vegetative development of the plants, irrespective of the photoperiodic conditions in which they were grown. Transcription of this gene is repressed in inflorescence apical meristems however, although not in flower primordia. The *SVP* gene has since been found

to code for a novel MADS-box domain protein that is believed to repress flowering in a dose-dependent manner, and act within an autonomous repression flowering pathway (Hartmann et al., 2000).

As MADS domain proteins are known to form functional homo- or heterodimers (Riechmann and Meyerowitz, 1997), it has been suggested that SVP and the floral repressor, MADS box protein, FLC, may interact to form such molecules. If SVP dimerisation is an essential functional requirement for *FLC* action, it would however be expected that the *svp* mutants would not show a vernalisation response, as down-regulation of the *FLC* gene is believed to be essential for plants to show early-flowering following such treatments. *svp* mutants only show a reduced response to vernalisation however, indicating that SVP may influence the flowering time of plants via interaction with other proteins besides FLC (Hartmann et al., 2000).

The EAF1 gene

The recessive *eaf1* mutants were isolated following Ds transposon mutagenesis of the early-flowering Nossen ecotype (Honma et al., 1993). These mutants flower earlier than wild-type plants in both long and short days, while retaining a photoperiod response. These mutants have an accelerated flowering response to vernalisation treatments when grown in short days. The *eaf1* mutants have since been found to be early-flowering due to a decrease in the length of both their juvenile and adult vegetative phases (Scott et al., 1999).

In short days, *eaf1* mutants also display phenotypes that are similar to those seen in some phytochrome and gibberellin response mutants, such as a pale green colour and elongated petioles and hypocotyls. The *EAF1* gene maps to a unique location on chromosome two however, away from genes disrupted in the early-flowering *phyB*, *clf*, and *elf3* mutants. The phenotype of *eaf1* mutants grown in short days, as well as the increased resistance of these lines to the gibberellin biosynthesis inhibitor, paclobutrazol, have led to the suggestion that gibberellin metabolism, or responsiveness to gibberellins, is altered in these lines (Scott et al., 1999). It has further been proposed that EAF1 may act as a negative regulator of gibberellin metabolism in a similar manner as the *SPY* or *RGA* gene products. Despite the similarity in the phenotypes displayed by *eaf1* and *spy* mutants, *EAF1* is not allelic to *SPY*, and *eaf1* mutants do not display the increased height or decreased fertility of the *spy* mutants (Scott et al., 1999).

1.4.10 Interactions amongst floral inhibitory genes

The presence of both an autonomous and a light-dependent, floral-repression pathway is suggested by the observation that Columbia seedlings flower after the production of four rosette leaves in complete darkness, compared to eight-to-ten leaves in the light. Constitutive floral repression is believed to be mediated primarily by the key gene *EMF*, whereas the primary role of the *TFL1*, *ELF1* and *ELF2* genes in the autonomous pathway is to extend the repression mediated by the *EMF* gene product (Martinez-Zapater *et al.*, 1994). Other genes, such as the *PHYB*, *ESD1*, *HY1*, *HY2*, and *ELF3* loci, are thought to act in a short day-mediated repression of flowering, perhaps by inhibiting gibberellin biosynthesis or signal transduction. The resulting limitation of gibberellins in plants grown in short days may alleviate any gibberellin-mediated repression of the *EMF* gene product and hence result in delayed flowering. As predicted by these models, *emf* mutants are found to be epistatic to all other early-flowering mutants tested (Coupland *et al.*, 1993; Martinez-Zapater *et al.*, 1994; Haughn *et al.*, 1995; Yang *et al.*, 1995a; Zagotta *et al.*, 1996).

Other interactions of interest are those involving the *elf3* mutant and early-flowering phytochrome mutants that have implicated the P_{fr} form of phytochrome in a short day floral repression pathway, possibly through interaction with gibberellin (Haughn *et al.*, 1995). When *elf3* alleles are combined with a severe *hy2* mutant allele, *hy2-200*, terminal flowers form on all primary and secondary inflorescences, suggesting that a signal transduction pathway defined by *ELF3* and *HY2* also influences the activity of FMI genes (Haughn *et al.*, 1995).

1.4.11 Interactions of genes that mediate floral repression and promotion

The hypothesis that *EMF* activity controls the switch from vegetative to reproductive development, as well as the reproductive to inflorescence transformation, has led to the proposal that *EMF* is central to a general repression of phase transitions. It is also now believed that the genes mutated in the late-flowering lines may normally act to promote flowering via their negative regulation of *EMF* activity. To assess these interactions, Haung and Yang (1998) analysed the interaction of late-flowering mutants involved in all three environmental response groups, with two weak *EMF* mutant alleles (*emf1-1*, *emf2-3*) and two strong alleles (*emf1-2*, *emf2-1*). The *emf1-1* mutant produces a small shoot with four-to-five leaves and one-to-three flowers, whereas *emf1-2* mutants form only carpelloid tissue. The weak *emf2-3* mutants has an inflorescence shoot that occasionally produces branches with sessile leaves and two-to-three flowers. The strong *emf2-1* mutant allele results in plants with a very short inflorescence with five-to-six sessile leaves and one-to-two flowers (Haung and Yang, 1998).

Following such an analysis, *emf1-1 fwa-1* double mutants are found to display an additive phenotype, producing more sessile leaves and no inflorescences after 45 days of growth. This suggested that the early appearance of these structures in *emf* mutants is dependent upon *FWA* activity. This supports the hypothesis that *FWA* represents a class of floral promotory gene which positively regulate inflorescence development, and repress vegetative development, through a pathway separate from those involving *EMF* and other flowering genes. *emf1-1 ft-1* double mutants also have an additive phenotype producing five times more sessile leaves than *emf1-1* single mutants. Double mutant plants that flower have a larger bolt than *emf1-1* plants, with many secondary branches and chimæric floral organs, suggesting an additional role for *FT* in flower organ development. *emf1-1 ld-1*, or *emf1-1 fca-1* mutants also have fewer plants bolting than in *emf1-1* populations and produce more sessile leaves but display no floral organ abnormalities. When *emf1-1* is combined with *fd*, *fve*, *fe*, or *fy*, similar phenotypes to *emf1-1 fca* and *emf1-1 ld* are observed, however these mutants display no internode or secondary branches. The *emf1-1 fpa* or *emf1-1 fha* double mutant phenotypes are similar to those of the *fd* and *fve* group but over 50% of plants flower. *emf1-1* is found to be completely epistatic to the *co* and *gi* mutations (Haung and Yang, 1998).

When the strong *emf1-2* allele was combined with these mutants, all double mutants resembled the *emf1-2* single mutant phenotype except the *emf1-2 fwa* combinations, which display an additive phenotype similar to that seen in *emf1 emf2* double mutants. Combinations of weak *emf2-3* alleles and most late-flowering mutants again result in additive phenotypes, although *emf2-3* is found to be epistatic to the *fy*, *fpa*, *co*, and *gi* lines. When the late mutants are combined with the strong *emf2-1* allele, all mutants show similarities to *emf2-1* mutants in their early development. Some combinations however, produce slightly later bolting plants (Haung and Yang, 1998).

The fact that no late-flowering mutations could cause rosette formation in *emf* mutants suggests that *EMF* activity is necessary for vegetative development and for normal function of genes affected in the late-flowering mutants, with the possible exception of the *FWA* gene (Haung and Yang, 1998). In addition, as the severe *emf* alleles are epistatic to most mutations that result in late-flowering plants, floral promotory genes are thought to interact directly with *EMF* to some extent, to accelerate flowering. As mentioned above, the *fwa* and *ft* mutations have the strongest effect on altering the *emf* phenotype, followed by those mutations (*fca*, *fy*, and *ld*) that disrupt photoperiod and vernalisation-sensitive pathways. These observations suggest a cascade of *EMF* suppression by genes mutated in the late-flowering mutants. Presumably, these genes also interact with other factors to promote flowering, as their functions are often required for the early floral bud formation of *emf* mutants. The additive nature of the phenotypes observed in double mutants of late-flowering

mutant lines and weak *emf* alleles may therefore be due to the lack of suppression of residual *EMF* activity in these lines. In addition, the complete epistasis conferred by *emf* alleles on *co* and *gi* mutants suggests that their role in promoting flowering is mediated exclusively by their suppression of *EMF* activity (Yang *et al.*, 1995a; Haung and Yang, 1998; Pineiro and Coupland, 1998).

Page *et al.* (1999) also analysed double mutant plants containing *emf1-1* and *fca-9* mutant alleles. These plants show *emf1-1* characteristics in early stages of growth, and an additive phenotype later in development. This indicates that *FCA* is required for the early transition to inflorescence development in *emf* mutants, and that *FCA* acts, not only to suppress *EMF*, but also in a pathway independently of *EMF*, to alter flowering time. Page *et al.* (1999) further suggest that *EMF* does not act in a central repression pathway but rather downstream of *CO* and *GI* in the long day-dependent flowering pathway. This theory is supported by the fact that *emf* mutants flower at the same time in long and short days (Haung and Yang, 1998; Page *et al.*, 1999).

When the *fld* mutant is combined with the *emf1-1* mutation, the early development of such plants is similar to *emf1-1* single mutants, as they produce no rosette leaves. Later development mimicks that of the *fld* mutant however, with many sessile leaves produced and no flower formation. *emf1-2 fld-2* double mutants have a very similar phenotype to *emf1-2* monogenic mutants, although they display a decrease in the number of stigmatic papillae and carpelloid tissue that are formed. The early appearance of floral organs in *emf* mutants is therefore predicted to be dependent upon the presence of the *FLD* gene product. In addition, *FLD* is predicted to promote flowering by decreasing *EMF* activity while promoting inflorescence development through its interactions with genes such as *LFY* and *AP1* (Chou and Yang, 1998).

The interaction of the *fld* mutation with the *elf1*, *elf2*, or *elf3* early-flowering mutants has also been examined. *fld-2 elf1*, *fld elf2* or *fld-2 elf3* double mutants flower at the same time as *fld-2* single mutants. *fld-2 elf3* double mutants also display the elongated hypocotyl phenotype of *elf3* mutants. The epistasis of *fld-2* to the *elf1*, *elf2*, and *elf3* mutant phenotypes indicates that a role for the *ELF1*, *ELF2*, and *ELF3* genes may be to negatively regulate *FLD*, and therefore indirectly maintain *EMF* activity (Chou and Yang, 1998).

The *esd-4* early-flowering mutant has also been shown to be epistatic to some late-flowering mutants (Simon and Coupland, 1996; reviewed in Coupland, 1995a). *esd-4 fve* double mutants for example, show a phenotype identical to *esd-4* single mutants suggesting that the *ESD-4* gene acts after *FVE*. In addition, the *ESD-4* gene is predicted to act after the *CO* gene in pathways mediating the timing of floral development, as the *esd-4* mutation completely

suppresses the late-flowering phenotype of *co* mutants in long days (Simon and Coupland, 1996; reviewed in Coupland, 1995a).

1.5 Genes controlling flowering time variation between late- and early-flowering ecotypes of *Arabidopsis*

There is a wide variation in the flowering times and physiological responses of ecotypes of *Arabidopsis* (Karlovska, 1974; Zhang and Lechowicz, 1994; Clarke *et al.*, 1995; Alonso-Blanco *et al.*, 1998). Late-flowering ecotypes for example, are thought to be adapted for growth at high latitudes and are strongly responsive to vernalisation (Burn *et al.*, 1994). Investigation of the natural flowering time variation amongst *Arabidopsis* ecotypes can reveal genes affecting flowering times not identified by the mutagenesis of the commonly used early-flowering ecotypes such as L. *erecta* and Columbia (Kowalski *et al.*, 1994; Martinez-Zapater *et al.*, 1994). Crosses between late-flowering, or medium-late-flowering ecotypes, and early-flowering ecotypes have for example, identified that one or two dominant genes are responsible for the late-flowering phenotype. Several other semi-dominant and recessive genes have also been shown to be involved in conferring 'lateness' on a range of ecotypes (Napp-Zinn, 1985; Martinez-Zapater *et al.*, 1991; Burn *et al.*, 1993b; Burn *et al.*, 1994; Clarke and Dean, 1994). Crosses between strongly vernalisation-responsive late-flowering ecotypes and early-flowering ecotypes also allow an estimate of the number of genes required for a strong vernalisation response. Napp-Zinn (1979) found that one dominant and three recessive genes were required for the vernalisation responsiveness of the late-flowering ecotype Stockholm following a cross with the early-flowering Limburg-5 ecotype. These four genes were described as being epistatic to those causing the weaker cold response of early ecotypes, although this epistasis was dependent upon the light intensity and other growth conditions of the plants (Napp-Zinn, 1979; Burn *et al.*, 1993b; Clarke and Dean, 1994; Koornneef *et al.*, 1994; Martinez-Zapater *et al.*, 1994).

The FRIGIDA (FRI) gene

The *FRI* (*FRIGIDA*) gene is a major dominant locus controlling flowering time in many late-flowering ecotypes from a variety of different geographical localities in Europe. Such ecotypes include the Stockholm (St), Le-O (Le-0), Pitztal (Pi), Innsbruck (In), Geneva (Ge-2), and Zurich (Zu-0) ecotypes from Switzerland, and the San-Feliu-2 (Sf-2) and Coimbra (Co-4) ecotypes from Spain and Portugal respectively. The *FRI* gene is also found to confer the strong vernalisation requirement in these late-ecotypes (Burn *et al.*, 1993b; Lee *et al.*, 1993; Burn *et al.*, 1994; Clarke and Dean, 1994; Martinez-Zapater *et al.*, 1994; Okada and Shimura, 1994; Amasino, 1996b; Aukermann and Amasino, 1996; Dennis *et al.*, 1996; Sanda *et al.*, 1997; Finnegan *et al.*, 1998; Sheldon *et al.*, 1999). This *FRI* gene also appears

to influence the sensitivity to far-red light of some late-flowering ecotypes, as several ecotypes containing this gene flower earlier and display no vernalisation response when grown in environments enriched with far-red light (Lee and Amasino, 1995). Plants containing *FRI* are also quite responsive to photoperiod, flowering much later in short days than in long days. The *FRI* gene is not allelic to any other late-flowering loci identified and maps to the top of chromosome four (Lee and Amasino, 1995; Aukermann and Amasino, 1996).

The repression of flowering conferred by the *FRI* locus was observed as early as 1962, by McKelvie following the isolation of an extremely late-flowering mutant - the *florens* (*F*) mutant. These plants take from six to 18 months to flower in long days or did not flower at all. Those that flower also contain a large number of axillary vegetative rosettes. When this mutant is crossed to wild-type L. *erecta*, the F2 ratio suggests that the 'lateness' of the *F* mutants is dependent on two unlinked loci, one of which is now believed to be an extremely late-flowering allele of the *FRI* gene (McKelvie *et al.*, 1962; Napp-Zinn, 1985; Lee *et al.*, 1993; Koornneef *et al.*, 1994; Peeters and Koornneef, 1996).

The role(s) of the *FRI* gene in floral repression were further investigated by Lee *et al.* (1993), who initially named the gene *FLA* (*FLOWERING ALTERED*). The *FLA/FRI* gene is identified as being the major locus conferring the late-flowering phenotype and vernalisation responsiveness of the Sf-2 and Le-0 ecotypes (Lee *et al.*, 1993). When these two ecotypes are crossed into the Columbia background, the F2 phenotypes suggests that only one gene confers the late-flowering phenotype. A number of intermediate flowering plants are observed in the F2 populations from this cross however, indicating that other genes besides *FLA/FRI* have some minor cumulative effects on the flowering times of the Sf-2 and Le-0 ecotypes. Vernalisation could completely abolish the effects of the dominant *FLA/FRI* alleles, suggesting that a major role of this gene is to render the plant dependent upon vernalisation for early-flowering (Burn *et al.*, 1993; Lee *et al.*, 1993). Lee *et al.* (1993) also speculated that several other strongly vernalisation responsive plants may contain a dominant suppressor of flowering similar to the *FRI* gene product. This hypothesis is supported by the presence of a single dominant gene that confers late-flowering and vernalisation responsiveness in both *Pisum* and Brassicacea species (Lee *et al.*, 1993).

Burn *et al.* (1993) examined the involvement of the *FRI* gene in conferring the 'lateness' of the ecotypes Pitztal and Innsbruck, by crossing them to three different early-flowering ecotypes. It was observed that Pitztal or Innsbruck crossed with Columbia give F1 plants that flower as late as the late-flowering parentals, while F1 plants from crosses involving L. *erecta* are intermediate flowering. The F1 plants from the crosses of Niederzenz with the Pitztal and Innsbruck however, flower later than the late-flowering parental lines. This

suggests that genes able to modify the effects of *FRI* are present in the L. *erecta* and Niederzenz backgrounds. In accordance with the observations of Lee *et al.* (1993), the range of flowering times within the F2 progeny from these crosses are large and skewed toward early-flowering. This indicates that the 'minor effect' genes causing this spread, are recessive (Burn *et al.*, 1993b).

Similar results were also obtained by Clarke and Dean (1994) who examined the late-flowering phenotype of Stockholm. They examined the flowering times of progeny from crosses involving the early-flowering Li-5 ecotype, the inbred Li-5 lines containing the Stockholm *FRI* allele (H51), and wild-type Stockholm. As expected, crosses of Stockholm to wild-type Li-5 plants produce a 3:1 late- to early-flowering ratio in the F2 progeny. When the H51 plants are crossed to L. *erecta* however, unexpected ratios in the F2 are found that can not be explained by any one or two gene models. This again suggests that modifiers of the *FRI* gene are present in the L. *erecta* ecotype (Clarke and Dean, 1994).

A more detailed examination of the role of the *FRI* gene in controlling flowering time in response to vernalisation, photoperiod, and light quality was performed by Lee and Amasino (1995). *FRI* from the Sf-2 ecotype was introgressed into the L. *erecta* and Columbia ecotypes for this study. Introgression of this gene into early-flowering ecotypes enabled the effects of this loci to be studied without the influence of other genes specific to late-flowering ecotypes, which may act to modify the expression of *FRI*. Introgression of the Sf-2 *FRI* allele (*FRI* [Sf-2]) into Columbia causes extremely late-flowering plants. L. *erecta* plants containing the *FRI* (Sf-2) allele are only slightly late-flowering however, unless they are combined with Sf-2 alleles of another locus, *FLC*, that is known to modify *FRI*. All plants containing the *FRI* (Sf-2) locus gave the same response to vernalisation as wild-type Sf-2, with longer vernalisation treatments required to saturate the early-flowering vernalisation response if these plants were grown in short days. These results indicated that while the presence of *FRI* does not change the ability of early-flowering ecotypes to respond to photoperiods, it does shift this response to later flowering. Lee and Amasino (1995) also concluded that the floral inhibitory effects of *FRI* are more readily regulated by vernalisation than by any inhibition conferred by short days (Lee and Amasino, 1995). Columbia::*FRI* and L. *erecta*::*FRI/FLC* plants also decrease their flowering time by up to 50% when given high far-red to red ratios of light, which is a response similar to that seen in wild-type Sf-2 plants. Vernalisation can dramatically decrease this far-red flowering responsiveness while having no effect on the increase in petiole length observed in these conditions. This result indicates that vernalisation may not affect far-red perception but rather acts downstream to help mediate responses to these light conditions (Lee and Amasino, 1995).

The similarities between the late-flowering and vernalisation responsive phenotypes of plants containing *FRI*, and those with mutations in the floral promotory *FCA*, *FPA*, *FVE*, *FY*, and *LD* genes indicates that *FRI* may act in the same pathway as these genes. *FRI* is predicted to act as a repressor in such a pathway however, whereas late-flowering trait genes are thought to accelerate flowering. The presence of dominant alleles of the *FRI* gene also creates obligate long day-requiring plants - similar to the effects of the *gal-3* mutation. Unlike plants containing *FRI* however, *gal-3* mutants are not responsive to vernalisation, and the delay caused by *gal-3* in long days is minimal (Lee and Amasino, 1995). It has been suggested that the presence of dominant *FRI* alleles however, creates a requirement for altered gibberellin metabolism, and the necessity for vernalisation, to show an early-flowering phenotype (Lee and Amasino, 1995; Amasino, 1996a; Aukerman and Amasino, 1996; Koornneef *et al.*, 1998; Gendall *et al.*, 1999).

The *FRI* gene has recently been isolated from cosmids that were able to 'rescue' the early-flowering phenotype in ecotypes such as Li-5 (Gendall *et al.*, 1999). The *FRI* gene is expressed at very low levels and encodes a novel protein, which has two small coiled-coil regions. Columbia *FRI* alleles carry two amino acid changes and a 164 bp insertion, and are predicted to be partially functional. The *FRI* allele from L. *erecta* contains a 375 bp deletion of the promoter region and is predicted to code for a non-functional out-of-frame protein. PCR amplification using primers that distinguish these alleles has shown that most late-flowering ecotypes carry an *FRI* allele similar to that seen in the Stockholm ecotype. The more temperate early-flowering ecotypes however, carry either the Columbia or L. *erecta* version of the *FRI* gene (Gendall *et al.*, 1999).

The FLOWERING LOCUS C (FLC) gene

As described above, when several late-flowering ecotypes are crossed to the early-flowering ecotype L. *erecta*, the late-flowering trait no longer segregates as a single dominant trait in the F1 and F2 progeny. The progeny of these crosses are instead skewed toward early-flowering, suggesting the presence of at least one other gene that could alter the effects of the *FRI* gene in this ecotype (Burn *et al.*, 1993b; Lee *et al.*, 1993; Lee *et al.*, 1994b; Lee and Amasino, 1995). To identify the number of genes involved in this suppression Lee *et al.* (1994b) crossed the late-flowering Sf-2 trait into L. *erecta*. The intermediate flowering phenotype of the F1 plants was attributed to two unlinked loci; *FRI*, and the modifier of *FRI*, *FLC* (*FLOWERING LOCUS C*). This *FLC* gene was found to be semi-dominant and exerts its effects on the *FRI* gene in a dose-dependent manner. The broad range of flowering times detected in the F2 progeny from crosses of late-flowering ecotypes and L. *erecta*, further suggests that late-flowering *FRI* and *FLC* alleles alone, can still confer some degree of 'lateness' to plants (Lee *et al.*, 1994b; Martinez-Zapater *et al.*, 1994).

In a screen to isolate suppressors of the *LD* flowering time gene, several genes allelic to the *L. erecta* version of the *FLC* gene were also found. This supports the finding that the *ld-3* mutants, potentially influencing the same floral pathway as *FRI*, flower late only in the absence of L. *erecta* versions of *FLC*. In contrast, the presence of late-flowering *FRI* alleles or *ld-3* mutant alleles in Columbia results in very late-flowering plants (Lee *et al.*, 1994b). This suggests that the L. *erecta* ecotype contains little active *FLC* gene product, and the extreme late-flowering conferred by *FRI* and *FLC* combinations requires non-L. *erecta* versions of this gene to be present. The presence of non-functional forms of the *FLC* in the L. *erecta* ecotype is further supported by the observations that when the *FLC* (Col) allele is introgressed into wild-type L. *erecta* their flowering times become similar to wild-type Columbia. This indicates that the main difference between these ecotypes is due to their different FLC alleles (Sanda and Amasino, 1996a; Aukerman and Amasino, 1996; Koornneef and Peeters, 1997). In addition, it has been observed that 'late' *FLC* alleles can be mutated to become L. *erecta*-like suppressor forms. It is therefore believed that due to the lack of an active floral repression allele of *FLC*, that *LD* and *FRI* may play a minor role, if any, in the flowering time of L. *erecta* (Lee *et al.*, 1994b, Aukerman and Amasino, 1996, Sanda and Amasino, 1996a).

Since many late mutants have been identified in L. *erecta*, active Columbia *FLC* alleles have been introduced into these mutant backgrounds to study the interactions of *FLC* with these mutations (Sanda and Amasino, 1996b). The *FLC* gene interacts synergistically with the *fca, fpa, fve* mutations to cause extremely late-flowering plants - similar to those seen when the *FRI* or *LD* loci are combined with late-flowering alleles of the *FLC* gene (Koornneef *et al.*, 1994; Aukerman and Amasino, 1996; Sanda and Amasino, 1996b). Six other mutants, *fwa, gi, ft, fha, fe,* and *fd,* defective in genes within the long day-dependent floral promotory pathway, show only a slight increase in their flowering times when combined with the *FLC* (Col) allele. According to the proposal that the *FLC* gene exerts its effects in a dose-dependent manner, the maximum delay in flowering displayed by most of the mutant lines is seen when active *FLC* (Col) alleles are present in the homozygous state. These observations suggest that genes within the autonomous flowering pathway, such as *FCA, FPA,* and *FVE,* may interact with *FLC* to exert their floral promotory roles via a repression of the inhibitory effects of *FLC*. The genes within the photoperiod-sensitive floral pathway are thought to have little interaction with *FLC* however, as the presence of an active *FLC* allele did little to alter the flowering time phenotype of mutants affected in these genes (Sanda and Amasino, 1996b).

The *FLC* gene has recently been cloned following the isolation of a late-flowering, transposon-tagged mutant of the C24 ecotype (Sheldon *et al.*, 1999; Smith, 1999). The defect in this mutant mapped to a region of chromosome five containing the quantitative trait

locus *FLF* previously shown to have large effects on flowering time. This mutant was therefore designated *flf-1*. *FLF* is now believed to be the same gene as *FLC* however, as it maps to the same chromosomal region as *FLC*, and acts as a semi-dominant represser of flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Sheldon *et al.*, 2000). *flf-1* mutant plants are extremely late-flowering, producing many more rosette and cauline leaves than wild-type plants. In addition, bolts in the *flf-1* mutants arise from the internodes between rosette leaves and these bolts only elongated slightly before forming aerial rosettes. This phenotype is similar to that seen in the *florens* (*F*) and *fld* mutants. It has been demonstrated that *flf-1* mutants have two-fold higher levels of expression of the *FLC* gene, and that this overexpression of *FLC* was directly related to the extreme late-flowering phenotype (Sheldon *et al.*, 1999).

Following cloning of the *FLC* gene, the protein product was found to show sequence similarity to members of the MADS box class of transcription factors (Sheldon *et al.*, 2000) It has therefore been proposed that the *FLC* MADS box gene may form homodimers, or heterodimers with other MADS box proteins or other unrelated proteins, to mediate its repression of flowering. Sheldon *et al.* (1999) also analysed the expression level of the *FLC* gene via Northern blot analysis. *FLC* transcripts are found to be expressed at low levels in reproductive tissues, and remain relatively constant throughout development in other tissues. These observations indicate that any reduction of *FLC* expression associated with the floral transition may be occurring in a limited number of cells, such as those at the apex. Interestingly, *FLC* is highly expressed in roots although there is no visible root phenotype in the *flf-1* mutants (Sheldon *et al.*, 1999).

It has also been observed that while vernalisation and gibberellin treatments could accelerate flowering of *flf-1* plants, a much longer period of vernalisation, and higher levels of gibberellin, are required to decrease the flowering time of *flf-1* lines to that of wild-type (Sheldon *et al.*, 1999). This indicates that *FLC* may be repressed by genes involved in the vernalisation pathway, and that *FLC* may act to antagonise, or lower the sensitivity of the plant, to the floral promotive effects of gibberellin. Sheldon *et al.* (2000) further confirmed the central role for *FLC* in the vernalisation response from several pieces of evidence. Firstly, vernalisation responsive, late-flowering mutants of several different ecotypes are all found to have elevated levels of *FLC* transcription, and in all cases, vernalisation results in a decrease in *FLC* levels. In addition, expression of an antisense *FLC* transcript in one of these mutants, *fca-1*, causes decreased *FLC* levels and results in plants that flowered as early as those given a 28 day vernalisation treatment. Secondly, two mutants, *vrn-1* and *vrn-2*, isolated in the *fca-1* background, and showing a decreased responsiveness to vernalisation, show high *FLC* levels. This suggests that the role of the *VRN 1* and *VRN 2* genes in initiating a vernalisation response may be mediated by their repression of *FLC* activity.

Confirming this, *vrn 1* and *vrn 2* mutants show a smaller decrease in *FLC* activity following cold treatment than *fca-1* mutants. Thirdly, it was noted that the level of expression of *FLC* is directly proportional to the duration of the cold treatment required, to decrease the flowering time of different ecotypes to that of early-flowering ecotypes. The slightly late-flowering C24 ecotype for example, shows a decrease in *FLC* expression after 14 days vernalisation, while in the very late-flowering Pitztal ecotype, 28 days vernalisation is required before a similar decrease in *FLC* activity is noted. Lastly, FLC protein, and *FLC* mRNA levels, are decreased by vernalisation treatments and remain repressed throughout the development of the vernalised plants. Like the vernalisation response, these changes in *FLC* levels must be reset in the next sexual generation by subsequent cold treatments (Sheldon *et al.*, 2000). It is now thought that the repression of *FLC* activity by vernalisation is mediated via the demethylation of the promoter region of *FLC* that may prevent binding of a transcription factor. Alternatively, the normally methylated promoter region of *FLC* may prevent the binding of a repressor protein. Following vernalisation, the demethylation of the promoter region of *FLC* may allow this repressor to bind and inhibit transcription (Finnegan *et al.*, 2000). Both of these models are supported by the observation that *FLC* transcript levels were decreased in early-flowering *MET1* antisense plants (Sheldon *et al.*, 1999).

These expression studies also support the observation from the double mutant analysis that only genes within the autonomous floral repression pathway, such as *FCA*, may exert their floral promotory effects via a repression of *FLC* activity. This proposal is further supported by the fact that late-flowering mutants defective in genes within the photoperiod-dependent pathway show no elevation of *FLC* levels above those seen in wild-type plants, and *flc-13* mutants still show a photoperiod response (Sheldon *et al.*, 1999; Sheldon *et al.*, 2000). It has also been proposed that the *FRI* gene product may act to repress flowering by promoting the activity of *FLC*. In support of this theory the *FLC* gene is found to be much more highly expressed in ecotypes containing late-flowering alleles of the *FRI* gene (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). It is noteworthy that the activation of *FLC* in L. *erecta* via an introduced Sf-2 *FRI* allele however, is much less than that seen in the Pitztal ecotype. Since it has been observed that there are no sequence differences at the amino acid level between FLC of Columbia, L. *erecta*, or C24 ecotypes, the differential regulation of the *FLC* gene within different ecotypes is believed to be due to differences in the promoter region (Sheldon *et al.*, 2000).

The AERIAL ROSETTE (ART) and ENHANCER OF AERIAL ROSETTE (EAR) genes

The Sy-O late-flowering ecotype forms vegetative rosettes of leaves in the axillary meristems, even after the primary meristem has bolted and initiated flower development. These axillary rosettes produce secondary branches in positions where a single cauline leaf

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would normally form. It is believed that the aerial rosettes form when the axillary meristems initiate at the floral transition stage but remain vegetative for longer than normal. It has since been found that this phenotype is dependent upon the interactions of dominant alleles of two genes, *ART (AERIAL ROSETTE)* on chromosome five, and *EAR (ENHANCER OF AERIAL ROSETTE)* on chromosome four. *EAR* has tentatively been identified as a new allele of *FRI*, as the introduction of Sf-2 *FRI* alleles into the earlier-flowering *ART* homozygotes, produce later flowering plants with aerial rosettes. In addition, other loci causing late-flowering, such as the *fca* mutant alleles, also cause further enhancement of the aerial rosette phenotype. As expected, vernalisation treatments could reverse the late-flowering aerial rosettes phenotype of the Sy-O ecotype, possibly via decreasing the activity of the *FRI* gene product (Koornneef and Peeters, 1997; Grbic and Bleeker, 1996).

The KRYOPHILA (KRY) and *FLOWERING KIRUNA (FKR) genes*

Other naturally occurring genes have also been found to be responsible for conferring either a vernalisation response, or late-flowering, in several different ecotypes. Karlovska (1974), found for example, several gene differences between early, medium, and late-flowering homozygote lines derived from natural ecotypes. In addition, Burn *et al.* (1993) examined the flowering time control in the medium/late Kiruna-2 ecotype and found that when it is crossed to the early-flowering ecotypes L. *erecta*, Niederzenz, or Columbia, all F1 plants flower early. The F2 population contains mainly early-flowering plants with approximately one quarter flowering as late as the Kiruna-2 parent. The F2 flowering time distribution was continuous however, suggesting that while one recessive gene may be controlling the majority of flowering time differences, several other genes may exert minor effects on this characteristic (Burn *et al.*, 1993; Martinez-Zapater *et al.*, 1994). The new recessive gene, termed *FKR (FLOWERING KIRUNA)* is not found to be allelic to any previously identified late-flowering mutant alleles and is believed to be involved in the promotion of flowering (Burn *et al.*, 1993b, Dennis *et al.*, 1996b).

Another locus, the recessive *KRYOPHILA* gene, is also believed to have significant effects on the flowering time of the late-flowering Stockholm ecotype. In 1994, Clarke and Dean characterised late-flowering inbred F2 lines (H55 and H51) from a cross between Stockholm and Limburg that are either singly homozygous for dominant alleles of the *FRI* locus, or recessive alleles of the *KRY* gene. From this analysis, the *FRI* gene was shown to be epistatic to the *KRY* gene except in conditions of low light intensity in which the loci acted additively. Furthermore, all lines show a decrease in flowering time following vernalisation, indicating that *FRI* and *KRY* may act together to confer a vernalisation requirement. The *KRY* locus has been confined to either chromosome five or three (Clarke and Dean, 1994).

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Floral induction in *Arabidopsis* is a complex process, involving the co-ordination of a large number of genes having either major, or relatively minor effects on the flowering time phenotype. Figure 1 attempts to assign the actions of several of the genes defective in flowering time mutants, as well as those genes responsible for the variation in flowering time in naturally occurring ecotypes, into the multiple proposed pathways to flowering. In this model, it is proposed that the *EMF* gene represses both the transformations from juvenile to adult vegetative growth and the adult vegetative to reproductive transition (Yang *et al.*, 1995a). Once the plant has passed through the juvenile vegetative stage however, other floral inhibitory and promotory genes are postulated to act in various pathways to determine the length of the adult vegetative stage that precedes floral induction. One of the main roles of genes within the constitutive floral repression pathways has been postulated to be in the promotion of the floral inhibitory gene *EMF* (Martinez-Zapater *et al.*, 1994). Genes within the autonomous flowering pathway are thought instead to inhibit the actions of late-flowering alleles of the floral repression gene, *FLC*, along with their role in promoting the floral transition directly (Sheldon *et al.*, 1999). As shown in Figure 1, late-flowering alleles of the *FRI* gene are also thought to act in a similar autonomous pathway. The main role of *FRI* in this pathway is suggested to promote, rather than inhibit, the action of the *FLC* gene (Michaels and Amasino, 1999). Long days are thought to promote flowering via the actions of genes within a long day-dependent flowering pathway, whereas the inhibition of floral induction in short days may be occurring via a short day dependent pathway. Although not shown in Figure 1, it is unclear whether this pathway is a separate entity, or whether genes mediating a delay in flowering in short days do so by a repression of genes within the long-day-dependent pathway. In Figure 1, two genes that are thought to be induced by long days, *FWA* and *FT*, are shown as promoting not only the floral induction process but also activating floral meristem identity genes (Araki *et al.*, 1998). A vernalisation-dependent floral promotory pathway is also thought to exist and be induced by cold treatments, while *FLC* activity is thought to be inhibited by these conditions (Sheldon *et al.*, 1999). In the model presented in Figure 1, the process of floral induction is proposed to be associated with elevated levels of, or sensitivity to, gibberellin within the apex. Following the transition to a reproductive program a sequential activation of the floral meristem identity genes, such as *LFY*, *AP1*, and *TFL*, and homeotic genes such as *AG*, *AP2*, and *AP3* occurs. This successive activation is then believed to lead to the production of floral meristems and ultimately flowers (Weigel and Meyerowitz, 1993).

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Flower formation

Homeotic genes (*AG, AP2, AP3, PI*)

AP1

CLF

TFL

LFY

EMF

Floral Induction;   Increasing levels of GA within the apex, or increasing responsiveness of the apex to GA. Activation of the Floral Meristem Identity genes (*LFY, API, TFL, UFO, CAL*)

FWA, FT

Long day-dependent floral promotion pathway.

(*CO, GI, FD, FE, FHA*)

Long days

EMF

Vernalisation-dependent floral promotion pathway.

Constitutive floral repression pathway.

(*ELG, ELF1, ELF2, SVP*)

Cold

FLC

FRI

ELF 3

Circadian clock input pathway

Short day-dependent floral repression pathway.

(*ESD2, ESD3, ESD4 , EFS*)

Autonomous floral promotion pathway.

(*LD, FLD, FCA FVE, FPA, FY*)

Adult vegetative stage

EMF

Juvenille vegetative stage

Figure 1.1

The placement of several flowering time loci within putative floral promotory and inhibitory pathways in *Arabidopsis*. Horizontal arrows represent floral promotory pathways or processes, while a horizontal line with a bar represent inhibitory steps. Vertical arrows indicate the developmental progression of *Arabidopsis* through the vegetative and reproductive phases.

Chapter 2

General materials and methods

2.1 Bacterial materials and methods

General media

LB Broth/Agar: 1% (W/V) Tryptone; 0.5% (w/v) Yeast extract; 1% (w/v) NaCl; +/- 1.5% Difco Bacto Agar, pH 7.5. The media was sterilised by autoclaving.

Mgl Broth/Agar: 0.5% (w/v) Mannitol; 0.1% (w/v) L-Glutamic Acid; 0.025% (w/v) KH_2PO_4; 0.01% (w/v) NaCl; 0.01% (w/v) $MgSO_4$; 0.001% (v/v) Biotin (0.01 g/100 ml stock); 0.5% Tryptone; 0.25% (w/v) Yeast extract; +/- 1.5% Difco Bacto Agar, pH 7.0. The media was sterilised by autoclaving.

SOB broth/Agar: 2% (w/v) Tryptone; 0.5% Yeast extract (w/v); 0.01 M NaCl; 0.0025 M KCl, +/- 1.5% Difco Bacto Agar, pH 7.0. The media was sterilised by autoclaving before the $MgSO_4$ (filter sterilised) was added to a final concentration of 0.02M.

SOC broth/Agar: 2% (w/v) Tryptone; 0.5% Yeast extract (w/v); 0.01 M NaCl; 0.0025 M KCl; +/- 1.5% Difco Bacto Agar, pH 7.0. The media was sterilised by autoclaving before the filter sterilised $MgSO_4$ and Glucose was added to final concentrations of 0.02M.

Terrific Broth: 1.2% (w/v) Tryptone; 2.4% (w/v) Yeast Extract; 0.004% (v/v) glycerol. pH 7.0. The media was sterilised by autoclaving before one tenth of the final total volume of a sterile solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 was added.

YEP broth/Agar: 1% (w/v) Yeast extract; 1% (w/v) Peptone; 0.5% (w/v) NaCl; +/- 1.5% Difco Bacto Agar, pH 7.0-7.5. The media was sterilised by autoclaving.

YMB broth/Agar: 0.05% (w/v) K_2HPO_4; 0.02% (w/v) $MgSO_4$; 0.01% (w/v) NaCl; 0.04% (w/v) Yeast extract; 0.01% (w/v) Mannitol; +/- Difco Bacto Agar, pH 7.0. The media was sterilised by autoclaving.

Antibiotics

Ampicillin: A 100 mg/ml stock solution of the sodium salt of ampicillin was prepared by dissolving in water. The solution was filter sterilised and stored at -20°C.

Kanamycin: A 100 mg/ml stock solution of kanamycin sulphate was prepared by dissolving in water. The solution was filter sterilised and stored at -20°C.

Rifampicin: A 100 mg/ml stock solution of rifampicin was prepared by dissolving in dimethyl sulfoxide. The solution was filter sterilised and stored at -20°C.

Media additives

X-gal (5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside): A 50 mg/ml stock solution of X-gal was prepared by dissolving the powder in N,N-Dimethyl-formamide (DMFO). The solution was filter sterilised, shielded from the light to prevent degradation, and stored at -20°C.

IPTG (isopropyl-β-thiogalactopyranoside): A 0.1 M stock solution of IPTG was prepared by dissolving the powder in sterile water. The solution was filter sterilised, shielded from the light to prevent degradation, and stored at -20°C.

General bacterial procedures

Bacterial cultures were inoculated with single bacterial colonies, or with glycerol stocks using appropriate sterile techniques. Liquid bacterial cultures of *E.coli* were grown with aeration in a New Brunswick shaker at 37°C overnight. Solid media was inoculated with single bacterial colonies using a sterile wire loop, or spread with bacterial cultures using a sterile glass rod, before being incubated at the correct temperature, for the appropriate length of time.

E. coli strains on solid media were maintained at 4°C for up to 1 month before requiring re-streaking. Glycerol stocks of bacterial cultures were made using a 1:1 (v/v) ratio of 100% Glycerol to bacterial culture. Stocks were either stored at -20°C for up to 6 months or at -70°C for long term storage.

2.1.1 Plasmid preparations

General solutions

Chloroform/isoamyl alcohol mixture: 24:1 (v/v) chloroform: isoamyl alcohol.

Phenol: Phenol was equilibrated with, and stored under, TE buffer pH 8.0 at -20°C.

Proteinase K: 10 mg/ml of proteinase K was dissolved in 50 mM Tris-Cl and 1 mM CaCl$_2$ and the solution stored at -20°C in 1 ml aliquots.

RNase: 10 mg/ml RNase was dissolved in 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl and boiled for 10-15 minutes to inactivate DNases. The solution was then stored at -20°C in 1 ml aliquots.

TE buffer: 10 mM Tris-Cl; 1 mM EDTA pH 8.0. The solution was sterilised by autoclaving.

E. coli minipreps

<u>Alkaline lysis miniprep</u>

This method is based on that described in Sambrook *et al.* (1989). 1.5 ml of an overnight culture was transferred to microcentrifuge tube and the cells pelleted by centrifuging the culture at 14,000 rpm for 30 seconds at 4°C. The broth was then removed by aspiration, and the bacterial pellet was resuspended in 100 µl of ice-cold Solution 1 (50 mM glucose; 25 mM Tris-Cl [pH 8.0]; 10 mM EDTA [pH 8.0]). 200 µl of freshly prepared Solution 2 (0.2 M NaOH; 1% SDS) was added and the contents of the tubes thoroughly mixed by inversion. 150 µl of a 5 M/3 M potassium acetate solution was added and the contents mixed by vortexing before being stored on ice for 3-to-5 minutes. Samples were then centrifuged at 14,000 rpm for 5 minutes at 4°C, and the supernatant removed to new tubes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the tube contents mixed by vortexing. Samples were then centrifuged at 14,000 rpm for 2 minutes and the upper aqueous layer removed to a new tube. The DNA was precipitated with the addition of 2 volumes of 100% ethanol, centrifuged at 14,000 rpm for 5 minutes at 4°C, and the supernatant removed with gentle aspiration. The DNA pellet was then rinsed with 70% ethanol before being dried at room temperature for 10 minutes and resuspended in 50 µl of TE buffer

Boiling plasmid miniprep

1.5 ml of a freshly grown overnight broth was centrifuged at 14,000 rpm for 15 seconds and the supernatant decanted. The cell pellet was then resuspended in 300 μl of STET (8% Sucrose; 50 mM Tris-Cl [pH 8.0]; 50 mM EDTA; 5% Triton X-100). 20 μl of a 10 mg/ml stock of lysozyme that was dissolved in 50 mM Tris-Cl (pH 8.0) was added to the cells and mixed in thoroughly. The solution was then incubated at room temperature for anywhere from 15 seconds to 10 minutes before being placed in a boiling water bath for 2 minutes. The mixture was then centrifuged at 14,000 rpm for 5 minutes and the gelatinous pellet removed. An equal volume of isopropanol:ammonium acetate (3:1) was added to the supernatant, mixed, and the DNA pelleted by centrifuging at 14,000 rpm for 5 minutes. The DNA pellet was rinsed with 70% ethanol and dried, before being resuspended in 50 μl of TE buffer containing RNase at a concentration of 50 μg/ml.

Modified alkaline lysis miniprep; for the preparation of sequencing templates

This method is based on that of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit Protocol, revision A, appendix A (Perkin Elmer). 4.5 ml of an overnight culture, grown in Terrific broth, was pelleted at 14,000 rpm for 1 minute and the supernatant removed. The bacterial pellet was then resuspended in 200 μl of GTE buffer (50 mM Glucose; 25 mM Tris-HCl [pH 8.0]; 10 mM EDTA [pH 8.0]), and 300 μl of freshly prepared 0.2 M NaOH; 1% SDS solution was added. The contents of the tube were mixed by inversion until the solution cleared and incubated on ice for 5 minutes. 300 μl of 3.0 M potassium acetate was then added and the tube contents again mixed by inversion before being incubated on ice for 5 minutes. The tubes were then centrifuged at 14,000 rpm for 10 minutes at room temperature and the supernatant transferred to a clean tube. RNase A was then added to a final concentration of 20 μg/ml and the tubes incubated at 37°C for 20 minutes. After the RNase treatment, 400 μl of chloroform was added to the supernatant, the layers mixed, and the tubes centrifuged for 1 minute to separate the phases. The aqueous layer was then removed to a new tube and the chloroform extraction repeated. The DNA was precipitated by adding an equal volume of 100% isopropanol, washed with 500 μl of 70% ethanol, and dried under vacuum for 3 minutes. The dried pellet was then resuspended in 32 μl of deionised water and the plasmid DNA re-precipitated by the addition of 8 μl of 4 M NaCl followed by 40 μl of 13% PEG$_{8000}$. The sample was thoroughly mixed and then incubated on ice for 20 minutes. The plasmid DNA was then pelleted by centrifugation at 14,000 rpm for 15 minutes at 4°C, the supernatant carefully removed, and the pellet rinsed with 500 μl of 70% ethanol. The pellet was then dried under vacuum for 3 minutes, resuspended in 20 μl of sterile double distilled water and stored at -20°C.

Magic™ minipreps; for the preparation of sequencing templates

This method is based on that of the Wizard™ Minipreps DNA Purification System Technical Bulletin, revision 1/94 (Promega). Cells from 3 ml of a freshly grown overnight culture were pelleted by centrifuging at 14,000 rpm for 1 minute. The cells were then resuspended in 200 μl of cell resuspension solution (50 mM Tris-Cl, pH 7.5; 10 mM EDTA; 100 μg/ml RNase A) and the suspension transferred to a microcentrifuge tube. 200 μl of cell lysis solution (0.2 M NaOH; 1% SDS) was added to the tube and the contents mixed by inverting the tube several times until the cell suspension cleared. 200 μl of neutralisation solution (2.55 M potassium acetate pH 4.8) was then added, and the contents again mixed by inversion. The tubes were then centrifuged at 14,000 rpm for 5 minutes and the cleared supernatant decanted into a new tube. 1 ml of the Magic minipreps DNA purification resin™ was then added to the sample and mixed in by inversion. For each miniprep, one Magic minicolumn™ was prepared by attaching the barrel of a 3 ml disposable syringe to the luer-lock extension of each minicolumn. 2 ml of column wash solution (200 mM NaCl; 20 mM Tris-Cl, pH 7.5; 5 mM EDTA; diluted 1:1 with 95% ethanol) was then pushed through the minicolumn. The minicolumn was then transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 20 seconds to dry the resin. The minicolumn was then transferred to a new microcentrifuge tube and 50 μl of TE buffer was added to the minicolumn. After 1 minute, the minicolumn was centrifuged at 14,000 rpm for 20 seconds to elute the DNA.

E. coli midiprep

The bacterial cells from a 30 ml to a 100 ml overnight culture were pelleted by centrifugation at 5,000 rpm for 10 minutes at 4°C, and resuspended in 2.5 ml of ice-cold solution 1 (25 mM Tris-Cl pH 8.0; 10 mM EDTA). 5 ml of freshly prepared solution 2 (0.2 M NaOH; 1% SDS) was added and the samples thoroughly mixed. The cells were then left on ice for exactly 5 minutes before 3.75 ml of ice-cold solution 3 (5 M/3 M potassium acetate) was added, and the cells left on ice for a further 10 minutes. Samples were then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was then removed to a new tube and 40 μl of 10 mg/ml RNase was added. The mixture was then incubated at 37°C for 30 minutes. Following RNase treatment, 20 μl of 10 mg/ml Proteinase K was added to the tubes and the samples were left at 37°C for a further 30 minutes. Half the final volume of isopropanol was added, and the samples centrifuged at 10,000 rpm for 15 minutes. The supernatant was removed and the pellet resuspended in 500 μl of TE buffer and transferred to a microcentrifuge tube. An equal volume of Phenol was added, the tube vortexed and then centrifuged at 14,000 rpm for 5 minutes. The upper aqueous layer was removed and transferred to a clean tube. This step was repeated once more. An equal volume of chloroform/iso-amyl alcohol (24:1) was then added to the solution, the tube vortexed and

centrifuged at 14,000 rpm for 5 minutes. The top layer was again transferred to a clean tube and this step was repeated once more. The DNA was then precipitated with 2.5 volumes of 100% ethanol and one tenth of the volume of 3 M sodium acetate. The DNA was left to precipitate for at least 1 hour at -20°C, or at -70°C for at least 30 minutes. The DNA was pelleted by centrifugation at 14,000 rpm for 30 minutes, washed with 70% ethanol, and after lyphilisation resuspended in 30-100 μl of TE buffer.

2.1.2 Bacterial transformation of E. coli

DMSO method

Preparation of competent cells

This method is based on that by Chung and Miller (1988). A 10 ml overnight culture of *E.coli* DH5α cells was grown at 37°C and 1 ml of this overnight culture was then used to inoculate a 100 ml LB broth. This culture was grown at 37°C with vigorous shaking until the optical density (OD_{600}) of cells was between 0.5 and 0.6. The bacterial cells were then pelleted at 3000 rpm for 10 minutes at 4°C and the broth removed. The cells were resuspended in one twentieth of the original volume of transformation and storage buffer (LB Broth [pH 6.1] containing 10% PEG [MW = 3,350], 5% DMSO and 20 mM Mg^{2+}? [10 mM $MgCl_2$ and 10 mM $MgSO_4$]) and incubated on ice for approximately 10 minutes. Competent cells were then snap frozen in liquid nitrogen and stored at-70°C for up to 6 months without loss of efficiency.

Transformation

0.2 ml aliquots of the competent cells were added to cold microcentrifuge tubes and mixed with between 5-15 ng of plasmid DNA, before the cells were returned to ice for 5-30 minutes. 0.2 ml of transformation and storage buffer, containing 20 mM of glucose, was then added to the transformation mix and the cells grown at 37°C for 1 hour with vigorous shaking. This step allows the expression of any antibiotic resistance genes contained in the plasmid. Following this incubation, 100 μl - to-200 μl aliquots of cells were spread onto plates containing IPTG (0.5 mM) and X-Gal (50 μg/ml) and the appropriate antibiotics.

TB method

Preparation of competent cells

This method is based on that by Inoue *et al.* (1990). *E.coli* DH5α cells were streaked onto LB agar plates and cultured overnight at 37°C. 10-12 large colonies (2-3 mm in diameter) were isolated and inoculated into 250 ml of SOB media contained in a 2 L flask. The cells were grown at 18°C with vigorous shaking (200-250 rpm) until the cells reached an optical density (OD_{600}) of 0.6 (approximately 48 hours). The cells were then placed on ice for 10 minutes, after which the culture was transferred a 500 ml centrifuge bottle, and the cells pelleted at 3000 rpm for 10 minutes at 4°C. The pellet was resuspended in 80 ml of TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl.), and incubated in an ice bath for 10 minutes before being centrifuged as previously described. The cell pellet was gently resuspended in 20 ml of TB, and DMSO was added, with gentle swirling, to a final concentration of 7%. After incubation in an ice bath for 10 minutes, the cell suspension was dispensed into 1 ml-to-2 ml aliquots and immediately chilled in liquid nitrogen. Frozen cells were stored at-70°C for at least 1 month without loss of competence.

Transformation

An aliquot of competent cells was thawed at room temperature before the cells were further aliquoted into 200 μl amounts, in 10 ml plastic screw top tubes, and placed in an ice bath. 5 ng-to-25 ng of plasmid DNA was added to each aliquot and the cells incubated in an ice-bath for 30 minutes. The cells were then heat-pulsed, without agitation, at 42°C for 30 seconds and immediately transferred to an ice bath. 0.8 ml of SOC solution was added to the samples and the tubes were incubated at 37°C with vigorous shaking for 1 hour. Following this incubation, 100 μl 1-to-200 μl aliquots of cells were then spread onto plates containing IPTG (0.5 mM) and X-Gal (50 μg/ml) and the appropriate antibiotics.

2.2 General plant methods

2.2.1 General solutions

Arabidopsis nutrient solution; 5 mM KNO_3; 2 mM $MgSO_4$.7H2O; 2 mM $Ca(NO_3).4H_2O$; 2.5 mM KPO_4 (pH 5.5); 50 μM EDTA (ferric sodium salt); 0.01% (v/v) Micronutrient soultion

Micronutrient solution; 70 mM H_3BO_3, 14 mM $MnCl_2.4H_2O$, 0.5 mM $CuSO_4.5H_2O$, 1 mM $ZnSO_4.7H_2O$, 0.2 mM $NaMoO_4.2H_2O$, 10 mM NaCl, and 0.01 mM $CoCl_2.6H_2O$

2.2.2 Growth of in vitro plants

Solid media

Germination medium (GM); 0.443% (w/v) MS medium powder, 1% (w/v) cane sugar, 0.05% (w/v) MES, 0.1% (w/v) phytagel. The pH of the media was adjusted to 5.8-5.9 before it was sterilised by autoclaving.

MS medium; 0.443% (w/v) MS medium powder, 3% (w/v) sucrose, and 0.18% (w/v) phytagel or 0.8% (w/v) agar. The pH of the media was adjusted to 5.8-5.9 before it was sterilised by autoclaving.

Seed sterilisation

Seed sterilisation was performed in a laminar air flow cabinet using sterile 1.5 ml microcentrifuge tubes and sterile Pasteur pipettes. Seeds were treated with 70% Ethanol for 2 minutes with constant, gentle inversion. Using a Pasteur pipette, the seeds were then transferred to another tube, this time containing a 10% (v/v) hypochlorite solution, and subjected to constant, gentle inversion for 10 minutes. Thereafter, the seeds were removed and washed in sterile MilliQ® water (MQ water) for 2 minutes (again with constant inversion). At least 4 washes in MQ water were required to completely remove all traces of the hypochlorite solution.

Seed sowing

In vitro sowing was performed in a laminar air flow cabinet using sterile Pasteur pipettes. After sterilisation, seeds were left in sterile MQ water at 4°C for at least 12 hours in order to break the seed dormancy. Prior to sowing, the seeds were aspirated with a pipette to evenly distribute them within the water to facilitate easy sowing of individual seeds onto the surface of the media. Porous tape (Micropore) was used to seal the plates to allow gaseous exchange to occur. *In vitro* plants were grown under fluorescent light ($\sim$60 μE/m^2/s) at a constant temperature (20°C) with a 16 hour photoperiod.

2.3 General molecular methods

2.3.1 General solutions

Agarose gel loading buffer (6X): 0.25% (w/v) bromophenol blue; 40% (w/v) sucrose. (Sambrook *et al.*, 1989).

Acrylamide gel loading buffer (non-denaturing): 50% (v/v) glycerol ; 1 X TBE buffer; 0.025% Bromophenol Blue; 0.025% Xylene Cyanol.

Acrylamide gel loading buffer (denaturing): 98% (v/v) deionised formamide; 10 mM EDTA (pH 8.0); 0.025% Bromophenol Blue; 0.025% Xylene Cyanol.

Chloroform/isoamyl alcohol mixture: 24:1 (v/v) chloroform:isoamyl alcohol.

Ethidium Bromide: A 10 mg/ml stock solution of ethidium bromide was prepared and stored in a light resistant container at 4°C.

Phenol: Phenol (Wako) was equilibrated in, and stored under TE buffer.

50 X Tris Acetate (TAE) Buffer: 2 M Trizma base; 1 M Glacial acetic acid; 0.05 M EDTA pH 8.0. The solution was sterilised by autoclaving.

20 X Tris Borate (TBE) buffer: 1.8 M Trizma base; 1.8 M Boric Acid; 0.04 M EDTA (pH 8.0). The solution was sterilised by autoclaving.

2.3.2 General DNA manipulation techniques

Quantification of DNA/RNA concentrations

Agarose plate

A 1% agarose gel solution containing ethidium bromide at a concentration of 0.5 µg/ml was prepared in a 9 cm Petri dish. 1 µl -to- 2 µl of DNA or RNA samples and standards were spotted onto the surface of the plate. The samples were then left to dry in the dark for at least 15 minutes at room temperature. The DNA or RNA was then visualised under UV light and the concentration of samples estimated.

Spectrophotometer readings

Spectrophotometer readings were measured using a Perkin-Elmer lambda-3 UV/Vis spectrophotometer. Optical density readings were taken at wavelengths of 260 nm and 280 nm. The readings taken at 260 nm allowed DNA/RNA concentrations to be estimated while the ratio of readings at 260 nm: 280 nm gave an estimate of the purity of the sample (an optimum of 1.8 for DNA samples and 2.0 for RNA samples). 1 ml of either sterile water or TE buffer, placed in a quartz cuvette, was first used to adjust the zero scale at the required wavelength. 1 μl of DNA/RNA sample resuspended in either sterile water or TE buffer was then added the 1 ml quartz spectrophotometer cuvette. The optical density readings at both 260 and 280 nm were then recorded for each sample. A further 1 μl of sample was then placed in the cuvette to check that the readings at both wavelengths were twice those recorded initially. The initial 260 nm readings were multiplied by 50 for DNA or 40 for RNA samples respectively to determine the concentration of the sample in μg/μl.

Restriction enzyme digests

The DNA to be digested was placed in a microcentrifuge tube containing the required amount of sterile water. One tenth of the total reaction volume, of the appropriate 10X restriction enzyme buffer was added to the tube. If required, Bovine Serum Albumin (BSA) was added to a final concentration of 1 mg/ml. 2-to-3 times the amount of enzyme required to digest 1μg of DNA in 1 hour was then added to the reaction providing this volume did not exceed one tenth of the final volume. The mixture was then incubated at the temperature recommended by the manufacturer of the enzyme, for a minimum of three hours. If possible the enzyme was heat inactivated by incubation at 65°C for 10-20 minutes. If heat inactivation was inappropriate the enzyme mixture was extracted with phenol/chloroform and the DNA ethanol precipitated overnight at -20°C.

Agarose gel electrophoresis of DNA samples

An appropriate quantity of agarose (0.8%- 3.5% [w/v]) was dissolved in 1 X TAE buffer by heating until no crystals remained. Once the agarose had cooled to 55°C, ethidium bromide was added to a final concentration of 0.5 μg/ml. The agarose solution was then poured into a horizontal (50-300 ml) gel apparatus and a well comb inserted. 1X TAE buffer was then added to the gel tank to completely cover the gel. One sixth of the total volume of loading buffer was added to each DNA sample and the samples loaded into the wells. Electrophoresis was performed for the appropriate length of time at a voltage ranging from 20-80 volts. DNA bands were viewed using a UV transilluminator.

Purification of DNA fragments from agarose gels.

<u>Phenol freeze method</u>

DNA bands of interest were cut from gels in the minimum amount of agarose using a sterile scalpel blade. Agarose slices were then crushed between pieces of parafilm and placed into a 1 ml syringe attached to a 27G needle. The agarose was forced through the needle into a clean microcentrifuge tube, and TE buffer was added to adjust the final volume of agarose mixture to 500µl. An equal volume of equilibrated phenol was added, and the mixture vortexed for 1 minute before being placed at -70°C for 1 hour. The samples were then centrifuged at 14,000 rpm for 20 minutes. The upper aqueous layer was removed to a clean microcentrifuge tube and an equal volume of chloroform was added. The tubes were then vortexed for 30 seconds before being centrifuged at 14,000 rpm for 5 minutes. This step was repeated once more. The DNA was then precipitated with one tenth of the volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, at -20°C overnight before being pelleted following centrifugation at 14,000 rpm for 30 minutes. The pellet was subsequently washed in 70% ethanol, dried under vacuum for 3 minutes, and resuspended in 10 µl of sterile water.

<u>GEL-SPIN™ DNA recovery kit</u>

This method is as per the GEL-SPIN™ DNA Recovery kit protocol (Worthington). DNA bands of interest were cut from the gels in the minimum amount of agarose. The agarose (up to 1 ml) was placed into a GEL-SPIN unit, containing a cationic exchanger that was placed inside a microcentrifuge tube. The tip of a plunger was wetted with sterile water and used to pick up a porous polyethylene filter. The filter was then placed on top of the agarose fragment and the agarose was pressed into the GEL-SPIN unit. The tube was centrifuged at 10,000 rpm for 2 minutes, 0.1 ml of GS solution (0.1 M Potassium Chloride) added to the GEL-SPIN unit, and the centrifugation repeated. The GEL-SPIN unit was then discarded and the DNA precipitated with 2.5 volumes of ethanol at -20°C for at least 1 hour. The samples were then centrifuged at 14,000 rpm for 30 minutes, washed in 70% ethanol and vacuum dried before the pellets were then resuspended in 15 µl of TE buffer.

Sequencing of cloned DNA fragments

This method is based on that of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit Protocol, revision A (Perkin Elmer). Plasmids containing DNA fragments to be sequenced were first extracted using the appropriate protocol. To set up the sequencing reactions, the following reagents were placed into 600 µl PCR tubes; 300-500 ng of double stranded plasmid DNA, 3.2 pmol of the appropriate primer (forward or reverse), 8 µl of

terminator premix (A-Dye Terminator; C-Dye Terminator; G-Dye Terminator; T-Dye Terminator); dGTP, dATP, dCTP, and dTTP nucleotides; Tris-HCl (pH 9.0); $MgCl_2$; thermal stable pyrophophatase; AmpliTaq (DNA polymerase) and enough sterile MQ water to give a final reaction volume of 20µl. The reaction mixture was then overlayed with 40 µl of mineral oil, and placed in a Perkin-Elmer 480 DNA thermal cycler preheated to 96°C. Thermal cycling was immediately commenced with the following cycling parameters; 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. After completion of the cycling, the tubes were then placed on ice.

Extension products from the reactions were purified by removing unincorporated nucleotides in the following manner; for each reaction a separate microcentrifuge tube was prepared containing 2 µl of 3 M Sodium acetate (pH 4.6) and 50 µl of 95% ethanol. The entire 20 µl of the sequencing reaction was then transferred to the tubes containing the ethanol solution, vortexed and left on ice for 10 minutes. The tubes were centrifuged at 14,000 rpm for 30 minutes, and the DNA pellet rinsed in 250 µl of 70% ethanol. The DNA pellet was then dried under vacuum for 3 minutes. The dried samples were the run on a ABI PRISM Genetic Analyzer in the Department of Microbiology, Menash University, Clayton.

Polymerase chain reaction

All reagents used for polymerase chain reactions were used exclusively for this purpose and all plastic and glassware for PCR use was handled only with gloved hands. In addition, a set of micropipettes were kept for PCR use only and periodically soaked in 0.1M HCl to denature any residual DNA. Filtered pipette tips were also used for all PCR applications. To set up a typical reaction the following reagents were added sequentially to sterile 600 µl PCR tubes; sterile MQ water to adjust the final reaction volume to either 25 or 50 µl, 10-150 ng of DNA template, one tenth of the final volume of 10 X Thermo buffer (Promega), $MgCl_2$ to a final concentration of between 1 and 3.5 mM; dNTP mix (10 mM of each dATP, dCTP, dGTP and dTTP) to a final concentration of 0.2 mM of each dNTP, and forward and reverse primers at a final concentration of between 0.2 µM and 0.4 µM. The reaction was then mixed and covered with 1-to-2 drops of mineral oil. Tubes were placed into a Perkin-Elmer 480 DNA thermal cycler preheated to 94°C and between 1 to 1.5 units of Taq DNA polymerase was added to each reaction. Cycling at the required parameters was immediately commenced. Reactions were stored at -20°C for up to six months.

Ligation of DNA

Following digestion by the appropriate restriction enzymes both the vector backbone and the DNA insert were extracted with phenol/chloroform and precipitated with one tenth of the volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. The vector was then phosphatased using shrimp alkaline phosphatase as per the United States Biochemical user manual. The shrimp alkaline phosphatase was then inactivated by incubation at 65°C for 10 minutes. The subsequent ligation reactions were performed in sterile 600 μl microcentrifuge tubes in a final volume of 20 μl. 1:1, 1:3 1:5 or 3:1 molar ratios of insert DNA to 50 ng of vector DNA were used in most ligation experiments. Once the appropriate amounts of vector and insert DNA had been added to the reaction, 10 X T4 DNA ligase buffer, T4 DNA ligase and sterile MQ water were also added. The reactions were gently mixed and incubated for 30 minutes at room temperature followed by three hours at 16°C. The ligation mixtures were stored at 4°C.

2.3.3 DNA extractions from plants

CTAB method

2 g-to-3 g of frozen plant tissue was ground to a fine powder in a clean mortar and pestle with liquid nitrogen. 2.5 ml of 2x extraction buffer (2% CTAB [cetyltrimethylammonium bromide] (w/v); 100 mM Tris-Cl pH 8.0; 20 mM EDTA pH 8.0; 1.4 M NaCl; 2% (β-mercaptoethanol[added just prior to use]), preheated at 65°C, was then added to the plant tissue in the mortar, and the mortar contents ground until thawed. The mixture was then transferred to a sterile 50 ml centrifuge tube and incubated at 65°C for 10 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, and the tubes shaken continuously for at least 5 minutes. The tubes were then centrifuged at 8000 rpm for 10 minutes at room temperature. The supernatant was removed and transferred to clean glass centrifuge tubes. An equal volume of chloroform: isoamyl alcohol (24:1) was again added and the tubes centrifuged as above. The supernatant was transferred to a new glass centrifuge tube and an equal volume of precipitation buffer (1% CTAB (w/v); 50 mM Tris-Cl pH 8.0; 10 mM EDTA pH 8.0; 1% (-mercaptoethanol [added just prior to use]) was added before the contents were mixed by inversion. Precipitation was allowed to proceed at room temperature for at least 1 hour.

Following the precipitation step, the samples were centrifuged at 8000 rpm for 10 minutes at room temperature. The supernatant was then discarded and the pellet resuspended in 2 ml of 1 M CsCl solution (50 mM Tris-Cl pH 8.0, 5 mM EDTA pH 8.0; 1 M CsCl), before 10 μl of a 10 mg/ml stock of ethidium bromide was added. 1.9 ml of a 5.7 M CsCl solution (CsCl

50 mM Tris-Cl pH 8.0; 5 mM EDTA pH 8.0; 5.7 M CsCl) solution was then added to a 5 ml ultracentrifuge tube, and the resuspended pellet was carefully added on top. Tubes to be placed in opposing holders of the Beckman ultracentrifuge rotor were then balanced to weigh within 0.001g of each other. The samples were then centrifuged at 36,000 rpm for 16 hours at room temperature (18-25°C). The ethidium bromide band that contained the DNA was then extracted into a clean microcentrifuge tube via an 18G needle attached to a 1 ml syringe that was inserted into the ultracentrifuge tube just below the DNA band. An equal volume of CsCl saturated isopropanol was then added to the DNA, the solution mixed by inversion, and the top ethidium-bromide-containing layer removed and discarded. This step was repeated several times until all traces of ethidium bromide were removed. The DNA was then precipitated with 3 volumes of 70% ethanol at -20°C overnight. The pellet was washed in 70% ethanol, dried under vacuum and resuspended in 30 µl of TE buffer.

Miniprep for the isolation of genomic DNA

3.5 ml of 20% SDS (w/v) was added to 50 ml of extraction buffer (100 mM Tris-Cl (pH 8.0); 50 mM EDTA; 500 mM NaCl; 10 mM (-mercaptoethanol [added just prior to use]) while a sterile mortar and pestle was cooled with liquid nitrogen. 2 g of plant tissue was ground to a fine powder in the mortar before 3 ml of the extraction buffer/SDS solution was added. The tissue/buffer mixture was again ground to produce a fine powder. The mixture was then transferred to a sterile 10 ml tube containing 2.5 ml of the extraction buffer/SDS mix and the tubes stored on ice until all samples were ground. The samples were then incubated at 65°C for 10 minutes, during which the tubes were shaken vigorously several times. 2 ml of 5 M potassium acetate was then added to the extracts and thoroughly mixed in. Following a further incubation on ice for 10 minutes, the samples were centrifuged at 10,000 rpm for 20 minutes. The supernatants were then poured through lint-free tissues into sterile centrifuge tubes. 0.6 of the volume of isopropanol was then added to the tubes and the samples gently mixed by inversion, before being spun at 10,000 rpm for 20 minutes to pellet the DNA. Following centrifugation the supernatant was removed and the tube inverted on a paper towel for 5 minutes to briefly dry the pellet. The inside of the tube was wiped with a lint-free tissue to remove any excess isopropanol, and the pellet resuspended in 700 µl of TE buffer. The DNA was then left for at least 2 hours at 4°C to ensure complete resuspension before being transferred to fresh tubes. RNase was subsequently added to each sample to a final concentration of 50 µg/ml, and the tubes incubated at room temperature for 30 minutes. The DNA was phenol-extracted and then precipitated with one tenth of the volume of 3M sodium acetate and 0.7 of the volume of isopropanol. The contents were gently mixed and the tube centrifuged at 10,000 rpm for 20 minutes. The supernatant was removed, the pellet washed in 70% ethanol, dried, and resuspended in 50-100 µl MQ water.

2.3.4 DNA-DNA hybridisation protocols.

Southern blot

This technique is adapted from Southern (1975) and based on the Hybond™-N+ membrane protocol, version 2 (Amersham). 10 μg-to-15 μg of DNA digested with the appropriate enzymes were run on an agarose gel and the gel photographed under UV light. The gel was then washed in 0.25 M HCl until the dyes in the loading buffer (xylene cyanol and bromophenol blue) had changed to a yellow colour. If fragments larger than 10 kb were to be examined the gel was left in the HCl for an additional ten minutes. The gel was rinsed in distilled water for 5 minutes and subsequently placed in denaturing buffer (1.5 M NaCl; 500 mM NaOH) so as to completely cover the gel, before being left for 30 minutes at room temperature with constant shaking. The gel was again rinsed in distilled water, placed in neutralisation buffer (1.5 M NaCl; 500 mM Tris-HCl (pH 7.2); 1 mM EDTA) and left at room temperature for 15 minutes with constant agitation. This neutralisation step was repeated once. The gel was then removed from the solution and was ready to be used for the capillary blotting process.

A large tray was filled with 1L of 20 X SSC (3 M NaCl: 300 mM Na$_3$citrate?) and a platform spanning the tray was prepared. This platform was covered with a wick made from three sheets of Whatman 3MM filter paper saturated with 20 X SSC. The prepared gel was flipped 180° so that the DNA samples at the bottom of the gel faced upwards and placed on the wick. Any air bubbles trapped beneath the gel were removed by rolling over the surface with a clean 10 ml glass pipette. A sheet of Hybond-N+ membrane was cut to the exact size of the gel and placed gently on top of the gel. Any air bubbles trapped between the gel and membrane were again squeezed out using a 10 ml glass pipette. Three sheets of 3MM Whatman paper, cut to size, were then placed on top of the membrane and any air bubbles removed. A stack of absorbent paper towel was then placed on top of the Whatman paper, before a glass plate supporting a 1 kg weight was placed on top of the paper towel. The transfer process was allowed to proceed for at least 12 hours. After blotting was complete, the apparatus was dismantled. The position of the wells of the gel were marked on the membrane, and the filter was washed briefly in 2 X SSC to remove any adhering agarose. The DNA was then fixed by either placing the membrane (DNA side down) on a UV light source for 2 minutes or baking the filter at 80°C for two hours. The membranes were then stored at room temperature until required.

Dot blots

The protocol used is based on HybondTM-N+ membrane protocol, version two (Amersham). DNA samples to be blotted were heated to 95°C and then chilled on ice briefly. An equal volume of 20 X SSC was added to the samples before they were dotted onto a pre-cut membrane in 2 μl aliquots. After all samples were placed on the membrane the filter was wet with denaturing solution for 5 minutes and then transferred onto filter paper soaked in neutralising solution for 1 minute. The membrane was blotted dry with filter paper and air dried and fixed as described previously.

Colony blots

This protocol is based on HybondTM-N+ membrane protocol, version 2 (Amersham). Bacterial colonies were incubated on LB plates at 37°C overnight. A sterile, pre-cut Hybond-N+ membrane was first pre-wet on a clean LB agar plate. This membrane was then carefully placed onto the agar surface of the initial plate containing the colonies. The membrane was marked using a sterile needle to allow correct orientation of the colonies. After 1 minute, the membrane was removed and placed colony side up onto a new LB plate. A second filter was then placed onto the original colony containing plate and marked in the same manner as the first to allow correct alignment. After 1 minute the duplicate filter was also placed, colony side up onto the surface of a new LB plate. LB agar plates containing filters, along with the original master plates, were incubated for 3-4 hours at 37°C until colonies of 0.5-1 mm were obtained.

Following this incubation membranes were placed onto filter paper and left to dry for ~10 minutes at room temperature. Filters were then placed, colony side up onto absorbent filter paper soaked in denaturing solution and left for 7 minutes. Membranes were then transferred onto new filter paper soaked in neutralising solution and left for 3 minutes. This step was repeated once. Filters were then briefly washed in 2 X SSC and left to air dry before being fixed as described previously.

Preparation of radioactively labelled DNA probes.

This method is based on the GIGA prime labelling kit protocol (Bresatec). 100 ng-to-200 ng of double stranded DNA was added to enough sterile MQ water so as to make a final volume of 6 μl, boiled for 5 minutes and chilled on ice. The DNA was then pulse spun, and 6 μl of the Decanucleotide solution (Tube 1), 6 μl of the nucleotide buffer cocktail (Tube 2A), 5 μl of α-32P-dATP and 1.5 μl of Klenow enzyme (8 u/μl) were added. The reaction was then incubated at 37°C for 30 minutes. Before removal of unincorporated nucleotides from the probe reaction, a purification column was prepared in the following manner. A small piece

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of sterile glass wool was placed at the bottom of a shortened Pasteur pipette. Sephadex G50 was then used to fill the column to within 1 cm of the top, with care taken to remove any bubbles formed. Column wash buffer (98 % (v/v) TE buffer; 0.1% (w/v) SDS; 0.1 M NaOH) was then used to fill the column before it was covered with parafilm and placed at 4°C until required.

The labelling reaction was stopped by the addition of 1 µl of 0.5 M EDTA, before 5 µl of a saturated solution of Orange G and 35 µl of a saturated solution of Dextran Blue dye was added to the reaction. The reaction was then loaded into the top of the Sephadex column and the washed through with column wash buffer. As the Dextran Blue dye co-migrates with large DNA fragments through the sephadex, the blue dye fractions containing the probe were collected. Fractions containing the orange dye contained unincorporated nucleotides and were discarded. The activity of the probe was checked using a scintillation counter.

Hybridisation protocol

This protocol is based on HybondTM-N+ membrane protocol, version 2 (Amersham). 100 µl of a 10 mg/ml stock of sheared herring sperm DNA was denatured by heating to 100°C for 5 minutes, briefly chilled and added to 50 ml of pre-hybridisation solution (5 X SSPE [0.9 M NaCl; 0.05 M Sodium phosphate; 0.005 M EDTA pH 7.7]; 5 X Denhardts solution [2% (w/v) Bovine Serum Albumin; 2% (w/v) Ficoll™; 2% Polyvinylpyrrolidone]; 0.5% (w/v) SDS). The pre-hybridisation solution was then added to a plastic bag or hybridisation tube containing the membranes and all bubbles were removed. The membrane was pre-hybridised for at least 1 hour at 65°C in a shaking water bath. Following pre-hybridisation, double stranded DNA probes were denatured by boiling for 2-3 minutes and added to the bag or tube containing the membrane in the pre-hybridisation solution. The probes were left to hybridise to the filters for at least 12 hours at 65°C in a shaking water bath. After hybridisation, the filters were incubated in wash solutions with constant shaking. Before proceeding to the next level of stringency in the wash solutions, filters were checked using a geiger counter for the presence of an adequate signal that would warrant further washes. The first two washes were in a solution of 2 X SSPE; 0.1% (w/v) SDS at room temperature for ten minutes. This was followed by a medium stringency wash of 1 X SSPE; 0.1% (w/v) SDS at 65°C for 15 minutes. The last two washes were in a high stringency solution of 0.1 X SSPE; 0.1% (w/v) SDS at 65°C for ten minutes. Following these washes, the filter was wrapped in plastic wrap and exposed to an X-ray film in an autoradiograph cassette at -70°C for the appropriate length of time. The X-ray films were developed in a Agfa-Gevamatic 60 X-ray machine, under NX-914 safety lights.

Membrane stripping protocol

For successful removal of probes, membranes were never allowed to dry during or after hybridisation and washing. To strip the membranes a solution of 0.5% (w/v) SDS was boiled and poured onto filters to be stripped and the solution allowed to cool to room temperature. The stripped filter was again exposed to X-ray film to determine if removal of the probe was successful.

Chapter 3

Characterisation of early-flowering mutant lines of the late-flowering Pitztal ecotype.

3.1 Introduction

The *EMF* gene is thought to be one of the most important loci controlling the repression of flowering in *Arabidopsis*, as *emf* mutants flower immediately after germination (Sung *et al.*, 1992; Yang *et al.*, 1995a; Bai and Sung. 1995). Indeed, the *EMF* gene is now thought to repress all stages of phase change in *Arabidopsis*. including the inflorescence to floral meristem transition (Yang *et al.*, 1995a). The main roles of several genes in the constitutive repression pathway such as the *TFL*, *ELF-1*, and *ELF-2* loci, are thought to be to extend the repression mediated by *EMF* in various environmental conditions (Martinez-Zapater *et al.*, 1994). The genes mediating a short day repression of flowering however, may act to inhibit the biosynthesis or signal transduction of gibberellin, a limiting hormone in short days, and thereby eliminate any gibberellin-mediated inhibition of *EMF* (Coupland *et al.*, 1993, Martinez-Zapater *et al.*, 1994, Haughn *et al.*, 1995; Yang *et al.*, 1995a; Zagotta *et al.*, 1996). Several of these early-flowering mutants including the phytochrome *hy-2* and *phy*-b mutants, or gibberellin signal transduction *spy* mutants, display pleiotrophic phenotypes such as pale-green foliage (*spy*, *elf-3*, *eaf1*), elongated hypocotyls (*spy*, *eaf1*, *phy-b*, *hy-2*, *elf-3*, *elg*), small plants size (*efs*) and homeotic transformations (*tfl*, *emf*) (Bai and Sung, 1995; Halliday *et al.*, 1996; Zagotta *et al.*, 1996; Silverstone *et al.*, 1998; Whitelam *et al.*, 1998; Scott *et al.*, 1999; Soppe *et al.*, 1999). These phenotypes suggest roles for several of these genes in various developmental processes outside of their involvement in the repression of the floral induction process.

Similar types of floral repression and promotory pathways are presumed to exist in both early- and late-flowering ecotypes, however the effects of these pathways may not become obvious in ecotypes with flowering times which are not much later than the time required for a plant to become competent to flower (Karlsson *et al.*, 1993). The presence of a vernalisation-responsive pathway in early-flowering ecotypes is, for example, only revealed if early-flowering plants are grown in non-inductive conditions. Identification of mutations that accelerate flowering in already early-flowering ecotypes has therefore necessitated the screening of plants grown in non-inductive short days, or under tissue culture conditions which delay flowering (Chandler *et al.*, 1996; Wilson and Dean, 1996

In this study a novel approach was taken to identify floral repression genes by attempting to isolate early-flowering mutants using a late-flowering ecotype background. There are believed to be several minor recessive genes, identified from an analysis of natural flowering time variations of *Arabidopsis*, that are important in controlling the flowering time of these late-flowering ecotypes (Lee *et al.*, 1993; Martinez-Zapater *et al.*, 1994; Aukermann and Amasino, 1996). Several more genes influencing this late-flowering phenotype have subsequently been identified following analysis of quantitative trait loci (Clarke *et al.*, 1995; Peeters and Koornneef, 1996; Stratton, 1998). The dominant *FRI*, and semi-dominant *FLC* loci however, are believed to be the major genes responsible for conferring the repression of flowering seen in late-flowering ecotypes. Mutations resulting in significantly earlier-flowering plants, in the late-flowering Pitztal ecotype, would therefore most likely affect either the activity of the *FRI* or *FLC* genes, or the activities of genes regulated by *FRI* or *FLC*. The presence of several other genes that influence floral induction in these ecotypes, including those controlling light perception or hormone signal transduction however, provide further candidate loci that may be affected when creating early-flowering mutants in late-flowering ecotypes.

3.2 Materials and methods

3.2.1 Seed stocks

Seed stocks were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC), the *Arabidopsis* Biological Resource Centre (ABRC) or from laboratory stocks.

Late-flowering Ecotypes		**Early-flowering ecotypes**	
Innsbruck	Lab. stock	Columbia:	Lab. stock
Nossen :	Donated by Dr. D. Bagnall	Niederzenz:	Lab. stock
Pitztal :	Lab. stock	Landsberg:	NASC # N1298
San-Feliu-2:	NASC # N1516	Landsberg *erecta:*	Lab. stock
Stockholm:	NASC # N1298		

3.2.2 Growth of soil plants

Dry *Arabidopsis* seeds were sown onto a 1:1 mixture of Debco Seed Raising Mix and Perlite moistened with *Arabidopsis* nutrient solution, covered in plastic wrap and placed at 4°C to break seed dormancy. After 24 hours, germinating seedlings were placed under constant fluorescent light (~60 $\mu E/m^2/s$), and maintained at a temperature of 18-21°C in greenhouse conditions. Alternatively, plants were placed in growth cabinets and grown under various light and temperature regimes as required. After seedlings had reached the cotyledon stage,

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the plastic wrap was removed, and the plants were watered every two to three days with *Arabidopsis* nutrient solution. Flowering times were measured as the number of days post-germination to the appearance of the floral meristem. The rosette leaf number was also recorded at the time of flowering.

3.2.3 Chemical mutagenesis of Arabidopsis seed

Chemical mutagenesis of the Pitztal ecotype was achieved by incubating approximately 10,000 seed of the Pitztal ecotype of *Arabidopsis* in a 40 mM solution of ethyl methanosulfonate (EMS) (Sigma). The seed was stirred in this solution for eight hours before the EMS solution was neutralised by decanting into a 10 M sodium hydroxide solution. The seed were then rinsed 8 times with 100 ml of MQ water over a period of 2 hours. To facilitate easy sowing, the mutagenised seed were resuspended in 500 ml of cooled 0.15% agar and dispensed with a pipette to a final density of approximately 100 seed per punnet.

3.2.4 Gamma-irradiation of Arabidopsis seed

Irradiation of approximately 10,000 seed of the Pitztal ecotype of *Arabidopsis* was carried out in the Gammacell 1000 Gamma Irradiator (Isomedix, Inc. New Jersey) with a Cesium-137 chloride source. The seed were exposed for an appropriate length of time to achieve a dose of either 75 or 100krads. To facilitate easy sowing, the mutagenised seed were resuspended in 500 ml of cooled 0.15% agar and dispensed with a pipette to a final density of approximately 100 seed per punnet.

3.2.5 Arabidopsis crosses

Under a binocular microscope, flowers of the recipient were hand-emasculated by removing all six stamens with fine forceps. Only unpollinated flowers of the most apical inflorescence were used. Any pollinated flowers within the inflorescence were removed before proceeding with the crosses. Anthers of mature flowers of the donor were removed, and the pollen applied to the stigmatic surface of the recipients.

3.3 Results

Flowering times of wild-type plants

Prior to proceeding with the mutagenesis experiments the flowering time of the late-flowering ecotype, Pitztal was determined under the conditions to be utilised for mutant screening. The mean flowering time for unvernalised wild-type Pitztal plants was found to be 99.07 ± 3.39 days. Vernalisation periods of 14 or 19 days decreased this flowering time to 71.74 ± 1.87 and 58.8 ± 2.21 days respectively, whilst a treatment of 21 days further decreased the flowering time to 35.2 ± 1.50 days. The range of flowering times observed in these populations also decreased with longer vernalisation periods (Figure 3.1). A similar decrease in the flowering time of Pitztal had been reported by Burn *et al.*, 1993b who demonstrated that a vernalisation treatment of 21 days decreased flowering times from 74.4 to 22.9 days. The flowering times of several early-flowering ecotypes, Columbia, L. *erecta*, and Niederzenz grown in these conditions were also examined. Unvernalised plants of these ecotypes had average flowering times of 21.9 ± 0.95, 21.8 ± 0.82, and 21.2 ± 0.71 days respectively, results consistent with those reported by several other researchers. These early-flowering ecotypes by definition are generally unresponsive to periods of vernalisation.

EMS and gamma-irradiation treatments of plants

The germination of seed either mutagenised with the chemical mutagen EMS, or irradiated with the Cs^{137} source, was markedly reduced compared to that of wild-type controls, with the EMS-treated seed displaying the lowest levels of germination (Table 3.1). In addition, a large proportion of M1 plants derived from mutagenised seed did not live part the cotyledon stage, with the highest mortality recorded for the gamma-irradiated seedlings (Table 3.1). In general, the gamma-irradiated plants were small in stature and many seedlings died at the later stages of development. Seedlings derived from EMS mutagenesis however, did yield a higher proportion of chlorophyll mutants, compared to those arising from irradiated seed.
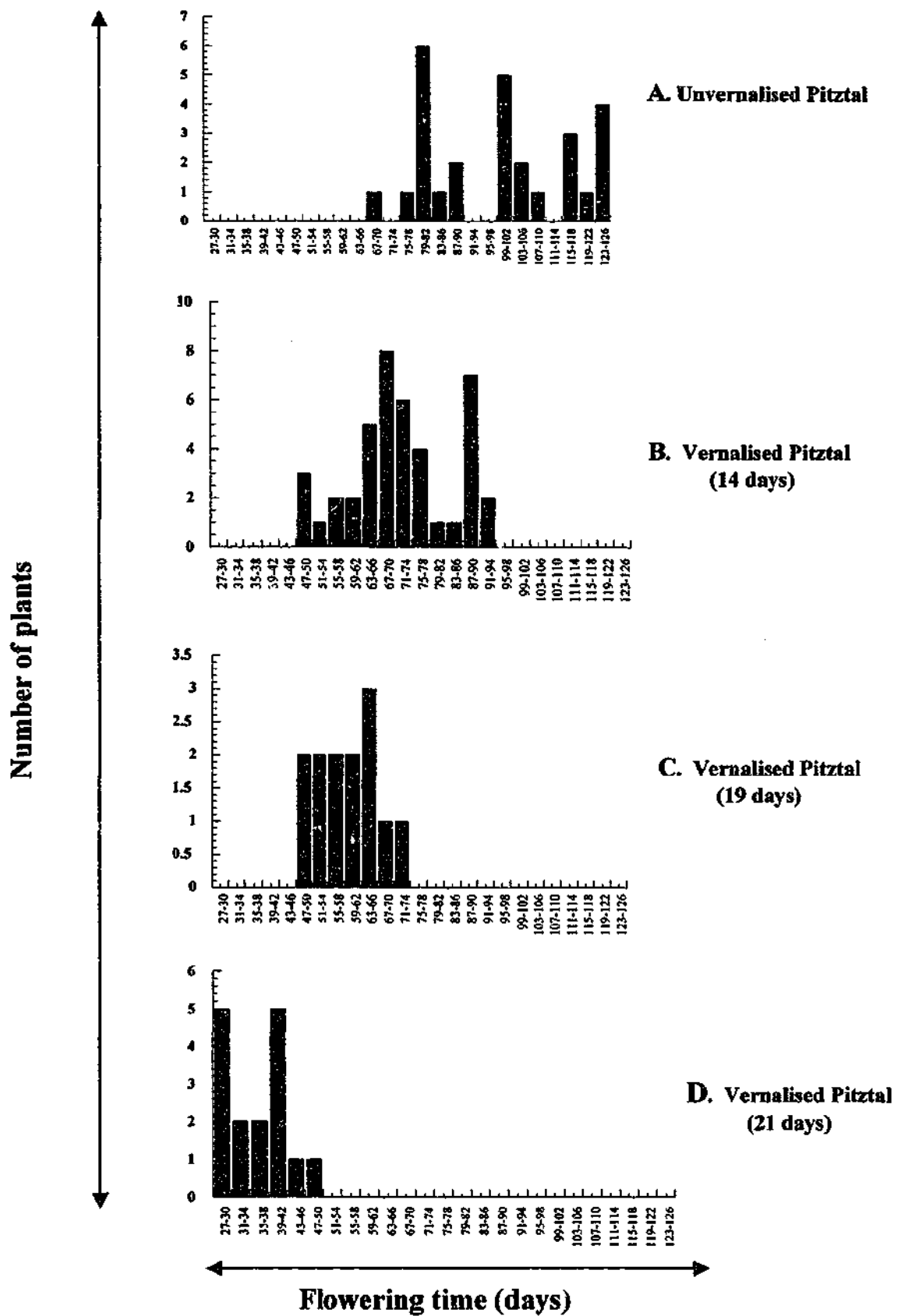
Figure 3.1

The flowering time (days) of unvernalised wild-type Pitztal plants (A), as well as those given either a 14, 19, or 21 day vernalisation treatment (B, C, and D).

Plant line	% germination	% mortality at the cotyledon stage.
M1 plants (EMS)	18.6	2.0
M1 plants (γ-radiation)	23.5	5.3
Pitztal control	69.3	0.0
L. *erecta* control	95.5	0.0

Table 3·1 : The percentage germination and the mortality at the cotyledon stage for M1 seedlings arising from either EMS or gamma-radiation treated seeds compared with those for wild-type Pitztal and L. *erecta* plants.

Figure 3.2 displays the ranges of flowering times for these M1 plants that were given a non-saturating vernalisation treatment of 14 days. The EMS-treated and gamma-irradiated M1 plants flowered within a similar range as wild-type Pitztal plants also given a two week cold treatment (Figure 3.1), although both mutagenised populations also contained plants flowering later and earlier than the wild-type population. No early-flowering plants were observed in the M1 populations. In general, the EMS M1 plants flowered slightly earlier than the gamma radiation-treated M1 plants, although the later flowering phenotype of the radiation-treated plants may be due to the slower growth rate observed amongst these M1 plants.

A. M1 plants (EMS)   **B. M1 plants (gamma-radiation)**
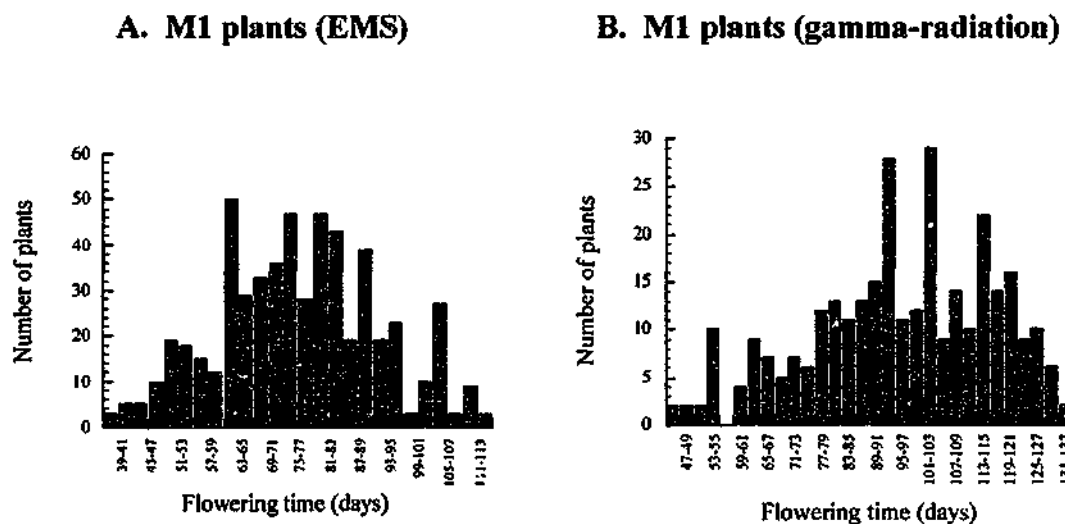
Figure 3.2

The flowering time (days) of the M1 plants grown from either EMS treated seed (A) or gamma-irradiated seed (B).

Screening for flowering time mutants

The non-vernalised progeny from the M1 plants were subsequently screened for early-flowering mutants that flowered in less that 35 days. This threshold period of 35 days for early-flowering was chosen as it was still significantly less than the flowering times (~45 days) of the earliest flowering Pitztal plants given a 19 day vernalisation treatment (Figure 3.1). 32 individual early-flowering mutant plants were originally obtained from the M2 population, with the earliest plants flowering in 22 days and the latest at 33 days. Of these 32 mutants, 19 were isolated from the EMS treated seeds, whilst 13 arose from the radiation treatment. The mean flowering times of the selected M2 plants for both treatments are summarised in Table 3.2. The range of flowering times amongst these early-flowering mutants was between 22 days and 33 days for the EMS derived M2 plants, and between 25 days and 29 days for the radiation-induced plants.

Plant line	Mean flowering time (days)
M1 plants (EMS)	77.14 ±0.67
Early-flowering mutant M2 plants (EMS)	25.90 ± 0.83
M1 plants (γ-radiation)	98.77 ± 1.17
Early-flowering mutant M2 plants (γ-radiation)	27.50 ± 1.59
Pitztal control (unvernalised)	99.07 ± 3.39
Pitztal control (21 days vernalisation)	35.18 ± 1.50
Columbia control	21.90 ± 0.95
L. *erecta* control	21.80 ± 0.82

Table 3.2: The average flowering time of the M1 and selected M2 plants arising from either EMS- or gamma radiation-treated seeds. The average flowering time is shown for unvernalised wild-type Pitztal plants, those given 21 days vernalisation treatment, and wild-type Columbia and L. *erecta* controls.

Several of the early-flowering plants created from either EMS treatment or radiation treatment did not produce viable seed in the M2 generation. Those plants that did, were allowed to self-fertilise in order to create M3 lines of mutants. These distinct mutant lines, or their progeny, were used in all subsequent experiments. Figure 3.3 shows the average flowering times of these M3 mutant lines compared to wild-type controls. The flowering times of these selected EMS (lines 390 -550) and gamma-radiation (lines 180-300) plants were found to be quite similar to the early-flowering Columbia controls, and dramatically earlier than those of the wild-type Pitztal control plants (Figure 3.3).

A. M3 mutant lines (EMS)

B. M3 mutant lines (gamma-radiation)
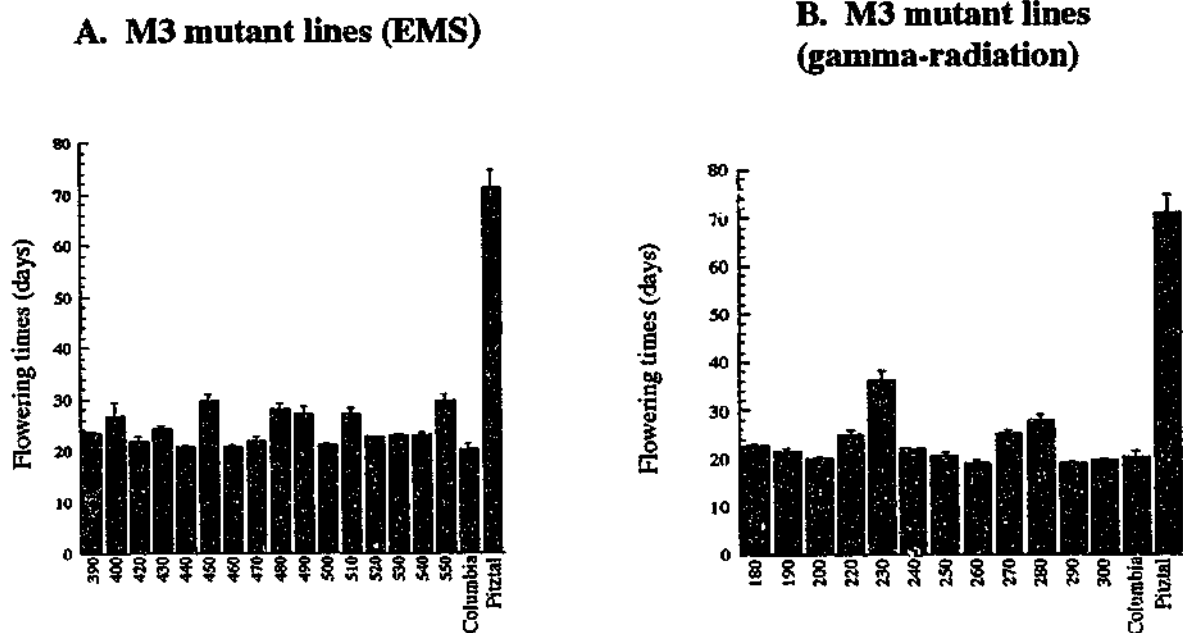
Figure 3.3

The average flowering time (days) of the putative M3 mutant lines derived from EMS treated seed (390-450) (A), or gamma-irradiated seed (180-300) (B), and those of wild-type Pitztal or Columbia controls.

EMS mutant lines

Some preliminary analysis of the EMS mutant lines was performed to determine if any of these mutant lines were temperature-sensitive. Subsequent temperature shift experiments may then have allowed for a determination of the developmental time of action of the genes mutated in any temperature-sensitive lines. Figure 3.4 shows the flowering time and rosette leaf number at flowering of these EMS lines grown at 21-22°C, or at 15°C, in continuous light . Three lines, 400, 430, and 490, were identified as being possible temperature-sensitive mutants as they displayed a slightly later flowering phenotype, and produced more rosette leaves, when grown at 15°C compared to 21°C. Temperature shift experiments on two of these lines demonstrated that the delayed flowering time phenotype of the 430 and 490 mutant lines at 15°C is only exhibited if the plants are shifted to these conditions before day four post germination (data not shown). This suggests that the timing of action of the genes controlling this phenotype in these two mutant lines is either at, or before, day four of growth.
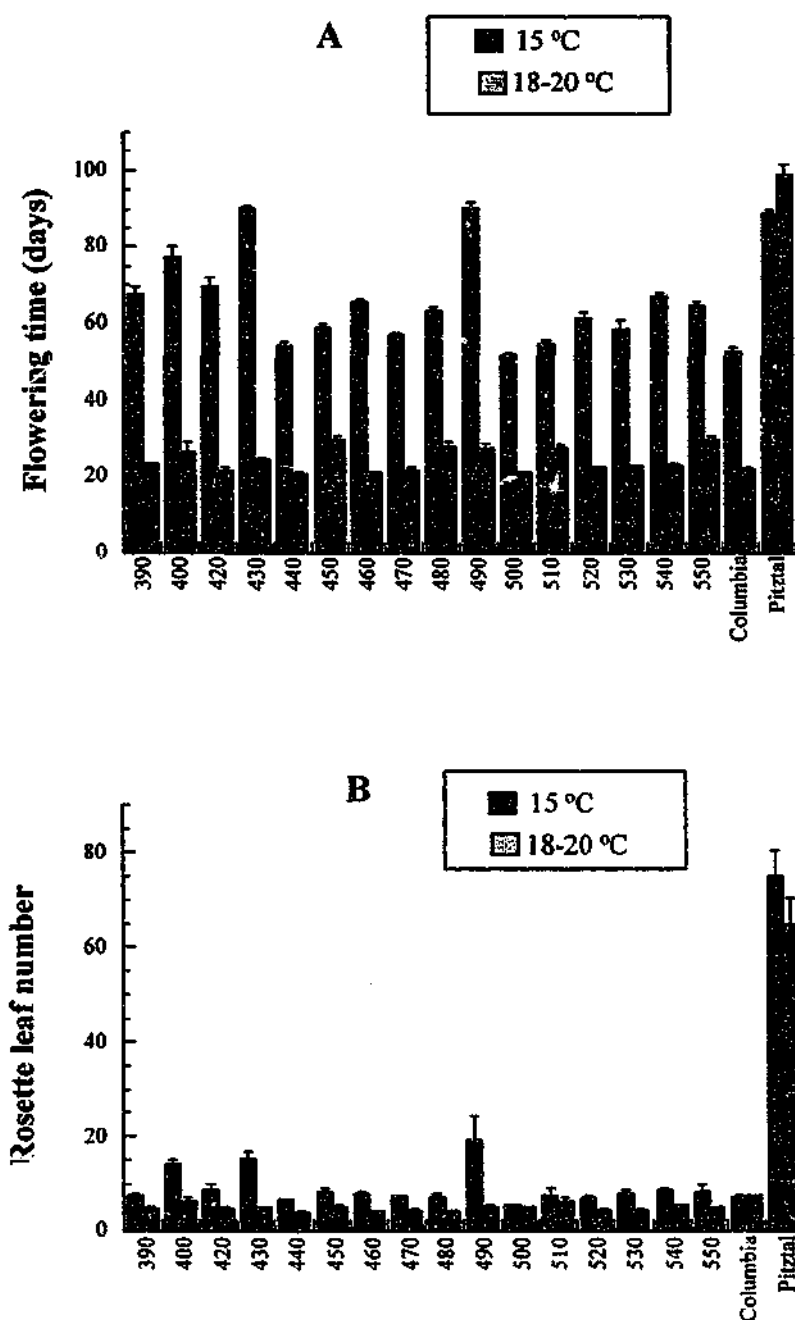
Figure 3.4

The average flowering time (A) and rosette leaf number at the time of flowering (B), of the EMS-induced mutant lines and wild-type Columbia and Pitztal controls was recorded in both greenhouse conditions (18-20 °C) and when grown at 15 °C. The EMS lines 400, 430, and 490 represent putative, temperature-sensitive, early-flowering mutants.

Gamma radiation-induced mutants

In order to eliminate the possibility of any contaminating seed in the M2 generation the Pitztal background of the putative radiation-induced mutants was examined. A Southern blot containing DNA extracted from the 12 original M3 lines, as well as from plants of the wild-type Pitztal, Columbia and L. *erecta* ecotypes, was hybridised with the RFLP marker, λ220, that maps to chromosome two. Bands specific to wild-type Pitztal DNA were detected in nine of the original putative mutant lines (220, 230, 240, 250, 260, 270, 280, 290, and 300), confirming a Pitztal background for these lines (Figure 3.5). DNA from mutant line 180 however, appeared to contain bands specific to the L. *erecta* ecotype, whereas bands specific to wild-type Columbia were detected in the 190 and 200 mutant lines. Lines 180, 190, and 200 were therefore not used in any further experiments. The remaining radiation-induced early-flowering mutant lines, which are now thought to be allelic, were subsequently termed *fler* (*floral late ecotype repressor*) mutants, as it is believed they contain a disruption in a locus mediating a repression of flowering within late-flowering ecotypes.

Figure 3.5

*Eco*RI digested DNA extracted from the original M3 radiation-induced mutant lines, wild-type Pitztal, Columbia and L. *erecta* was hybridised with RFLP marker (λ220) that maps to chromosome 2. Band 1 appeared to be specific to the L. *erecta* ecotype, bands 2 and 3 to the Columbia ecotype, and band 4 to the Pitztal ecotype.

Lane	DNA	Lane	DNA	Lane	DNA
1	Mutant line 180	6	Mutant line 240	11	Mutant line 290
2	Mutant line 190	7	Mutant line 250	12	Mutant line 300
3	Mutant line 200	8	Mutant line 260	13	Wild-type Pitztal
4	Mutant line 220	9	Mutant line 270	14	Wild-type Columbia
5	Mutant line 230	10	Mutant line 280	15	Wild-type L. *erecta*

The morphology of several of the radiation-induced M3 plants, as well as some EMS-induced M3 plants, are shown in Figure 3.6. These selected early-flowering lines were often quite similar in appearance to early-flowering ecotypes, particularly in terms of the number of rosette and cauline leaves produced (Figures 3.6). The maximum bolt height of these lines was more similar however, to that seen in wild-type Pitztal plants, and the radiation-induced lines in particular were able to produce very tall plar*s as displayed by the mutant line 290 in Figure 3.4. Three of the radiation lines (250, 260, and 300) also exhibited a pleiotropic phenotype. The rosette leaves of these plants were pale green in colour and curled downward around the mid-rib. The radiation-induced line 260 displayed the strongest phenotype while leaves of the 250 and 300 lines had less severe changes (Figure 3.6). The severity of these phenotypes however, did appear to lessen in subsequent generations.

General physiological analysis of the radiation-induced fler mutant lines

The two most common measures of the timing of floral induction in *Arabidopsis* are the recording of the time (in days) before a 1 cm bolt is observed, or to count the number of rosette leaves at the time of flowering. These two measures are believed to positively correlate with each other such that plants with large numbers of rosette leaves are also late-flowering. A strong correlation between these two variables has, for example, been recorded in the L. *erecta* ecotype (Koornneef *et al.*, 1991; Martinez-Zapater *et al.*, 1994). In order to establish that such a relationship existed for the radiation-induced *fler* mutant lines, and several wild-type ecotypes, in the present greenhouse conditions, the flowering time and rosette leaf number at the time of flowering, were plotted against each other in scatter plots (Figure 3.7). A strong positive correlation between these factors was noted for all *fler* mutant lines and ecotypes tested. The rosette leaf number at the time of flowering was therefore considered a relatively accurate measure of flowering time for subsequent experiments. This measurement has the advantage over recording flowering time in days, as relationships between the rosette leaf number and flowering of plants is generally unaffected by environmental factors that may result in a temporal delay in growth rate.

The flowering time and rosette leaf number of the nine remaining *fler* mutants was therefore recorded under the conditions to be used for subsequent experiments (Figure 3.8). Whilst all mutant lines flowered much earlier than wild-type Pitztal plants, mutant line 230 flowered slightly later than the other mutants and the early-flowering ecotype controls. The rosette leaf numbers of each line, including line 230, were similar to those of the L. *erecta*, Columbia, and Niederzenz controls however, and much lower than the leaf number of the wild-type Pitztal control. This suggests some delay in the growth rate of mutant line 230 in this experiment.
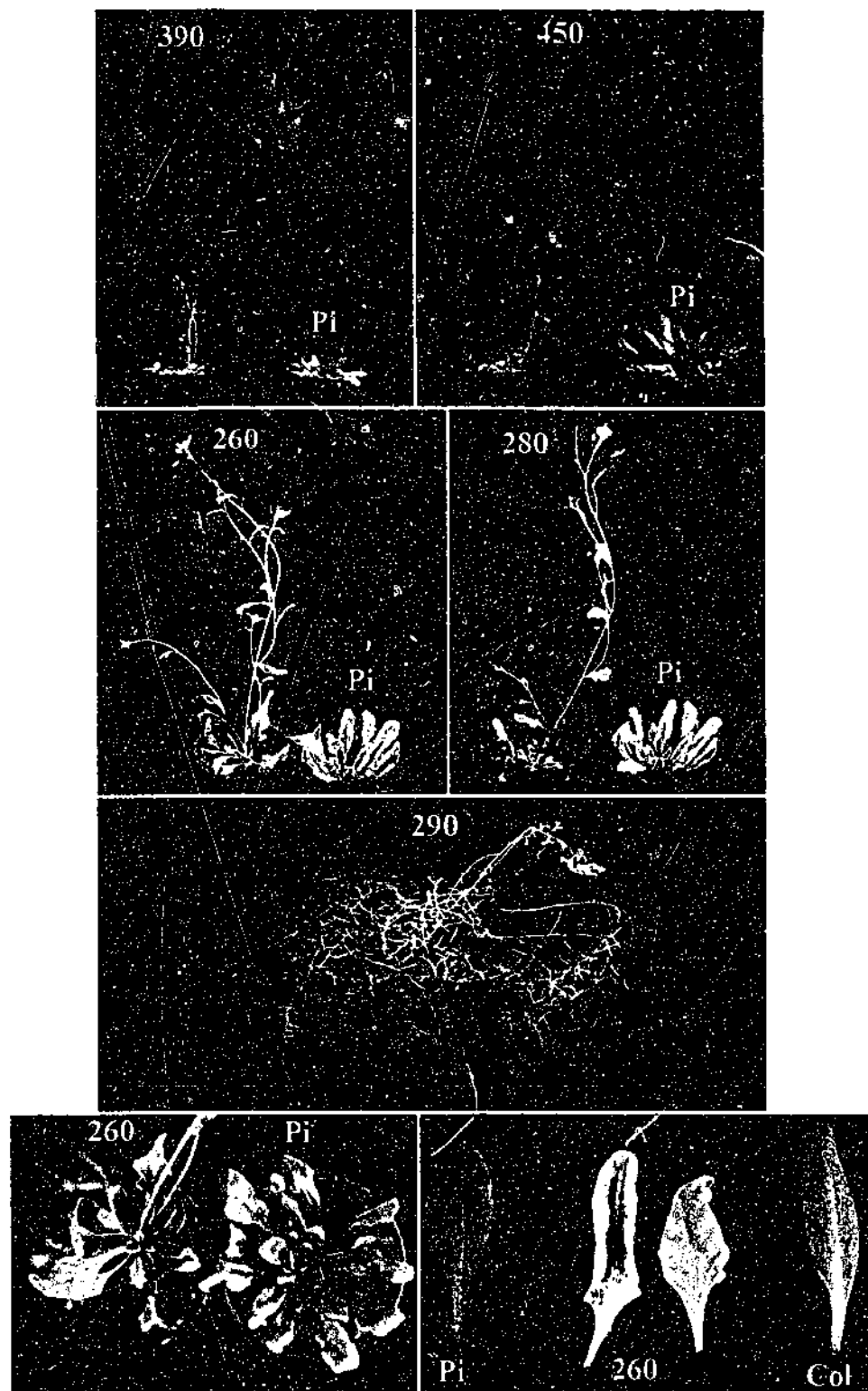
Figure 3.6

The early-flowering phenotypes of the EMS-induced (390 and 450), or radiation-induced (260 and 280), mutant plants compared with wild-type Pitztal (Pi), approximately 30 days following germination (panels 1 to 4). The phenotype of the radiation-induced mutant line 290 at a later stage of development (panel 5). The rosette and individual leaf phenotypes of the 260 mutant line that exhibits the 'curly' phenotype, compared to rosettes of wild-type Pitztal and leaves of wild-type Pitztal and Columbia (Col) plants (panels 6 and 7).
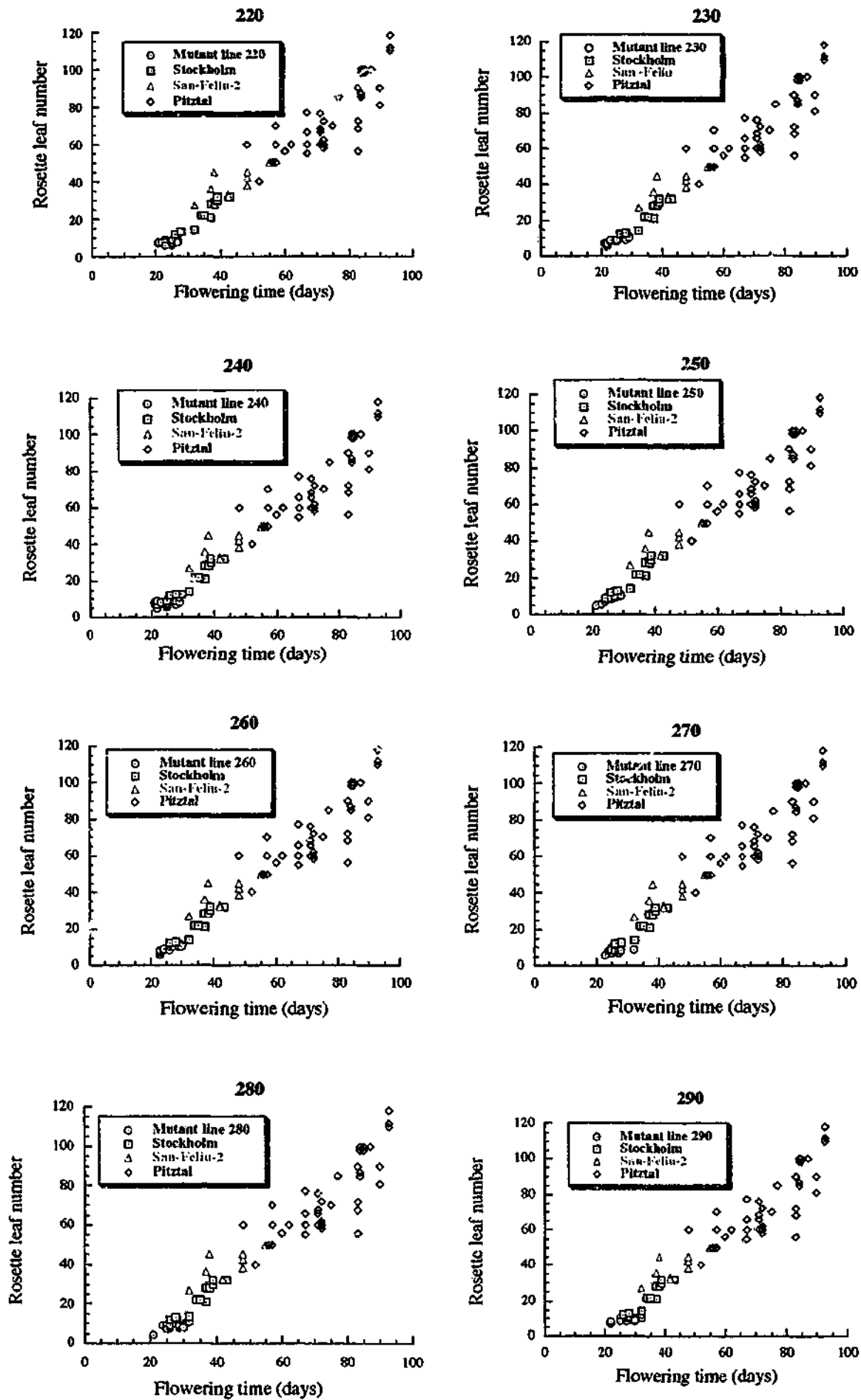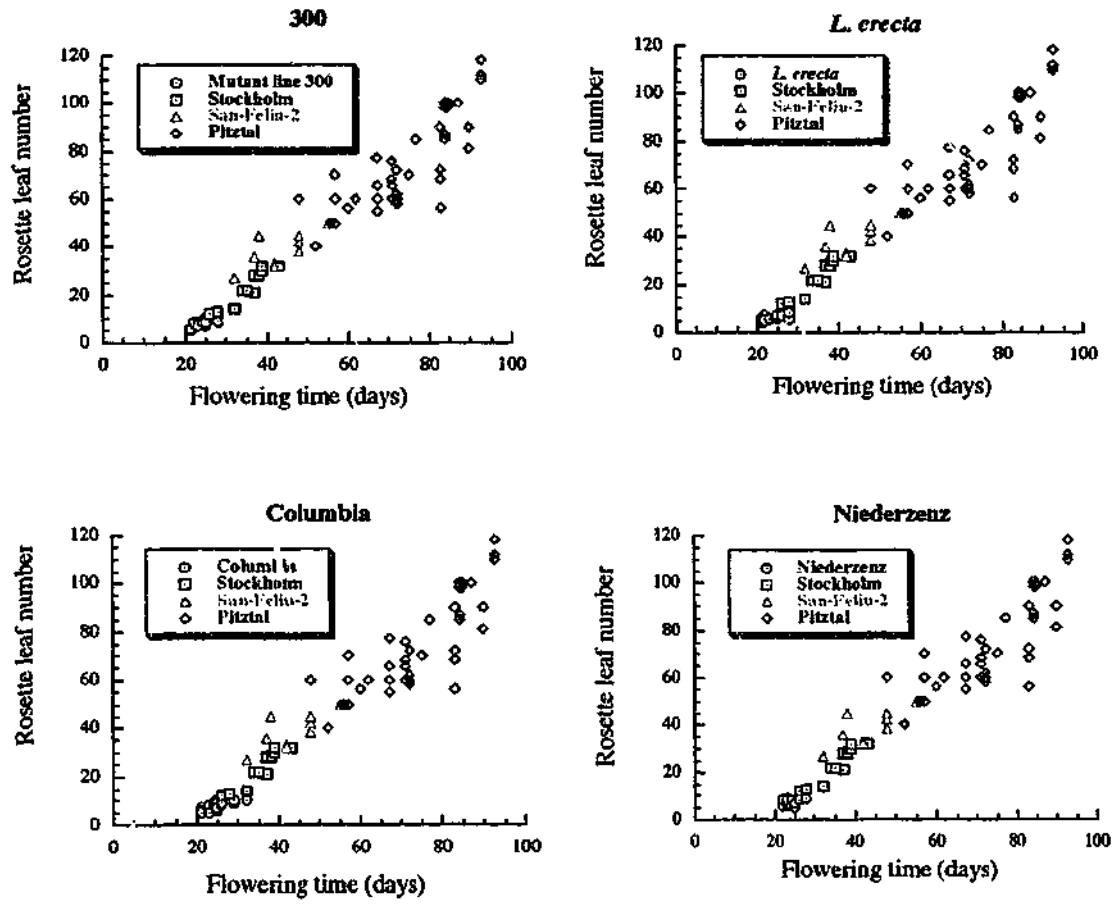
Figure 3.7

The correlation between flowering time and rosette leaf number, at the time of flowering, of mutant and control plants grown in standard greenhouse conditions.

A

Flowering time (days)

220 230 240 250 260 270 280 290 300 *L. erecta* Columbia Niederzenz Pitztal

B

Rosette leaf number

220 230 240 250 260 270 280 290 300 *L. erecta* Columbia Niederzenz Pitztal
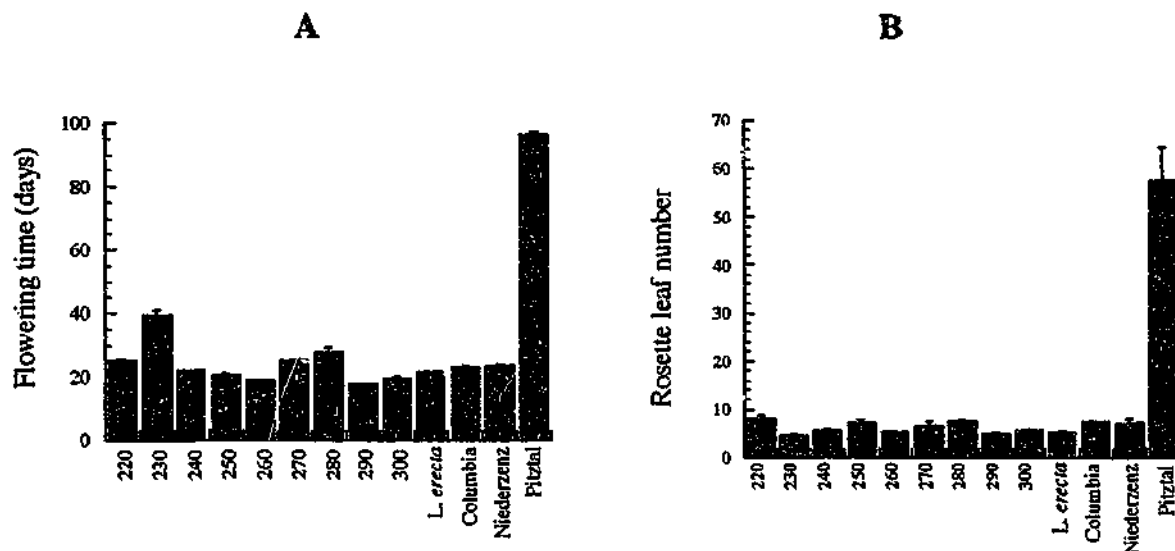
Figure 3.8

The average flowering times (A) and rosette leaf number at the time of flowering (B) of the radiation-induced *fler* mutant lines grown in standard greenhouse conditions, at 18°C to 22°C with continuous light.

Subsequently, the growth rate of all the *fler* mutant lines was examined to determine if their rate of leaf production, or other aspects of growth, were different from that of wild-type Pitztal. Figure 3.9 presents the results of a comparison of the average fresh weight of five individual plants of each mutant line and wild-type controls. Plants were harvested every two days, from day 10 post germination up to day 20 post germination. None of the mutant lines appeared to show a drastic deviation in growth patterns compared to any of the early- or late-flowering ecotype controls. The mutant lines did have slightly more variation in their weights however, although this was generally only seen in plants older than 14 days.

The growth rate of the mutants was also compared to control plants by measuring the rate of leaf production, or the plastochron, of the *fler* mutants and wild-type plants (Figure 3.10). To measure this variable, the number of leaves greater than 3mm in length was recorded daily for each mutant line and control plants, from day 10 post germination up to day 20 post germination. Again, no major differences in this variable were detected between the mutant lines and any of the control lines. These results indicate that there is little, if any, difference in the growth rate of the mutant lines, and that an accelerated growth rate is not a causal factor in the early-flowering phenotype of these plants.

220

Fresh weight (mg) vs Days post germination
Legend: Mutant line 220, Pitzal, San-Feliu-2, *L. erecta*, Columbia

230

Fresh weight (mg) vs Days post germination
Legend: Mutant line 230, Pitzal, San-Feliu-2, *L. erecta*, Columbia

240

Fresh weight (mg) vs Days post germination
Legend: Mutant line 240, Pitzal, San-Feliu-2, *L. erecta*, Columbia

250

Fresh weight (mg) vs Days post germination
Legend: Mutant line 250, Pitzal, San-Feliu-2, *L. erecta*, Columbia

260

Fresh weight (mg) vs Days post germination
Legend: Mutant line 260, Pitzal, San-Feliu-2, *L. erecta*, Columbia

270

Fresh weight (mg) vs Days post germination
Legend: Mutant line 270, Pitzal, San-Feliu-2, *L. erecta*, Columbia

Figure 3.9

The fresh weight (mg) of each *fler* mutant line (220-300), wild-type Pitztal, San-Feliu-2, Columbia, and L. *erecta*, recorded every two days, from day 10 to day 20 post germination.
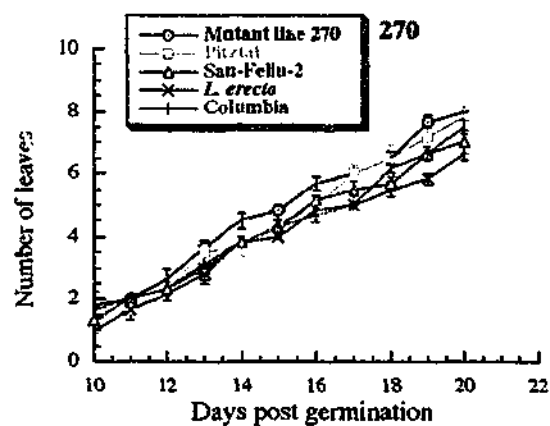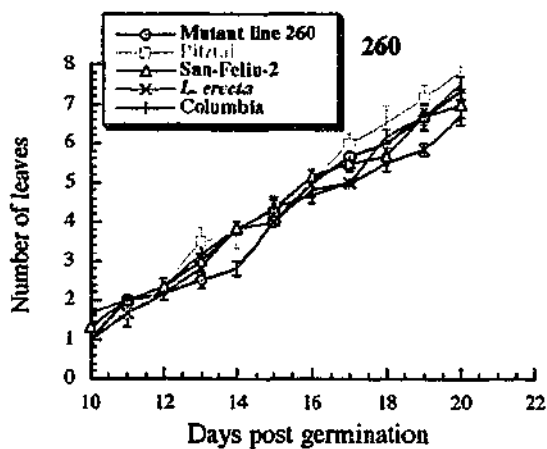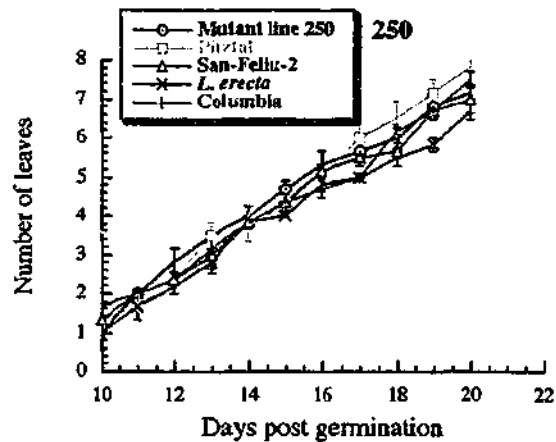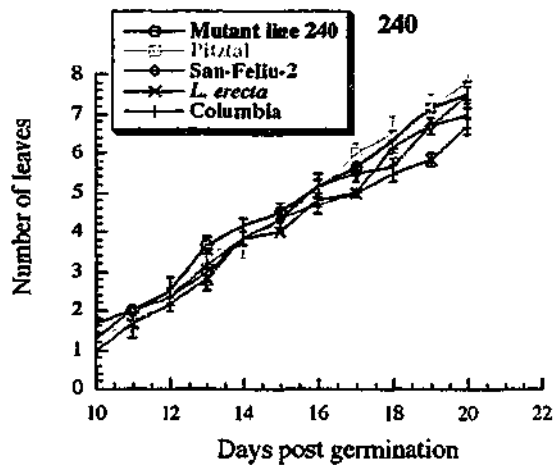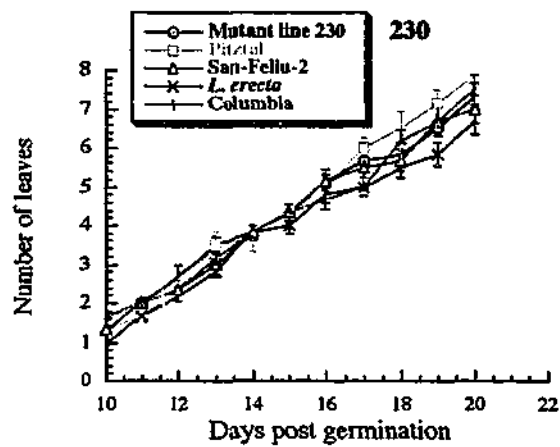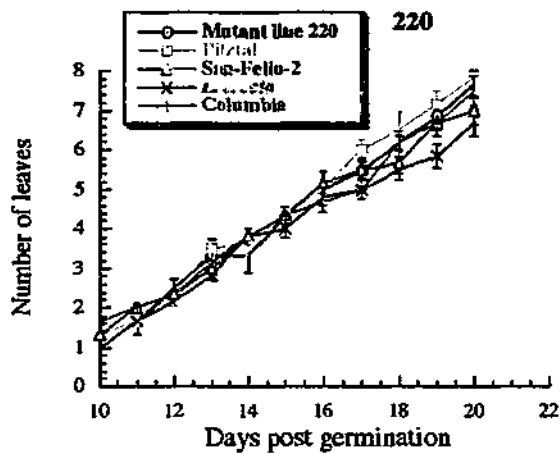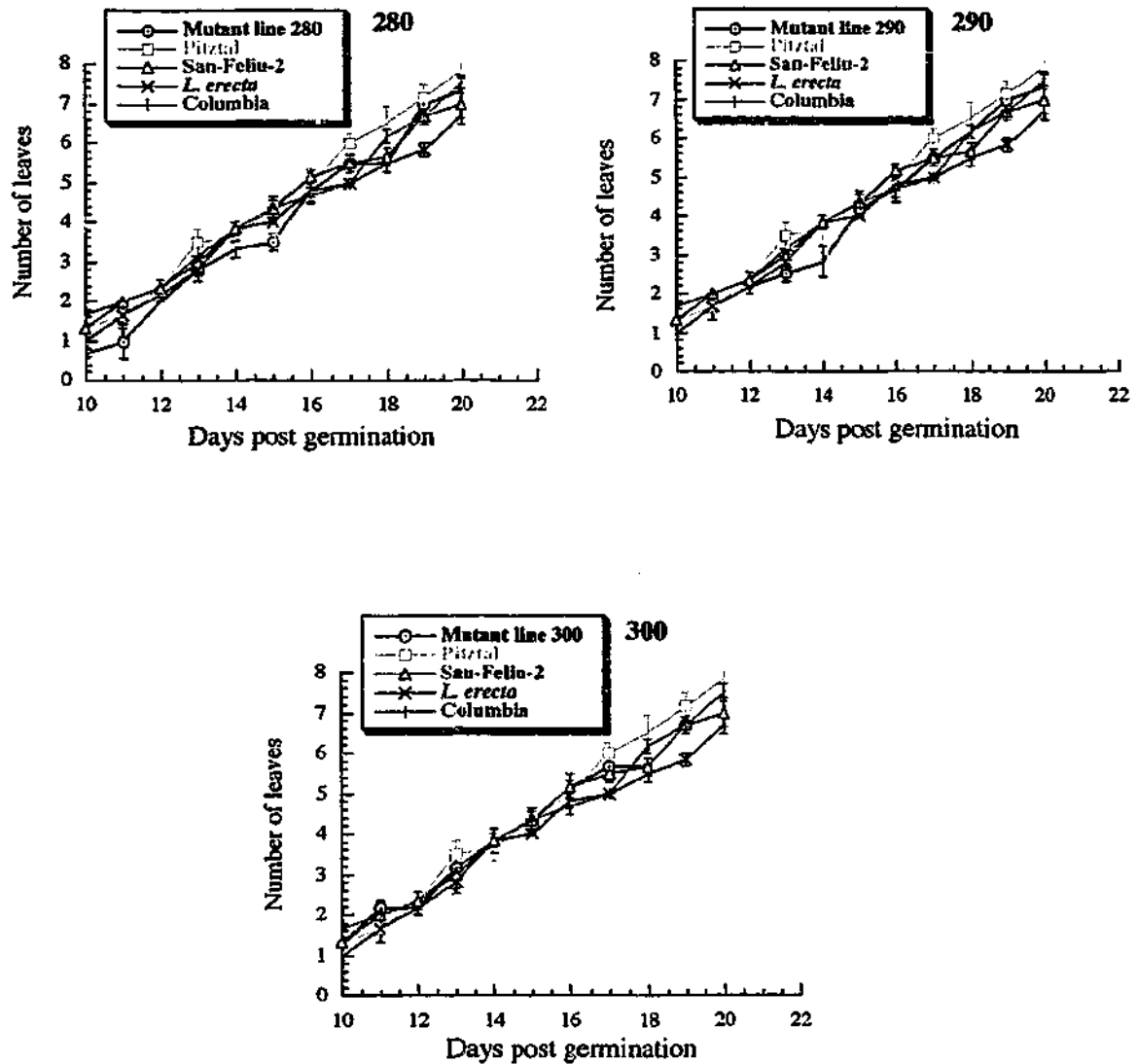
Figure 3.10

The average number of leaves greater than 3mm in length of each *fler* mutant line (220-300), wild-type Pitztal, San-Feliu-2, Columbia, and L. *erecta*, recorded every day, from day 10 to day 20 post germination.

Morphological descriptions of the radiation-induced fler mutant lines

The growth pattern of the *fler* mutants and wild-type controls was also visually recorded from day 14 post-germination up to day 45 post-germination. At day 14 most of the mutant lines were similar in appearance to wild-type Pitztal (Figure 3.11). Two of the mutant lines, 220 and 230, however possessed leaf hairs, a phenotype that also appears occasionally in wild-type Pitztal populations, and is present in the San-Feliu-2, L. *erecta*, Columbia, and Niederzenz lines. The remaining mutant lines were glabrous like the majority of wild-type Pitztal plants. Line 260 displayed its characteristic pale green colour and a slight serration of the leaves, even at this early stage of development. Line 300 was also slightly pale green in colour. At this stage the 220 and 280 *fler*, mutant lines showed the more compact rosette structure, which may be due to shorter leaf petioles, commonly observed in these lines. Mutant line 240 was slightly delayed in its development at this time, however this phenotype did not persist in subsequent generations.

At day 21 post-germination, floral buds and small bolts were evident in several *fler* mutant lines (230, 250, 270, and 300) and the wild-type L. *erecta* and Niederzenz plants (Figure 3.12). Only leaf primordia could be visually detected at the apex of the wild-type Pitztal and San-Feliu-2 lines at this time. The serration of the leaf margins of the 260 *fler* mutant line was also becoming more evident at this stage of development, and some leaf epinasty was visible. While the rosettes of mutant lines 220 and 280 remained quite compact, those of several other mutant lines were becoming quite large. Mutant line 300 was also found to occasionally possess larger leaves than the wild-type Pitztal control as can be seen in this example (Figure 3.12).

By day 28 post-germination, all of the mutant lines and the wild-type, early-flowering ecotype controls had bolted to varying degrees (Figure 3.13). The late-flowering wild-type Pitztal and San-Feliu-2 plants however remained vegetative. No gross differences between the structure of the bolts of the mutant lines, and those of the early-flowering ecotype plants was seen, and in all lines an indeterminate inflorescence meristem was created. The curling of the leaves in mutant line 260 was now obvious. At a later stage of development, day 33, several of the mutant lines (230, 250, 270, 280, 290, and 300) had produced secondary lateral meristems in the axils of their rosette leaves, giving rise to more leaves near the base of these plants (Figure 3.14). As expected, the wild-type Pitztal and San-Feliu-2 ecotypes had not begun to produce any reproductive structures at this stage. The leaves of all mutant lines had now also begun to display epinasty, a phenotype similar to that seen in wild-type Pitztal plants at the later stages of development.
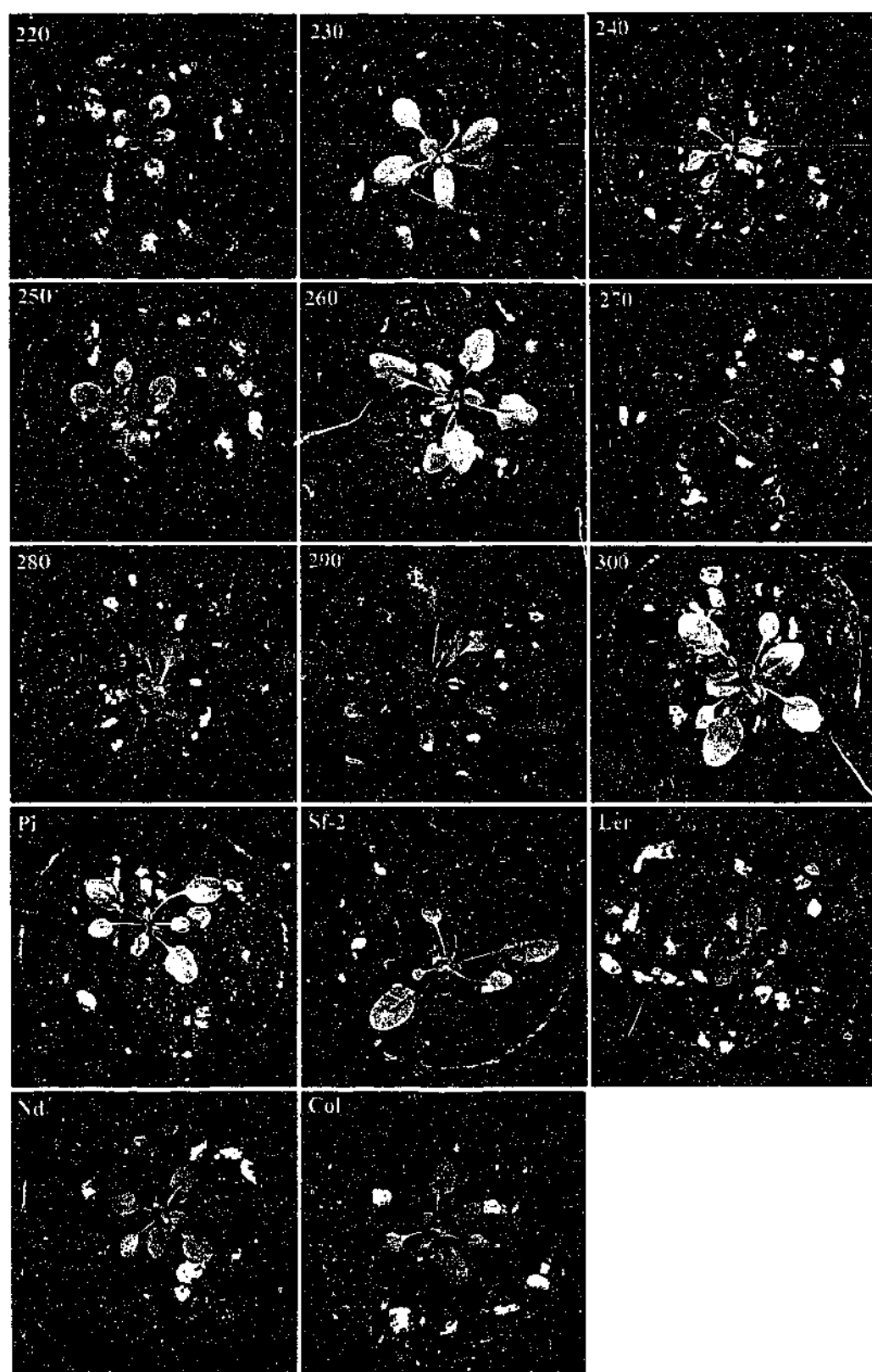
Figure 3.11

The growth pattern of the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col), 14 day post-germination.
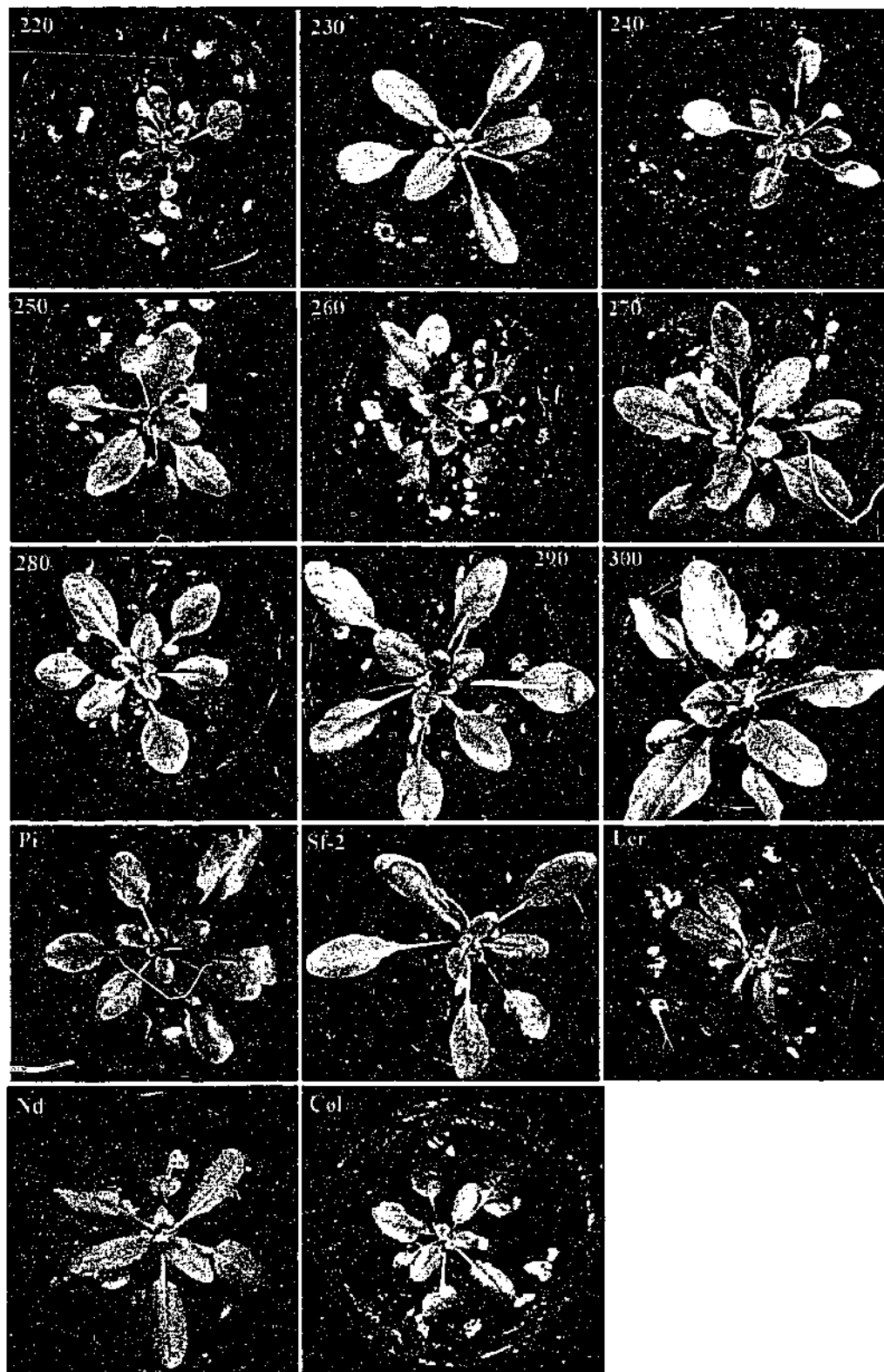
Figure 3.12

The growth pattern of the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col), 21 day post-germination.

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Figure 3.13

The growth pattern of the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col), 28 day post-germination.

Figure 3.14

The growth pattern of the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col), 33 day post-germination.

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At day 45 post-germination, Pitztal and San-Feliu-2 plants still remained vegetative, while all *fler* mutant lines, and early-flowering ecotype controls, had produced numerous flowers and siliques and had started to undergo senescence (Figure 3.15). The curled rosette leaves in the wild-type Pitztal control plants was quite evident at this stage of development. The San-Feliu-2 and Pitztal control plants took approximately 60 and 80 days respectively to produce any bolts (Figure 3.16), by which stage all mutant plants had fully senesced and set seed. It was also noted that the late-flowering control plants had many more secondary lateral bolts than the mutant lines or early-flowering ecotype controls.

To determine if any homeotic transformations had occurred within the flowers of the early-flowering mutant lines, a visual record of the floral structures was undertaken. Figure 3.17 illustrates the presence of the correct number of all floral organs within the flowers of all the *fler* mutant lines, although occasionally fewer numbers of a stamens were observed in the flowers of some lines. In addition, no obvious alterations in the structure or organisation of the floral structures within the flowers was noted. Furthermore, the size of the flowers were similar to that of the wild-type Pitztal controls, whereas L.*erecta* flowers were consistently smaller that those of other wild-type ecotypes, a phenotype characteristic of this line (Figure 3.18). The size of all structures contained within the mutant flowers (sepals, petals, stamens, and gynoecium) were also found to be of a similar size to those of the Pitztal plants. The glabrous sepals of wild-type Pitztal and all the mutant lines, except those of lines 220 and 230, can be clearly seen.

The seeds from all the radiation-induced mutant lines were also examined and compared to those of wild-type Pitztal, Columbia and L. *erecta* plants (Figure 3.19). All seeds from the mutant lines were similar in size to those of wild-type Pitztal, and slightly larger than those of the Columbia and L.*erecta* plants. There was however a rather high percentage of non-viable seeds, characterised by their dark and shrivelled appearance, detected in the seed population of two of the *fler* mutant lines (250 and 270) (Table 3.3). This seed phenotype became less severe in subsequent generations of these mutant lines, particularly for line 250. It is unclear if these non-viable seed were a consequnce of multiple mutations present in such lines or were directly related to the mutation causal to the early flowering phenotype of the mutants.
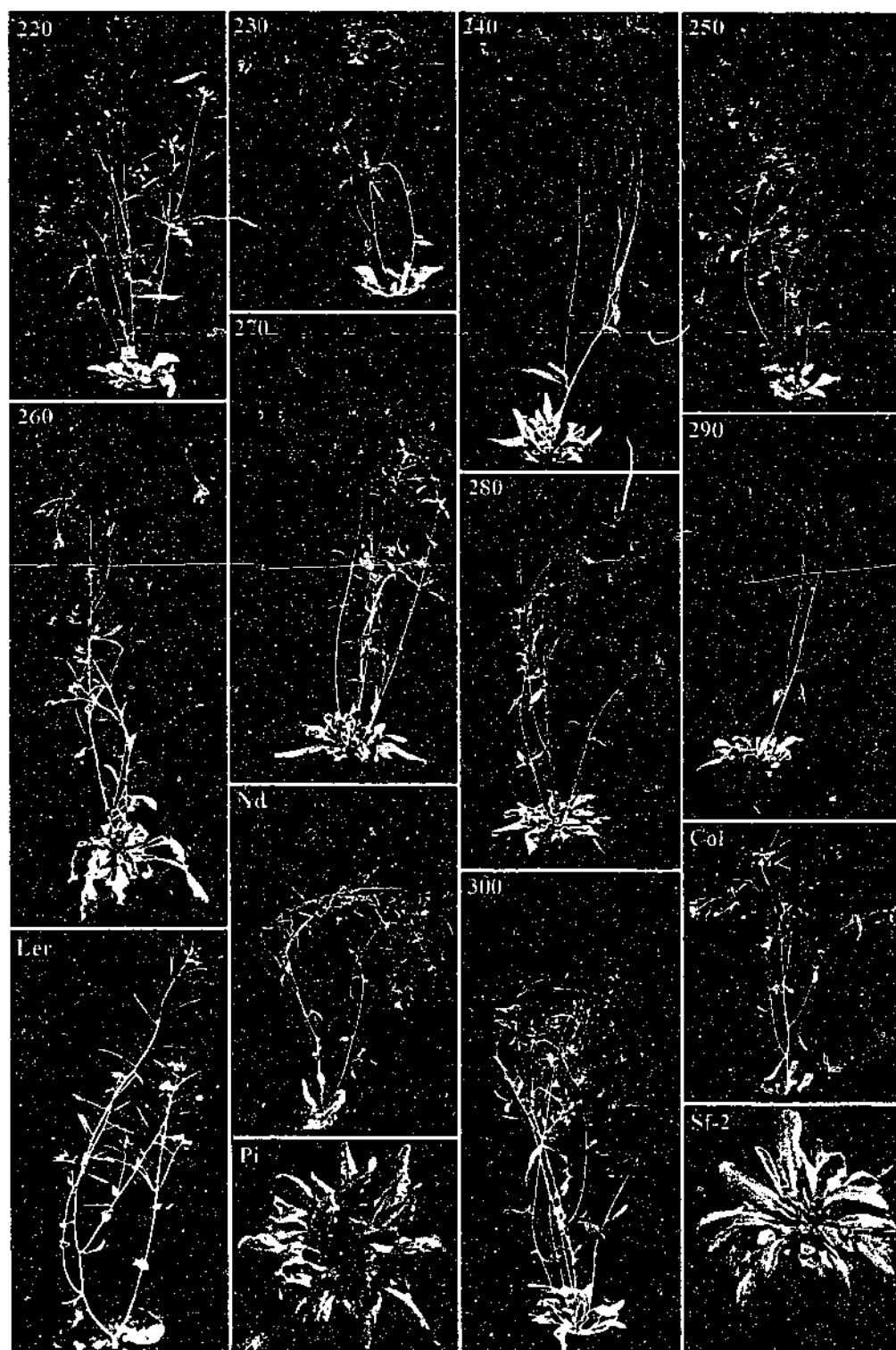
Figure 3.15

The growth pattern of the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col), 45 day post-germination.
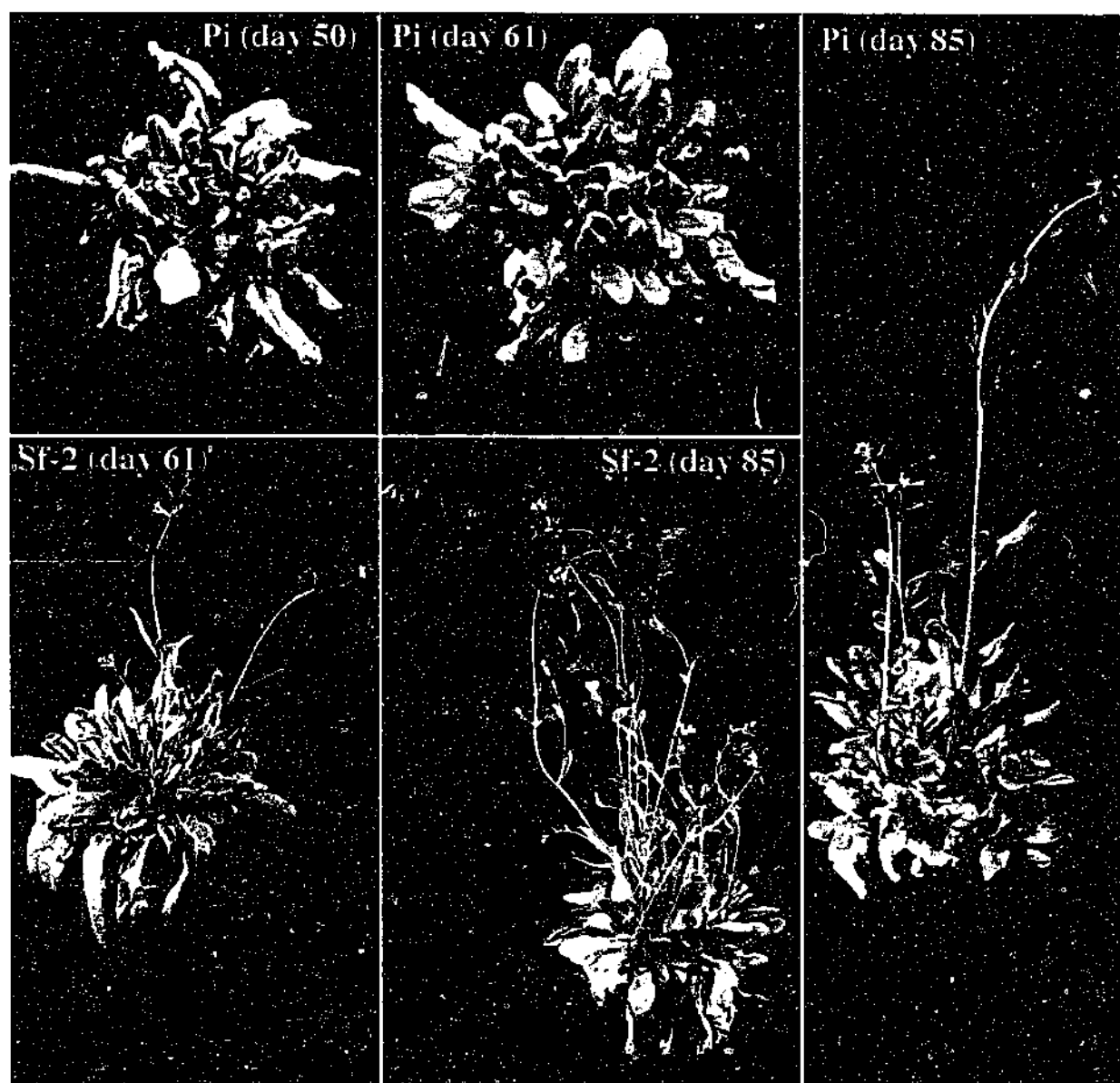
Figure 3.16

The growth pattern of the wild-type Pitztal (Pi) plants at day 50, 61, or day 85 post-germination, and wild-type San-Feliu-2 (Sf-2) plants at day 61 and day 85 post-germination.
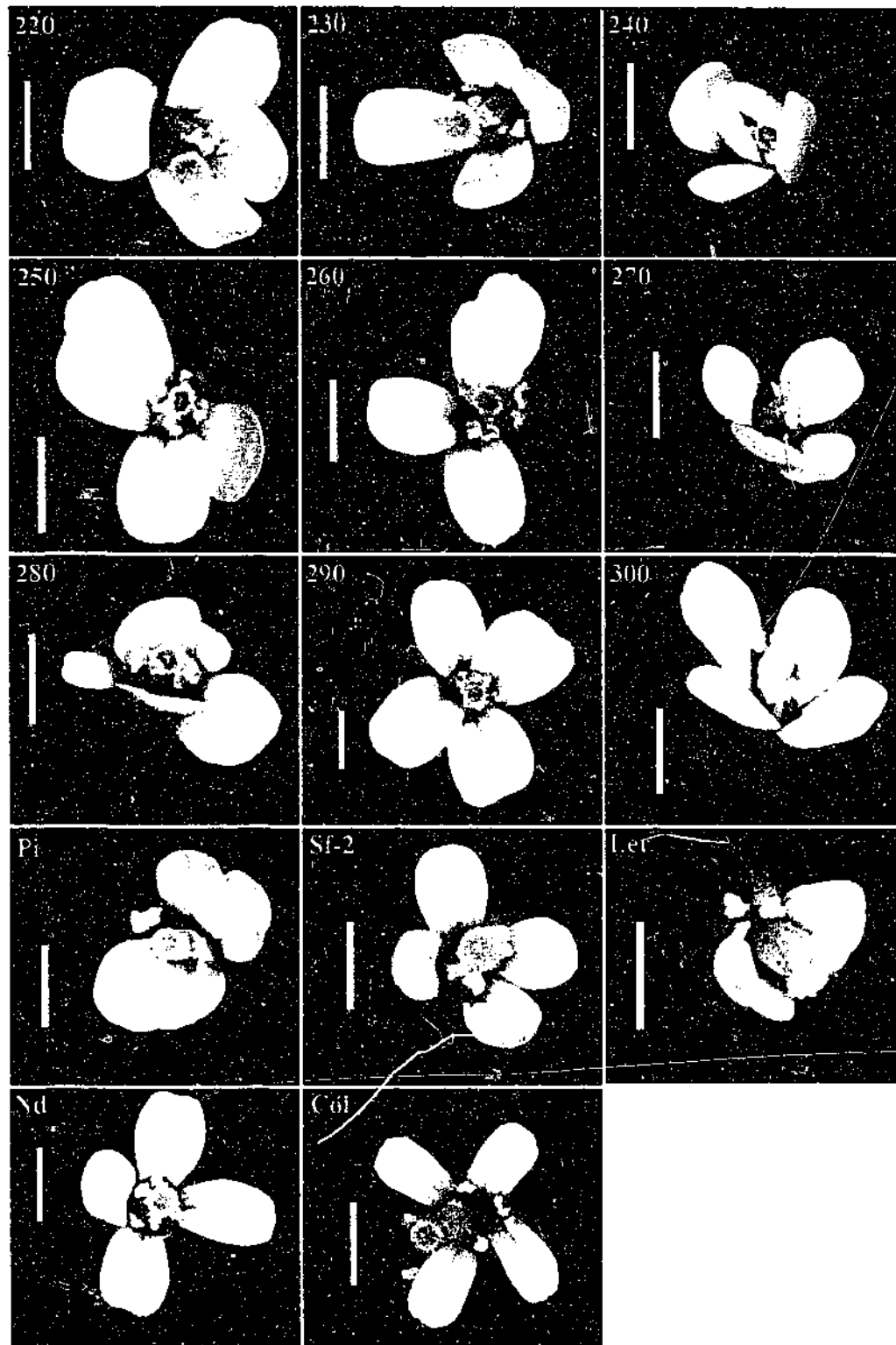
Figure 3.17

A top view of the floral structures from the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col). The bar represents 1mm.
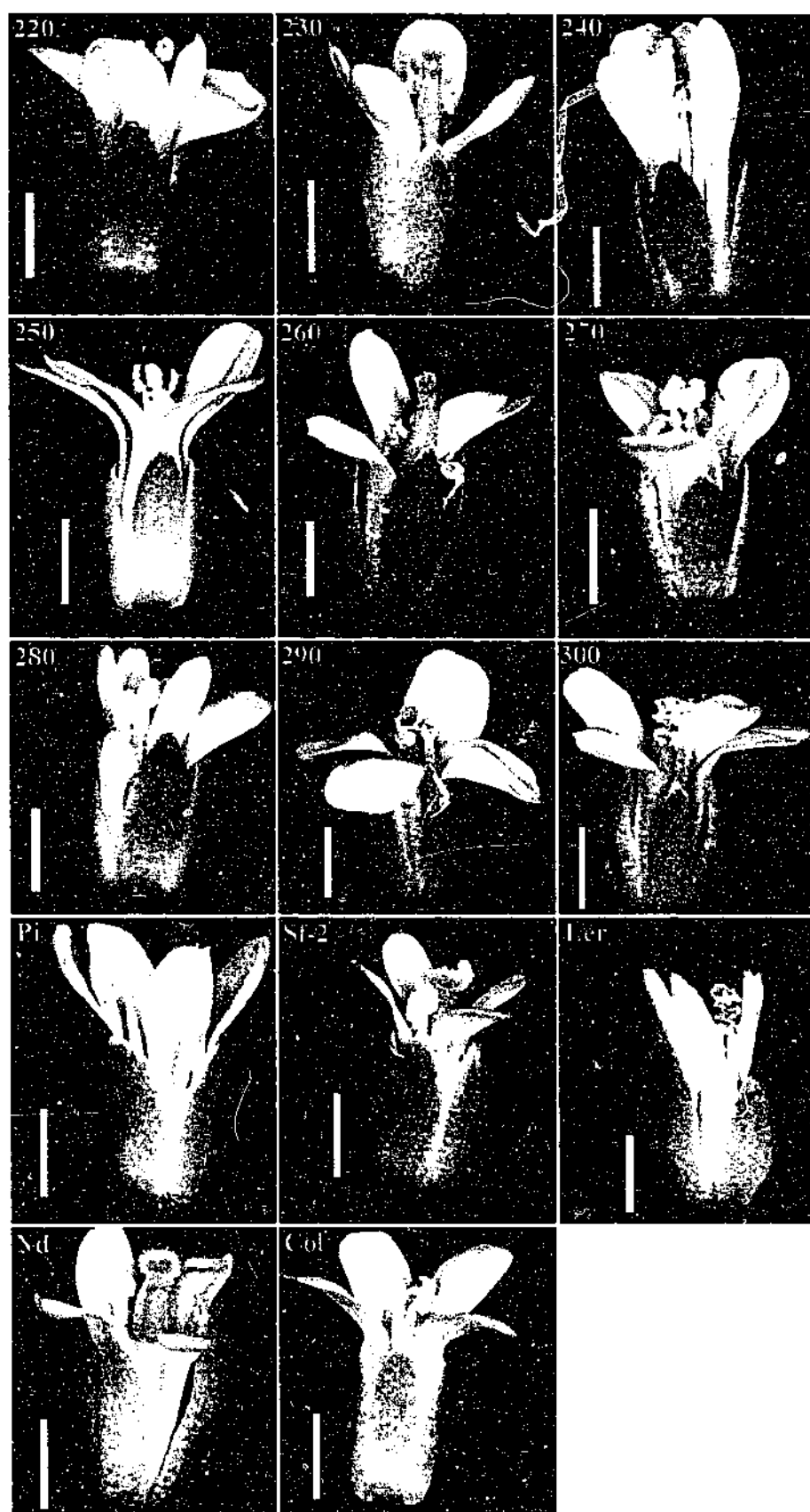
Figure 3.18

A side view of the floral structures from the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), San-Feliu-2 (Sf-2), L. *erecta* (Ler), Niederzenz (Nd), and Columbia (Col). The bar represents 1mm.
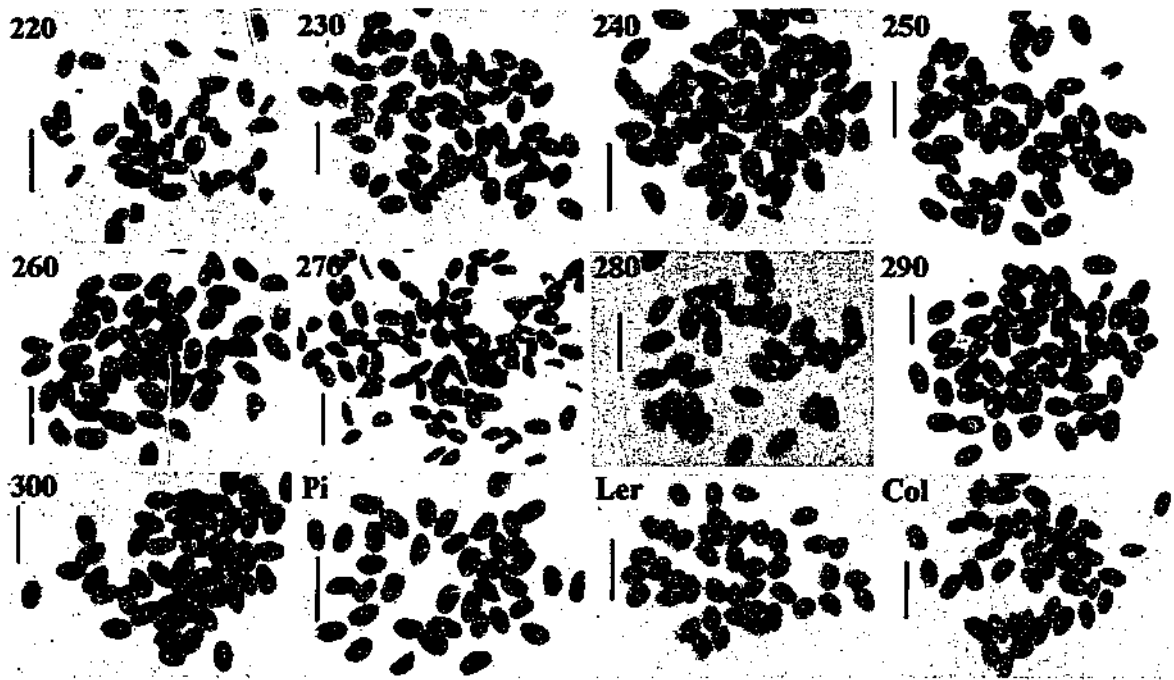
Figure 3.19

The seed phenotype of the nine radiation-induced *fler* mutant lines (220-300), wild-type Pitztal (Pi), L. *erecta* (Ler), and Columbia (Col). The length of the bar in each image represents 1 mm.

Table 3.3

Plant line	Number of seeds	Percentage of non-viable seed
Mutant line 220	109	11.9
Mutant line 230	100	0
Mutant line 240	86	2.6
Mutant line 250	80	34.3
Mutant line 260	93	4.8
Mutant line 270	137	64.2
Mutant line 280	101	0
Mutant line 290	89	2.5
Mutant line 300	95	1.4
Wild-type Pitztal	100	0
Wild-type L.*erecta*	100	0
Wild-type Columbia	100	0

Segregation analysis of the early-flowering mutant phenotype in crosses of the fler lines with wild-type Pitztal

Figure 3.20 presents the average flowering times, and rosette leaf numbers at the time of flowering, of the F1 progeny from crosses of the nine *fler* mutant lines to wild-type Pitztal. These crosses were initially performed in order to determine the recessive, semi-dominant, or dominant nature of the mutant phenotype. Reciprocal crosses of the 300 *fler* mutant line were also performed to gain more information about the inheritance of the mutant phenotype in this line that was to be used in subsequent gene isolation experiments. The 300 (a) cross, shown in Figure 3.20, represents the cross in which the mutant line 300 was the female parent (similar to the other mutant line crosses) and 300(b) represents the reciprocal cross. The flowering time and leaf number of control crosses of wild-type Pitztal crossed to L. *erecta*, Columbia and Niederzenz were also examined.

All the F1 progeny from crosses of the mutant lines to Pitztal flowered at similar times to that of the wild-type Pitztal controls, and much later than the selfed mutant lines. The rosette leaf number of these progeny was also considerably greater than those of the selfed mutant lines. The average leaf number of several of these F1 plants however, were either lower (Pitztal crossed with 220, 240, 270, 280, 290, 300(a)), or higher (Pitztal crossed with 260), than wild-type Pitztal control plants. These results suggest that while the mutant phenotype is almost fully recessive to the late-flowering Pitztal phenotype, there appears to be other genes segregating in these mutant lines that can influence flowering times. Alternatively, the mutated gene responsible for the early-flowering phenotype in several *fler* lines may be semi-dominant. This appears unlikely however, as no intermediate flowering plants were found in the initial M1 populations of these plants. The slight differences in the average leaf number of the reciprocal crosses of mutant line 300 and wild-type Pitztal may also suggest that some maternally inherited genes may be affecting the flowering time of this mutant line

To establish the numbers of mutated genes that affect flowering time within each mutant line, the F1 from the crosses of the mutants to Pitztal were allowed to self and the flowering time and rosette leaf number of the F2 were analysed. Figures 3.21 presents the distributions of the flowering times of these F2, as well as those of the F2 progeny from the Columbia, L. *erecta*, or Niederzenz control crosses with Pitztal. Figures 3.22 illustrate the rosette leaf number distributions of these same crosses. The pattern of the flowering time and leaf number distributions of all F2 progeny from the mutant line crosses were similar, with approximately one quarter of these plants flowering at similar times to the early-flowering mutant lines. While the rest of the F2 plants exhibited delayed flowering and most flowered as late as wild-type Pitztal controls, there was a significant proportion of intermediate flowering plants in these populations, similar to those seen in the distribution of the Pitztal
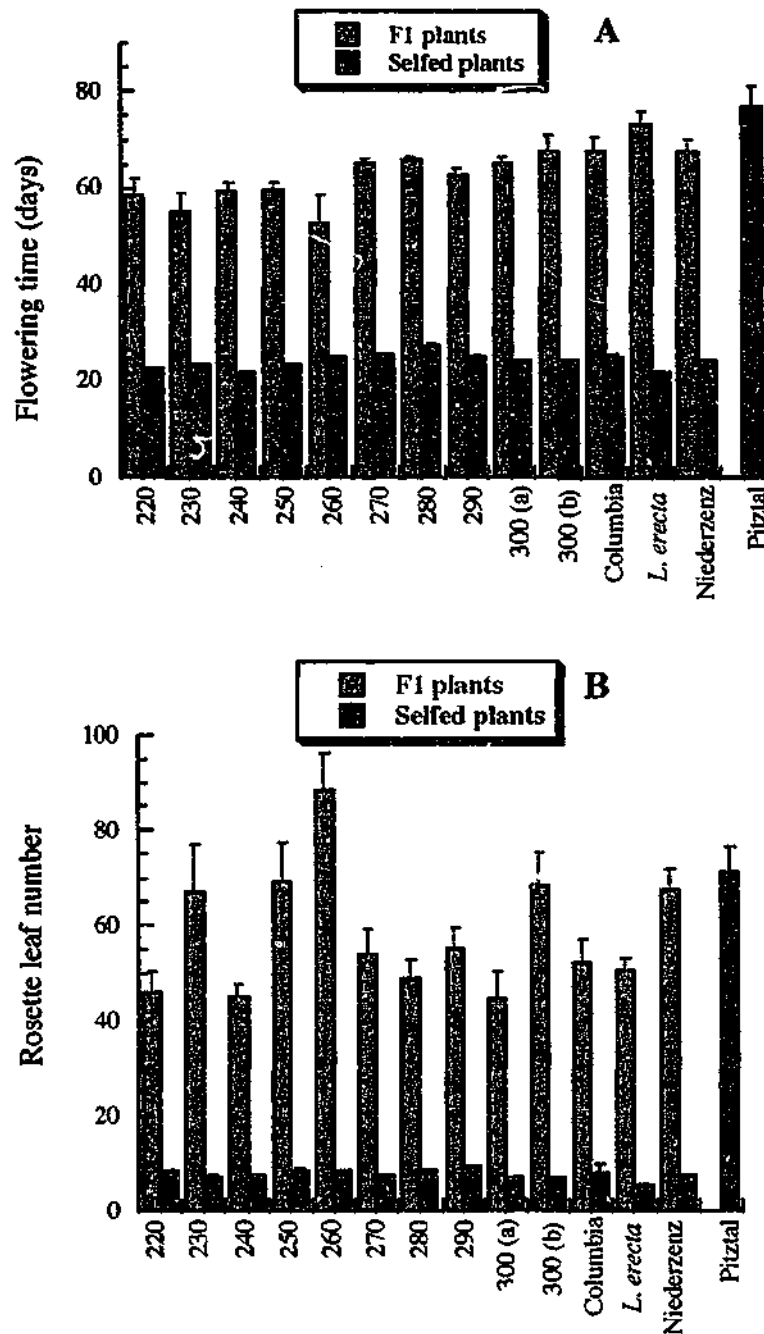
Figure 3.20

The average flowering time (**A**), and the rosette leaf number at the time of flowering (**B**), of the F1 progeny produced from crosses between the *fler* mutant lines, wild-type Columbia, *L. erecta*, and Niederzenz with wild-type Pitztal. The average flowering time, and rosette leaf number at the time of flowering, of the selfed mutant lines, wild-type Pitztal, Columbia, *L. erecta*, Niederzenz were also recorded as controls.

220 X Pitztal (F2)

230 X Pitztal (F2)

240 X Pitztal (F2)

250 X Pitztal (F2)

260 X Pitztal (F2)

270 X Pitztal (F2)

280 X Pitztal (F2)

290 X Pitztal (F2)
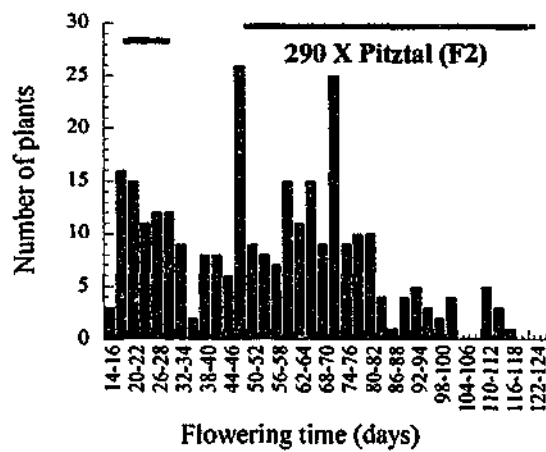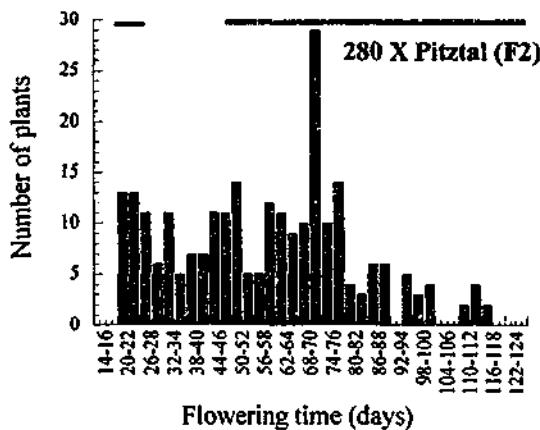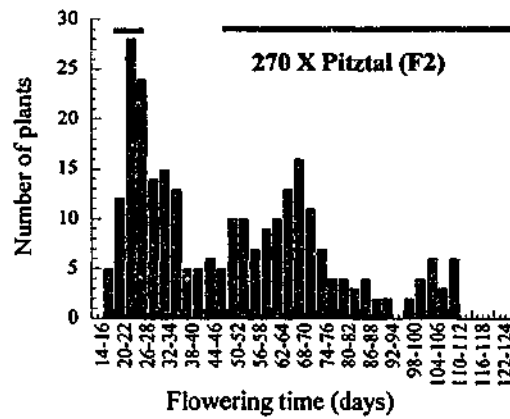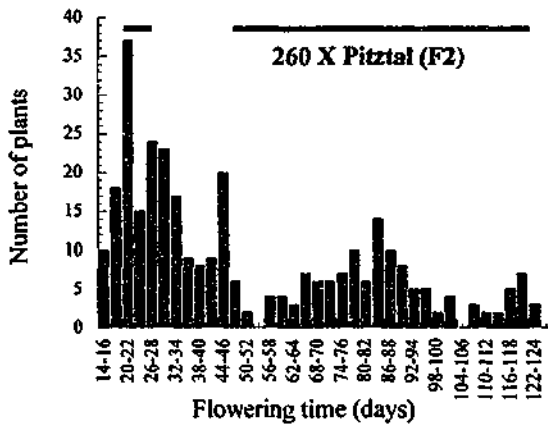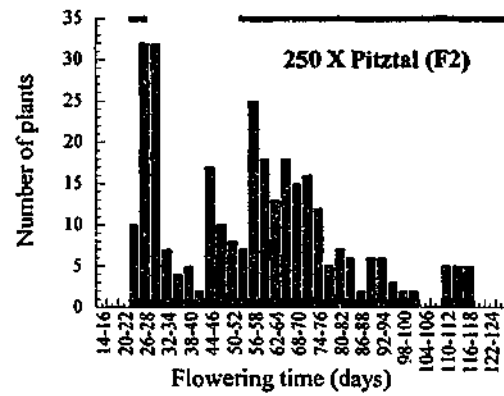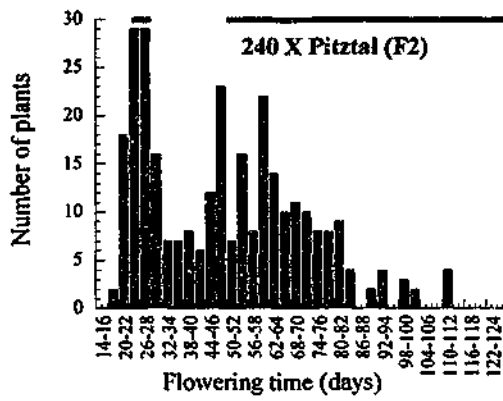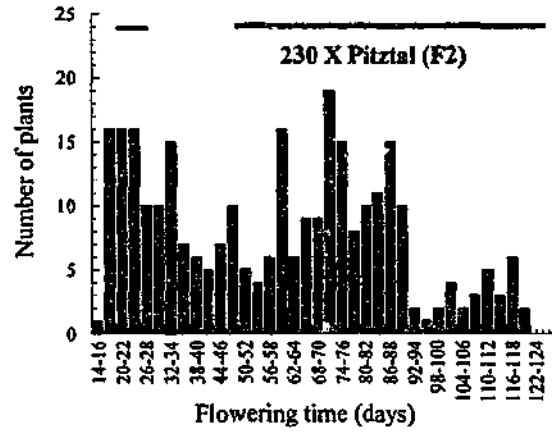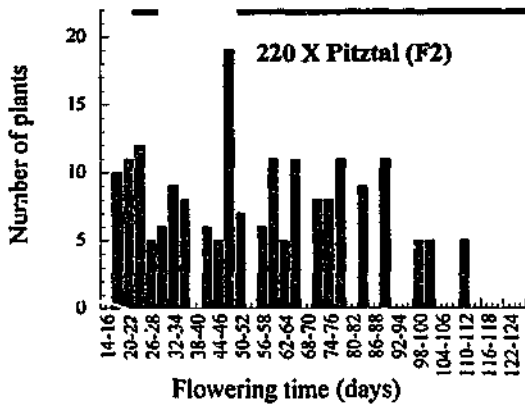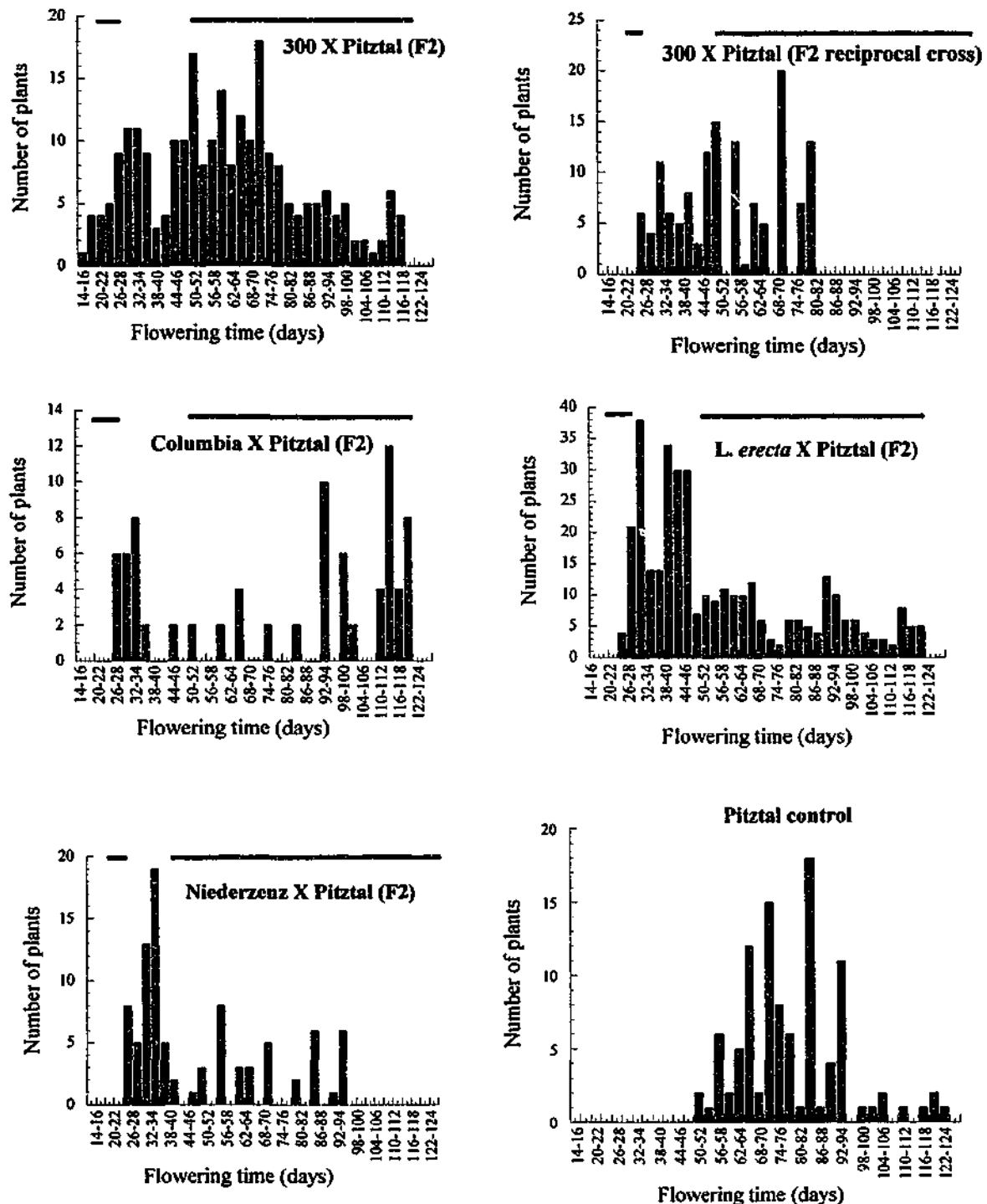
Figure 3.21

The flowering times of the F2 progeny from the *fler* mutants, wild-type L. *erecta*, Columbia, and Niederzenz, crossed with wild-type Pitztal. The red bar on each histogram represents the range of flowering time of wild-type Pitztal control plants, while the black bar represents the range of flowering times of the *fler* mutants, wild-type L. *erecta*, Columbia, or Niederzenz.
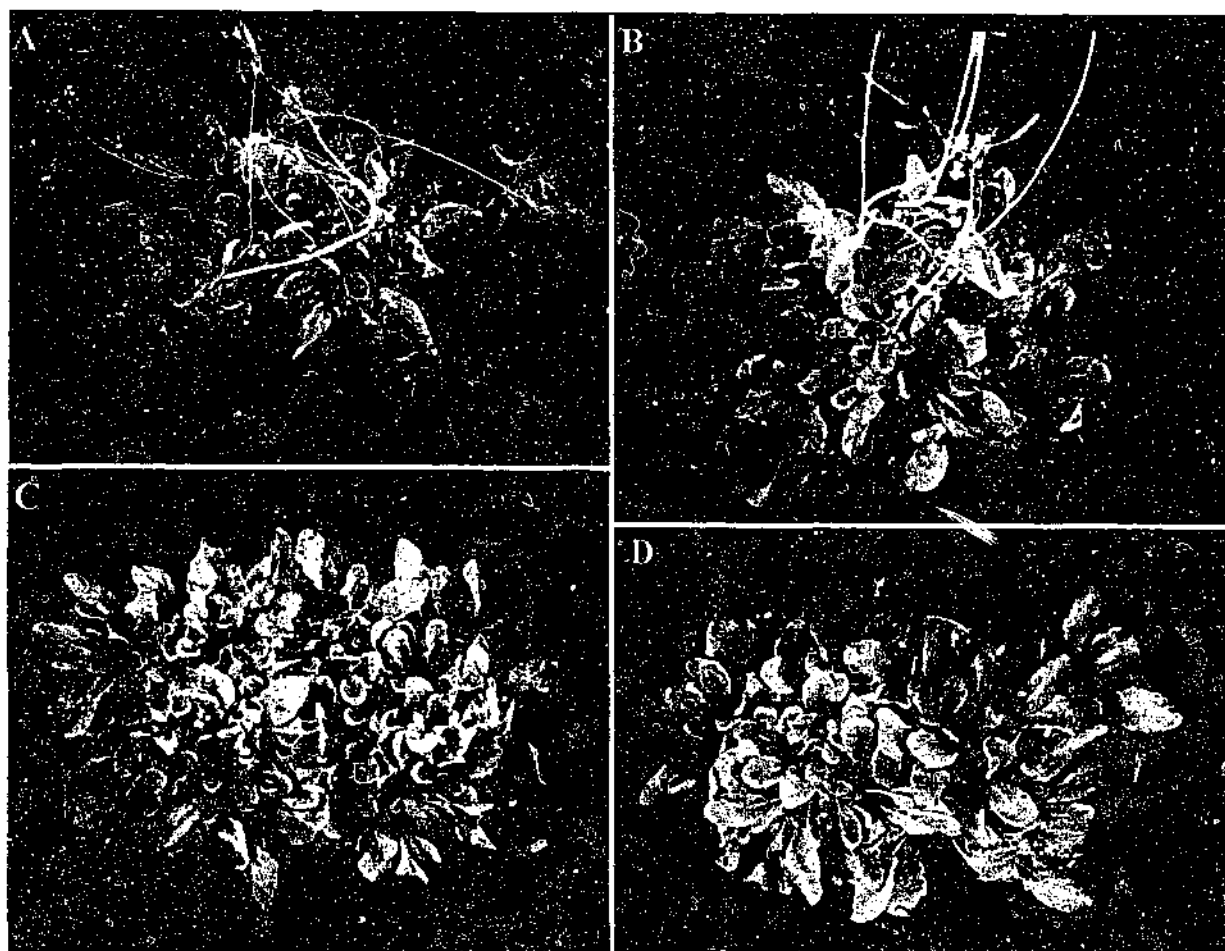
220 X Pitztal (F2)

230 X Pitztal (F2)

240 X Pitztal (F2)

250 X Pitztal (F2)

260 X Pitztal (F2)

270 X Pitztal (F2)

280 X Pitztal (F2)

290 X Pitztal (F2)

126
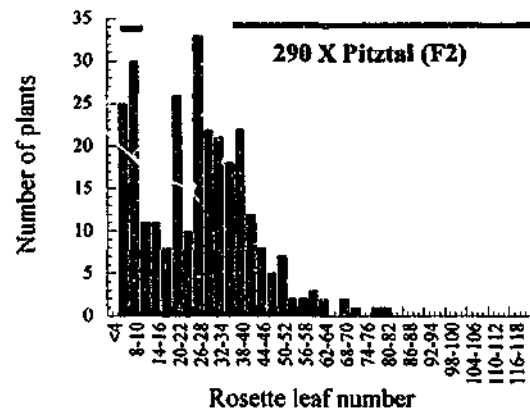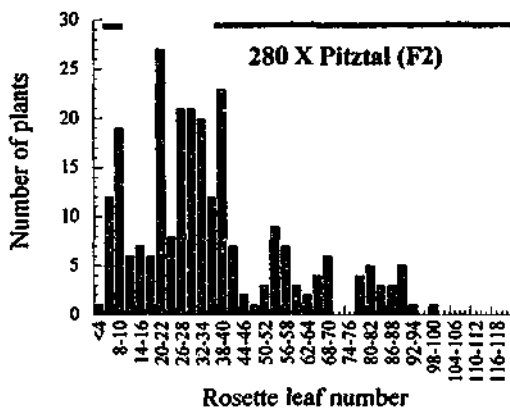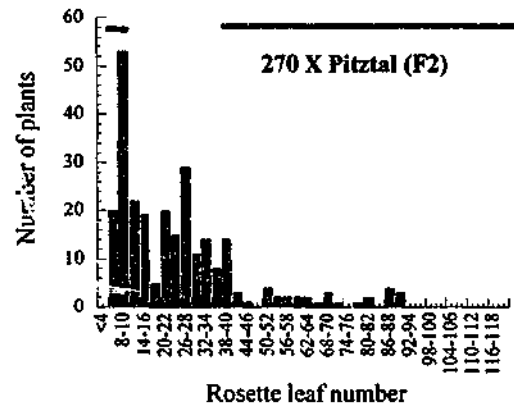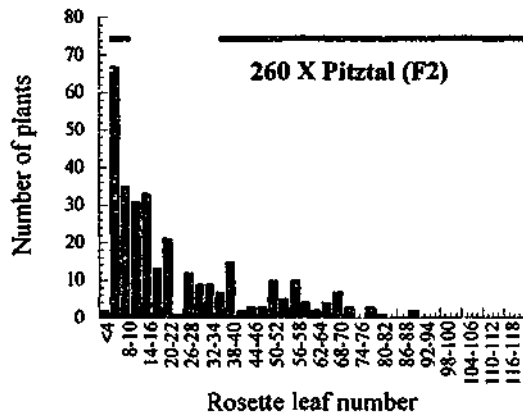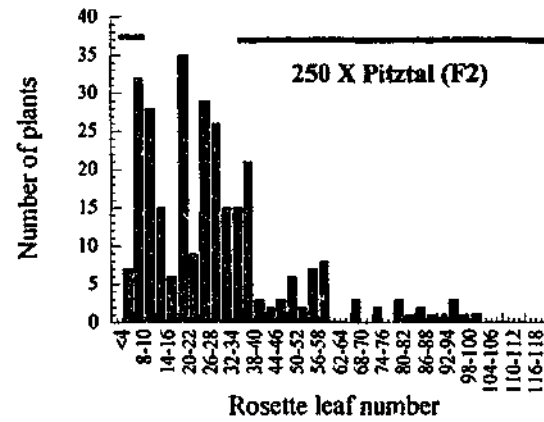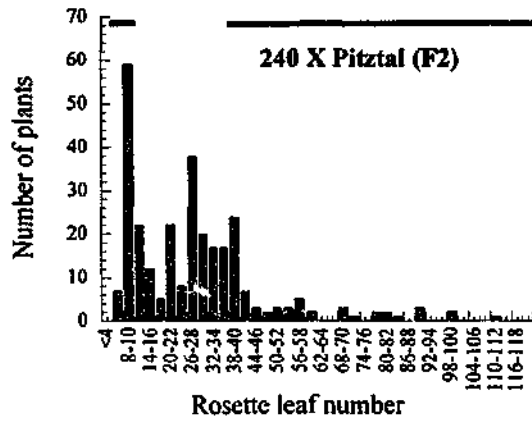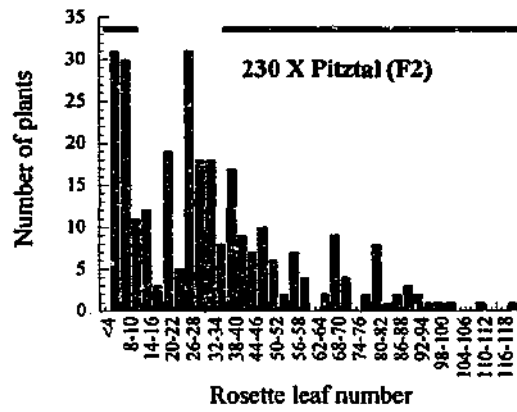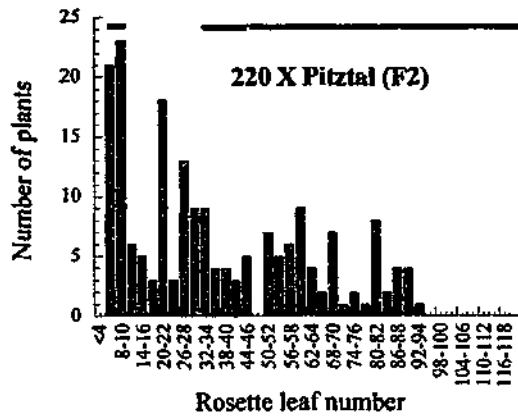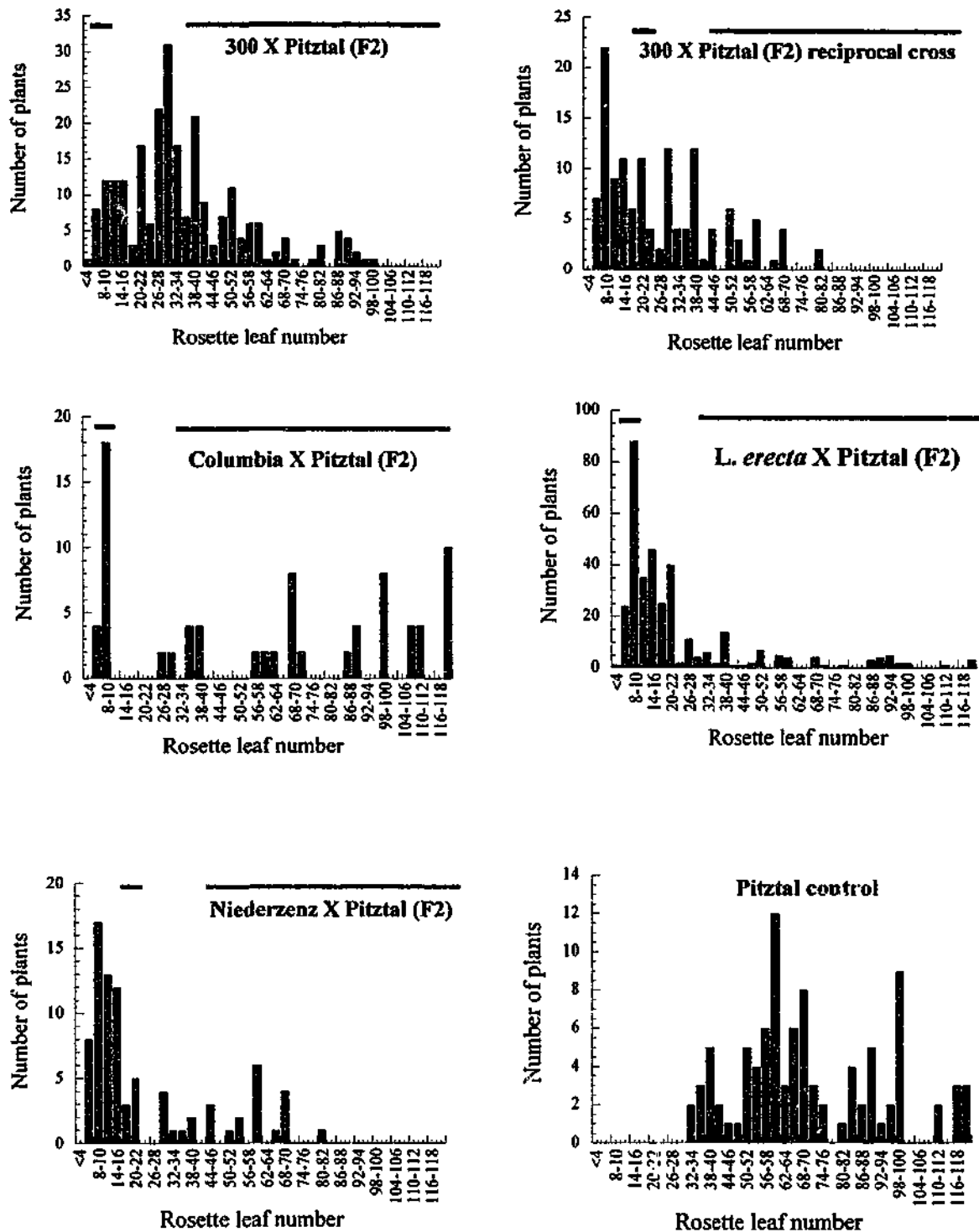
Figure 3.22

The rosette leaf number at the time of flowering of the F2 progeny from the *fler* mutants, wild-type L. *erecta*, Columbia, and Niederzenz, crossed with wild-type Pitztal. The red bar on each histogram represents the range of rosette leaf number at the time of flowering of wild-type Pitztal control plants, while the black bar represents the range of rosette leaf numbers of the *fler* mutants, wild-type L. *erecta*, Columbia, and Niederzenz.

and L. *erecta* cross. Intermediate-flowering plants were considered to be those flowering earlier than any wild-type Pitztal plants and later than any mutant lines (*ie.* between 29 and 50 days or between 12 to 32 rosette leaves). While the F2 populations did not appear to fit an exactly 1:3 ratio of early-flowering to late-flowering plants, due to the presence of intermediate flowering plants that create a continuous distribution, the ratio of early to intermediate to late flowering did also not appear to segregate as a 1:2:1 ratio as would have been expected if a single semi-dominant locus was segregating in these crosses. Chi-squared analysis performed on the flowering times, or rosette leaf numbers, of the F2 population plants appear to confirm these observations as none of the distributions conformed to a 1:2:1 ratio ($p \leq 0.01$). In addition, only four of the twelve F2 flowering distributions (220, 230, Columbia and Niederzenz crossed to Pitztal) fit a 3:1 ratio of delayed-flowering (considered to be plants that flowered later than 28 days and have greater than 12 rosette for the purposes of this analysis) to early-flowering plants ($p \geq 0.05$) as the distributions tended to be skewed toward earlier flowering. The 260 x Pi F2 progeny appeared to deviate the most from either ratio with an almost 1:1:1 ratio of early to intermediate to late flowering plants observed.

Distributions of F2 progeny similar to those found for the crosses of Pitztal to L. *erecta* and Pitztal to the mutant lines have been observed by several other researchers in crosses of L. *erecta* with other late-flowering ecotypes such as Stockholm (Clarke and Dean, 1994). The skewing of these distributions toward earlier flowering is thought to be due to the presence of the L. *erecta* versions of *FLC* which modifies the phenotypes conferred by the segregation of the dominant late-flowering *FRI* locus. The presence of the intermediate-flowering plants in distributions of late-flowering ecotypes that were crossed with L. *erecta*, are therefore thought to be due to the interactions of the late- and early-flowering alleles of *FRI* and *FLC*, as well as the ability of *FRI* and *FLC* alleles individually, being able to confer some degree of lateness (Lee *et al.*, 1993; Burn *et al.*, 1993b; Lee *et al.*, 1994b; Martinez-Zapater *et al.*, 1994). Researchers have however also shown that crosses of late-flowering lines to ecotypes such as Columbia, which contain 'late-flowering' versions of *FLC* can also result in F2 progeny with a large range of flowering times that were again often skewed toward early-flowering. In addition, these distributions appear to be continuous as some plants do not fall into discrete late or early-flowering classes. The authors suggest that segregation of multiple recessive genes having minor effects on flowering time are responsible for this large spread in flowering times (Lee *et al.*, 1993; Burn *et al.*, 1993b). These patterns described also correspond to those observed in the F2 progeny from the radiation-induced mutants crossed to Pitztal. Therefore, it appears that while one major dominant or semi-dominant gene may be responsible for the altered flowering time phenotype of the mutants, the activity of several other minor genes influencing this trait, may be altered following the disruption of the predominant flowering locus in these lines.

Alternatively, the removal of the activity of such a locus may simply allow the minor effects these genes have in influencing flowering time to be more noticeable in the mutant plants.

In order to more definitively determine the presence of a single, major, flowering-time gene affected in the *fler* mutants, the F1 progeny from crosses of the radiation-induced mutants and Pitztal were backcrossed to either wild-type Pitztal or the relevant mutant line. Figure 3.23 shows th flowering time distributions of these backcrosses. The rosette leaf number distributions of these crosses are illustrated in Figure 3.24. It was predicted that all Pitztal backcrosses would produce delayed flowering plants if one or more major dominant or semi-dominant loci had been disrupted in the *fler* mutant lines. In backcrosses to the early-flowering mutant lines an approximate 1:1 segregation of early- to late-flowering times would be expected if a single dominant loci had been mutated in the *fler* lines.

While chi-squared analysis confirmed that all backcrosses to the mutant lines did segregate in a 1:1 delayed flowering (again taken as plants that flowered later than 28 days and have greater than 12 rosette leaves for the purposes of this analysis) to early-flowering ratio, intermediate-flowering plants were also observed in both types of backcrosses. In addition, the distributions of these progeny were invariably skewed toward earlier flowering. The majority of the Pitztal backcrosses (excluding the 240 and 250 backcrosses) for example, had plants that flowered earlier than any observed wild-type Pitztal. Furthermore, the majority of plants from the Pitztal backcrosses flowered at times similar, or only slightly later, than the earliest recorded flowering times of wild-type Pitztal with no very late-flowering plants being observed. Similarly, the 'late-flowering' segregants from the mutant line backcrosses flowered only as late, or even earlier, than some of the earliest flowering Pitztal controls. In addition, the early-flowering segregants of these crosses flowered in some cases even earlier than plants of the selfed mutant lines. The 1:1 segregation of the mutant backcross progeny suggests that one locus, having major effects on flowering time, has been altered in all the mutant lines and is causal to the early-flowering phenotype. The tendency toward early-flowering in the backcross progeny is however further suggestive of the segregation of several other minor effect flowering time genes present in the polymorphic Pitztal and mutant backgrounds.

Figure 3.25 shows some examples of plants produced from the backcross of the F1 progeny to Pitztal or the relevant mutant line. The segregation of early- and late-flowering plants is clearly seen in panels A and B which represent plants from a cross of mutant line 260 or 280 with Pitztal, backcrossed to mutant line 260 or 280. The absence of any early-flowering segregants in the backcrosses of these F1 plants to Pitztal is demonstrated in panels C and D.
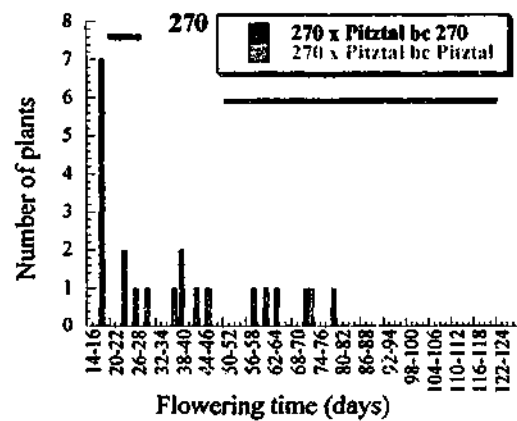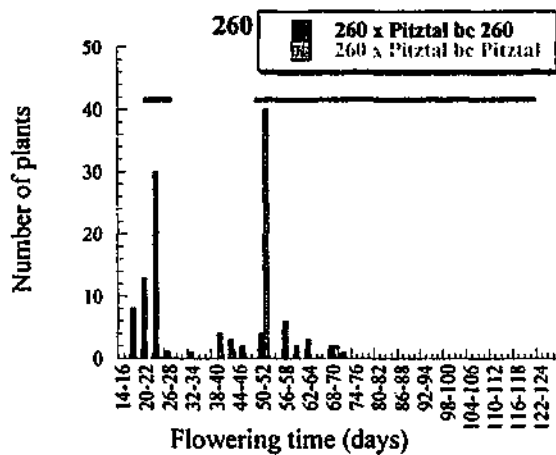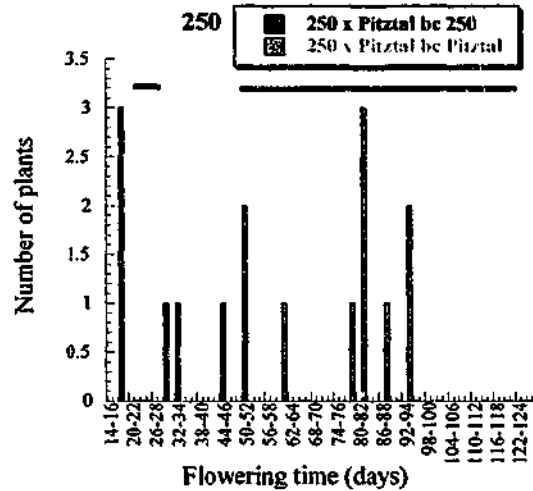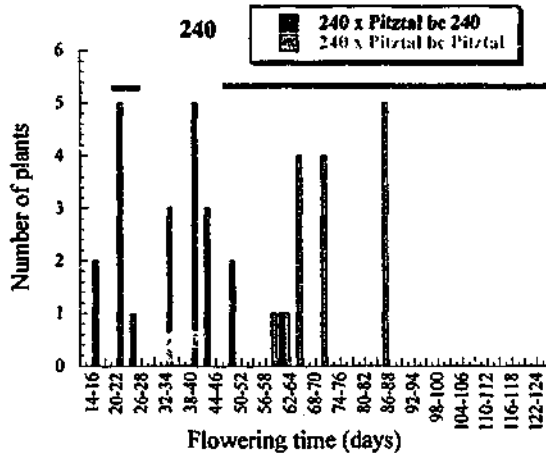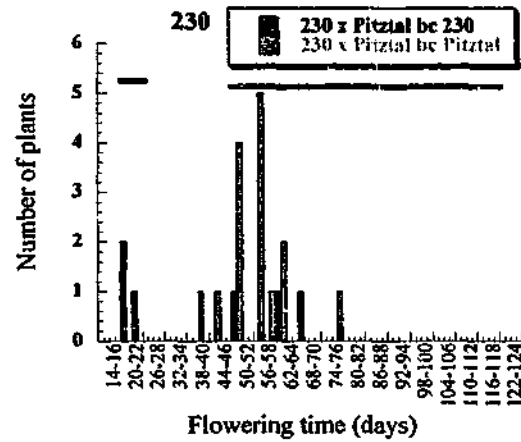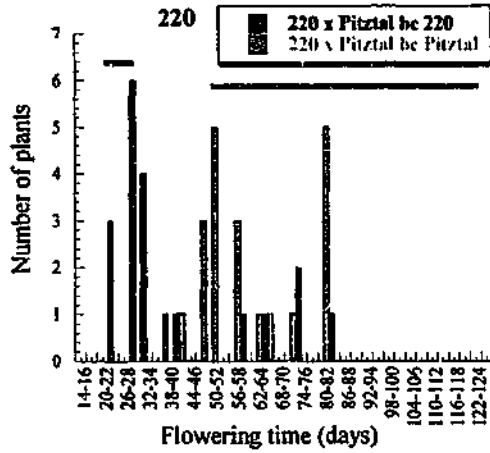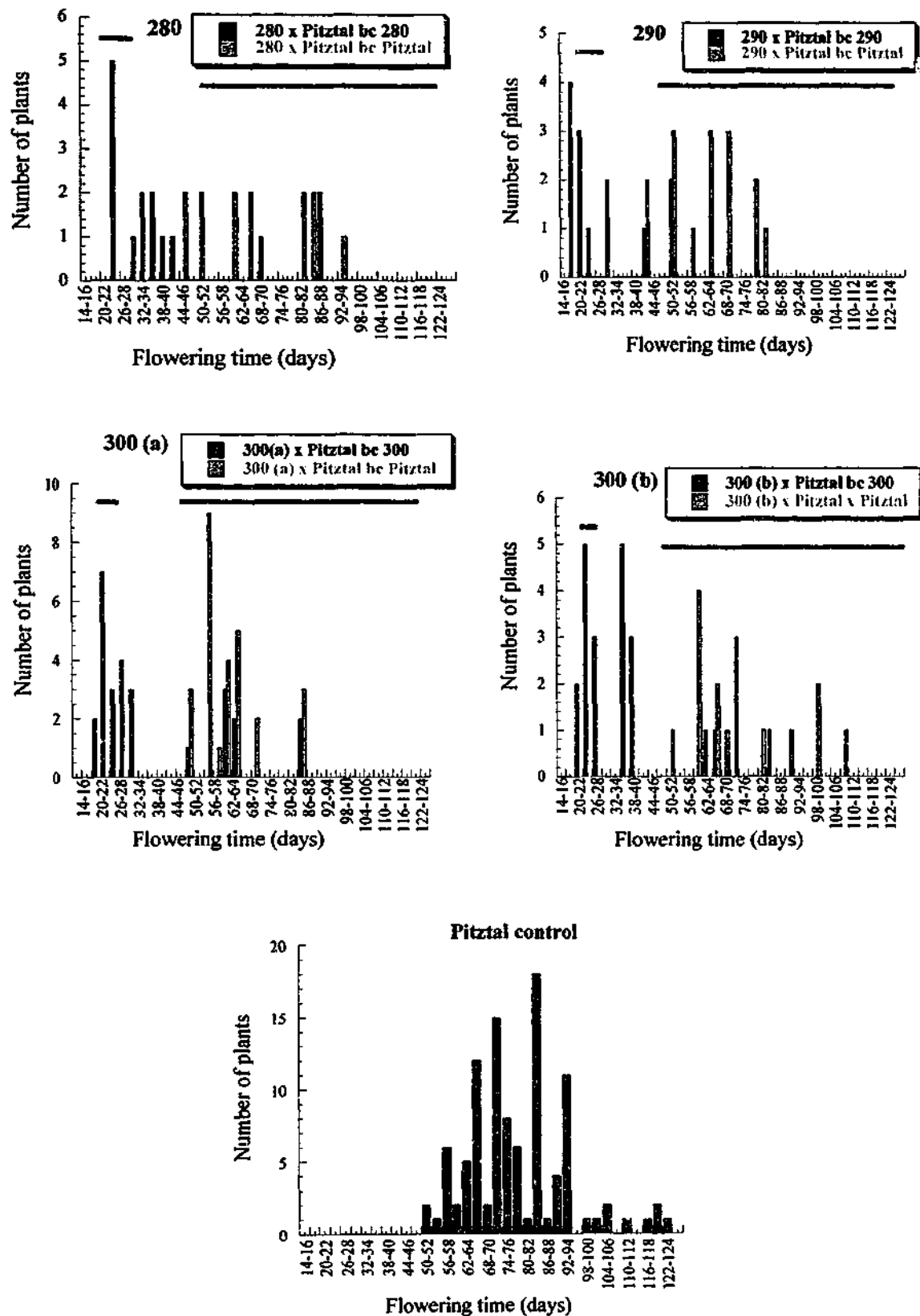
Figure 3.23

The flowering times of the F1 progeny from the *fler* mutants crossed with wild-type Pitztal, backcrossed to either Pitztal or the appropriate mutant line. The red bar on each histogram represents the range of flowering time of wild-type Pitztal control plants, while the black bar represents the range of flowering times of the *fler* mutants.
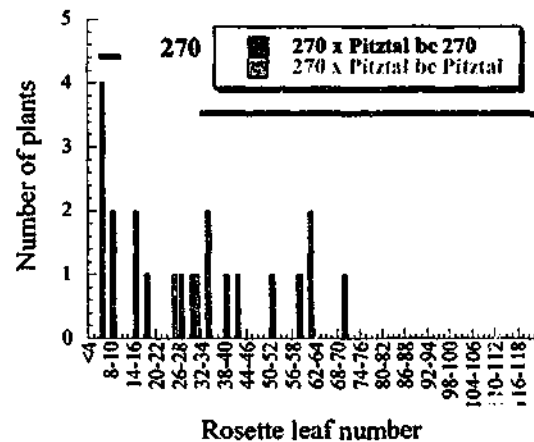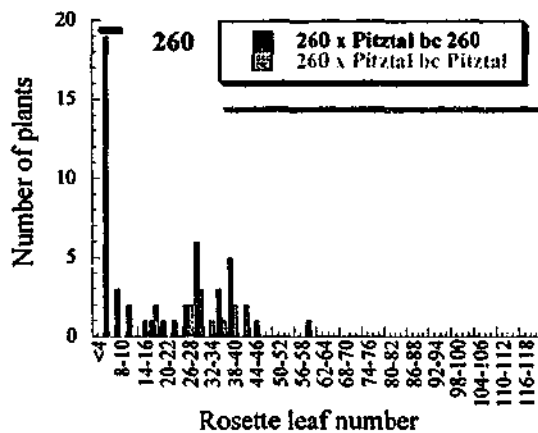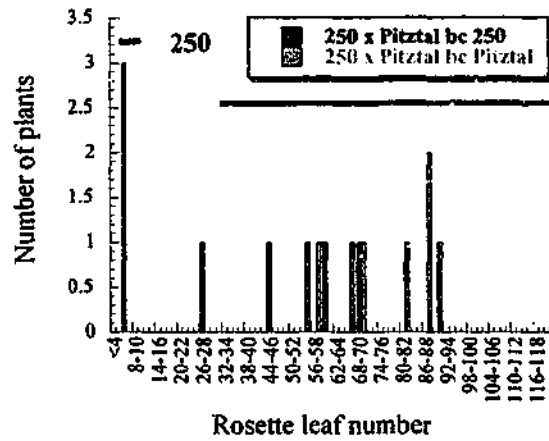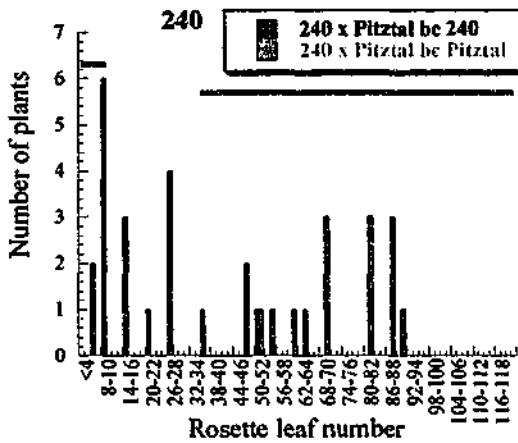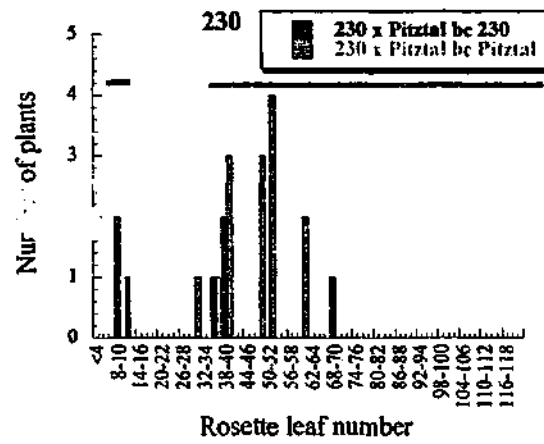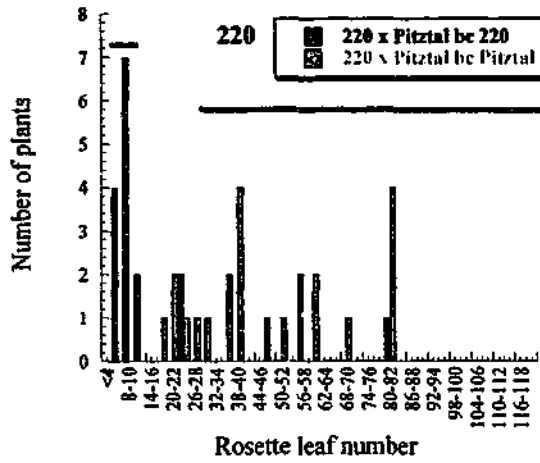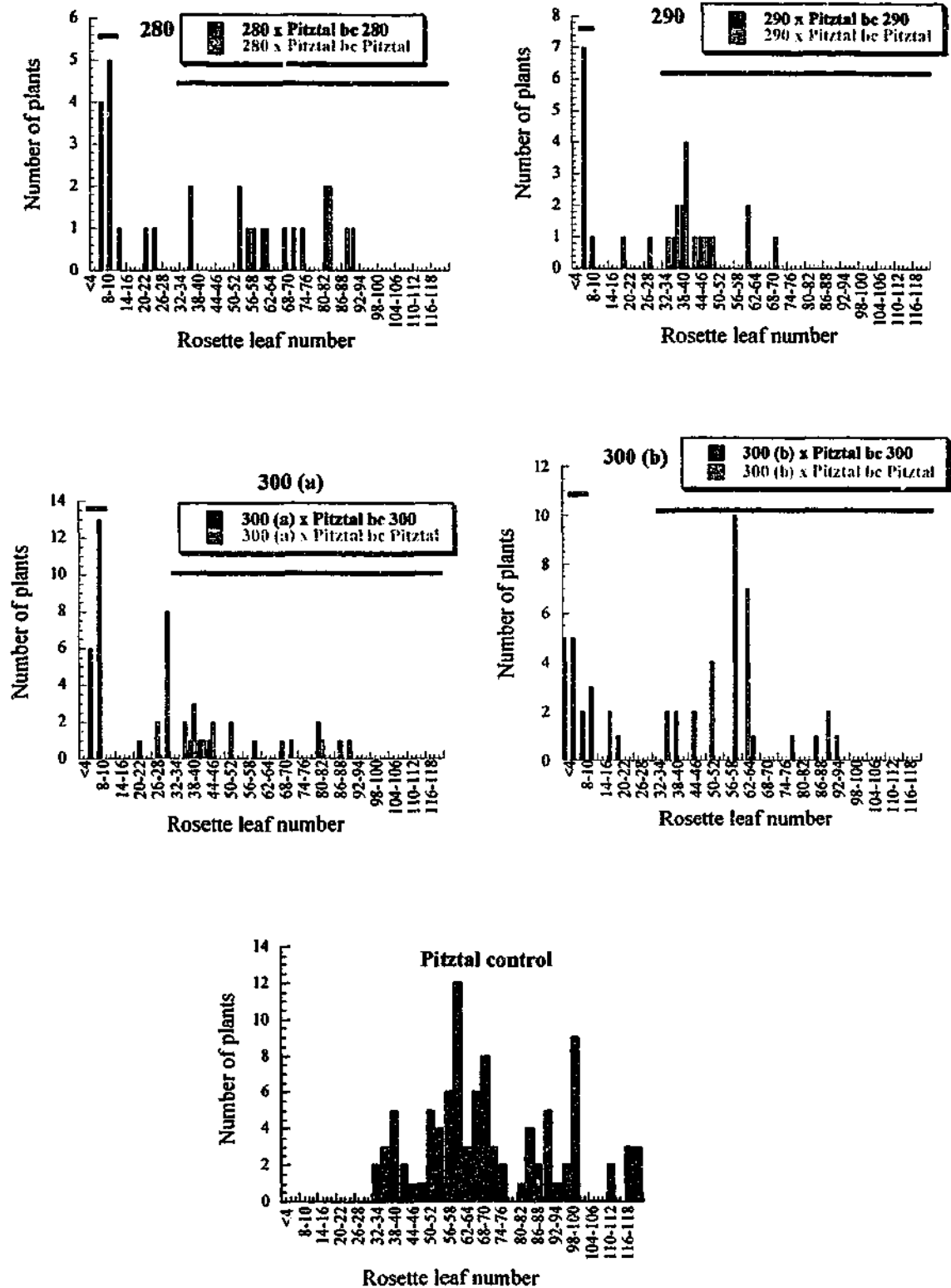
Figure 3.24

The rosette leaf number at the time of flowering of the F1 progeny from the *fler* mutants crossed with wild-type Pitztal, backcrossed to either Pitztal or the appropriate mutant line. The red bar on each histogram represents the range of rosette leaf numbers at the time of flowering of wild-type Pitztal control plants, while the black bar represents the range of rosette leaf numbers of the *fler* mutants.

Figure 3.25

The Fl progeny of the *fler* mutant lines 260 and 280 crossed to wild-type Pitztal and backcrossed to the mutant lines or wild-type Pitztal plants.

(A) Mutant line 260 X Pitztal backcrossed to mutant line 260

(B) Mutant line 280 X Pitztal backcrossed to mutant line 280

(C) Mutant line 260 X Pitztal backcrossed to wild-type Pitztal

(D) Mutant line 280 X Pitztal backcrossed to wild-type Pitztal

Complementation tests

To establish the allelic realtionship between the mutated genes within the *fler* mutant lines, crosses between each of the nine lines were performed. To detect any maternal inheritance effects, reciprocal crosses using the same two parental lines were also established. It was believed that the crosses between mutant lines carrying mutations in the same gene should result in only early-flowering F1 progeny. Combinations of mutant lines carrying alterations at different loci were expected to produce F1 plants that flowered somewhat later than the parental lines. As all the mutated loci within the early-flowering mutants lines were believed to be recessive, any delay in flowering in the heterozygous F1 plants is presumed to be the result of the presence of wild-type Pitztal alleles at either loci.

Table 3.4 summarises the average flowering time and rosette leaf number at the time of flowering of these crosses. The data along the diagonal of the table is the flowering time, and rosette leaf number at the time of flowering, of the selfed mutant lines, used as controls in this experiment. Using the leaf number data rather than flowering times, which can be artifactually delayed, some preliminary groupings of mutants putatively affected in the same gene were made, as both the original and reciprocal crosses of these lines gave only early-flowering progeny. Further analysis of some other crosses involving these lines indicated some contradictions between these groupings as intermediate rather than earlier flowering plants were observed in some F1 populations. Despite these inconsistencies it was noted that all crosses did produce F1 progeny which flowered with 10 leaves or less in at least one of the two reciprocal crosses, suggesting that all the mutant lines are allelic. It is unclear as this stage whether the intermediate flowering F1 plants observed are due to the segregation of 'minor effect' flowering times genes within the mutant lines, or whether some *fler* mutant lines are not null mutants, thereby allowing the combination of the residual activity of affected genes in these parental lines to delay flowering in the F1 progeny.

The variation in the morphology of the mutants also indicates that the different *fler* lines are likely to be polymorphic at a number of loci. The most striking example of this is the differences between mutant lines 220 and 260. The *fler* mutant line 220 plants are dark green in colour and have hirsute leaves and meristems, whereas plants of line 260 are pale green in colour, displays leaf epinasty, and are completely glabrous. The mutant line 220 is also of particular interest as when this line is used as the male in crosses with six of the remaining eight lines, the F1 progeny flowers later than the selfed mutant lines. When this line is the female parent however, the flowering times of the F1 plants do not appear to differ from the flowering times of the relevant selfed mutant lines. This may suggest, at least for line 220, some involvement of maternally inherited genes in the resultant mutant phenotype.

Mutant line	220 (M)	230 (M)	240 (M)	250 (M)	260 (M)	270 (M)	280 (M)	290 (M)	300 (M)	Σ leaf number
220 (F)	24.9 ± 0.8 6.7 ± 0.2	21.1 ± 0.3 7.0 ± 0.3	23.3 ± 0.9 7.2 ± 0.5	25.3 ± 1.4 8.8 ± 0.8	23.7 ± 0.4 7.8 ± 0.4	23.1 ± 0.6 7.1 ± 0.4	28.6 ± 0.5 8.5 ± 0.4	19.6 ± 0.7 6.8 ± 0.3	24.2 ± 1.3 8.5 ± 1.2	7.71
230 (F)	21.4 ± 0.3 7.3 ± 0.4	36.3 ± 1.7 7.9 ± 0.7	28.6 ± 0.2 9.8 ± 4.3	18.0 ± 0.6 5.9 ± 0.6	20.3 ± 0.6 7.0 ± 1.5	21.0 ± 1.9 11.4 ± 3.1	20.2 ± 0.4 6.0 ± 0.2	19.9 ± 0.4 6.0 ± 0.2	20.5 ± 0.4 6.1 ± 0.3	7.43
240 (F)	32.7 ± 0.7 10.8 ± 0.5	31.2 ± 0.6 13.0 ± 0.9	21.9 ± 0.3 7.6 ± 0.2	30.1 ± 0.7 9.9 ± 0.5	22.5 ± 0.9 9.5 ± 1.8	20.1 ± 0.2 6.1 ± 0.2	21.9 ± 0.2 7.4 ± 0.2	19.4 ± 0.4 5.9 ± 0.4	20.7 ± 0.3 6.4 ± 0.2	8.63
250 (F)	33.6 ± 1.3 13.0 ± 1.1	22.2 ± 0.9 6.9 ± 0.3	25.5 ± 0.4 7.71 ± 0.6	20.4 ± 0.7 6.6 ± 0.2	18.5 ± 0.5 5.5 ± 0.2	26.8 ± 0.6 7.9 ± 0.5	27.3 ± 0.8 7.8 ± 0.2	21.3 ± 0.6 6.6 ± 0.3	29.1 ± 1.0 10.7 ± 0.3	8.26
260 (F)	21.0 ± 0.3 7.7 ± 0.4	25.5 ± 0.5 9.7 ± 0.4	23.0 ± 0.7 9.0 ± 0.3	20.3 ± 0.3 6.7 ± 0.2	18.9 ± 0.5 5.3 ± 0.2	20.2 ± 0.8 6.6 ± 0.6	19.5 ± 0.4 5.6 ± 0.4	22.0 ± 0.9 9.0 ± 1.0	24.0 ± 1.3 9.0 ± 2.1	7.91
270 (F)	22.4 ± 0.5 10.0 ± 0.9	21.0 ± 0.0 7.7 ± 1.5	32.3 ± 0.4 10.9 ± 0.4	30.6 ± 0.5 8.1 ± 0.3	22.8 ± 0.6 10.4 ± 1.2	24.2 ± 0.7 7.1 ± 0.5	32.2 ± 0.4 10.1 ± 0.4	26.8 ± 1.7 12.8 ± 1.1	24.9 ± 0.3 7.0 ± 0.3	9.65
280 (F)	35.0 ± 0.0 24.0 ± 3.1	21.7 ± 0.6 9.0 ± 0.6	21.7 ± 0.2 7.7 ± 0.2	30.1 ± 0.7 9.5 ± 0.5	24.7 ± 0.4 11.6 ± 0.5	32.1 ± 0.8 10.8 ± 0.6	27.9 ± 1.3 7.9 ± 0.5	25.7 ± 0.7 12.1 ± 0.8	30.4 ± 0.6 10.5 ± 0.4	11.90
290 (F)	32.4 ± 0.4 10.6 ± 0.4	25.7 ± 0.4 8.8 ± 0.2	20.5 ± 0.3 6.2 ± 0.2	23.8 ± 0.3 7.1 ± 0.2	22.0 ± 0.6 6.5 ± 0.6	21.9 ± 0.9 7.2 ± 0.5	21.8 ± 0.2 7.1 ± 0.2	24.6 ± 0.3 8.3 ± 0.2	23.6 ± 0.5 7.4 ± 0.3	7.61
300 (F)	31.0 ± 0.9 17.3 ± 1.4	26.4 ± 0.4 8.9 ± 0.4	21.9 ± 0.6 8.3 ± 0.5	30.7 ± 0.6 8.9 ± 0.4	33.1 ± 1.1 18.3 ± 1.5	23.8 ± 1.0 8.3 ± 0.9	22.0 ± 0.5 7.8 ± 0.4	21.7 ± 0.7 7.3 ± 0.3	19.5 ± 0.5 6.6 ± 0.2	10.64
Σ leaf number	12.59	8.88	8.35	8.11	9.58	8.18	7.53	8.31	9.03	--

Table 3.4

The flowering times (top), and rosette leaf number at the time of flowering (bottom), for the F1 progeny of crosses between two *fler* mutant lines. The female parental line for each cross (F) is represented along the horizontal axis of the table. The figures highlighted on the diagonal axis represent the flowering time and rosette leaf number of the selfed mutant lines. Figures below the diagonal represent the average flowering times and leaf numbers observed from reciprocal crosses of the same two mutant plants.

The summed leaf number of the crosses in which line 220 was the male parent was 100.7, the highest recorded, while the same parameter was almost the lowest (61.7) when summed between crosses in which this line was the female parent (Table 3.4). These results again suggest that there may be some disruptions within maternally inherited genomes segregating amongst the mutant lines. Furthermore, it appears as though these genes may have either promotory affects on the flowering time of the mutants, as in the case of line 220, or inhibitory effects, such as in line 280, which has one of the largest summed leaf numbers amongst crosses in which it was used as the female (95.2). The presence of the 'floral inhibitory' 280, and the absence of the 'floral promotory' 220, maternally inherited genomes may, for example, explain why the highest LN (24.0) observed amongst all of these FI plants is from the 280 (F) X 220 (M) cross.

As mentioned the lack of complementation observed in all of these crosses does suggest all the nine *fler* lines are allelic, however in view of some anomolous results in the complementation anslysis it was decided to analyse each mutant line separately in subsequent experiments.

Responses of the mutant lines to photoperiod and vernalisation

All ecotypes of *Arabidopsis* have been shown to behave as facultative long day plants, although their long day requirement for early-flowering can vary (Martinez-Zapater *et al.*, 1994). Between 12-18 hours light is generally considered long day conditions, while 8-10 hours light constitutes short days. Several late-flowering ecotypes can also respond to vernalisation treatments with a very significant reduction in their flowering times and rosette leaf number. Early flowering ecotypes do not show a significant response to vernalisation in long days due to their already accelerated flowering times in these conditions (Kakutani *et al.*, 1995). The delay in flowering of these ecotypes, caused by short day conditions, can however often be at least partially overcome by vernalisation treatments. This suggests the presence of a vernalisation-responsive pathway in both late- and early-flowering ecotypes (Kakutani *et al.*, 1995; Amasino, 1996a). The flowering times of the mutant lines in response to different photoperiods, as well as to vernalisation treatments, was therefore examined to determine if these responses deviated from those of wild-type Pitztal or early-flowering ecotype controls.

Figure 3.26 illustrates the flowering time, and rosette leaf number at the time of flowering, of the *fler* mutant lines and Columbia, L. *erecta*, Niederzenz, and Pitztal plants grown in either 24 hours, 16 hours, or 8 hours light. While the flowering time of all *fler* lines was slightly delayed in 16 hours light compared to 24 hours light, the leaf number at the time of flowering of these plants was quite similar. The mutant lines did exhibit a strong delay in

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their flowering times, with a concomitant increase in the number of leaves at the time of flowering, when grown in short days (8 hours light). These responses were not however as extreme as those shown by wild-type Pitztal under these conditions. Such results indicate that the mutant lines still retain the ability to respond to short day conditions, and that the flowering delay noted is similar in magnitude to that shown by all the early-flowering ecotype control lines.

The response of the mutant lines to a 21 day vernalisation treatment and subsequent growth in either long (16 hours light), or short (8 hours light) day conditions was also tested. As expected vernalisation, treatments had little effect on the already early-flowering phenotype of the mutant lines, wild-type Columbia, L. *erecta*, or Niederzenz plants grown in long days. In contrast, the late-flowering Pitztal controls grown in long days did respond strongly to these vernalisation treatments, manifested by a dramatic reduction in their leaf number and flowering times (Figure 3.27).

In short day conditions, the delayed flowering times and increased leaf number of the mutant lines were also not drastically reduced by the 21 day vernalisation treatment. Some mutant lines (230, 250, 260, and 300), like the Columbia controls, did exhibit a slight reduction in leaf number and flowering time after vernalisation, although these plants were still later flowering than those grown in long day conditions. The other mutant lines appeared to behave similarly to the L. *erecta* and Niederzenz controls with vernalisation having little, if any, effect on their flowering times. Again, only the Pitztal control plants showed a strong response to vernalisation that reduced their flowering times and leaf numbers so that they now flowered at similar times to the mutant lines and early-flowering ecotype controls. Vernalised Pitztal plants however still flowered later if subsequently grown in short days compared to long day conditions (Figure 3.28). These results suggest that at least under the present growth conditions, 21 days vernalisation does not fully compensate for the delay of flowering time caused by short days in the Pitztal ecotype.

Crosses of mutant lines to early-flowering ecotypes

As postulated, the early-flowering mutant lines in this present study may potentially carry mutations in either the *FRI* or *FLC* genes, or both. If the mutations lay within the *FRI* gene then crosses of these lines to early-flowering ecotypes, which do not carry late-flowering alleles of *FRI* (Gendall *et al.*, 1999) would be expected to only produce early-flowering progeny. Alternatively, if the mutant phenotype was due to a disruption in the *FLC* gene, then crosses to Columbia and Niederzenz ecotypes would be expected to yield some slightly later flowering F1 progeny as these ecotypes carry alleles of *FLC* that cause late- or very late-flowering respectively, in combination with Pitztal alleles of *FRI* (Burn *et al.*, 1993b).
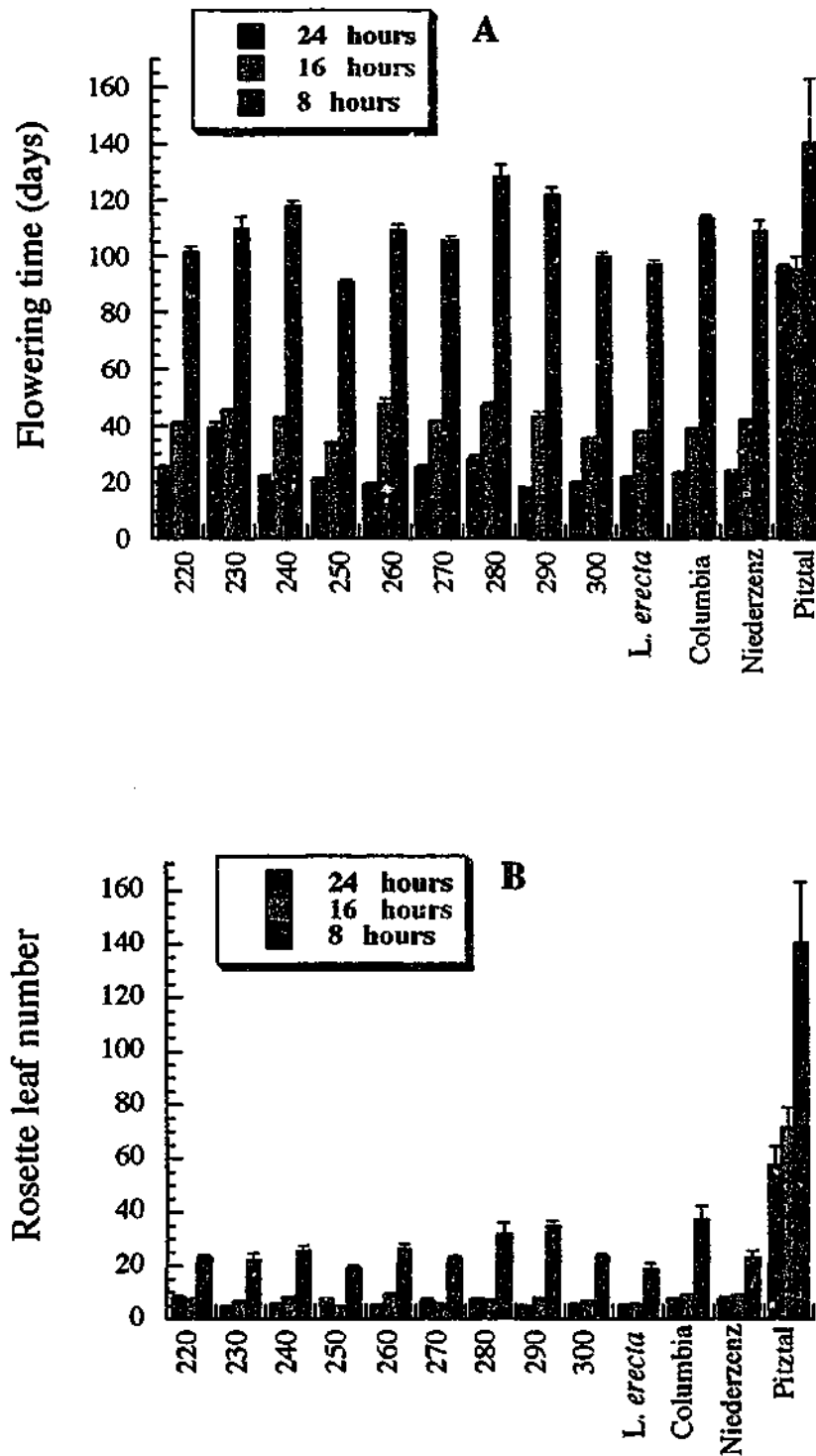
Figure 3.26

The average flowering time (A), and rosette leaf number at the time of flowering (B), of the radiation-induced *fler* mutant lines, wild-type L. *erecta*, Columbia, Niederzenz, and Pitztal grown in either 24, 16, or 8 hours light.
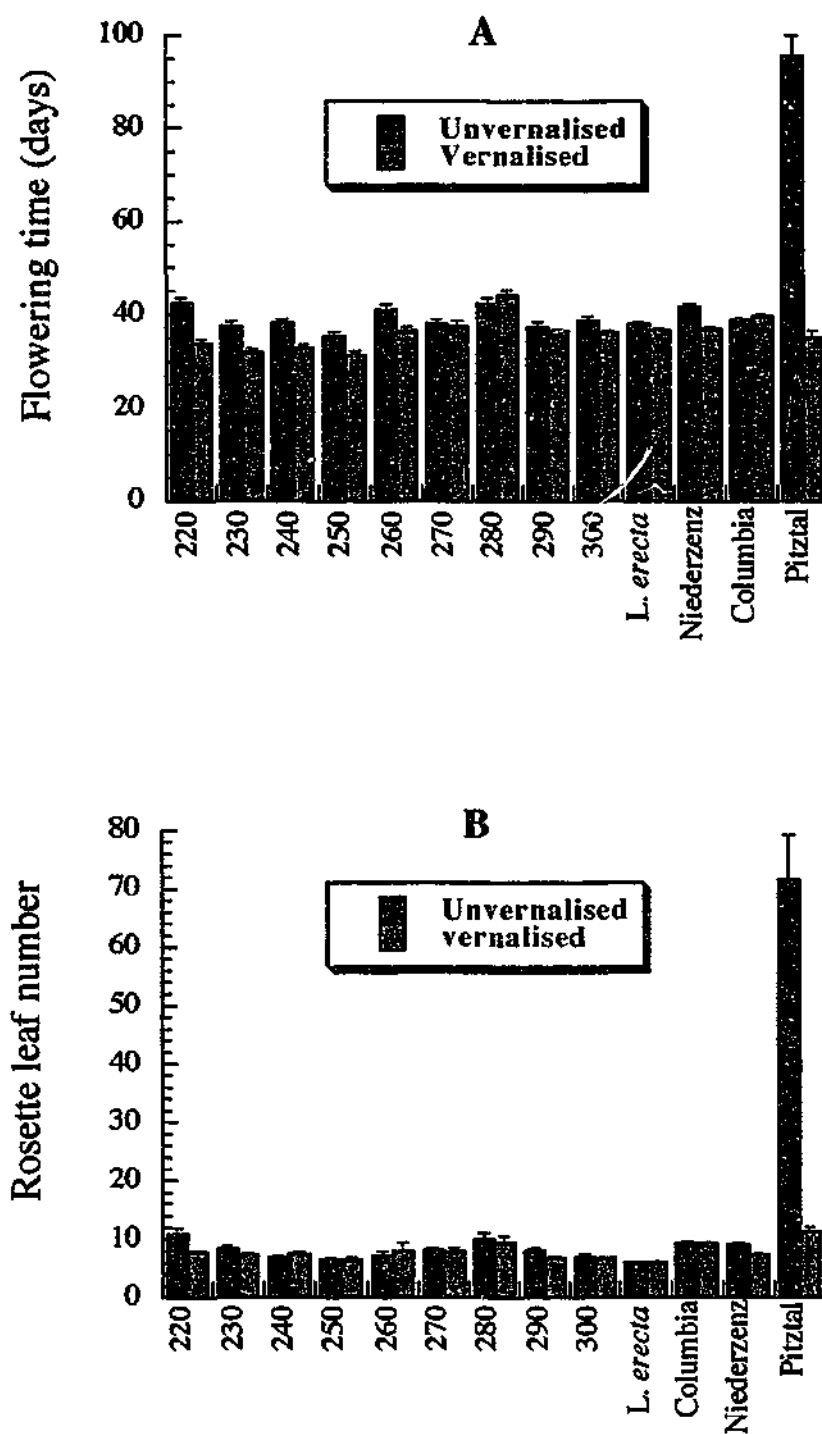
Figure 3.27

The average flowering time (A), and rosette leaf number at the time of flowering (B), of vernalised or unvernalised radiation-induced *fler* mutant lines, wild-type L. *erecta*, Columbia, Niederzenz, and Pitztal grown in long days (16 hours light).
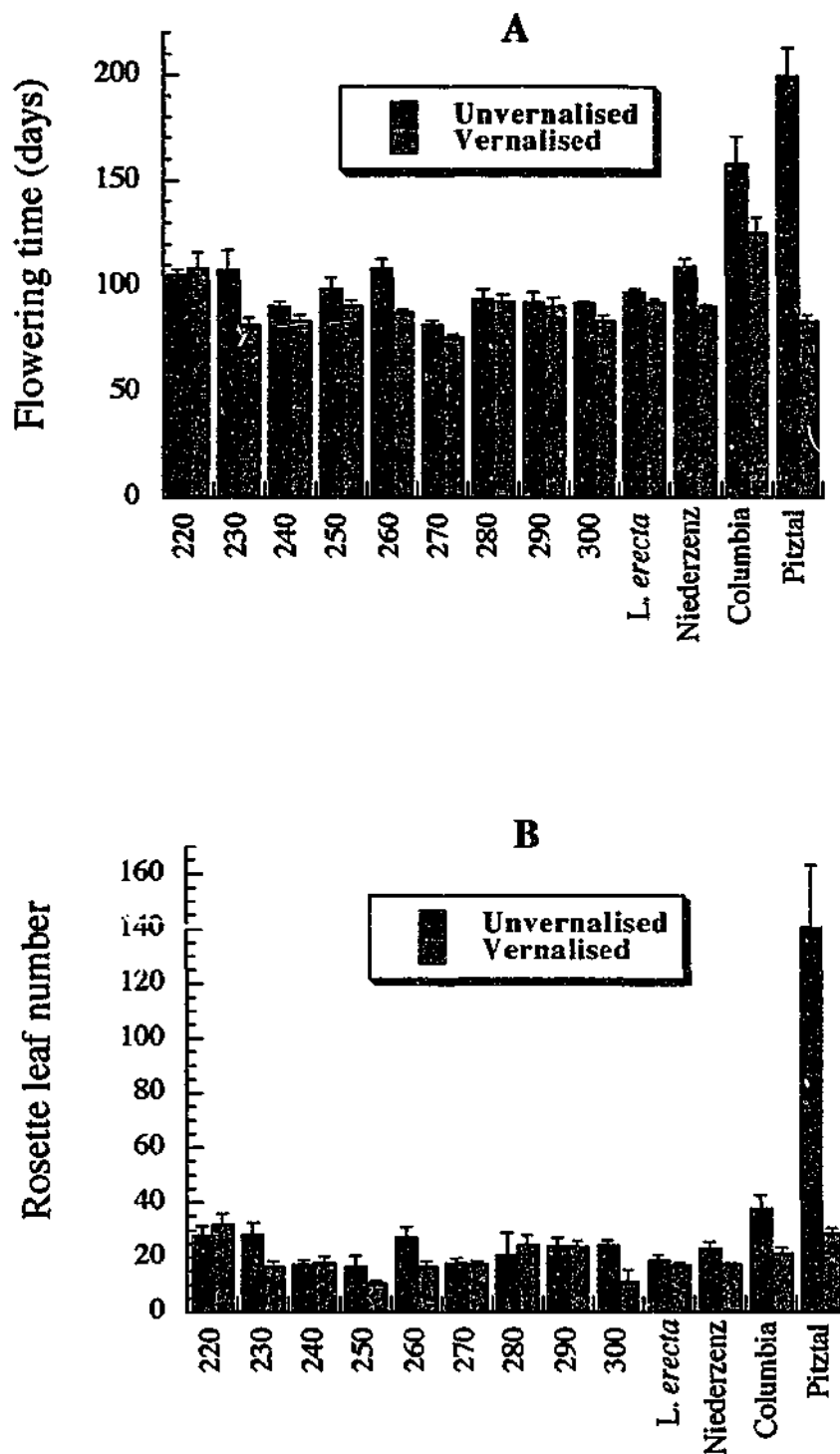
Figure 3.28

The average flowering time (A,) and rosette leaf number at the time of flowering (B), of vernalised or unvernalised radiation-induced *fler* mutant lines, wild-type L. *erecta*, Columbia, Niederzenz, and Pitztal grown in short days (8 hours light).

As *FLC* is only semi-dominant however, such heterozygous F1 plants would be predicted to flower at intermediate times. Crosses of the radiation-induced lines (putatively affected in the *FLC* gene) with the L. *erecta* ecotype would be predicted to result in early-flowering plants since this ecotype does not possess the *FLC* allele capable of causing extremely late flowering in conjunction with Pitztal alleles of *FRI* (Burn *et al.*, 1993b; Lee *et al.*, 1993; Lee *et al.*, 1994b; Lee and Amasino, 1995).

Figures 3.29 and 3.30 illustrate the flowering time, and rosette leaf number at the time of flowering, respectively, of the F1 progeny from crosses of the nine *fler* mutant lines to either wild-type Columbia, L. *erecta*. or Niederzenz. The flowering times and leaf numbers of the selfed mutant lines and the corresponding controls are also presented. No evidence of any delay in the F1 progeny from any crosses were detected, suggesting that the mutation causing early-flowering in *fler* plants is unlikely to be in the *FLC* gene. Mutations in the *FRI* gene or other loci that may also cause such early-flowering plants in combination with early-flowering ecotype backgrounds should be considered. While similar interactions between *FRI* and *FLC* as those described above may be occurring within the F1 plants, the effects of these combinations may be masked by the presence of mutated alleles at other loci in the mutant lines that cause epistatic early-flowering. Such genes may act upstream or downstream of the actions of *FRI* and *FLC* to allow repression of flowering in wild-type Pitztal.

The F1 progeny from the initial crosses were also backcrossed to either the corresponding mutant line or to the relevant early-flowering ecotype parent. Again, if the mutations in the mutant lines were in the *FRI* gene then no segregation in flowering time may be expected, as all relevant *FRI* alleles in these crosses would confer early-flowering. Alternatively, if *FLC* mutations were present in the mutant lines, some segregation of early and late-flowering plants in the backcrosses to the wild-type Columbia and Niederzenz ecotypes (which contain *FLC* alleles capable of conferring late flowering when combined with at least one Pitztal copy of the *FRI* gene) may be detected. No such late or intermediate flowering plants were detected in the progeny of any of these backcrosses and the average flowering time and rosette leaf number at the time of flowering of these plants are presented in Figures 3.31, 3.32 and 3.33. These results once more suggest mutations causing early-flowering in the Pitztal mutant lines are more likely to be in the *FRI* gene, rather than in the *FLC*, locus. In fact, several backcrosses resulted in F1 plants that flowered slightly earlier than plants of the selfed mutant lines. This phenotype may be due to the presence of more early-flowering ecotype versions of genes that also affect flowering times of *Arabidopsis* in these F1 plants compared to the mutant line parental plants.
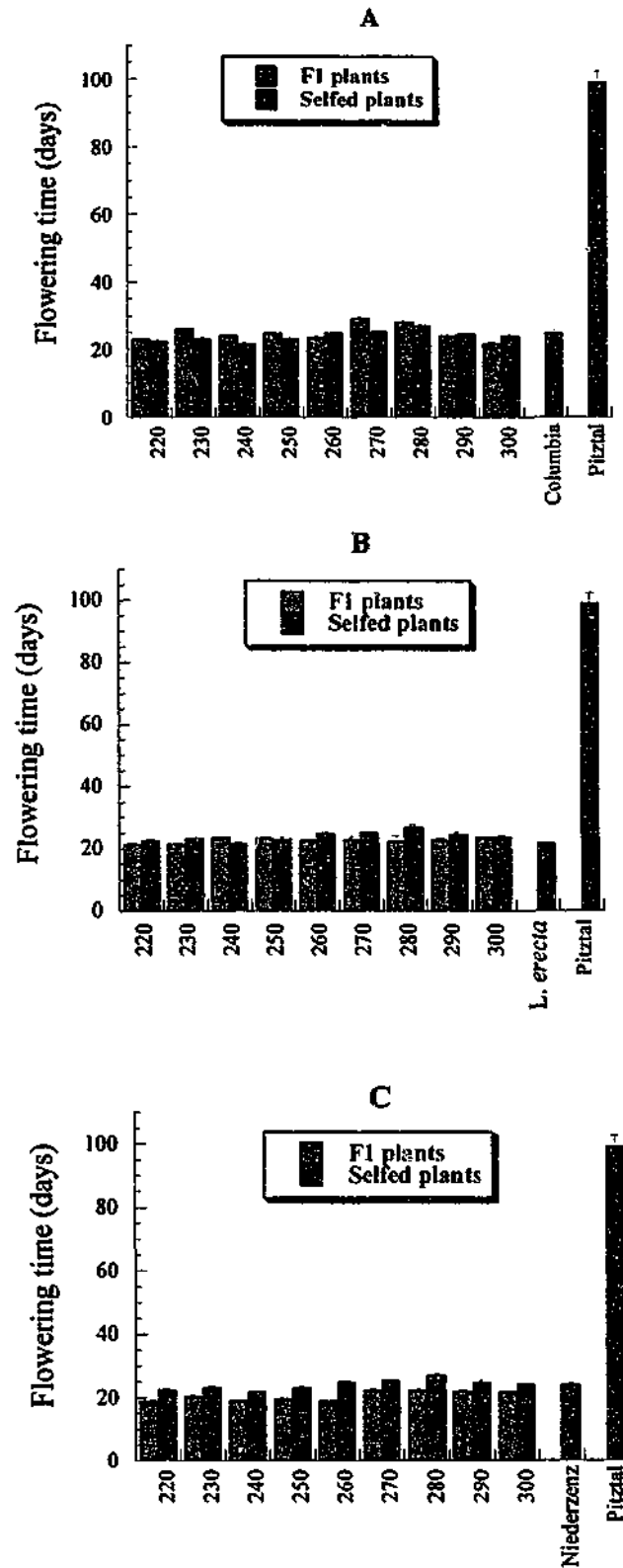
Figure 3.29

The average flowering time of the F1 plants from crosses of the *fler* mutant lines with
wild-type Columbia (A), L. *erecta* (B), or Niederzenz (C), grown in continuous light.
The average flowering time of the selfed *fler* lines, wild-type Pitztal and either wild-
type Columbia, L. *erecta,* or Niederzenz were also recorded as controls.

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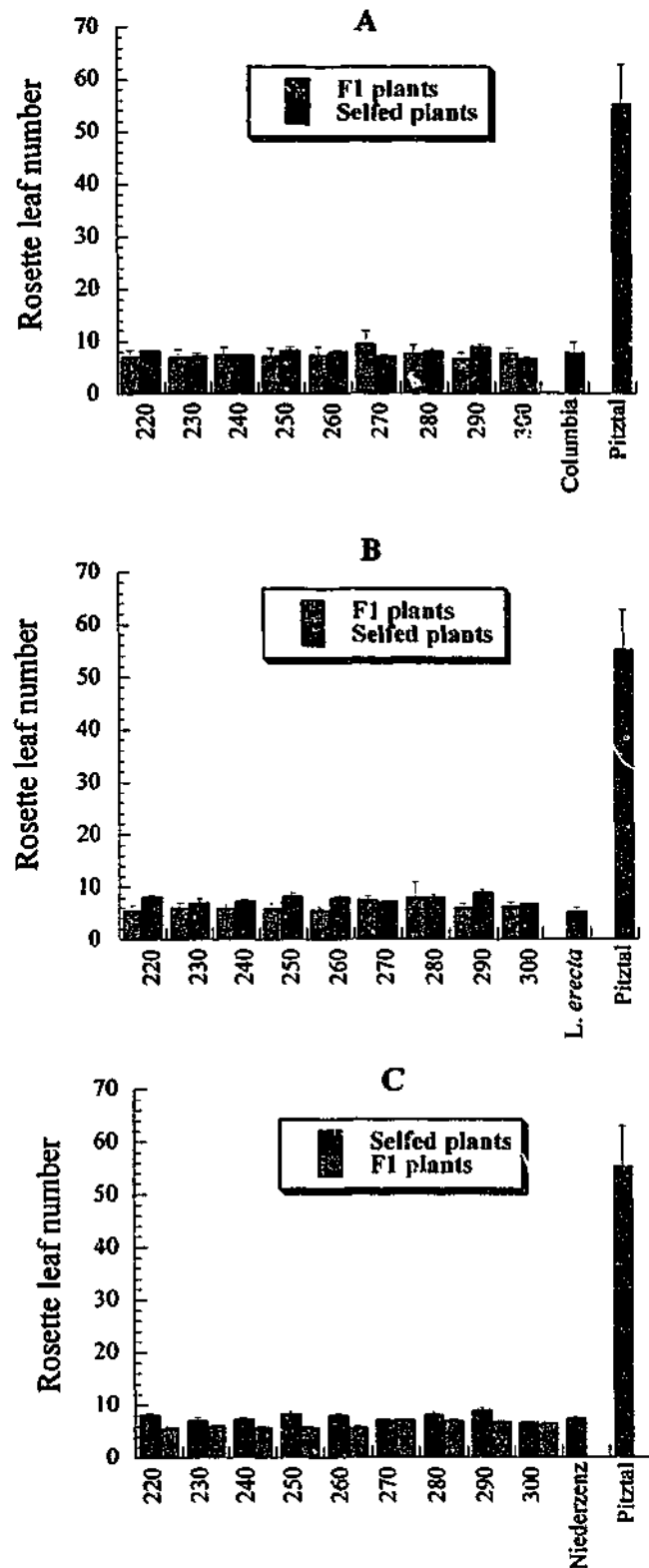
Figure 3.30

The average rosette leaf number at the time of flowering of the F1 plants from crosses of the *fler* mutant lines with wild-type Columbia (A), L *.erecta* (B), or Niederzenz (C) grown in continuous light. The average rosette leaf number at the time of flowering of the selfed *fler* lines, wild-type Pitztal and either wild-type Columbia, L. *erecta*, or Niederzenz were also recorded as controls.
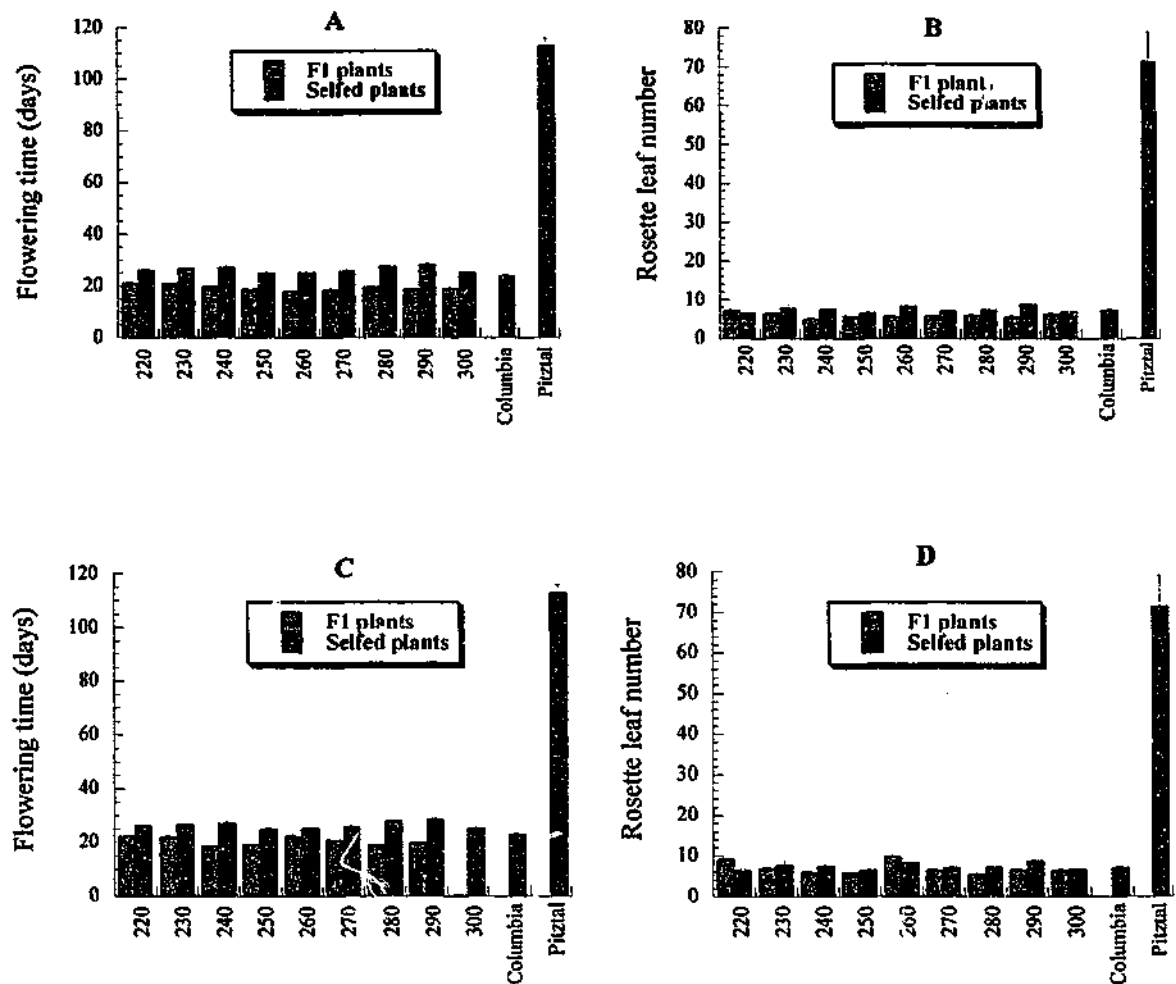
Figure 3.31

The F1 plants from crosses of the *fler* mutant lines with wild-type Columbia were backcrossed to either wild-type Columbia, or the appropriate mutant line. The average flowering time, or rosette leaf number at the time of flowering, of the F1 progeny from these Columbia backcrosses (**A** and **B**), or the mutant line backcrosses (**C** and **D**), were subsequently recorded. The average flowering time, or rosette leaf number at the time of flowering, of the selfed mutant lines, wild-type Columbia and Pitztal were also recorded as controls.
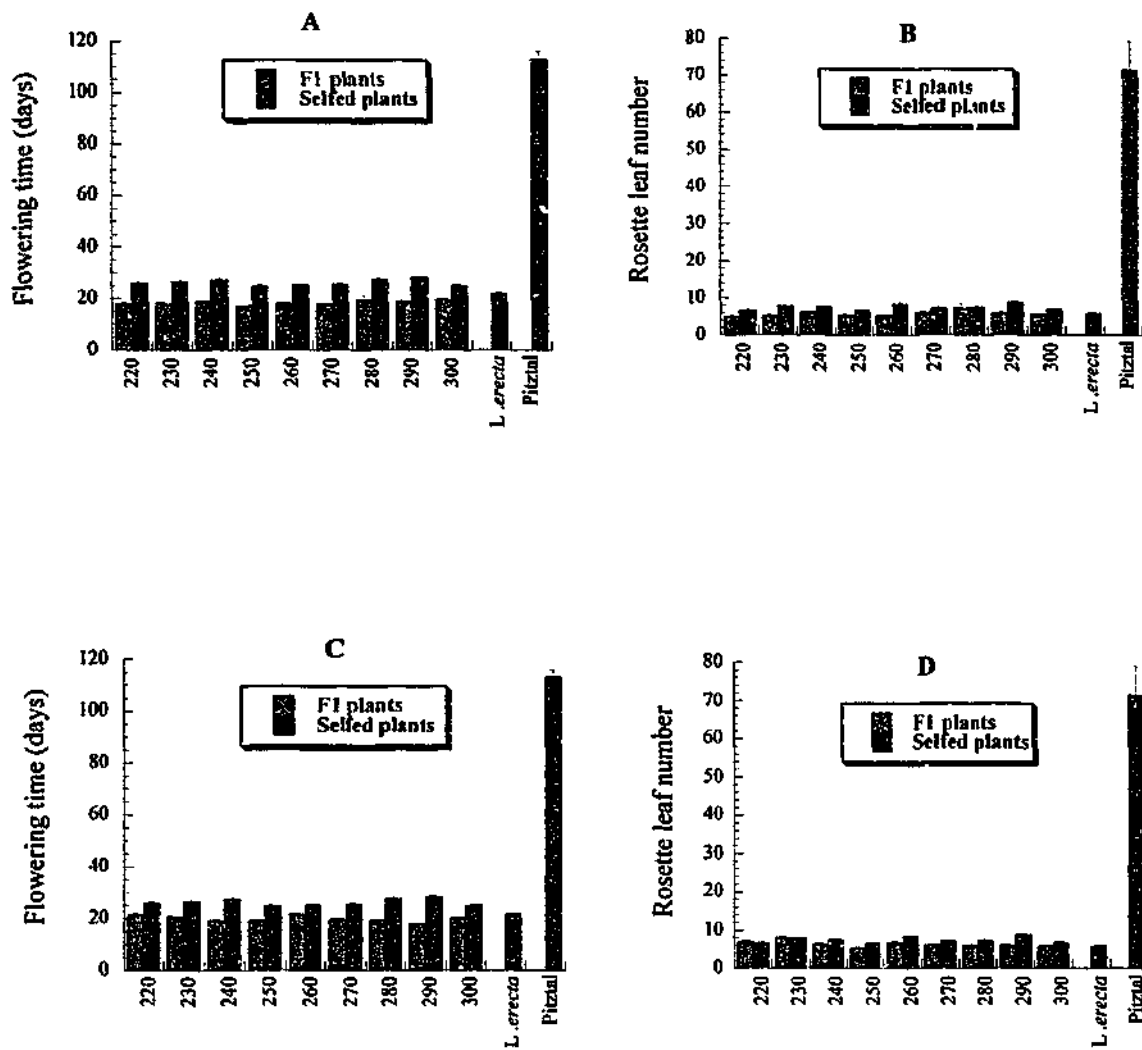
Figure 3.32

The F1 plants from crosses of the *fler* mutant lines with L. *erecta* were backcrossed to either L. *erecta*, or the appropriate mutant line. The average flowering time, or rosette leaf number at the time of flowering, of the F1 progeny from these L. *erecta* backcrosses (A and B), or the mutant line backcrosses (C and D), were subsequently recorded . The average flowering time, or rosette leaf number at the time of flowering of the selfed mutant lines, wild-type L. *erecta* and Pitztal were also recorded as controls.
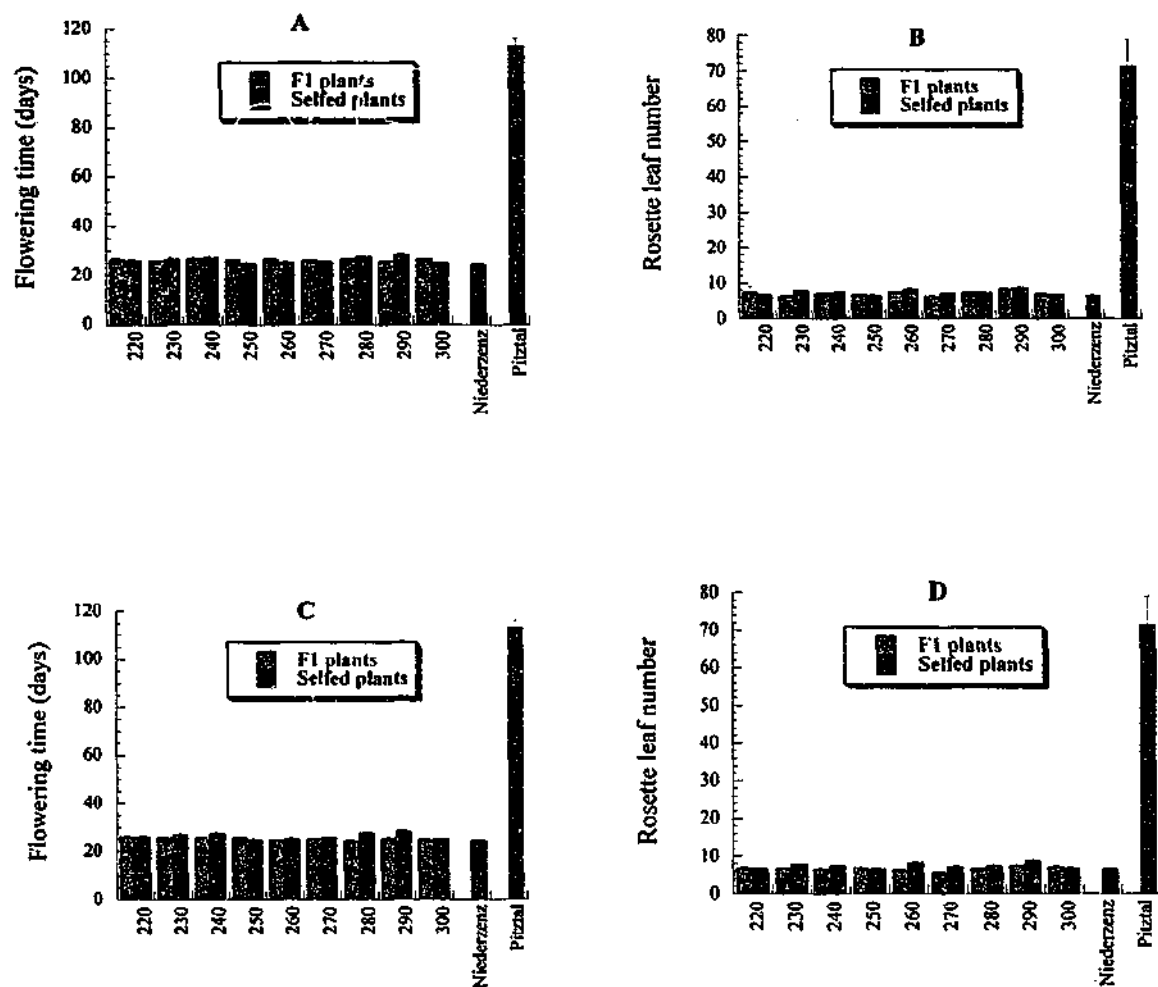
Figure 3.33

The F1 plants from crosses of the *fler* mutant lines with wild-type Niederzenz were backcrossed to either wild-type Niederzenz, or the appropriate mutant line. The average flowering time, or rosette leaf number at the time of flowering, of the F1 progeny from these Niederzenz backcrosses (**A** and **B**), or the mutant line backcrosses (**C** and **D**), were subsequently recorded. The average flowering time, or rosette leaf number at the time of flowering, of the selfed mutant lines, wild-type Niederzenz and Pitztal were also recorded as controls.

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Crosses of mutant lines to other late-flowering ecotypes

The radiation-induced mutants lines were also crossed to two other late-flowering ecotypes, Stockholm and San-Feliu-2. The average flowering time and rosette leaf number at flowering of the F1 progeny from these crosses is illustrated in Figure 3.34. As in Pitztal, the *FRI* gene in these two ecotypes has previously been shown to be the major dominant locus conferring the late-flowering phenotype (Lee *et al.*, 1993; Clarke and Dean, 1994; Lee and Amasino, 1995). A further recessive gene *KRYOPHILA* was also found to have significant effects on the flowering time of the Stockholm ecotype, although the *FRI* gene was found to be completely epistatic to the *KRY* gene except in certain light conditions (Clarke and Dean, 1994). In the present greenhouse growth conditions, the Stockholm ecotype flowered earlier and with less rosette leaves than did San-Feliu-2, which in turn produced less leaves and flowered earlier than did wild-type Pitztal plants.

When the mutant lines were crossed to the Stockholm ecotype the F1 plants flowered at very similar times or slightly later, and after the production of a similar or marginally increased, number of rosette leaves as the Stockholm control plants. Even the latest of these F1 plants were earlier flowering (like wild-type Stockholm) and produced less leaves than wild-type Pitztal. The alleles of the genes causing early flowering in the mutant lines therefore appear to be fully recessive to any genes causing late-flowering in Stockholm. In several mutant lines however, other genes, possibly conferring late flowering in wild-type Pitztal, appear to be acting additively with the Stockholm 'late-flowering' genes to create F1 plants that flower later than Stockholm. These genes may potentially be modifiers of the *FRI* gene product, similar to those proposed to be present in the Niederzenz ecotype (Burn *et al.*, 1993b). A similar situation was observed in the F1 progeny of the mutant lines crossed to wild-type San-Feliu-2 plants. Several F1 plants appeared to produce more rosette leaves than the wild-type San-Feliu-2 ecotype control although their flowering times were similar. In addition, the increase in leaf number of these F1 plants compared to San-Feliu-2 controls was not as large as those observed for the mutant line and Stockholm crosses. This smaller deviation may be attributable to the fact that the flowering times initially of wild-type Pitztal and San-Feliu-2 plants are closer than those of Pitztal and Stockholm. Similar *FRI* modifier genes, presumably from the Pitztal background, that exert their late-flowering phenotype in the F1 plants of the mutants crossed to Stockholm may therefore already be active in wild-type San-Feliu-2 plants.
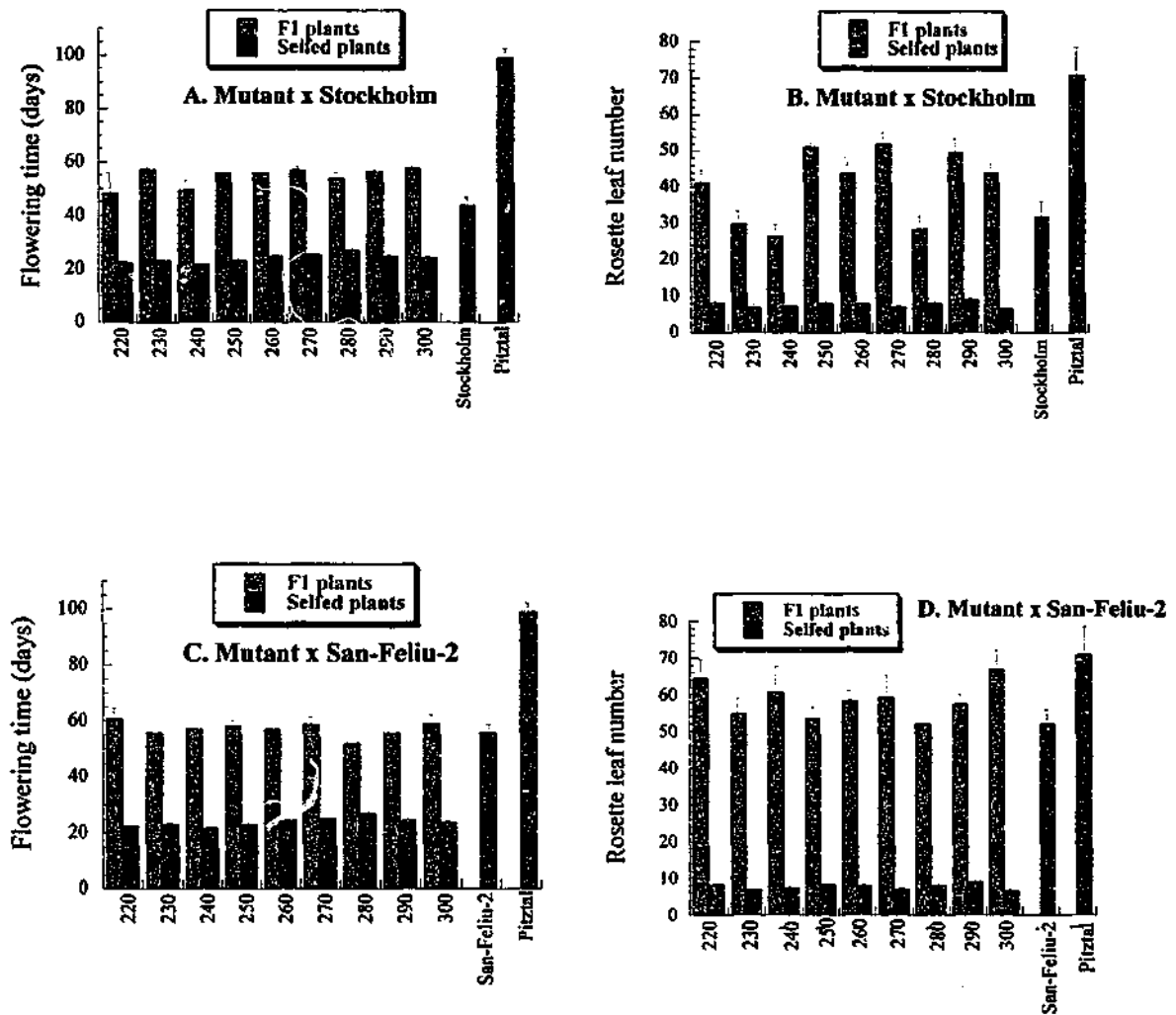
Figure 3.34

The average flowering time (**A**), or rosette leaf number at the time of flowering (**B**), of the F1 progeny of the *fler* mutant lines crossed with wild-type Stockholm. The average flowering time (**C**), or rosette leaf number at the time of flowering (**D**) of the F1 progeny of the *fler* mutant lines crossed with wild-type San-Feliu-2.

Hypocotyl length of the mutant lines

Several previously characterised early-flowering mutants (the eight *hy-1* to *hy-8* lines) isolated in early-flowering ecotypes, were initially isolated on the basis of the elongated hypocotyl phenotype of light-grown seedlings (Pepper and Chory, 1997; Devlin *et al.*, 1998; Whitelam *et al.*, 1998). These *hy* mutants have mutations affecting the production of various photoreceptors including phytochrome or cryptochrome, or in the downstream transduction of signals collected from such receptors (Pepper and Chory, 1997; Devlin *et al.*, 1998; Whitelam *et al.*, 1998). To determine if the mutants used in the present study represent similar mutations in the Pitztal background, the hypocotyl length of both light- and dark-grown seedlings of the mutant lines were examined in collaboration with Dr. D. Bagnall (CSIRO, Canberra, Australia). Two mutant lines (270 and 290) showed some elongation of their hypocotyls when grown in the light compared to the Pitztal controls (Figure 3.35). This suggests that these plants may be putative photoreceptor mutants. The difference in the hypocotyl length of these mutants compared to wild-type Pitztal plants is not however maintained in dark-grown seedlings. Interestingly, several mutant lines 250, 280, and 300 actually exhibited shorter hypocotyls than wild-type Pitztal plants when grown in the light. Mutant line 300 was particularly light sensitive and produced a very short hypocotyl (2.93mm compared with 7.23mm for Pitztal). The short hypocotyl phenotype of two of these lines, 280 and 300 however, was again not maintained in dark-grown seedlings. The 250 *fler* mutant line did however, still produce a short hypocotyl even when seedlings were grown in the dark. This phenotype shows similarities to the constitutively photomorphogenic (*cop*) or de-etiolated (*det*) mutants that show light-induced growth patterns even in plants grown in the dark (Chory *et al.*, 1989; Deng *et al.*, 1991). Mutant line 250 however still displayed some dark growth responses and did not show reduced apical dominance, or day-neutral flowering responses characteristic of the *cop* and *det* mutants.
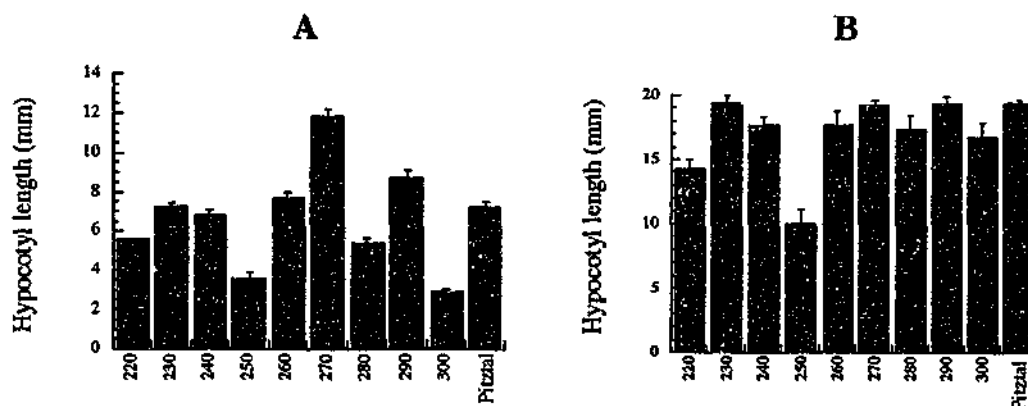
Figure 3.35

The average hypocotyl length of light-grown (A) or dark-grown (B) seedlings for the radiation-induced *fler* mutant lines and wild-type Pitztal.

3.4 Discussion

The overall morphology, growth characteristics, and flowering responses of the radiation-induced mutant lines, in conjunction with RFLP analysis, indicates early-flowering mutations have been created in the late-flowering Pitztal ecotype. Growth rate experiments have also shown little difference in either the rate of leaf production, or fresh weight over time, of these mutant lines compared to wild-type Pitztal. The evidence therefore suggests that these mutations have inactivated a floral repression pathway that is present and active in wild-type Pitztal. The segregation analysis of the flowering time and leaf number of the F1 and F2 plants from backcrosses of the present mutant lines crossed with wild-type Pitztal and other late-flowering ecotypes, strongly suggests one dominant floral repression gene has been disrupted in each mutant line. Furthermore, while the mutant lines still exhibit a short day response, they no longer show the extreme inhibition of flowering time of Pitztal plants in short days. The similarity of these mutant phenotypes to those of the early-flowering ecotypes like L. *erecta* and Columbia suggest that while the short day repression pathways may still be active in the mutant lines, some major floral repression factor present in late-flowering ecotypes is not active.

Good candidates for the floral repression genes that may be altered in the *fler* mutants are the dominant, or semi-dominant, *FRI* or *FLC* genes that are primarily responsible for the flowering time variation between late and early-flowering ecotypes. The *FRI* gene is believed to be the major dominant genes responsible for the late-flowering of many ecotypes including Pitztal, Stockholm and San-Feliu-2. This gene also confers both the strong vernalisation and far-red light responsiveness of such plants (Lee and Amasino, 1995; Aukerman and Amasino, 1996, Dennis *et al.*, 1996; Sanda *et al.*, 1997; Finnegan *et al.*, 1998). It is believed that the *FRI* gene product acts as a floral repressor, possibly in the same autonomous flowering pathway as the *FCA, FPA, FVE, FY,* and *LD* genes (Lee and Amasino, 1995) and may interact with the *EMF* gene to promote vegetative development (Aukerman and Amasino, 1996, Koornneef and Peeters, 1997). Several different alleles of *FRI* have now been shown to be associated with the different flowering times observed amongst various ecotypes. *FRI* alleles from late-flowering ecotypes such as Stockholm and Pitztal for example, are thought to be fully functional and confer very late-flowering, while alleles of this locus in early-flowering ecotypes such as L. *erecta* are thought to be non-functional (Burn *et al.*, 1993b; Lee and Amasino, 1995; Gendall *et al.*, 1999). The expression of the *FRI*-conferred late-flowering phenotype is also believed to be reliant upon the presence of particular alleles of the semi-dominant locus, *FLC*. It is further postulated that it is the levels of the *FLC* transcript present in a plant which determines its flowering time phenotype and that the main role for *FRI* is in upregulating expression of *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Sheldon *et al.*, 2000). The expression

of the *FLC* gene has also been shown to be repressed by the activity of genes within the autonomous flowering pathway as well as following vernalisation treatments. Genes within the long-day dependant pathway however do not appear to influence *FLC* expression (Sheldon *et al.*, 1999). The differential expression of *FLC* in late and early ecotype is thought to be due to either sequence changes in the promoter region of this gene, or via differences in the regulation of this gene by different alleles of genes such as *FRI* or *FCA* (Sheldon *et al.*, 2000). The *FLC* gene is proposed to repress flowering by blocking the activity of, or response to, GA within the apex of the plant which delays the induction of GA regulated floral meristem identity genes (Sheldon *et al.*, 1999).

The severely decreased or completely absent response to a 21 day vernalisation treatment in the mutant lines, even in non-inductive conditions, suggests some disruption in a vernalisation-reliant pathway in these plants, a process with which both the *FRI* and *FLC* genes are believed to be intimately involved (Lee and Amasino, 1995; Sheldon *et al.*, 2000). The fact that the mutant lines flower comparatively early even in short days, compared to Pitztal, may help to explain the lack of a very strong vernalisation response in such plants, however several mutant lines showed no vernalisation response, and vernalised plants of some lines flowered later than unvernalised controls. The disrupted vernalisation response of the mutant lines does implicate *FLC* mutations in these lines, as levels of the *FLC* gene are proposed to be crucial in mediating a vernalisation response. The lack of a vernalisation response seen in the *fler* mutants may also be explained however if a decrease in the activity of the regulatory *FRI* gene occurs in ecotypes such as Pitztal following vernalisation, and that this results in the decreased *FLC* activity detected after such treatments (Sheldon *et al.*, 2000). A disruption in the normal function of the *FRI* gene in the *fler* mutants lines, may therefore result a constitutive decrease in the activity of *FLC* in the mutants, resulting in mutant plants that no longer require a vernalisation treatment for early-flowering. Strong evidence for a direct role of *FRI* in a vernalisation response has previously been provided following the introgression of 'late' *FRI* gene copies into the early-flowering ecotypes Columbia and L. *erecta*. Columbia plants, for example, which also contain an active copy of *FLC*, flowered extremely late and responded strongly to a 30 to 40 day vernalisation treatment with early-flowering, only after the introduction of an Sf-2 *FRI* gene copy (Lee and Amasino, 1995). Like the *flc* mutants that have thus far been identified (Sheldon *et al.*, 2000), the *fler* mutants did show some photoperiod response although this was less than that seen in wild-type Pitztal plants. Earlier flowering of the mutant lines in long day conditions would however still be expected if the mutants were affected in the *FRI*, as genes involved in the long day floral promotory pathway are thought to have little effect on both the *FLC* gene and *FRI* activity in wild-type Pitztal (Lee and Amasino, 1995; Sheldon *et al.*, 1999). It is also worth noting that the F2 progeny from crosses of the *fler* lines with wild-type Pitztal segregated in a more similar pattern to the F2 progeny from a cross of L. *erecta* with Pitztal

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than the F2 progeny from a cross of Columbia with Pitztal. This suggests that the *fler* lines may have a *FRI/FLC* genotype similar to that predicted for the *L. erecta* ecotype. The progeny of the F1 backcrosses to the mutant lines do not behave in a similar manner as would be predicted for a L. *erecta* genotype however, with a 1:1 ratio of delayed to early-flowering plants being detected rather than a 3:1 ratio of early- to late-flowering plants.

Analysis of the *fler* mutant lines has revealed that, in addition to the potential disruptions in the *FRI* or *FLC* loci, there are other genes segregating within these lines which are affecting the flowering time and morphology of the plants. The results indicate that interactions between a number of 'minor effect' flowering genes present in the mutant lines and other ecotypes can influence flowering time. For example, some mutant lines produced F1 plants when crossed to either wild-type Pitztal, Stockholm, or San-Feliu-2 that flowered considerably earlier or later than the late-flowering parental lines. At this stage it is unclear whether the alleles of such minor flowering-time genes, present in the *fler* mutant lines are interacting with the *FRI* or *FLC* gene products, or with other loci to affect flowering time. It also remains to be determined whether these minor effect genes are additional mutations to the major disruption in the *fler* lines or represent natural polymorphisms present within the Pitztal lines and in different *Arabidopsis* ecotype populations. Genes which are known to interact with *FRI* and have effects on flowering time, such as the *KRY* and *FKR* genes are possible candidates for the genes that influence the flowering time phenotype of the F1 and F2 plants from these late-ecotype and *fler* mutant line crosses (Clarke and Dean, 1994; Dennis *et al.*, 1996). The segregation of genes able to modify the *FRI /FLC* mediated late-flowering phenotype has also previously been observed by Burn *et al.* 1993(b) in crosses of Pitztal with L. *erecta* or Niederzenz that resulted in progeny that flowered earlier or later respectively than wild-type Pitztal. While the complementation analysis indicated that similar genes were mutated amongst the *fler* lines, the presence of intermediate flowering F1 plants also suggests the presence of genes, other than those primarily responsible for the mutant phenotype, and having slight effects on flowering time, are segregating in the *fler* mutant lines. The fact that such intermediate flowering plants were often only seen in one of the two reciprocal crosses for any given pair of *fler* lines, may also indicate that some of these 'minor effect' genes may be maternally inherited.

The major *fler* mutation may also potentially be allelic to mutations known to cause accelerated flowering in early-flowering ecotypes. The presence of a short day response in the *fler* mutants however, suggests that the mutation is probably not in the genes *EFS*, *ESD-2*, *ESD-3*, *ESD-4*, or *ELF-3* that are associated with the floral repression pathway acting in short days in early-flowering ecotypes. The presence of a distinct vegetative phase in the current mutant lines also indicates that these plants do not result from a disruption in the major floral repression gene *EMF*, as mutations at this locus result in photoperiod insensitive

plants that flower immediately following germination (Sung *et al.*, 1992). The *FRI* gene product has been proposed to act by mediating a promotion of the *EMF* gene in late-flowering ecotypes (Aukerman and Amasino, 1996, Koornneef and Peeters, 1997). It is therefore possible that the *fler* mutation may result in an absence of this *FRI*-mediated extension of *EMF* expression in the *fler* mutant plants. This could result in a decrease in activity of the *EMF* gene similar to that which would be expected in early-flowering ecotypes which are thought to contain very low amounts, or none, of the *FRI* gene product.

Like the *fler* mutants, other early-flowering mutants (*elg*, *elf-1*, *elf-2*, *tfl-1*, *eaf-1*, and *svp*) have been identified which exhibit some day-length response, and are thought to act in a constitutive floral repression process in early-flowering ecotypes. These mutants flower earlier than wild-type ecotype controls even in short day conditions, like the Pitztal mutant lines. However, the radiation-induced *fler* mutants do not display other characteristics of some of the mutants in this group. For example, none of the characteristics of *tfl* mutants, such as a reduction in plant height, disruption in floral organ number or organisation, or production of a terminal flower (Alvarez *et al.*, 1992) are seen in any of the *fler* mutant lines. In addition, while some *fler* mutants do show similar phenotypes to those displayed by the *elg* mutants created in the *ga-4* background, such as an elongated hypocotyls in white light, the increased petiole length associated with the *elg* mutants is not seen in these *fler* lines (Halliday *et al.*, 1996).

The recently identified early-flowering *pif* (*photoperiod insensitive flowering*)(*elf-3*) mutants, also show some curling of the leaf margins (Hicks *et al.*, 1996b), a phenotype similar to that seen in some of the *fler* mutant lines, however the increase in leaf and inflorescence size associated with *pif* plants was not seen in these *fler* mutant lines (Hicks *et al.*, 1996b). Another mutation conferring a similar leaf curling phenotype is the *curly leaf* (*clf*) mutation. The leaves of *clf* mutants are narrow and curl upwards around the longitudinal axis of the leaf (hyponasty). These mutants were also found to be early-flowering, particularly in short days (Goodrich *et al.*, 1997). The leaf curling observed in the *fler* mutant line 260, and to some extent in lines 250 and 300 however, tended to be curled down around the midrib and tips of the leaves. Furthermore, none of the floral abnormalities or homeotic transformations seen in *clf* mutants (Goodrich *et al.*, 1997) were observed in the *fler* mutant lines.

The pale green colour and curled leaves of some of the radiation-induced *fler* mutant lines are also reminiscent of some of the phenotypes of mutants disrupted in genes involved in gibberellin production or signal transduction. Gibberellin overexpression *spy* mutants, as well as the early-flowering *eaf-1* mutants, for example, demonstrate early-flowering and are pale green in colour (Jacobsen and Olszewski, 1993; Jacobsen *et al.*, 1996; Scott *et al.*,

1999). The *fler* lines displaying this lack of pigmentation (250, 260, and 300) however, do not show either the partial male sterility seen in the *spy* mutants, nor the elongated hypocotyl phenotype exhibited in both the *spy* and *eaf-1* mutants. Indeed, in contrast to these gibberellin overexpression mutants, mutant lines 250 and 300 display short hypocotyls. The pale-green phenotypes observed in some mutant lines may result from alterations in genes controlling chlorophyll production or function, that in turn may induce a stress-related early-flowering response. The probability of a disruption in chloroplast-related function which influences floral induction in some *fler* mutants, is further supported by the suggestion from the complementation analysis, that some lines contain maternally inherited genes that are influencing the flowering time of such plants. The mutant lines for which a strong maternal effect on flowering time is suggested (220 and 280) are not however those showing a visible reduction in pigmentation.

Interestingly, some characteristics of gibberellin-deficient mutants such as a decrease in fertility and viable seed production was noted in line 220. Both mutant lines 220 and 280 exhibit some other phenotypic characteristics similar to those seen in some gibberellin-deficient or -insensitive mutants, such as a compact rosette structure and a slightly darker green colour (Peng and Harbend, 1997; Cowling *et al.*, 1998). However, these mutants are late-flowering and show decreased apical dominance (Peng and Harbend, 1997; Cowling *et al.*, 1998). As the *FRI* and *FLC*-mediated floral repression system is postulated to involve a disruption in either the regulation of, or response to, gibberellins (Sheldon *et al.*, 2000), a disruption in this process in the *fler* mutants may account for the phenotypes observed in the mutants that are similar to those seen in plants with an alteration in their gibberellin regulated processes.

The two *fler* mutant lines (270 and 290) that show an elongated hypocotyl phenotype when compared to wild-type Pitztal, do not however exhibit a visible lack of chlorophyll, a phenotype also associated with early-flowering *phy-b* mutants, that are disrupted in the production of phytochrome and show an elongated hypocotyl phenotype (Halliday *et al.*, 1994; Whitelam and Devlin, 1997; Devlin *et al.*, 1998, Whitelam *et al.*, 1998). In addition, while the *fler* mutant lines do display delayed flowering in short days like the *phy-b* mutants, they do not exhibit the decreased rate of leaf production also shown by *phy-b* mutants (King and Bagnall, 1996). Phytochrome overexpression mutants also show some similarities to the *fler* mutant lines. *PHY-A* overexpression lines, for example, flower earlier than control plants, although these plants become day neutral (King and Bagnall, 1996; Whitelam *et al.*, 1998).

In contrast to the elongated hypocotyl phenotypes displayed by the phytochrome mutants, the *fler* mutant lines 250, 280, and 300 exhibited shorter hypocotyls in light-grown seedlings

and in one case (250) in dark-grown seedlings. This de-etiolated phenotype of mutant line 250 in dark grown plants is suggestive of the phenotypes conferred by mutations in the *COP*, *DET*, or *FUS* loci, which are involved with signal transduction of phytochrome-mediated responses. Mutations in these loci result in photomorphogenic development even in the absence of light. The det*1-1* mutants also display photoperiod insensitive flowering (Chory *et al.*, 1989; Deng *et al.*, 1992; Chory *et al.*, 1996; Pepper and Chory, 1997). While other aspects of development in constant dark conditions were not examined for mutant line 250, the short hypocotyl phenotype of light-grown seedlings of this line are not similar to *cop* or *fus* mutants which show little phenotypic alterations in the light (Deng *et al.*, 1991; Chory *et al.*, 1989; Chory *et al.*, 1992; Chory, 1996). *det1* mutants in contrast are smaller and paler than controls in white light (Chory *et al.*, 1989). The pale green colour of the some *fler* mutants lines did not however appear to be associated with smaller plants or exclusively to those exhibiting a short hypocotyl.

It has recently been demonstrated that the endogenous circadian clock may also be involved in the control of hypocotyl elongation of *Arabidopsis* (Dowsan-Day and Milar, 1999). Several mutants disrupted in the endogenous circadian rhythm have previously been described in *Arabidopsis*. Using automated video imaging Dowsan-Day and Milar (1999) were able to show that hypocotyl elongation was controlled by circadian rhythms, with a period of hypocotyl growth arrest detected at subjective dawn and a period of elongation at subjective dusk. The authors also demonstrate that the aberrant hypocotyl phenotype in several clock mutants may be a direct result of a disruption in the circadian clock rather than a simple pleiotrophic effect of the mutation (Dowsan-Day and Milar, 1999). One of the *Arabidopsis* clock mutants, *elf-3*, results in earlier flowering plants and displays an elongated hypocotyl phenotype and arrhythmic expression of several circadian clock regulated genes in light grown plants (Hicks *et al.*, 1996; Dowsan-Day and Milar, 1999). A possible explanation therefore for the short hypocotyl phenotype observed in some of our early-flowering *fler* mutants may be a disruption in the circadian rhythm of these plants that results in a disruption of the hypocotyl elongation period at the transition from light to dark and also has affects on flowering time. The absence of this short hypocotyl phenotype in dark-grown plants of two of the *fler* lines suggest that, like the *elf-3* mutants, detection of these circadian disruptions is dependant upon some light-induced signals in these mutants.

Despite the similarities the *fler* mutants have with some accelerated-flowering mutants isolated in early-flowering ecotypes, it remains most likely that the *fler* mutation represents a disruption of the major floral repression pathway, associated with the *FRI* and *FLC* genes, which operates in late-flowering ecotypes. Crosses of the *fler* mutant lines to the early-flowering ecotypes L. *erecta* and Columbia, whose differences in flowering time have been found to be primarily due to different *FLC* alleles (Sanda and Amasino, 1997; Ackerman and Amasino, 1996; Koornneef and Peeters, 1997), however suggest the *FLC* gene is not

disrupted in the mutant lines as no intermediate flowering plants were observed in the F1 progeny from the Columbia crosses. In addition, while the presence of intermediate flowering plants within the F2 population from crosses of the *fler* mutants to wild-type Pitztal suggest disruptions in a semi-dominant locus like *FLC*, the distribution of early to intermediate to late-flowering plants does not follow the expected 1:2:1 ratio. Instead the late-flowering phenotype of the F1 plants and the almost 3:1 ratio of delayed flowering to early-flowering plants observed in the F2 progeny from such crosses, support the hypothesis that the *FLER* gene, disrupted in the mutant lines, may be allelic to the *FRI* gene. Several other genes affecting either hormone or light perception, and/or their signal transduction may however also be disrupted in the radiation-induced mutant lines, either directly through the mutagenesis process, or indirectly, as a result of alterations in the *FRI* gene.

Chapter 4

Microsatellite mapping and genetic analysis of the mutation causal to the early-flowering phenotype of selected *fler* mutant lines.

4.1 Introduction

The construction of both physical and genetic marker maps of the *Arabidopsis* genome has facilitated the mapping of genes affecting numerous traits in this species. The markers used in these mapping techniques include RFLPs (Restriction Fragment Length polymorphisms), RAPDs (Random Amplified Polymorphic DNAs), or more recently SSLPs (Simple Sequence Length Polymorphisms). Microsatellites are a type of SSLP consisting of tandem repeats of one to two bases that are embedded in unique sequences. Microsatellites are thought to occur randomly, and quite abundantly throughout most eukaryotic genomes and have been found in a wide range of plants species including potato, corn and soybean (Hearn *et al.*, 1992; Bell and Ecker, 1994). The presence of unique sequences flanking the tandem repeats enables the length of such sequences to be rapidly assessed following PCR amplification from small amounts of tissue, and subsequent gel electrophoresis. This allows rapid genotyping of samples, in contrast to RFLP techniques which require larger scale DNA preparations and a hybridisation step (Bell and Ecker, 1994). Microsatellite markers have been found to exhibit more length variation amongst several different ecotypes of *Arabidopsis* than do RFLP markers. In humans these microsatellite polymorphism have been found to be highly stable and therefore useful for the genetic analysis of populations, allowing construction of high resolution maps to identify loci conferring susceptibly to various genetic diseases (Hearn *et al.*, 1992).

Markers such as RAPDs and CAPS (Co-dominant cleaved Amplified Polymorphism Sequences) also have several disadvantages compared to microsatellites. RAPD markers for example, are usually dominant and are therefore not as efficiently mapped as co-dominant markers in F2 populations, due to the decreased chance of detecting heterozygous recombinant genotypes (Reiter *et al.*, 1992). CAPS marker phenotypes are, like microsatellite markers, also analysed via PCR amplification. The generation of novel CAPS markers however requires the prior knowledge of the ecotype-specific sequence of the chromosomal region to which the gene is to be mapped, and the time-consuming process of generating an RFLP within that region (Konieczny and , 1993; Bell and Ecker, 1994).

In 1994, Bell and Ecker assigned 30 microsatellite markers to the five *Arabidopsis* chromosomes using the Lister and Dean recombinant inbred lines (Lister and Dean, 1993). They also described the length polymorphism obtained when each repeat region was amplified in six different early-flowering ecotypes. The majority of markers, particularly those containing GA repeat regions, were found to be highly polymorphic between all six ecotypes (Bell and Ecker, 1994).

Mapping of the *fler* mutation in the mutant lines to any chromosomal region, using the L. *erecta* and Columbia recombinant inbred (RI) lines, was not possible as this requires the prior isolation and cloning of the gene of interest in order to probe the various RI lines (Lister and Dean, 1993). In addition, as no RFLP maps of various late-flowering ecotypes exist, mapping of the mutation using RFLP techniques was also believed to be impractical. Using early-flowering ecotypes in a RFLP based mapping strategy was also not a feasible option as following the early-flowering mutant phenotype in the already early-flowering ecotype background is not possible. It was therefore decided to use some of the microsatellite marker regions described by Bell and Ecker to examine the linkage relationship between the mutated gene(s) that results in the early-flowering phenotype of the *fler* mutant lines, and chromosomal regions containing the flowering time genes *FRI* and *FLC*. As mentioned earlier, these two genes are believed to be the two dominant and semi-dominant loci that confer the late-flowering phenotype of ecotypes such as Pitztal, San-Feliu-2 and Stockholm (Lee *et al.*, 1993; Lee and Amasino, 1995; Sheldon *et al.*, 1999).

The first step in the microsatellite marker mapping exercise involved crossing the mutant lines with two other late-flowering ecotypes, Stockholm and San-Feliu-2. This would allow the early-flowering phenotype of the mutant lines to be recognised within the F2 progeny population, in order to establish linkage between this phenotype and the microsatellite regions examined. The initial microsatellite primers chosen were the nga249 primers that amplified a region on chromosome five closely linked to the *FLC* gene, and the nga8 and nga12 primers that amplified repeat regions on chromosome 4 near the *FRI* gene. The mapping technique also requires the presence of a length polymorphism for these microsatellites between the Pitztal mutant background and either the San-Feliu-2 or Stockholm ecotypes. Once such a variation is established the genotypes of both the late- and early-flowering F2 progeny from the relevant cross are analysed. If there is close linkage between a particular region and the mutation resulting in the early-flowering phenotype of the mutant lines, then all the early-flowering F2 progeny would be expected to show a Pitztal microsatellite marker genotype. If the mutation is completely unlinked to the *FRI* or *FLC* regions, then a random distribution of Pitztal and San-Feliu-2 or Stockholm genotypes would be expected in such plants. One further possibility is that the linkage of the mutation to the microsatellite marker is low enough so as to allow some recombination to occur. Early-flowering F2 plants that have a non Pitztal homozygous genotype (*ie.* heterozygous or

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homozygous for the San-Feliu-2 band), or late-flowering F2 progeny that have a Pitztal genotype, may represent these infrequently occurring recombinant plants.

In a separate attempt to more conclusively determine if the mutated gene within the mutant lines was the *FRI* gene, the mutant lines were crossed to L. *erecta* plants that contain an introgressed San-Feliu-2 ecotype version of the *FRI* gene. In 1994, Lee and Amasino reported on the creation of such lines, as well as L.*erecta* plants containing an introgressed copy of a San-Feliu-2 version of the *FLC* gene, or San-Feliu-2 versions of both the *FRI* and *FLC* genes. It had previously been reported that introduction of a late-flowering allele of the *FRI* gene into the Columbia ecotype resulted in late-flowering plants, however when such alleles were introduced into L. *erecta* plants little change in the flowering time phenotype was detected. This observation is believed to be due to the lack of *FLC* activity within the L. *erecta* ecotype (Sheldon *et al.*, 2000) that does not allow the late-flowering phenotype of the *FRI* allele to be expressed. The introgressed L. *erecta* lines confirmed this result, as only the L. *erecta* lines containing both *FRI* and *FLC* alleles from the late-flowering San-Feliu-2 ecotype exhibited an extreme delay in their flowering times (Lee *et al.*, 1994b; Lee and Amasino, 1995). These lines are useful for an analysis of the effects of the *FRI* and *FLC* gene as they should eliminate the presence of any other 'background' genes influencing the flowering time phenotype of late-flowering ecotypes. An analysis of the flowering times of the F1 and F2 progeny from a cross of the mutant lines to L. *erecta* (San-Feliu-2 *FRI*) plants was therefore performed to determine whether the *fler* early-flowering lines contained mutations in the *FRI* gene.

4.2 Materials and Methods

4.2.1 PCR directly from Arabidopsis plant tissue

This PCR method is based on that presented in Klimyuk *et al.* (1993). A small piece of plant tissue (a 5 mm-long root tip or a 2 mm^2 piece of leaf tissue) was placed in a sterile microcentrifuge tube containing 40 µl of 0.25 M NaOH (freshly diluted from a 10 M stock) and incubated in a boiling water bath for exactly 40 seconds. The pH of the sample was then neutralised by the addition of 40 µl of 0.25 M HCl and 20 µl of 0.5 M Tris-HCl (pH 8.0)/0.25% (v/v) Triton-X 100. Tubes were then boiled for an additional two minutes. The tissue samples could then be used for PCR immediately or stored at 4°C for several weeks. If samples were stored they were again incubated at 100°C for two minutes prior to use in the PCRs.

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4.2.2 Genomic DNA miniprep from Arabidopsis for PCR

This method is based on that by Edwards *et al.* (1991). Two rosette leaves (~0.5 cm in length) were ground in a microcentrifuge tube with a sterile plastic pestle for about 10 seconds. 400 μl of extraction buffer (200 mM Tris-HCl (pH 7.5); 250 mM NaCl; 25 mM EDTA; 0.5% (w/v) SDS) was then added and the sample was again ground briefly and vortexed for five seconds. This sample can be left at room temperature until all the remaining tissue samples are ground. All samples were then centrifuged at 14,000 rpm for one minute at room temperature. 300 μl of the supernatant was subsequently transferred to a clean microcentrifuge tube and 300 μl of isopropanol was added, the sample mixed, and left at room temperature for two minutes. The tubes were then centrifuged at 14,000 rpm for five minutes, the supernatant decanted, and the pellet left to air dry for 15-20 minutes, before the DNA was resuspended in 100 μl of TE buffer. Between 2-to-4 μl of the DNA suspension was used in subsequent PCR reactions.

4.2.3 Acrylamide gel electrophoresis for microsatellite marker analysis

The PCR samples amplified using the microsatellite marker primers were run on 8% denaturing polyacrylamide gels prepared in the following manner. The plates, spacers and combs to be used were thoroughly cleaned with 2% SDS and ethanol, dried, and assembled in the casting rack as described in the Biorad users manual. For an 8% gel the following components were added to a sterile 50 ml centrifuge tube; 4.2 g of urea, 2.7 ml of a 30% acrylamide solution (29% acrylamide and 1% N,N'-methylene-bisacrylamide) and 2 ml of 5 X TBE buffer. The final volume was adjusted to 10 ml with sterile distilled water, and the urea crystals were completely dissolved before 80 μl of 10% APS and 10 μl of TEMED solutions were added. The gel solution was then poured into the apparatus, the well comb placed between the plates and the gel was left to polymerise for 45 to 60 minutes. 8 μl of the PCR samples were mixed with 1.6 μl of loading dye (50% (w/v) sucrose; 50 mM EDTA; 0.1% (w/v) Bromophenol blue; 0.1% (w/v) Xylene cynol) and loaded on the gel.

After electrophoresis was complete, the gels were stained with silver stain by the following procedure. Gels were first washed in 10% ethanol for five minutes before being placed into a solution of 1% HNO_3. The gels were left in this solution for the amount of time required for the blue dyes in the loading buffer to turn yellow. The gels were then placed in a large volume (500 ml) of a 0.012 M $AgNO_3$ solution for 20 minutes with constant agitation. Following this incubation the gels were rinsed in sterile distilled water for two to three minutes to remove any excess silver. The gels were then gently agitated in developing solution (3% (w/v) Na_2CO_3; 0.06% formaldehyde; 2 μg/ml $Na_2S_2O_3.5H2O$) until the image reached the required intensity and immediately rinsed briefly in sterile distilled water followed by a five minute rinse in distilled water. The stain was fixed following a five

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minute wash in 10% acetic acid and a further brief rinse in sterile distilled water. Gels were then photographed immediately or left to dry overnight.

4.3 Results.

Microsatellite marker mapping was initiated using the nga12 markers that that map near the *FRI* gene on chromosome four, and the nga249 markers that map near the *FLC* gene on chromosome five. Several attempts were made to optimise PCR conditions by altering annealing temperature, $MgCl_2$ concentrations, and the method of genomic DNA template preparation. The banding pattern initially obtained with nga12 markers was too complex to be useful (Figure 4.1). A simpler banding pattern was produced following optimisation, however no consistent pattern could be obtained in reactions containing nga 12 forward and reverse primers (Figure 4.2). A single band was reproducibly obtained using nga249 markers (Figures 4.1 and 4.2). However, electrophoresis on high resolution gels of the PCR products obtained from the Pitztal, Stockholm, and San-Feliu-2 ecotypes using these markers revealed no detectable size difference. This indicated no polymorphism in this region exists between the three late-flowering ecotypes (Figure 4.3).
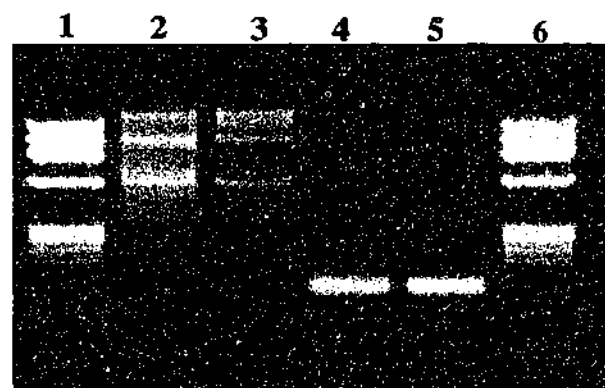
Figure 4.1

The PCR products from reactions containing either nga12 or nga249 microsatellite marker primers and 2μl of Pitztal tissue extract, analysed on a 4% agarose gel. Lanes 1 and 6 contain φX174 *Hae*III markers. Lanes 2 and 3 contain the products from reactions utilising the nga12 primers, while lanes 4 and 5 contain the products from reactions containing the nga249 primer pairs.
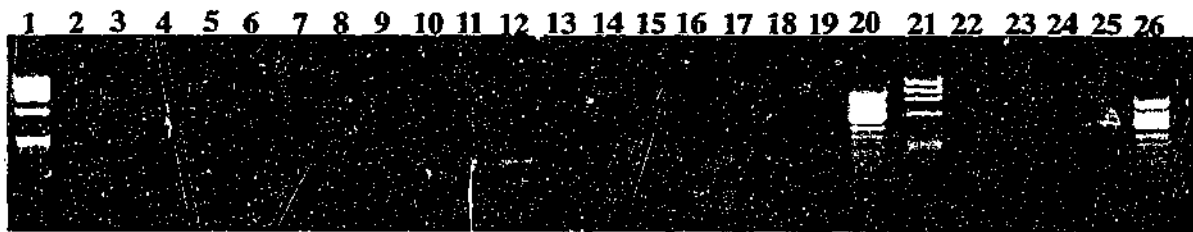
Figure 4.2

The PCR products of reactions containing either nga12 or nga249 microsatellite primers and DNA extracted from either Pitztal (Pi), Stockholm (St), San-Feliu-2 (Sf-2) ecotypes, or F1 plants of various mutant and late-ecotype crosses, were analysed on a 4% agarose gel.

Lane	DNA	Lane	DNA	Lane	DNA
1	φX174 HaeIII	10	50ng 280xSf-2 (nga12)	19	100ng 300x St F1 (nga 249)
2	50ng Pi (nga 12)	11	100ng 280xSf-2 (nga 12)	20	φX174 HaeIII
3	100ng Pi (nga 12)	12	50ng Pi (nga 249)	21	φX174 HaeIII
4	50ng St (nga 12)	13	100ng Pi (nga 249)	22	50 ng 280xSf-2 F1 (nga 249)
5	100ng St (nga 12)	14	50ng St (nga 249)	23	100ng 280xSf-2 F1 (nga 249)
6	50ng Sf-2 (nga 12)	15	100ng St (nga 249)	24	negative control (nga 12)
7	100ng Sf-2 (nga 12)	16	50ng Sf-2 (nga 249)	25	negative control (nga 249)
8	50ng 300xSt F1 (nga 12)	17	100ng Sf-2 (nga 249)	26	φX174 HaeIII
9	100ng 300xSt F1 (nga 12)	18	50ng 300xSt F1 (nga 249)		
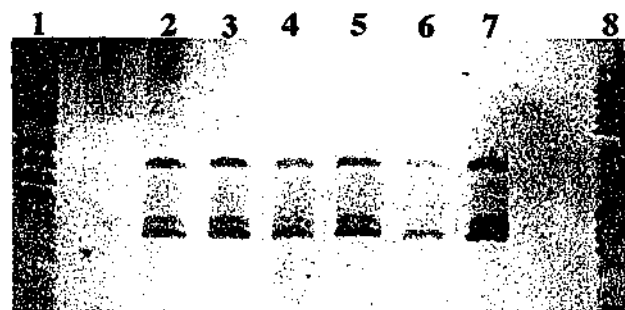
Figure 4.3

The PCR products amplified using microsatellite primers nga 249 were run on an 8% polyacrylamide gel. Lanes 1 and 8 contain φX174 HaeIII markers, lanes 2, 3 and 4 contain PCR products using 50 ng of Pitztal, Stockholm, or San-Feliu-2 template DNA while lanes 5, 6 and 7 contain 100ng of these templates respectively.

New microsatellite markers, nga8, that mapped to a very similar region as nga12 and *FRI* were used in an attempt to detect a polymorphism between the Pitztal and Stockholm and the San-Feliu-2 ecotypes in this region. After several trials, polymorphisms between the Pitztal ecotype and the Stockholm and San Feliu-2 ecotypes were detected in PCR samples utilising these nga8 microsatellite marker primers (Figure 4.4). The polymorphism between Pitztal and the San-Feliu-2 ecotypes appeared to be more distinguishable on the 4% agarose gels, over a range of salt concentrations, than that observed between the Pitztal and the Stockholm ecotypes. Before further analysis could be initiated however it was important to confirm that the nga8 primers would detect a Pitztal band in the mutant lines to be used. Therefore these primers were used to amplify bands from DNA extracted from wild-type Pitztal, Stockholm, San Feliu-2, Columbia and L. *erecta* ecotypes, as well as the nine radiation induced early-flowering lines. In order to also test the sensitivity of this method in detecting plants heterozygous for the Pitztal and San-Feliu-2 or Stockholm bands, reactions were performed that contained DNA extracted from either the F1 plants from a cross of mutant line 300 with Stockholm, or F1 plants from a cross of mutant line 280 with San-Feliu-2. Figure 4.5 displays the results of this analysis.
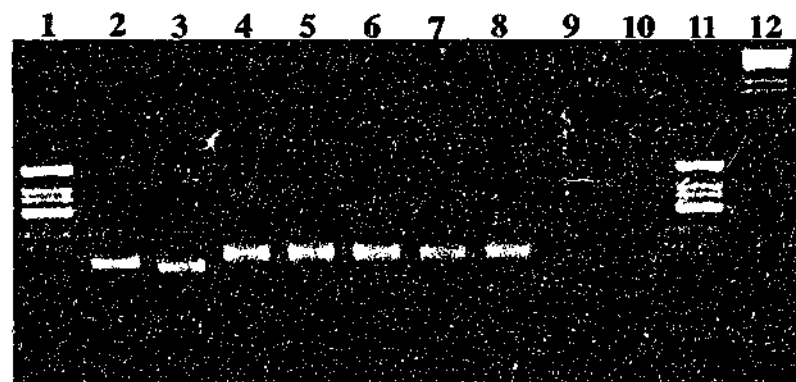
Figure 4.4

The PCR products amplified using the nga8 primers were run on a 4% agarose gel. Lanes 1 and 11 contain φX174 *Hinf*I markers and lane 12 contains λ *Bste*II markers. Lanes 2 and 3 contain 50ng of Pitztal and Stockholm template DNA respectively. Lanes 4, 5, and 6 contain PCR products obtained using 25 ng, 50 ng, and 75 ng of San-Feliu-2 template DNA respectively and 1.5 mM $MgCl_2$. Lanes 7, 8, and 9 contain PCR products obtained using 25 ng, 50 ng, and 75 ng of San-Feliu-2 template DNA respectively and 2 mM $MgCl_2$. Lane 10 contains a negative PCR control.

Whereas the polymorphism between Pitztal and Stockholm plants was difficult to distinguish, the products generated from Pitztal and San-Feliu-2 plants were quite distinct and a clear difference in the size of the two bands amplified in the F1 plants of the cross between mutant line 280 and San-Feliu-2 plants could be seen (lane 7). The intensity of the San-Feliu-2 band however, was less than that of the Pitztal fragment. Little difference was observed in the sizes of the bands produced in the heterozygous F1 plants of the mutant line 300 crossed to Stockholm plants (lane 6). The banding pattern of all mutant lines, except line 230, was similar to that of the Pitztal controls and different from those amplified from all other ecotypes examined. It is unclear as to why amplification of DNA extracted from mutant line 230 also contains a smaller band of a similar size to that seen in the Columbia ecotype. It is felt that this most likely resulted from contamination of the template DNA rather that a difference between the DNA sequence of this region in this line, compared to that detected in wild-type Pitztal plants.
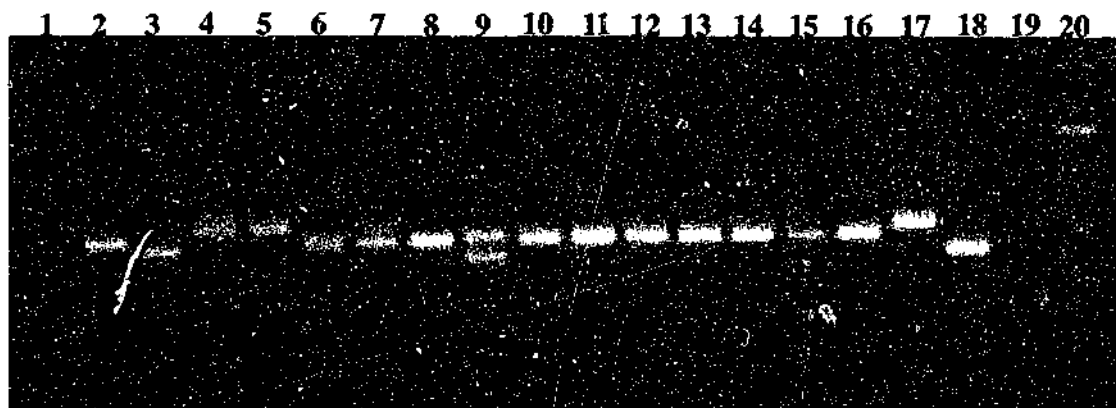
Figure 4.5

PCR products amplified using microsatellite primers nga8 were analysed on a 4% agarose gel. Lanes 1 and 20 contain ϕX174 *Hinfl* markers and lane 19 a negative PCR control. Lanes 2 and 3 contain Pitztal and Stockholm DNA templates respectively, while lanes 4 and 5 contain San-Feliu-2 DNA. Lanes 6 contains DNA extracted from F1 plants of a cross of mutant line 300 to Stockholm plants, and lane 7 contains DNA from the F1 progeny of a cross of the *fler* line 280 with San-Feliu-2. Lanes 8 to 16 inclusive contain DNA from the nine mutant lines 220 to 300 and lanes 17 and 18 contain DNA from either L. *erecta* or Columbia ecotype plants. Lane 19 contains a negative PCR control.

Microsatellite marker mapping in the region near the FRI gene

In order to test for linkage between the *fler* mutation and the *FRI* gene, *fler* mutant lines were crossed with the late-flowering ecotypes and DNA extracted from the F2 progeny for the analysis. The initial mapping experiments concentrated on a cross of the mutant line (300) to the San-Feliu-2 ecotype. DNA was extracted from a small piece of leaf tissue of 113 F2 progeny plants, about 28 of which would be predicted to be early-flowering. The F2 plants were left to grow following the removal of the tissue required for the DNA extraction, in order for their flowering times and leaf number at the time of flowering to be recorded. PCR amplifications using the nga8 primers were then performed on the purified DNA samples. No amplification could be obtained from 2 of these 113 samples. The results of this analysis are displayed in Figures 4.6(A to I). A summary of the rosette leaf number phenotypes of the F2 population as well as their corresponding microsatellite marker genotypes is provided in Figure 4.7. It was noted that the F2 progeny segregated in an approximately 3:1 ratio of delayed-flowering to early-flowering plants, with 88 plants flowering with greater than 10 rosette leaves and 25 flowering with lower rosette leaf numbers. All of these early-flowering F2 plants displayed a Pitztal banding pattern following microsatellite marker analysis. All three microsatellite genotypes were seen amongst the late flowering F2 progeny however, with 24% displaying a Pitztal homozygote pattern rather than the predicted San-Feliu-2 homozygous or an heterozygous banding patterns. This mapping analysis was subsequently performed on a sample of 31 F2 plants from a cross of mutant line 280 and the San-Feliu-2 ecotype. A very similar distribution of genotypes to those found from the mutant line 300 cross was observed amongst these F2 plants (Figure 4.8). Again all early-flowering plants exhibited a Pitztal homozygote pattern and later flowering plants showed all three microsatellite marker genotypes.

The absence of any Sf-2 homozygous genotypes amongst the early-flowering F2 progeny from both crosses does suggest linkage between the mutation resulting in the early-flowering phenotype and the region amplified by the nga8 microsatellite marker primers. While a significant number of Pitztal homozygotes were observed within the late-flowering progeny, the allele frequency indicates some limitations in the ability of this technique to detect the difference between plants that are heterozygous in the nga8 region and those homozygous for the Pitztal banding pattern with preferential amplification of the Pitztal sequence occurring in heterozygous plants. This is supported by the observation that while approximately 25% (26/111 for the 300 line cross and 6/31 for the 280 cross) of F2 plants displayed a San-Feliu-2 homozygous pattern, greater than 25% (47% and 54% respectively for the 300 or 280 mutant crosses) showed a Pitztal homozygous pattern, and less than 50% (30% and 26% respectively for the 300 and 280 mutant crosses) displayed a heterozygous pattern, indicating many of the heterozygous plants were being scored as Pitztal homozygotes. Indeed it can be seen that in even in several of the plants that do show an heterozygous genotype that the San-Feliu-2 band appears to be much weaker in intensity.

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Figure 4.6

The *fler* mutant line 300 was crossed to San-Feliu-2 plants and DNA was extracted from the F2 progeny. nga8 microsatellite marker primers were then used in a PCR amplification. DNA from wild-type Pitztal, San-Feliu-2, Columbia, L. *erecta*, and mutant line 300 was also amplified using these primers. All reactions were run on 4% agarose gels to determine the sizes of the products. The phenotypes and microsatellite genotypes of the samples are shown in the legend below each figure. FT refers to flowering time and LN to the rosette leaf number at the time of flowering.
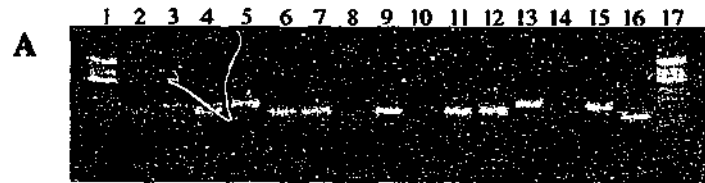
Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinfl* markers	--	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 19	42	80	Sf-2 homozygote
6	300 x Sf-2 F2 plant 52	48	65	Pi homozygote
7	300 x Sf-2 F2 plant 37	34	38	Pi homozygote
8	300 x Sf-2 F2 plant 36	21	8	Pi homozygote
9	300 x Sf-2 F2 plant 13	21	8	Pi homozygote
10	300 x Sf-2 F2 plant 6	21	8	Pi homozygote
11	300 x Sf-2 F2 plant 10	19	6	Pi homozygote
12	300 x Sf-2 F2 plant 41	23	8	Pi homozygote
13	300 x Sf-2 F2 plant 38	58	98	Sf-2 homozygote
14	Negative control	--	--	--
15	wild-type L. *erecta* (Ler)	22	6	Ler. homozygote
16	wild-type Columbia (Col)	25	8	Col. homozygote
17	φX174 *Hinfl* markers	--	--	--

Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinfl* markers	--	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 10	19	6	Pi homozygote
6	300 x Sf-2 F2 plant 36	21	8	Pi homozygote
7	300 x Sf-2 F2 plant 13	21	8	Pi homozygote
8	300 x Sf-2 F2 plant 6	21	8	Pi homozygote
9	300 x Sf-2 F2 plant 41	21	8	Pi homozygote
10	300 x Sf-2 F2 plant 44	26	9	Pi homozygote
11	300 x Sf-2 F2 plant 51	74	98	Sf-2 homozygote
12	300 x Sf-2 F2 plant 53	26	12	Pi homozygote
13	300 x Sf-2 F2 plant 42	68	156	Sf-2 homozygote
14	300 x Sf-2 F2 plant 46	28	16	Heterozygote
15	φX174 *Hinfl* markers	--	--	--
16	φX174 *Hinfl* markers	--	--	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant 43	53	92	Pi homozygote
21	300 x Sf-2 F2 plant 33	48	70	Sf-2 homozygote
22	300 x Sf-2 F2 plant 31	39	25	Pi homozygote
23	300 x Sf-2 F2 plant 32	68	96	Sf-2 homozygote
24	300 x Sf-2 F2 plant 26	80	72	Heterozygote
25	wild-type L. *erecta* (Ler)	22	6	Ler. homozygote
26	wild-type Columbia (Col)	25	8	Col. homozygote
27	Negative control	--	--	--
28	φX174 *Hinfl* markers	--	--	--
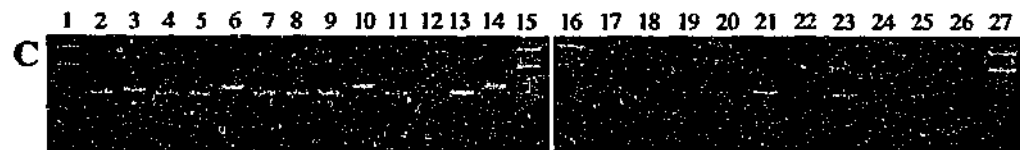
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Figure 4.6 cont.

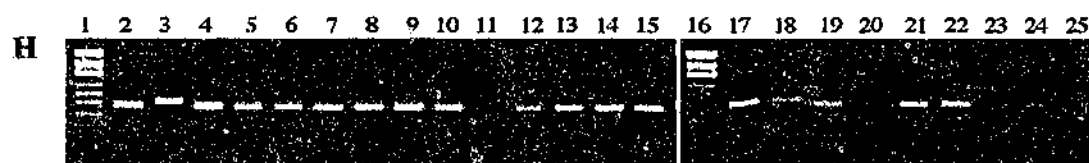| Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinfl* markers	–	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 28	34	44	Heterozygote
6	300 x Sf-2 F2 plant 45	54	75	Sf-2 homozygote
7	300 x Sf-2 F2 plant 39	28	11	Pi homozygote
8	300 x Sf-2 F2 plant 22	30	12	Pi homozygote
9	300 x Sf-2 F2 plant 40	48	61	Pi homozygote
10	300 x Sf-2 F2 plant 35	65	56	Sf-2 homozygote
11	300 x Sf-2 F2 plant 29	48	82	Pi homozygote
12	300 x Sf-2 F2 plant 5	50	65	Sf-2 homozygote
13	300 x Sf-2 F2 plant 1	45	38	Pi homozygote
14	300 x Sf-2 F2 plant 2	61	60	Sf-2 homozygote
15	φX174 *Hinfl* markers	--	--	–
16	φX174 *Hinfl* markers	–	--	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant 8	61	97	Pi homozygote
21	300 x Sf-2 F2 plant 4	68	96	Heterozygote
22	300 x Sf-2 F2 plant 12	59	82	Sf-2 homozygote
23	300 x Sf-2 F2 plant 7	42	50	Pi homozygote
24	300 x Sf-2 F2 plant 15	59	80	Heterozygote
25	300 x Sf-2 F2 plant 14	42	35	Pi homozygote
26	Negative control	--	--	--
27	φX174 *Hinfl* markers	–	--	--

Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinfl* markers	–	--	–
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 43	53	92	Pi homozygote
6	300 x Sf-2 F2 plant 33	48	70	Sf-2 homozygote
7	300 x Sf-2 F2 plant 31	49	25	Pi homozygote
8	300 x Sf-2 F2 plant 32	68	96	Sf-2 homozygote
9	300 x Sf-2 F2 plant 14	42	35	Pi homozygote
10	300 x Sf-2 F2 plant 7	42	50	Pi homozygote
11	300 x Sf-2 F2 plant 13	21	8	Pi homozygote
12	300 x Sf-2 F2 plant 12	42	35	Sf-2 homozygote
13	300 x Sf-2 F2 plant 15	59	80	Heterozygote
14	300 x Sf-2 F2 plant 18	54	72	Heterozygote
15	φX174 *Hinfl* markers	–	--	--
16	φX174 *Hinfl* markers	--	--	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant 20	68	69	Heterozygote
21	300 x Sf-2 F2 plant 11	61	130	Sf-2 homozygote
22	300 x Sf-2 F2 plant 73	69	89	Pi homozygote
23	300 x Sf-2 F2 plant 74	69	82	Sf-2 homozygote
24	300 x Sf-2 F2 plant 60	71	80	Sf-2 homozygote
25	300 x Sf-2 F2 plant 58	34	25	Sf-2 homozygote
26	Negative control	--	--	--
27	φX174 *Hinfl* markers	--	--	--

Figure 4.6 cont.

E

Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinf*I	--	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 16	21	9	Pi homozygote
6	300 x Sf-2 F2 plant 19	72	80	Sf-2 homozygote
7	300 x Sf-2 F2 plant 52	78	65	Pi homozygote
8	300 x Sf-2 F2 plant 37	34	12	Pi homozygote
9	300 x Sf-2 F2 plant 36	21	8	Pi homozygote
10	300 x Sf-2 F2 plant 38	58	98	Sf-2 homozygote
11	300 x Sf-2 F2 plant 3	27	10	Pi homozygote
12	300 x Sf-2 F2 plant 51	74	98	Sf-2 homozygote
13	300 x Sf-2 F2 plant 41	22	8	Pi homozygote
14	300 x Sf-2 F2 plant 53	25	12	Pi homozygote
15	φX174 *Hinf*I	--	--	--
16	φX174 *Hinf*I	--	--	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant 42	78	156	Sf-2 homozygote
21	300 x Sf-2 F2 plant 44	25	9	Pi homozygote
22	300 x Sf-2 F2 plant 61	30	11	Pi homozygote
23	300 x Sf-2 F2 plant 49	66	156	Heterozygote
24	300 x Sf-2 F2 plant 25	26	9	Pi homozygote
25	300 x Sf-2 F2 plant 68	66	120	Sf-2 homozygote
26	Negative control	--	--	--
27	φX174 *Hinf*I	--	--	--
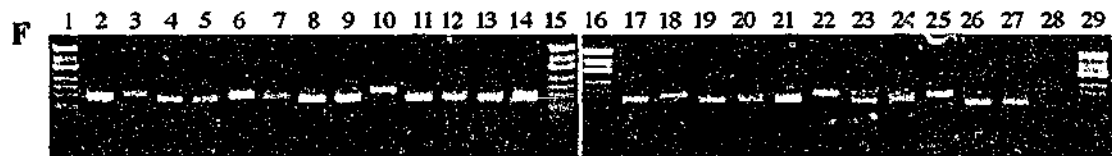
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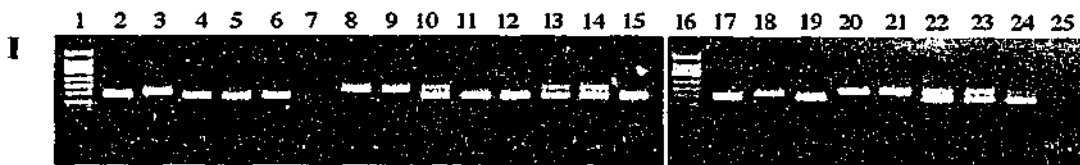| Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinf*I	--	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 24	24	6	Pi homozygote
6	300 x Sf-2 F2 plant 80	77	153	Heterozygote
7	300 x Sf-2 F2 plant 64	24	7	Pi homozygote
8	300 x Sf-2 F2 plant 76	61	82	Heterozygote
9	300 x Sf-2 F2 plant 78	58	62	Heterozygote
10	300 x Sf-2 F2 plant 62	42	40	Sf-2 homozygote
11	300 x Sf-2 F2 plant 79	34	12	Pi homozygote
12	300 x Sf-2 F2 plant 66	59	80	Pi homozygote
13	300 x Sf-2 F2 plant 17	20	6	Pi homozygote
14	300 x Sf-2 F2 plant 71	45	48	Heterozygote
15	φX174 *Hinf*I	--	--	--
16	φX174 *Hinf*I	--	--	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant 57	34	40	Pi homozygote
21	300 x Sf-2 F2 plant 63	26	10	Pi homozygote
22	300 x Sf-2 F2 plant 77	59	63	Sf-2 homozygote
23	300 x Sf-2 F2 plant 67	61	98	Heterozygote
24	300 x Sf-2 F2 plant 9	61	98	Heterozygote
25	300 x Sf-2 F2 plant 65	34	19	Sf-2 homozygote
26	300 x Sf-2 F2 plant 23	28	12	Pi homozygote
27	300 x Sf-2 F2 plant 48	59	86	Heterozygote
28	Negative control	--	--	
29	φX174 *Hinf*I	--	--	--

Figure 4.6 cont.
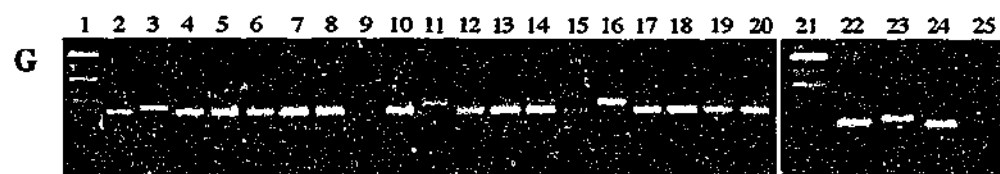
Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinfl*	--	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 45A	41	55	Heterozygote
6	300 x Sf-2 F2 plant 40A	22	6	Pi homozygote
7	300 x Sf-2 F2 plant 54A	39	22	Pi homozygote
8	300 x Sf-2 F2 plant 1A	29	9	Pi homozygote
9	300 x Sf-2 F2 plant 51A	78	60	Sf-2 homozygote
10	300 x Sf-2 F2 plant 4A	51	38	Heterozygote
11	300 x Sf-2 F2 plant 43A	73	120	Sf-2 homozygote
12	300 x Sf-2 F2 plant 52A	33	25	Pi homozygote
13	300 x Sf-2 F2 plant 59A	31	12	Heterozygote
14	300 x Sf-2 F2 plant 63A	40	45	Heterozygote
15	300 x Sf-2 F2 plant 49A	22	6	Pi homozygote
16	300 x Sf-2 F2 plant 46A	43	26	Sf-2 homozygote
17	300 x Sf-2 F2 plant 2A	40	21	Heterozygote
18	300 x Sf-2 F2 plant 75A	20	8	Pi homozygote
19	300 x Sf-2 F2 plant 6A	29	12	Heterozygote
20	300 x Sf-2 F2 plant 48A	29	12	Heterozygote
21	φX174 *Hinfl*	--	--	--
22	wild-type Pitztal (Pi)	96	88	Pi homozygote
23	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
24	Mutant line 300	26	9	Pi homozygote
25	Negative control	--	--	--

Lane no:	Sample	FT(days)	LN	Genotype
1	φX174 *Hinfl*	--	--	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 55A	69	62	Pi homozygote
6	300 x Sf-2 F2 plant 64A	37	12	Pi homozygote
7	300 x Sf-2 F2 plant 57A	22	6	Pi homozygote
8	300 x Sf-2 F2 plant 14A	44	53	Heterozygote
9	300 x Sf-2 F2 plant 19A	47	63	Heterozygote
10	300 x Sf-2 F2 plant 17A	27	6	Pi homozygote
11	300 x Sf-2 F2 plant 27A	65	50	--
12	300 x Sf-2 F2 plant 65A	44	58	Pi homozygote
13	300 x Sf-2 F2 plant 33A	44	62	Pi homozygote
14	300 x Sf-2 F2 plant 38A	47	43	Heterozygote
15	300 x Sf-2 F2 plant 53A	31	10	Pi homozygote
16	φX174 *Hinfl*	--	--	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant 25A	28	6	Pi homozygote
21	300 x Sf-2 F2 plant 32A	57	91	Heterozygote
22	300 x Sf-2 F2 plant 7A	39	35	Heterozygote
23	300 x Sf-2 F2 plant 26A	72	78	Pi homozygote
24	300 x Sf-2 F2 plant 8A	30	12	Pi homozygote
25	Negative control	--	--	--

Figure 4.6 cont.

| Lane no:	Sample	FT (days)	LN	Genotype
1	φX174 *Hinfl*	--	–	--
2	wild-type Pitztal (Pi)	96	88	Pi homozygote
3	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
4	Mutant line 300	26	9	Pi homozygote
5	300 x Sf-2 F2 plant 30A	23	7	Pi homozygote
6	300 x Sf-2 F2 plant 37A	27	6	Pi homozygote
7	300 x Sf-2 F2 plant 47A	40	30	--
8	300 x Sf-2 F2 plant 34A	72	92	Sf-2 homozygote
9	300 x Sf-2 F2 plant G	50	48	Sf-2 homozygote
10	300 x Sf-2 F2 plant Q	56	64	Heterozygote
11	300 x Sf-2 F2 plant 36A	39	33	Pi homozygote
12	300 x Sf-2 F2 plant 13A	25	5	Pi homozygote
13	300 x Sf-2 F2 plant C	33	30	Heterozygote
14	300 x Sf-2 F2 plant J	56	83	Heterozygote
15	300 x Sf-2 F2 plant A	23	7	Pi homozygote
16	φX174 *Hinfl*	--	–	--
17	wild-type Pitztal (Pi)	96	88	Pi homozygote
18	wild-type San-Feliu-2 (Sf-2)	66	53	Sf-2 homozygote
19	Mutant line 300	26	9	Pi homozygote
20	300 x Sf-2 F2 plant P	45	28	Sf-2 homozygote
21	300 x Sf-2 F2 plant 35A	57	83	Sf-2 homozygote
22	300 x Sf-2 F2 plant 3A	53	88	Heterozygote
23	300 x Sf-2 F2 plant 50A	40	29	Heterozygote
24	300 x Sf-2 F2 plant 23A	51	33	Heterozygote
25	Negative control	--	--	–
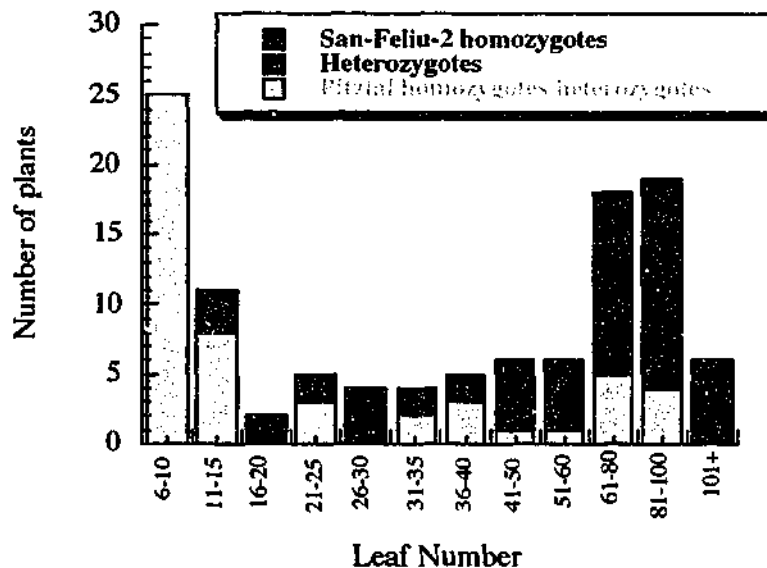
Figure 4.7

Histogram displaying the number of F2 progeny from a cross between the *fler* mutant line 300 and the San-Feliu-2 ecotype that exhibit the different nga8 microsatellite marker genotypes, plotted against the corresponding leaf number phenotypes.
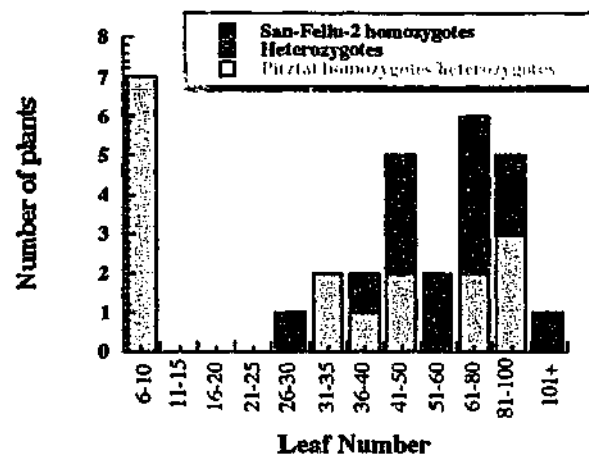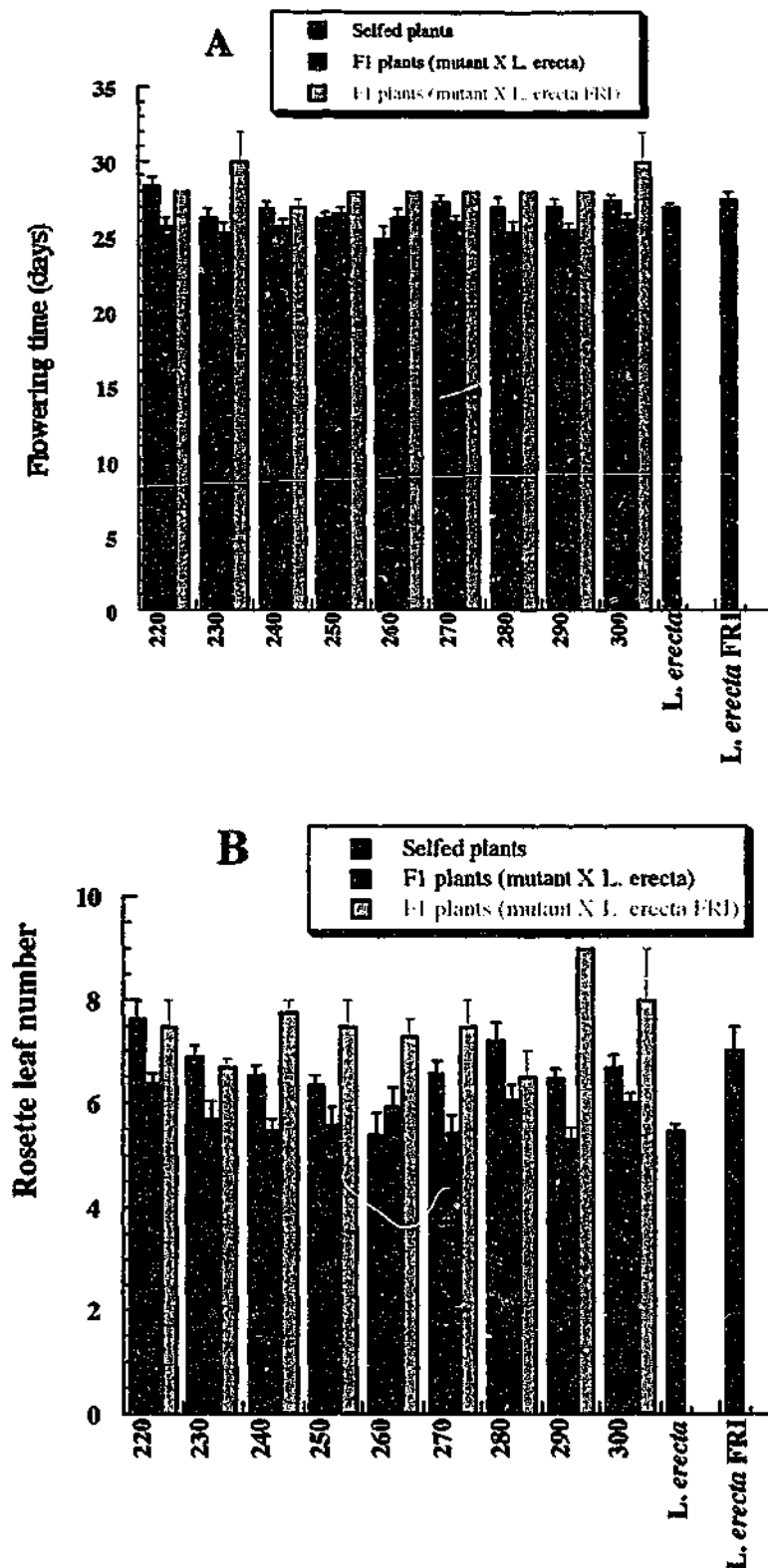
Figure 4.8

The number of F2 progeny from the mutant line 280 and San Feliu-2 ecotype cross exhibiting the different

nga8 microsatellite marker genotypes plotted against the corresponding leaf number phenotypes.

Introduction of the San-Feliu-2 FRI gene into the early-flowering mutant lines


As the microsatellite marker analysis suggested that the mutation resulting in the early-flowering phenotype may be linked to a chromosomal region near the *FRI* gene it was decided to determine if the mutant lines were defective in the *FRI* gene by crossing the *fler* lines to L. *erecta* plants containing an introgressed San-Feliu-2 *FRI* allele (Lee *et al.*, 1994b). This L. *erecta* (*FRI*) line flowers only slightly later, and with a marginally broader range than wild-type L. *erecta* plants. The early-flowering phenotype of any *fler* mutant lines containing a mutation within the *FRI* gene would be complemented following the introduction of the San-Feliu-2 version of the *FRI* gene via these crosses. Complementation of an *FRI* mutation would be expected to delay flowering significantly and result in plants flowering at intermediate times due to the presence of a functional Pitztal version of the *FLC* gene within the F1 progeny. The F2 progeny would then be expected to contain both early, intermediate and a late-flowering plants in an approximately 7:6:3 ratio.

The flowering time and rosette leaf number at the time of flowering of the F1 and F2 plants from crosses of the nine *fler* mutants with the L. *erecta* (*FRI*) line were recorded (Figures 4.9, 4.10, and 4.11). The F1 plants from these crosses all flowered early, at very similar times to the L. *erecta* (*FRI*) controls and only slightly later than the selfed mutant line plants or F1 plants from a cross of the mutant lines to wild-type L. *erecta* (Figure 4.9). The F2 progeny however, contained several intermediate flowering and late-flowering plants (Figures 4.10 and 4.11). In addition, none of these F2 populations segregated in a 7:6:3 ratio of early to intermediate to late-flowering plants (p≤ 0.05) with many more early-flowering plants being observed than expected. As a control the rosette leaf number at the time of flowering of the F2 progeny from crosses of the *fler* lines with L. *erecta* were also recorded (Figure 4.12). Interestingly, several intermediate flowering plants and some late-flowering plants were also observed amongst these plants.
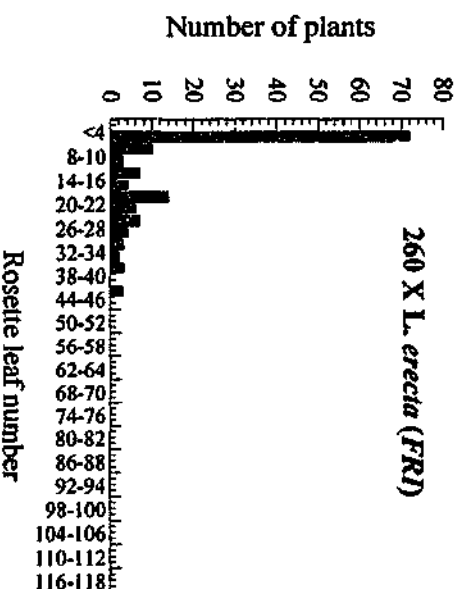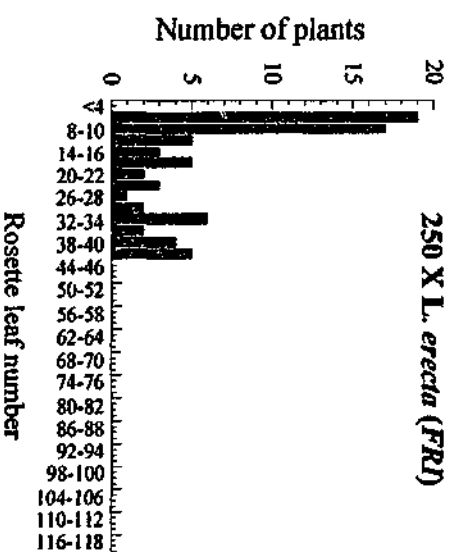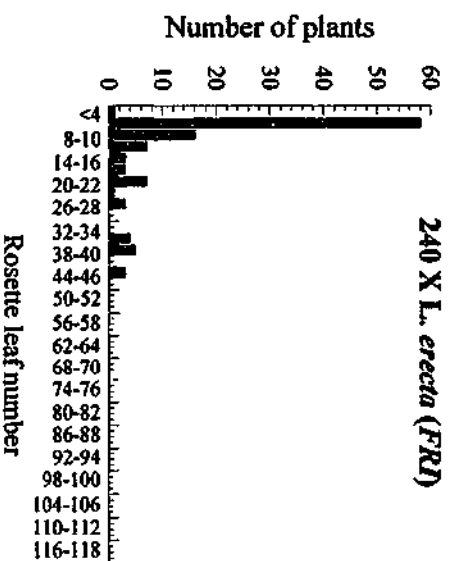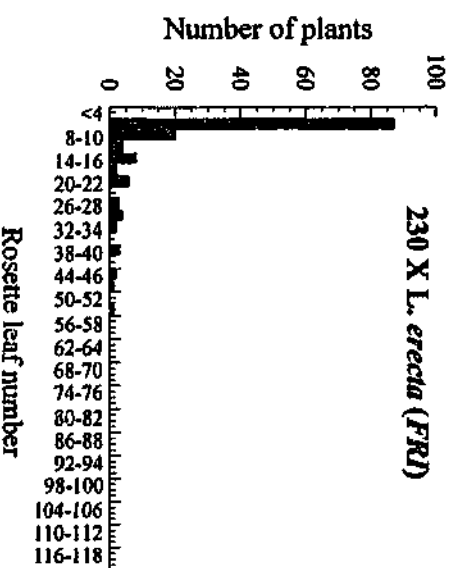
Figure 4.9

The flowering times (A), and rosette leaf number at the time of flowering (B), of the F1 plants from a cross of the *fler* lines to L. *erecta* plants containing a San-Feliu-2 allele of the *FRI* gene was compared to those of the F1 plants of *fler* lines crossed to L. *erecta* plants, or to selfed mutant line plants.
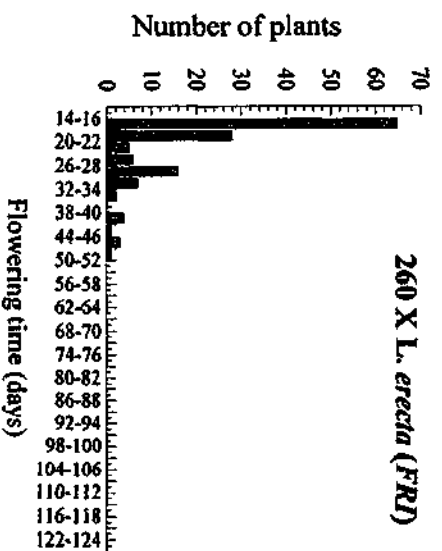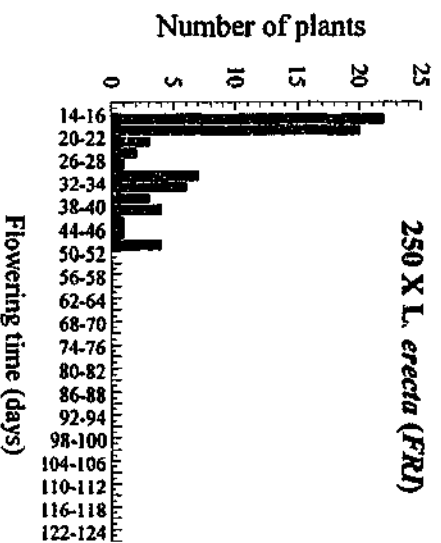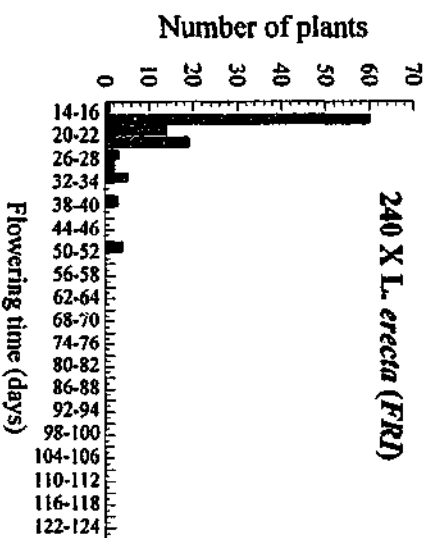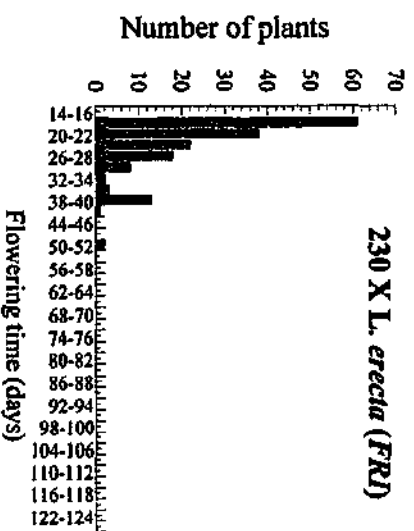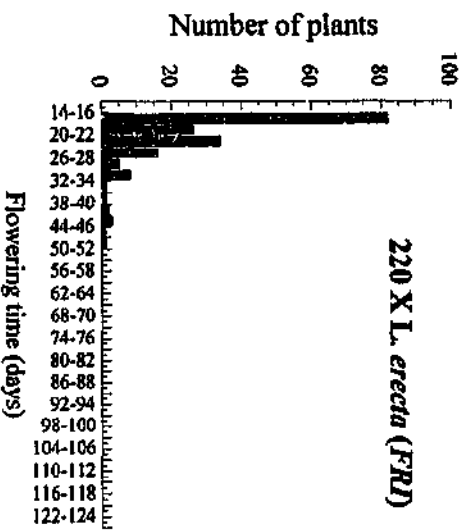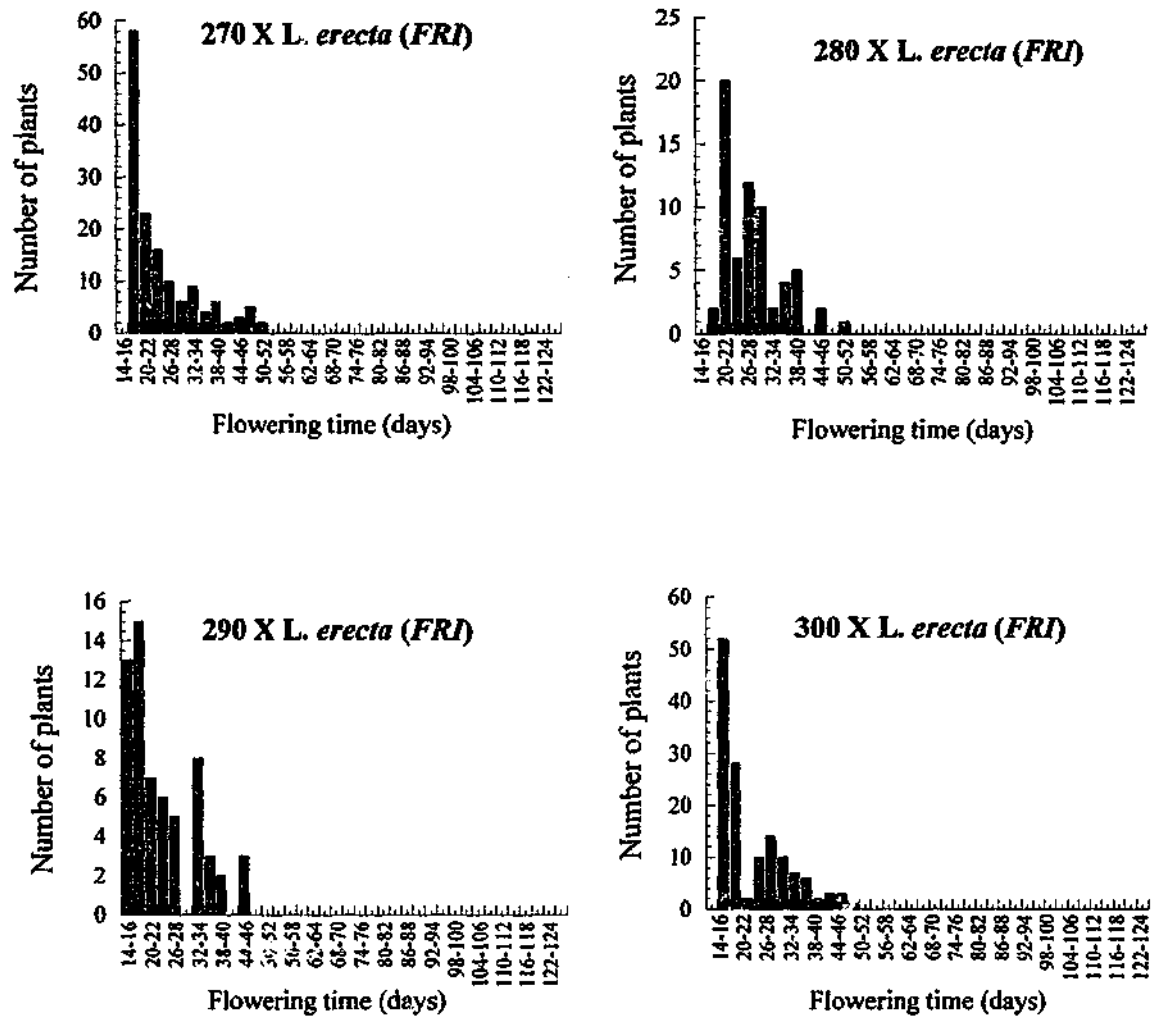
Figure 4.10

The flowering time of the F2 progeny from the *fler* mutant lines crossed with the L. *erecta* line containing an introgressed San-Feliu-2 allele of the *FRI* gene.

240 X L. erecta (FRI)

220 X L. erecta (FRI)

250 X L. erecta (FRI)

230 X L. erecta (FRI)

260 X L. erecta (FRI)

Number of plants

Rosette leaf number
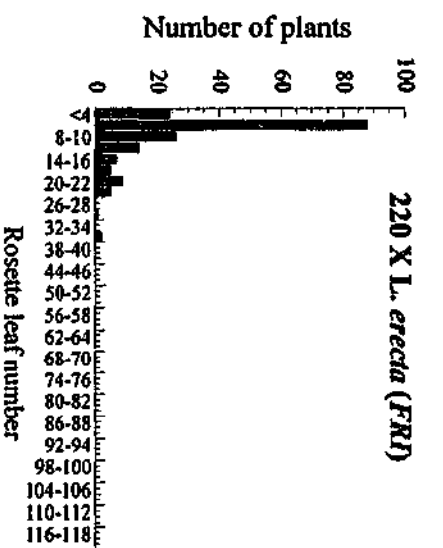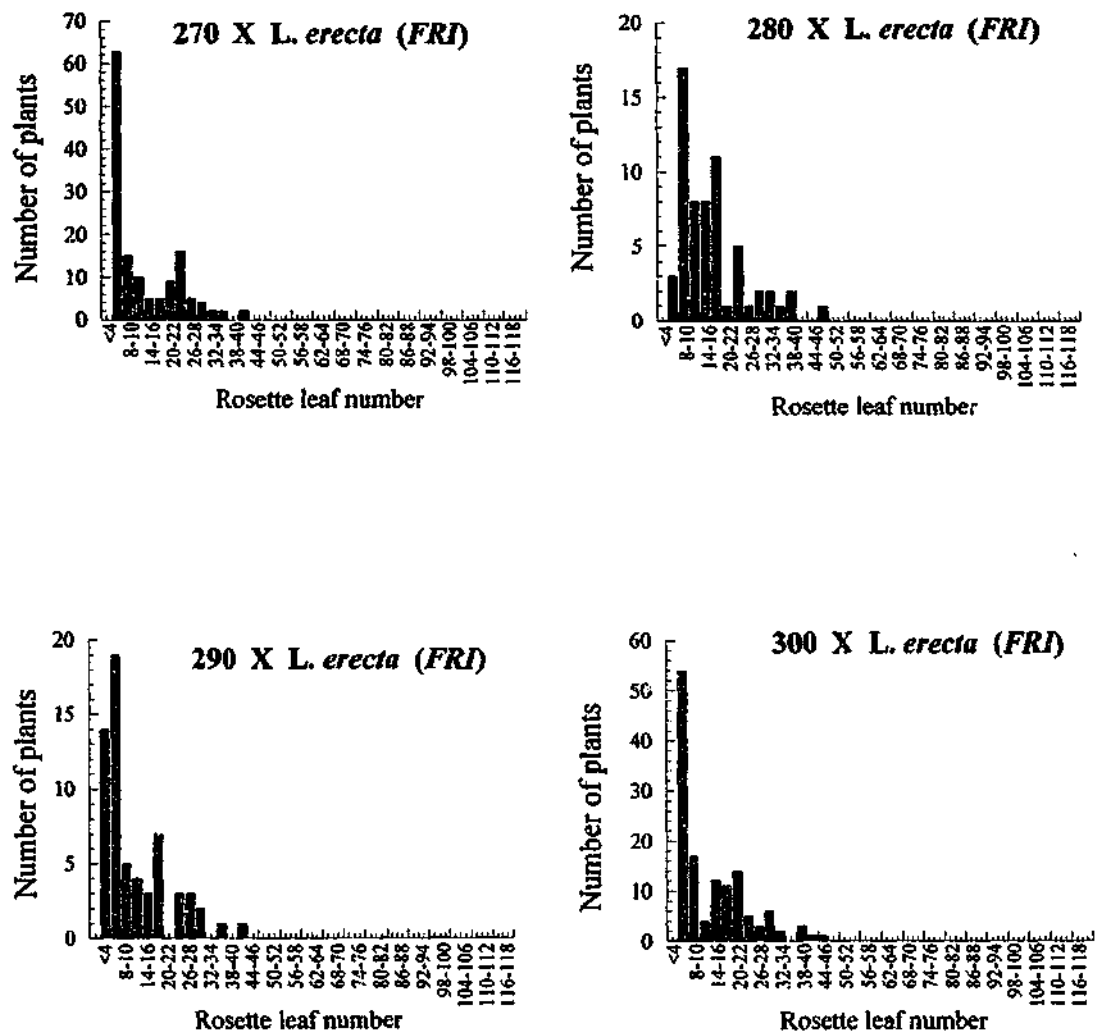
Figure 4.11

The rosette leaf number at the time of flowering of the F2 progeny from the *fler* mutant lines crossed with the
L. *erecta* line containing an introgressed San-Feliu-2 allele of the *FRI* gene.

Number of plants

240 X L. erecta

Rosette leaf number

Number of plants

220 X L. erecta

Rosette leaf number

Number of plants

260 X L. erecta

Rosette leaf number

Number of plants

250 X L. erecta

Rosette leaf number

Number of plants

230 X L. erecta

Rosette leaf number
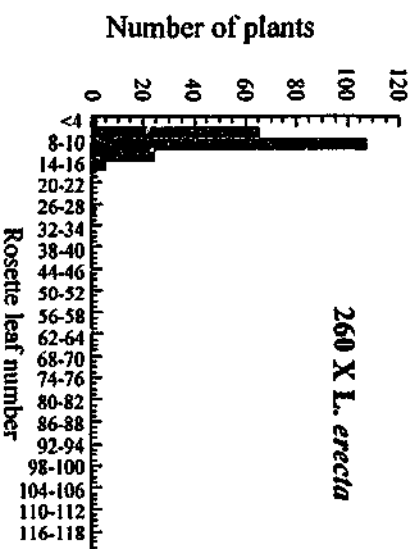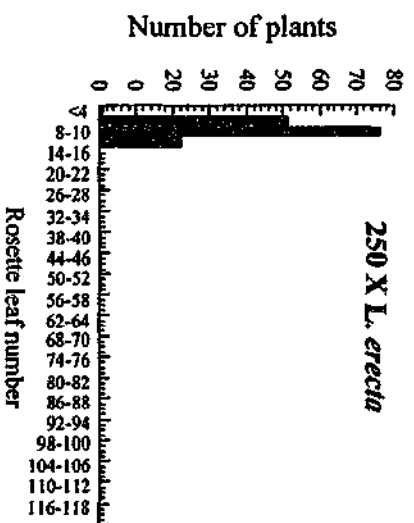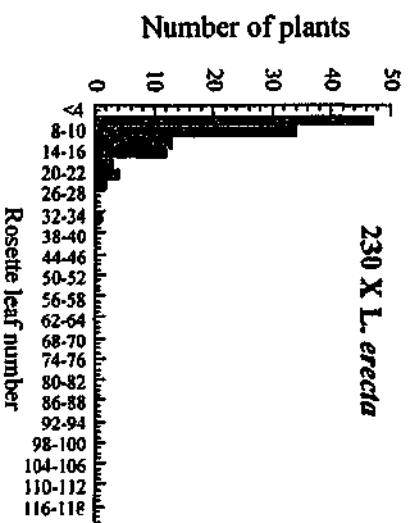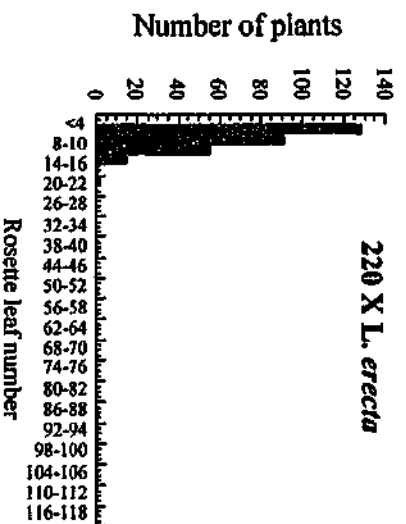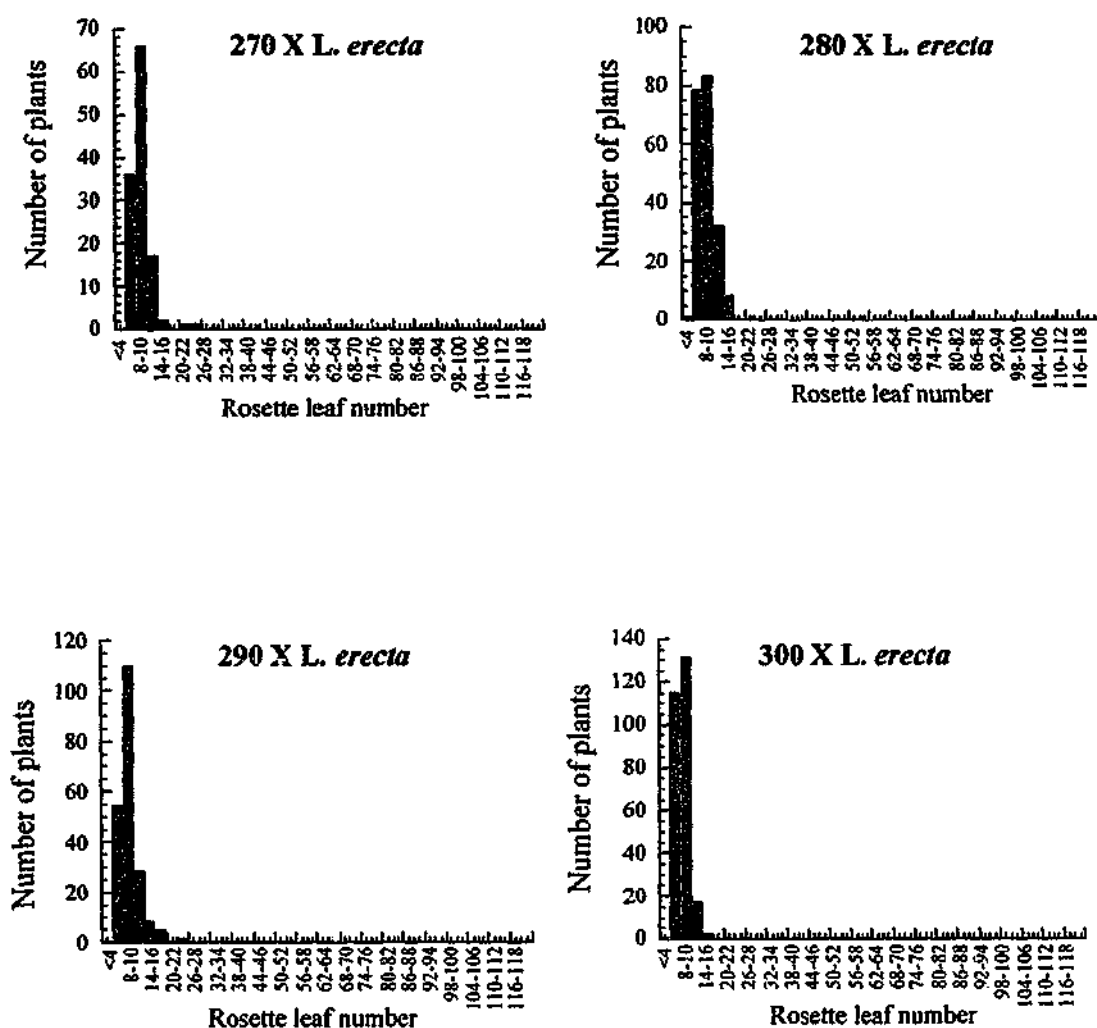
Figure 4.12

The rosette leaf number at the time of flowering of the F2 progeny from the *fler* mutant lines crossed with the wild-type L. *erecta* line.

4.4 Discussion

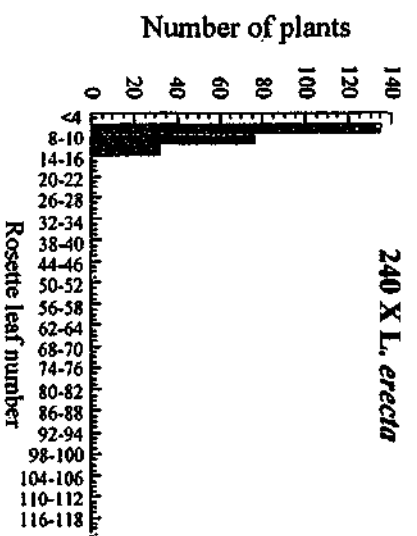In this study, microsatellite marker analysis was used in an attempt to link the *fler* mutation with the chromosomal region containing the nga8 microsatellite marker. This marker was chosen as it maps close to the *FRI* locus (approximately 10 cM away based on the classical genetic map), and displayed a size polymorphism between Pitztal and San-Feliu-2 DNA in this region. The information gained from this analysis was however limited by the inability to distinguish all heterozygote F2 progeny plants from those plants that are homozygous for Pitztal DNA in this region. This appeared to be due to the much weaker, and sometimes completely absent, amplification of San-Feliu-2 specific nga8 DNA sequences, in reactions containing both Pitztal and San-Feliu-2 template DNA. This limitation in the analysis resulted in a much larger than expected proportion of Pitztal homozygotes, and a corresponding decrease in the numbers of heterozygotes, being observed in the F2 population. The amplification of the San-Feliu-2 band did not appear to be affected in plants homozygous for San-Feliu-2 DNA in the nga8 region as strong amplification was seen amongst such samples. In support of this, the proportion of San-Feliu-2 homozygous genotypes detected (~25%) was in agreement with what would be expected in the F2 progeny from selfed heterozygous F1 plants. Despite the limitation in interpreting these results, the lack of any San-Feliu-2 homozygous genotypes amongst the early-flowering F2 progeny suggests that the *fler* mutation is linked to the region on chromosome 4 containing the nga8 markers and the *FRI* gene. This result also suggests that the most likely explanation for the presence of Pitztal homozygotes amongst the later-flowering plants is due to the limitations of this PCR based mapping procedure and does not indicate the *FLER* gene is unlinked to nga8.

While no recombinant plants were detected amongst the early-flowering F2 plants the small sample size may have negated the ability to identify such individuals. In addition, due to the inherent problems with this analysis, some of the Pitztal homozygotes detected in this early-flowering population may have represented recombinant heterozygous plants. The *GA1*, *HY4* and *DET1* loci, whose gene products can affect the flowering time of *Arabidopsis* , also map close to the nga8 markers. The *fler* mutants however, do not exhibit the daylength insensitive flowering of the *det1* lines (Pepper and Chory, 1997), the late-flowering phenotype of *ga1* plants (Wilson *et al.*, 1992), nor the elongated hypocotyl phenotypes of the *hy4* mutants (Johnson *et al.*, 1994). Four other flowering time genes map some distance away from the nga8 markers (between 22 and 54 cM away based on the classical genetic map). In addition, mutations in three of these genes (*FCA*, *FWA*, and *FD*) result in late-flowering plants (Martinez-Zapater *et al.*, 1994), while mutants of the *VRN2* locus result in plants that show a complete loss of a vernalisation response in long and short days, phenotypes not exhibited by the *fler* lines.

While the mapping data suggests linkage of the mutation resulting in the early-flowering phenotype of the *fler* lines 280 and 300 with the chromosomal region containing the *FRI* gene, it remains unclear as to whether the a mutation in the *fler* lines is in the *FRI* gene itself or in a separate linked loci. To help answer this question the nine radiation lines were crossed to a L. *erecta* line containing a late-flowering allele of the *FRI* gene from the San-Feliu-2 ecotype (Lee *et al.*, 1994b). If the mutant lines were mutated in the *FRI* gene then the F1 plants from a cross of the mutant lines to the L. *erecta* (Sf-2 *FRI*) plants may be expected to flower at intermediate times and the F2 progeny to segregate in a 7:6:3 ratio of early to intermediate to late-flowering plants.

The fact that the F1 plants from such crosses flowered at very similar times to those observed for the L. *erecta* (Sf-2 *FRI*) parent, and only slightly later than the early-flowering mutant line parents, suggests that none of the nine radiation-induced lines are affected in the *FRI* gene. The presence of some late and intermediate flowering plants in the F2 progeny does however appear to suggest that some interaction with the mutant genotype can occur following the introduction of the Sf-2 late-flowering version of the *FRI* gene into this genetic background. Interestingly, a conclusion that the *fler* mutation represents a lesion in the *FRI* gene would contradict what has formerly been observed in this study and by other researchers, in that the *FRI* gene would appear to be acting as a recessive gene in these crosses and therefore only able to exert its affects in the homozygous state in some of the F2 progeny. In crosses of either the *fler* mutant lines or wild-type L. *erecta* to wild-type Pitztal, for example, it has previously been shown that all the F1 progeny flower late, indicating a dominant flowering time gene is disrupted in the early-flowering mutant or early-flowering ecotypes such as L. *erecta*. In the crosses of the mutants with the L. *erecta* (*FRI* [Sf-2]) line however, the early-flowering background of either the mutant lines or L. *erecta* appears epistatic to the late-flowering effects of the introgressed San-Feliu-2 version of the *FRI* gene. In addition, the small proportion of the later-flowering plants within the F2 progeny does not appear to suggest a segregation of only one recessive flowering time gene amongst such plants. In support of the theory that the mutant lines are not disrupted in the *FRI* gene, the F2 progeny from the control, mutant lines to L. *erecta* crosses, also contain delayed-flowering plants indicating that the later-flowering plants observed in the L. *erecta* (*FRI*) crosses are not solely due to the presence of the San-Feliu-2 *FRI* allele.

The presence of delayed flowering plants in the F2 population from both the mutant lines crossed to L. *erecta* or L. *erecta* (*FRI*) plants may also indicate that the *FLER* lesion is not in the *FLC* locus, as activity of FLC is thought to be required for a significant delay in flowering, and the L. *erecta* *FLC* allele is not thought to respond to activation by late-flowering alleles of *FRI*. In addition, the suggestion from the microsatellite analysis that the *FLER* gene does map to chromosome 4 supports the proposal that the *FLER* locus is not allelic to *FLC*. In conclusion it appears as if the *fler* mutation is behaving in an epistatic

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manner to the *FRI/FLC* mediated floral repression system in the early-flowering ecotype crosses, although not in wild-type late-flowering ecotypes. This suggests the possibility that there may be a requirement for an active *FLER* and *FLC* allele in at least one of the parental plants to set up the *FRI/FLC* floral repression system in the progeny.

Chapter 5

Isolation of differentially expressed sequences between early-flowering mutant line 300 and wild-type Pitztal using cDNA subtraction.

5.1 Introduction

Subtractive hybridisation techniques can be used to isolate differentially expressed genes in two populations of cDNA, or structurally altered DNA sequences in genomic DNA. These subtractive techniques, involving genomic or cDNA populations that contain sequences of interest (tester DNA) being subtracted against another population of DNA (driver DNA), are followed by an extensive analysis of enriched sequences in an attempt to isolate any differentially expressed genes.

Genomic DNA subtractions have been used to successfully isolate genes that have been disrupted by deletions in several mutant organisms (Straus and Ausubel, 1990; Sun *et al.*, 1992; Weiland *et al.*, 1992; Darrase *et al.*, 1994). This technique relies on the hybridisation and physical separation of genomic DNA fragments common to both wild-type and mutant, leaving wild-type versions of the DNA disrupted in the mutant genome. Sequences can then be amplified by PCR and cloned for further analysis (Straus and Ausubel, 1990; Sun *et al.*, 1992; Wieland *et al.*, 1992). The main limitation of genomic subtraction techniques is the requirement of a significant structural disruption in genes of interest in the mutant genome, such as a large deletion or chromosomal rearrangement (Sun *et al.*, 1992). The detection of these mutations is also complicated if the genetic background of the mutants is already quite polymorphic. Furthermore, as this technique operates at the DNA level it cannot be used to isolate genes with only quantitative differences in expression between two nucleic acid populations.

Other techniques, such as differential display, are however specifically designed to isolate genes showing differential expression at the mRNA level. Differential display involves first isolating mRNA from the two populations of RNA containing the differentially expressed genes. First strand cDNA is then synthesised using oiligo-dT anchor primers. These primers contain two bases at the 5' end of the chain of dTTPs. The first of these bases may be an dATP, dGTP, dCTP or dTTP while the second can only be an dATP, dGTP or dCTP. Second strand cDNA is then amplified using both the anchor primer and a random 10 mer.

The use of anchor primers in this amplification results in approximately one twelfth of the total mRNA sequences present in either population, being represented in the products of the PCR. Amplification of a subset of mRNA sequences is required to reduce the complexity in the cDNA population and allow a comparison by gel electrophoresis. The PCR products of the two populations are therefore run, in tandem, on a sequencing acrylamide gel. Any difference in banding pattern, in particular the presence or absence of bands, may represent sequences differentially expressed between the two populations (Liang and Pardee, 1992; Liang et al., 1993; Oh et al., 1995). One of the advantages of differential display is that only small amounts of mRNA (~0.2µg) are required. Liang et al. (1993) noted that differential expression or amplification of bands can vary however, depending upon the initial amount of input RNA in the reactions. Differential display should therefore not be used as an accurate measure of quantitative differences in the expression of genes. In addition, if degenerate anchor primers are used to amplify the cDNA, rare messages, which may not amplify if there is a 2 to 3 bases mismatch in the primer sequence, will be under represented in the resulting cDNA. Furthermore, the use of degenerate primers may increase the amount of false positive bands detected (Liang and Pardee, 1992. Liang et al., 1993).

cDNA subtraction techniques have also been utilised to isolate differentially expressed sequences in several organisms. Many of these techniques are based around the construction of subtractive cDNA libraries. The aim of creating such libraries is to enrich the cDNA with differentially-expressed target genes prior to screening. This is achieved via subtraction steps to remove sequences common to the two populations of interest (Sargent and Dawid, 1983; Hedrick et al., 1984; Duguid and Dinauer, 1989; Hara et al., 1991). The subtractive steps in these procedures involve several rounds of hybridisation of mRNA isolated from the two populations of interest, and subsequent physical separation of the single stranded differentially expressed cDNA molecules. Methods to separate these single-stranded cDNA molecules have included the use of latex beads with attached oligo-dT molecules. These beads are used to prime cDNA production from mRNA of a driver population of RNA. Following the hybridisation steps the tester-driver heterohybrids (representing sequences common to both driver and tester populations), along with any single-stranded or double-stranded driver molecules, can be removed from the sample by centrifugation of the attached latex beads (Hara et al., 1991). Other techniques to separate molecules of interest from common sequences have included utilising streptavidin-coated beads to remove biotynylated driver homohybrids and tester-driver heterohybrids (Duguid and Dinauer, 1989). Alternatively, selective chromatography that will remove cDNA-mRNA hybrids and free mRNA has also been utilised. The problem with these latter approaches is that the unhybridised cDNA recovered is often very dilute and requires amplification via PCR (Wu et al., 1994). Another important factor in these subtractive techniques is the hybridisation

environment. Denaturing of the single-stranded DNA prior to the annealing steps, for example, can allow the formation of hairpin loop structures to occur. These structures cause short double-stranded regions in the 5' end of these molecules that prevent their subsequent recovery. The stability and integrity of the RNA or cDNA template initially used is also crucial for the subsequent integrity and length of any cDNA molecules synthesised (Wu *et al.*, 1994). A major disadvantage of these subtractive cDNA techniques is the lack of equalisation of low and high abundance transcripts during the subtraction. Therefore, the original disproportionate nature of high and low abundance sequences present in the original RNA population is maintained in the subtractive cDNA libraries. This makes the isolation of rare differentially-expressed transcripts unlikely (Gurskaya *et al.*, 1996).

A cDNA subtraction technique that eliminates the need for multiple hybridisation steps or physical separation of double- and single-stranded cDNA of interest has recently been developed (Gurskaya *et al.*, 1996). This cDNA subtraction technique also incorporates an equalisation step during the hybridisation steps. A schematic diagram of this technique is presented in Figure 5.1.

The cDNA subtraction technique involves the following steps; tester and driver cDNA are both digested with a frequently-cutting, restriction enzyme. The tester cDNA is then divided into two proportions and each tester population is ligated with a different cDNA adaptor. An excess of driver cDNA is then added to each sample, the cDNA denatured, and then left to anneal together. In this first hybridisation step, four different types of molecules are formed in both sample 1 and 2. These are the single-stranded tester molecules (type a molecules in Figure 5.1), double-stranded tester homohybrids (type b molecules in Figure 5.1), double-stranded tester-driver heterohybrids, representing sequences common to both tester and driver populations, (type c molecules in Figure 5.1), or double-stranded and single-stranded driver molecules (type d molecules in Figure 5.1).

After the initial hybridisation, mixing sample 1 and 2 without denaturing allows hybridisation of the type a molecules from both samples, thereby forming type e molecules that represent the differentially-expressed target sequences (Figure 5.1). The addition of an excess of fresh denatured driver cDNA at this stage, ensures annealing of the high abundance transcripts in the initial single-stranded tester populations with homologous sequence in the driver cDNA population. Following a fill in reaction to create primer annealing sites, and an initial round of PCR, type e target molecules are exponentially amplified while all other molecules (a, b, c or d) undergo a lower level of amplification, or no amplification (Figure 5.1). High and low abundance sequences unique to the tester population are equalised, as the majority of high abundance unique sequences will form homodimers during the first hybridisation (type b molecules). These b molecules then

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undergo a lower level of amplification during PCR as they tend to form a loop structure that prevents primer binding (Figure 5.1) (Siebert *et al.*, 1995; Gurskaya *et al.*, 1996). Gurskaya *et al.* (1996) predicts that following the equalisation and hybridisation steps, approximately 30% of all target molecules will be represented by sequences that were initially expressed at low abundance in the tester cDNA population. A second round of PCR, utilising nested primers specific to the adaptor sequences, is subsequently used to decrease any background primer artefacts from the first PCR, and enrich the sample for the target differentially expressed molecules. Gurskaya *et al.* (1996) reported a 200 fold enrichment for rare transcripts, and a dramatic decrease in the abundance of highly expressed transcripts utilising this technique.

Similar cDNA subtraction techniques have since been used to successfully isolate differentially expressed genes amongst cDNA populations in both animal and plant systems. Examples include genes specifically expressed in rodents during cold stress (Salvatore *et al.*, 1998), or those fed high salt diets (Wang *et al.*, 1999), or in mouse mutants deficient in a factor critical for lung development (Maekawa *et al.*, 1999). In plants this cDNA subtraction technique has been utilised to isolate genes such as those specifically expressed during somatic embryogenesis in carrots (Sato *et al.*, 1995), or in the floral shoot apices of *Arabidopsis* (Abe *et al.*, 1999), as well as male specific transcripts in *Silene latifolia* (Lebel-Hardenack *et al.*, 1997).

The early-flowering *fler* mutant lines used in this study were created following radiation treatments, which have been shown to cause large deletions or chromosomal rearrangements at the DNA level (Sankaranarayanan, 1991). It was therefore hoped that these early-flowering mutants may have a significant disruption in a gene affecting flowering time which in turn could affect the expression of several genes associated with floral induction in these lines. Differential display or subtractive techniques were hence thought to be potentially useful procedures to isolate genes associated with the early-flowering phenotype. Initially, the genomic subtraction method was used in an attempt to isolate genes disrupted in one of the radiation-induced mutant lines. Following the subtraction procedure, approximately 40 independent clones were obtained. These clones contained sequences representing wild-type Pitztal fragments of genes putatively deleted in the mutant line. Southern blot analysis revealed that 38 of these 40 sequences represented fragments of DNA with no major structural anomalies between the mutant and wild-type genomes. Two sequences did exhibit a difference in the size of bands detected on a southern blot of mutant and wild-type Pitztal DNA. Subsequent experiments revealed however, that this difference was probably due to the presence of some polymorphisms in the Pitztal background used to create the lines.
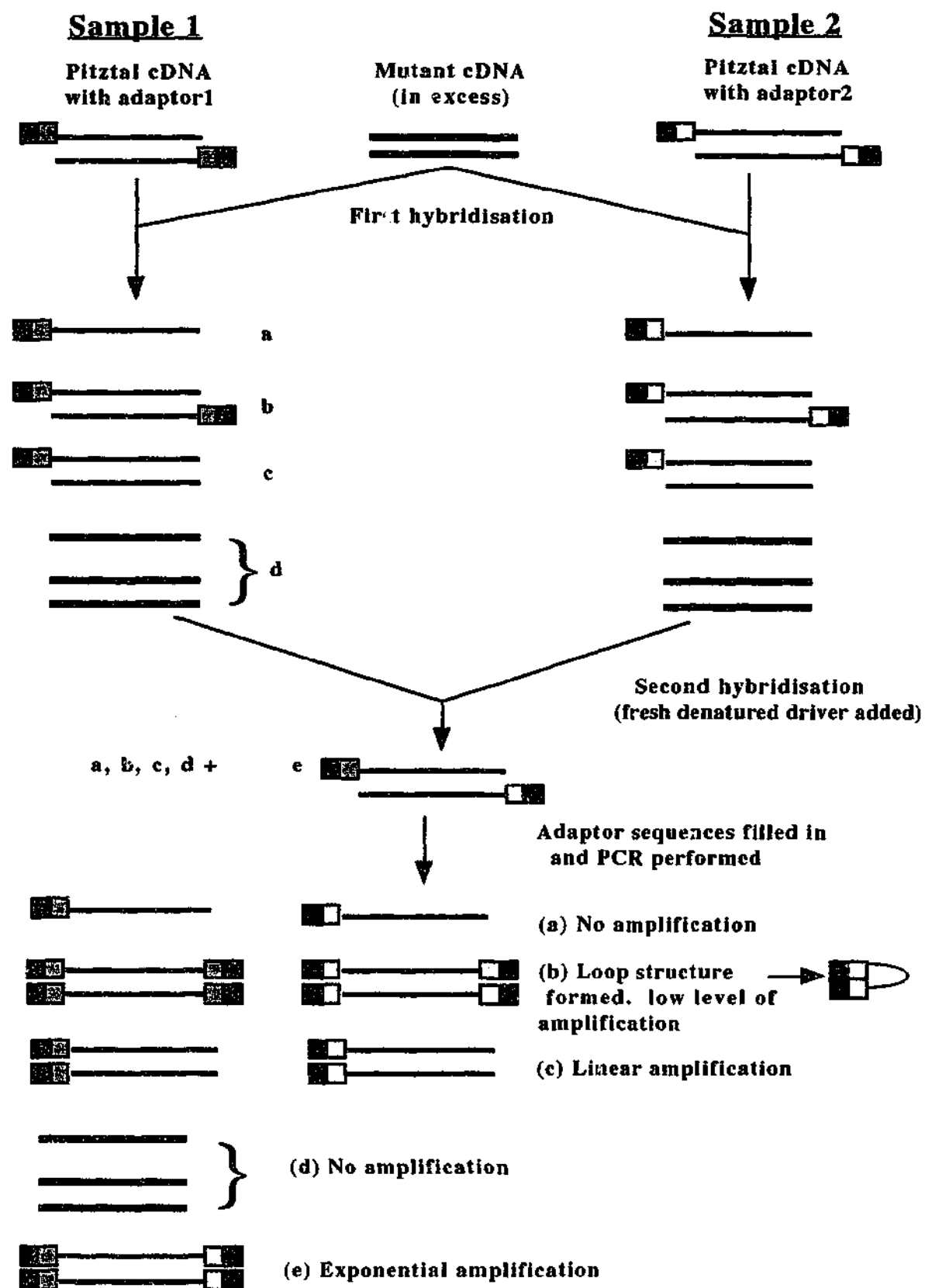
Figure 5.1

Schematic diagram of the cDNA subtraction procedure (adapted from CLONETECH PCR-Select[TM] cDNA Subtraction Kit user manual). The red and blue boxes represent the binding sites on adaptor 1 and two of PCR primers 1 and 2 respectively. The green and yellow boxes represent the binding sites on adaptor 1 and 2 of nested primers 1 and 2 .

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The differential display method was then trialed in order to isolate any genes with differential expression between mutant and wild-type Pitztal plants. The reproducibility of this method was not adequate however, and several false positive bands were present in each experiment. Subsequently, the cDNA subtraction method based on that proposed by Gurskaya *et al.* 1996, was used in an attempt to isolate genes exhibiting a quantitative or qualitative difference in their expression between wild-type Pitztal and the *fler* lines. This technique has been used to successfully isolate at least one sequence with dramatically different expression patterns between the early-flowering mutant line 300 and wild-type Pitztal.

5.2 Materials and methods

5.2.1 RNA manipulation techniques

Gloves were worn for all RNA procedures. A separate set of chemicals, glassware, equipment and consumables were kept for RNA work only. All RNA solutions were prepared using RNase free water that had been treated with diethyl pyrocarbonate (DEPC) and autoclaved. All glassware and vessels that were used for RNA work were washed successively (with intermittent rinses in DEPC treated water) with the following solutions before being used; 0.1% SDS, 0.1 M NaOH (equipment soaked for one hour at this step), Chloroform and 100% Ethanol.

5.2.2 Mini prep isolation of RNA

A 10 ml centrifuge tube containing 750 μl of TLES buffer (100 mM Tris (pH 8.0), 100 mM LiCl, 10 mM EDTA, 1% (w/v) SDS), and 750 μl of RNA grade phenol was prepared for each extraction, and placed in an 80°C waterbath. 0.5 g of plant tissue was ground under liquid nitrogen in a mortar and pestle, and then added to centrifuge tubes containing TLES and phenol. The samples were vortexed for 30 seconds. 750 μl of chloroform/isoamyl alcohol (24:1) was added to the tubes and the samples vortexed again for 30 seconds. The mixture was divided into two microcentrifuge tubes and centrifuged at 10,000 rpm for 5 minutes. The supernatant of each sample was subsequently removed to a clean microcentrifuge tube, and an equal volume of 4 M LiCl was added. Samples were then kept at 4°C overnight before a 30 minute centrifugation at 10,000 rpm at 4°C. The pellet was resuspended in 250 μl of RNase-free MQ water. 25 μl of NaAc (pH 6.0) and 500 μl of absolute ethanol was added, and the RNA precipitated overnight at -20°C. Following

precipitation, the RNA was pelleted by centrifugation at 10,000 rpm for 20 minutes, washed in 70% ethanol, and resuspended in 50-100 μl of RNase-free MQ water.

5.2.3 Isolation of mRNA

This method is based on the Dynal dT-streptavidin magnetic beads user manual. 100 μg of total RNA, resuspended in 100 μl of RNase free MQ water, was used to prepare approximately 1 μg of mRNA. 200 μl aliquots of thoroughly mixed beads were prepared for each 100 μl aliquot of total RNA (100 μg) to be used. The microcentrifuge tube containing the beads was placed in a magnet to separate the beads from the storage solution, and the supernatant removed. The beads were then washed once in 200 μl of 2 X binding buffer (20 mM Tris-HCl (pH 7.5); 1.0 M LiCl; 1 mM EDTA) and the supernatant again removed. The beads were then resuspended in 100 μl of 2 X binding buffer. The RNA sample was heated to 65°C for two minutes before it was added to the equilibrated beads. The RNA and bead mixture was left incubate for six minutes at room temperature. Again the supernatant was removed using the magnet, and the beads were washed twice in 1 X washing buffer (10 mM Tris-HCl (pH 7.5); 0.15 M LiCl; 1 mM EDTA). To elute the mRNA the beads were resuspended in 15 μl of elution buffer (2 mM EDTA), and heated to 95°C for one minute. The supernatant containing the mRNA was then immediately removed to a clean microcentrifuge tube.

5.2.4 Preparation of radioactively labelled cDNA probes

Single stranded cDNA probes were prepared from mRNA attached to magnetic beads that were prepared as described in section 5.2.3. The beads were washed 3 times in 1 X washing buffer at the step preceding elution. The beads were then washed twice in reverse transcription buffer (50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM MgCl$_2$; 10 mM DTT), and resuspended in 16.5 μl of reverse transcription buffer. The following reagents were added to the sample; 2 μl of 0.7 mM dNTPs, 20 units of RNase inhibitor and 200 units MoMLV reverse transcriptase. The reaction was then incubated at 42°C for one hour with constant shaking to keep the beads in suspension. Following reverse transcription, the reaction mix was removed by placing the microcentrifuge tube in the magnet. The beads were then resuspended in 20 μl of elution buffer, and the mRNA-cDNA duplex was heated to 95°C for 1 minute, placed in the magnet, and the mRNA removed and discarded. The heat treatment was repeated once more. The beads were then washed once in TB buffer (50 mM KCl; 20 mM Tris-HCl (pH 8.4); 1.5 mM MgCl$_2$, 0.1 mg/ml BSA), resuspended in 20 μl of TB buffer, heated to 95°C for 2 minutes and the supernatant removed. The cDNA bead mixture was then resuspended in 6 μl of MQ water and placed on ice before the following

reagents were added; 6 µl of giganucleotide solution, 6 µl of nucleotides, 5 µl of α-^{32}P-dATP, and 1 µl of Klenow enzyme. The reaction was incubated at 37°C for 30 minutes. The reaction mixture was then heated to 95°C for 2-to-3 minutes and immediately placed in the magnet to collect the second strand cDNA. The labelled cDNA was purified through a sephadex G50 column as described in section 2.3.4 (preparation of radioactively labelled DNA probes).

5.2.5 Northern blot analysis

This method is based on that described in Davis *et al.* (1986) (section 11-4). All gel tanks, trays, and combs were soaked with 1% SDS solution for one hour prior to use, and rinsed thoroughly with RNase free water. To prepare the gel; 1% (w/v) agarose was dissolved in 1 X MOPS buffer (0.2 M 3-[N-morpholino]propanesulfonic acid (MOPS); 0.05 M NaAc; 0.01 M EDTA) in a clean RNase-free flask. The agarose was allowed to cool to ~60°C before 5.5% (v/v) of a 37% formaldehyde solution was added. The agarose was then poured into a horizontal electrophoresis apparatus and the gel allowed to solidify for at least 1 hour before use.

RNA in 1-to-5 µl aliquots was mixed with 20 µl of freshly prepared RNA loading buffer (for 1.5 ml add 0.72 ml formamide, 0.16 ml 10 X MOPS buffer, 0.26 ml 37% formaldehyde, 0.18 ml water, 0.1 ml 80% glycerol, and 0.08 ml of a saturated solution of bromophenol blue) and 0.5µl of ethidium bromide (10 mg/ml). The RNA samples were then heated to 65°C for 8 minutes before being loaded. Electrophoresis was performed at a voltage ranging from 20-80 volts and RNA bands were visualised using a UV transilluminator.

Gels were blotted in a similar manner as for Southern blots (section 2.3.4 [Southern blots]) except that the RNA gels were not washed before beginning the capillary blot transfer. In addition, the filters were not washed following transfer. RNA was fixed onto the filters via exposure to UV illumination for 2 minutes.

5.2.6 Hybridisation protocols

This method is based on the Clontech ExpressHyb™ Hybridisation solution user manual (PT1190-1). Membranes to be hybridised were placed into plastic bags that were heat-sealed on three sides. Pre-warmed ExpressHyb™ solution (~10 ml for a 10 X 15 cm membrane) was added to the bag containing the membranes, and the bag completely sealed after all bubbles were removed. The membrane was pre-hybridised for 30 minutes at 68°C in a shaking water-bath. Following pre-hybridisation, double stranded cDNA probes were

denatured by heating to 100°C for five minutes before being added to 10 ml of fresh ExpressHyb™ solution. This solution was then immediately added to the bag containing the membrane (NB. Single stranded cDNA probes were not denatured before adding to the ExpressHyb™ solution). The probes were left to hybridise to the filters for one hour at 68°C in a shaking water bath. After hybridisation the filters were then incubated in wash solution 1 (2X SSC; 0.05% (w/v) SDS) at room temperature for 10 minutes. This wash was repeated three to four times. If necessary, filters were incubated twice in wash solution 2 (0.1X SSC; 0.1% (w/v) SDS) for 20 minutes at 50°C.

Following the washes the filter was wrapped in plastic wrap and exposed to an X-ray film at -70°C for the appropriate length of time. The X-ray films were developed in a Agfa-Gevamatic 60 X-ray machine, under NX-914 safety lights. Following autoradiography membranes were stripped as described in section 2.3.4 (membrane stripping protocol) and re-probed with a Ubiquitin probe to check for equal loading of RNA samples.

5.2.7 Phosphoimaging

As an alternative to autoradiography, Northern blot filters were phosphoimaged. Powder-free gloves were always used to handle the phosphoimaging screens and any residual background signal was erased from the screen by exposure to bright illumination for at least ten minutes. The radioactive membranes were wrapped in plastic wrap and placed RNA side up in the phosphoimaging cassette. The screen was placed face down on top of the membrane and left to expose in the cassette at room temperature, for the appropriate length of time. Following exposure, the screen was placed into the STORM phosphoimager and the image scanned into a computer ready for analysis. The signal intensities for each gene were quantified relative to those of *ubiquitin* using the ImageQuant software.

5.2.8 cDNA subtraction

This method is based on that described in the CLONETECH PCR-Select cDNA Subtraction Kit User Manual

First strand cDNA Synthesis

For each sample to be used (*ie.* tester and driver samples), 2 µg of mRNA dissolved in 2-to-4 µl of sterile MQ water was placed in a 0.5 ml microcentrifuge tube, 1 µl of cDNA synthesis primer (10 µM), and if required MQ water was added to give a final volume of 5 µl The tube was incubated at 70°C in a thermal cycler for 3 minutes, and then cooled on ice for 2 minutes. To the reaction tube 2 µl of the 5X first-strand buffer (250mM Tris-HCl (pH 8.3);

30mM MgCl$_2$; 375 mM KCl), 1 µl of dNTPs (10mM each), 1 µl of diluted α-^{32}P-dCTP, and 1 µl of MMLV reverse transcriptase (200 units/µl) were added. The reactions were incubated at 42°C for 1.5 hours and subsequently placed on ice ready for second strand synthesis.

Second strand cDNA synthesis

To the first strand synthesis reaction tube 48.4 µl of sterile water, 16 µl of 5X second-strand buffer (500 mM KCl; 50 mM NH$_4$SO$_4$; 25 mM MgCl$_2$, 0.75 mM β-NAD; 100 mM Tris-HCl [pH7.5]; 0.25 mg/ml BSA), 1.6 µl of dNTPs (10 mM each), 4 µl of 20X Second-Strand enzyme cocktail (DNA polymerase I, 6 units/µl; RNase H, 0.2 units/µl; E. *coli* DNA ligase, 1.2 units/µl) were added. The tubes were then incubated at 16°C for two hours. 2 µl of T4 DNA polymerase was added, and the tubes incubated for a further 30 minutes at 16°C. To terminate second strand synthesis, 4 µl of a 20 X EDTA/Glycogen mixture (0.2M EDTA; 1mg/ml glycogen) was added. The cDNA was subsequently extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with the addition of one tenth volume of 4M NH$_4$OAc and 2 volumes of 95% ethanol. The cDNA was subsequently digested with the *Rsa*I enzyme. The enzymatic reaction was terminated with a mixture of EDTA and glycogen and the cDNA was again phenol-chloroform extracted.

Ligation of adaptors

1.5 µl of each *Rsa*I digested tester cDNA sample was diluted with 7.5 µl of sterile MQ water. 2 µl of this diluted cDNA was then combined with 2 µl of either adaptor 1, adaptor 2, or 2 µl of both adaptor 1 and 2. 4 µl of a master mix containing 5X ligation buffer, T4 DNA ligase, and water, in a ratio of 1:2:1 was added to each reaction. The reaction volumes were made up to 10 µl with sterile MQ water if required, and the reactions were incubated at 16°C overnight. The ligation reaction was stopped with a mixture of EDTA and glycogen. The samples were then placed at 72°C for 5 minutes to inactivate the ligase.

First hybridisation

In two separate tubes, 1.5 µl of the tester cDNA, ligated to either adaptor 1 or 2, was added to 1.5 µl of the *Rsa*I digested driver cDNA and 1 µl of the 4X hybridisation buffer. The two samples were overlayed with oil and incubated in a thermal cycler for 1.5 minutes at 98°C, and subsequently for 8 hours at 68°C.

Second hybridisation

1µl of the *Rsa*I digested driver cDNA was added to 1 µl of 4X hybridisation buffer and 2 µl of sterile MQ water, the sample overlayed with mineral oil, and denatured at 98°C for 1.5 minutes before being allowed to cool to 68°C. The two hybridisation sample prepared in the first hybridisation step were then combined into one tube and 1µl of fresh denatured driver cDNA added. The sample was mixed and centrifuged briefly before being left to incubate at 68°C overnight. 200 µl of dilution buffer (20 mM HEPES-HCl; 50 mM NaCl and 0.2 mM EDTA) was then added to the mixture and the sample heated to 75°C for 7 minutes. The hybridised samples were initially amplified using PCR primers 1 and 2 and subsequently using nested PCR primers 1 and 2. These primers are homologous to the adaptor sequences ligated to the tester cDNA samples.

5.2.9 Acyrlamide gel electrophoresis of cDNA subtraction PCR products

cDNA subtraction products labelled with α-^{32}P-dATP were run on 6% non-denaturing polyacrylamide gels using the following procedure. The glass plates of the electrophoresis apparatus were thoroughly cleaned using a scourer pad and a solution of 2% SDS and rinsed thoroughly in tap water. The plates were then cleaned twice with 80% ethanol and allowed to dry. One plate was then coated with Sigmacote™ and left to dry before the plates and spacers were assembled together. For a 6%, 125 ml, non-denaturing polyacrylamide gel the following components were added to a 500 ml flask; 18.75 ml of a 40% acrylamide solution (38.6 % acrylamide and 1.3% N,N'-methylene-bisacrylamide) and 12.5 ml of 10X TBE buffer. The final volume was adjusted to 125 ml with sterile distilled water and the gel solution was filtered through a glass filter sheet. Any bubbles were removed from the solution by placing the mixture under a vacuum. The acrylamide solution was then placed on ice while 1.2 ml of a 10% APS solution and 125 µl of TEMED were added and thoroughly mixed. The solution was then poured into the glass plate apparatus being careful to avoid creating any bubbles. The well comb was placed into the gel and the gel left to polymerise for at least 1 hour before use.

When the gel was ready to be used, the well comb and bottom spacer were removed and any unwanted acrylamide removed from the wells using a fine pasteur pipette. The assembled gel was placed in the vertical gel tank and any bubbles formed between the glass plates were removed using an 18G needle and syringe. The gel was pre-run at 1000V for 15-20 minutes before samples were loaded. Radioactive markers to be run on these gels were created by amplifying the pUC119 cloning vector, using specifically designed primers, in the presence of α-^{33}P-dATP. The radioactively-labelled vector sequences were then double digested with the *ScRF*I and *Ssp*I restriction enzymes to give the required fragment sizes.

Samples to be electrophoresed were mixed with 6 X loading buffer (50% [v/v] glycerol; 1X TBE buffer; 0.025% bromophenol blue; 0.025% xylene cyanol). Electrophoresis was performed for the appropriate length of time at a voltage ranging from 1000-1500 Volts. Following electrophoresis, the gel was dried under a vacuum at 80°C for 1 hour before being exposed to X-ray film at -70°C.

5.2.10 *Isolation of PCR bands from acrylamide gels*

PCR products of interest were cut from the dried acrylamide gel, and the gel fragments soaked in 100 µl of sterile MQ water at room temperature for 20 minutes. The samples were then vortexed briefly, sealed with parafilm, and boiled for 15 minutes to elute the DNA. The samples were again vortexed and allowed to cool to room temperature. The DNA was then stored at 4°C for two days and vortexed occasionally. Following this incubation, the samples were centrifuged at 13,000 rpm for 3 minutes and the supernatant removed to a new tube. 10 µl of 3 M NaAc (pH 6.0), 2.5 µl of glycogen (20 mg/ml), and 450 µl of absolute ethanol was added to the tubes and the DNA left to precipitate overnight at -20°C. The samples were then centrifuged at 4°C for 30 minutes and the pellets washed in ice-cold 70% ethanol, dried and resuspended in 10 µl of sterile MQ water. All sequences obtained from the subtraction were analysed using the Australian National Genome Information Service (ANGIS).

5.2.11 *Chlorophyll assay*

0.5 g of leaf tissue was placed into a 20 ml glass centrifuge tube containing 10 ml of 80% acetone, and approximately 0.1 g of $CaCO_3$. The tubes were then kept on ice for the remainder of the procedure. The leaf sample were ground, in the dark, using a hand-held homogeniser before the samples were filtered to remove any debris. The final volume of the extraction was then made up to 12.5 ml with 80% acetone. The optical density of the samples are measured at 645 nm and 663 nm, the peak absorption wavelengths of chlorophyll b and a respectively. The relative amounts of chlorophyll a and b in mg/g of fresh weight are then calculated based on the equations for green leaf extracts proposed by Machlachlan and Zalik, 1963.

5.3 Results

Isolation of mRNA and cDNA from mutant and wild-type, soil-grown, Pitztal plants

RNA was extracted from mutant line 300 and wild-type Pitztal plants at the cotyledon, 2-leaf, 4-leaf, and 6-leaf stages of development, as well as from flowering mutant plants, and from Pitztal plants harvested at the time of flowering of the mutant. RNA was extracted from whole seedlings at the cotyledon stage, and from leaf tissue at all other stages. Harvesting of mutant and wild-type tissue at the equivalent developmental stages was performed at the same time. It was estimated that approximately 100 μg of this total RNA would be required to produce 1 μg of mRNA. Therefore, 60 μg of total RNA from each developmental stage, for both mutant and wild-type Pitztal, was pooled together to give 300 μg of RNA in total. mRNA was produced from this total RNA as detailed in section 5.2.3. Figure 5.2 demonstrates the results of this mRNA extraction. The presence of a large amount of rRNA in the supernatant of this extraction (lanes 3 and 4 in gel A and lane 3 in gel B) suggests an efficient extraction of the rRNA away from the mRNA. Approximately 1 μg of the 3 μg of mRNA extracted in total, was also examined by gel electrophoresis to assess the integrity of the sample (lane 5 in gel A and lane 4 in gel B). First and second strand cDNA was then created from the remaining 2 μg of mRNA template, for both mutant and wild-type Pitztal samples, as well as from the from the human skeletal muscle control polyA+ RNA provided in the CLONETECH cDNA subtraction kit. Mutant, control, and wild-type Pitztal cDNA samples were subsequently digested with the *RsaI* restriction enzyme and complete digestion of the cDNA was confirmed.
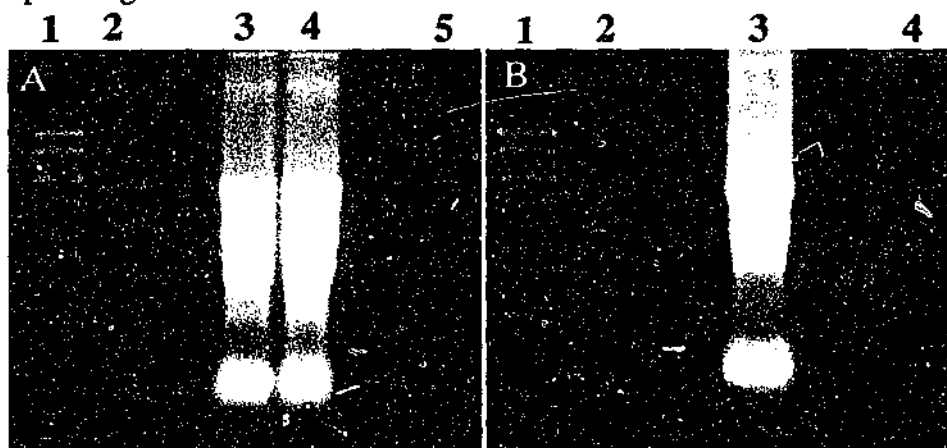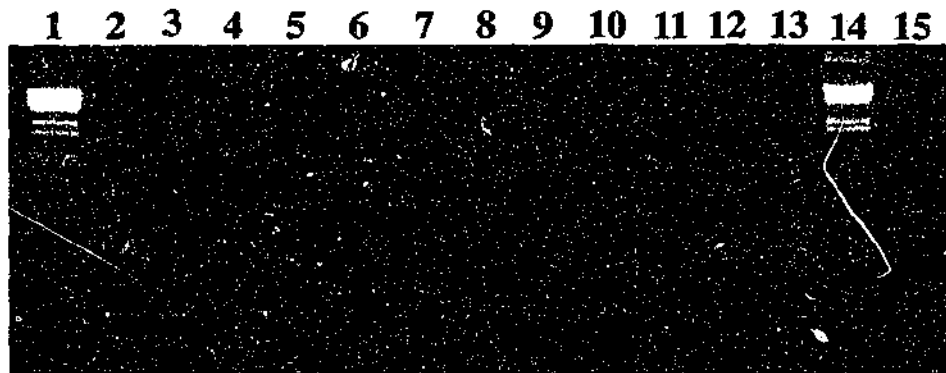

Figure 5.2

The results of the extraction of mRNA from total RNA for both the Pitztal samples (gel A) and mutant samples (gel B). Lane 1 in each gel contains RNA markers and lane 2 contains 0.5 μg of the total RNA. Lane 3 in gel B, and lanes 3 and 4 in gel A contain the total RNA present in the supernatant following extraction of the mRNA. Lane 4 in gel B and lane 5 in gel A contain ~1μg of the extracted mRNA.

Adaptor ligation

For each of the wild-type Pitztal cDNA samples (tester cDNA), and the skeletal muscle control cDNA spiked with φX174 *Hae*III markers (control tester cDNA), three different ligation reactions were set up. The first reaction involved the ligation of the two types of tester cDNA to adaptor 1, the second involved ligating tester cDNA to adaptor 2, and the third involved ligating the tester cDNAs to both adaptors. Before proceeding with the hybridisation steps of the subtraction it was important to determine the efficiency of these ligation reactions. A ligation efficiency test was therefore utilised to determine that at least one quarter of the tester molecules contained adaptor sequences. This test involved amplifying tester cDNA using both a gene specific primer and a primer specific to the adaptor sequence (PCR primer 1 or 2). If the adaptor ligation has been successful, the PCR product amplified using one gene specific and one adaptor specific primer, should be of a similar intensity as the PCR product amplified using two gene specific primers. The *G3PDH* (glyceraldehyde-3-phosphate dehydrogenase) gene specific primers were used to analysis the efficiency of the skeletal muscle control cDNA ligation reactions. The results of this analysis are presented in Figure 5.3. The PCR products using two gene specific primers (lanes 10 and 11) were found to be of a similar intensity as the products of PCR amplifications using only one gene specific primer (lanes 2 to 9). This suggests the ligation of adaptor sequences has been successful for the skeletal muscle control tester cDNA. As an adequate set of *Arabidopsis* gene-specific primers was not available at that time, Pitztal tester cDNA that was ligated to both adaptors was amplified using PCR primers 1 and 2 (lanes 12 and 13). The results suggest successful ligation of the adaptor sequences to Pitztal tester cDNA as a large range of cDNA bands was amplified from this PCR.
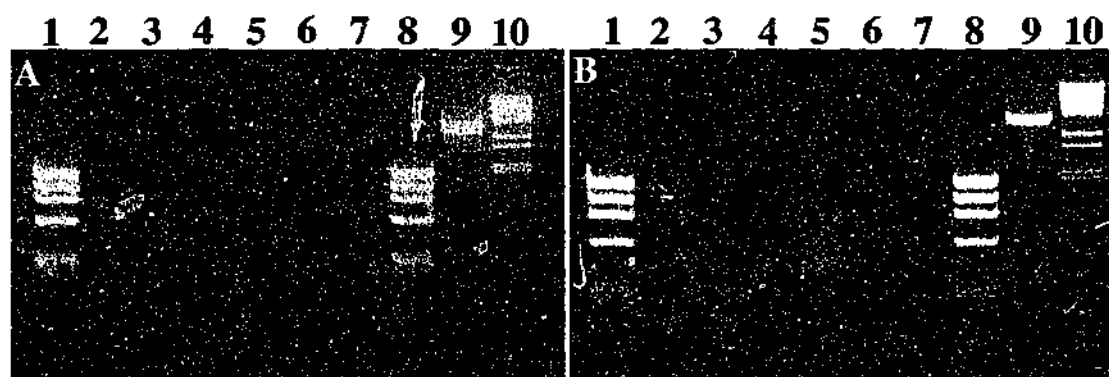

Figure 5.3

Ligation efficiency test for the control skeletal muscle tester cDNA and wild-type Pitztal tester cDNA. Lanes 1 and 14 contain λ *Bst*EII markers. Lanes 2 to 9 contain skeletal muscle cDNA amplified using the *G3PDH* primer and either PCR primer 1 (lanes 2, 3, 6 and 7), or PCR primer 2 (lanes 4, 5, 8, and 9). Lanes 10 and 11 contain skeletal muscle cDNA amplified using both the 3' and 5' *G3PDH* primers. Lanes 12 and 13 contain Pitztal cDNA amplified using PCR primer 1 and 2. Lane 15 contains a negative PCR control.

cDNA subtraction

The first and second hybridisation steps were performed for both the mutant and Pitztal cDNA samples and for the skeletal muscle control tester and driver cDNA. The results of the PCR amplifications of the products of the subtraction are presented in Figure 5.4. These PCR products (lanes 2 and 4 in gels A and B) were compared to the appropriate tester unsubtracted cDNA amplified using either PCR primers 1 and 2, or nested primers 1 and 2 (lanes 3 and 5 in gels A and B). A control sample of successfully subtracted skeletal muscle cDNA provided in the CLONETECH cDNA subtraction kit was also amplified as a further control reaction (lanes 6 in each gel). It appears as though the subtraction was successful for the skeletal muscle cDNA as the only remaining bands in the subtracted sample represent bands corresponding to ϕX174 *Hae*III markers. There are also distinct bands present in the plant subtraction sample (lane 1 in gels A and B) compared to the smear of fragments amplified in the unsubtracted Pitztal tester cDNA.
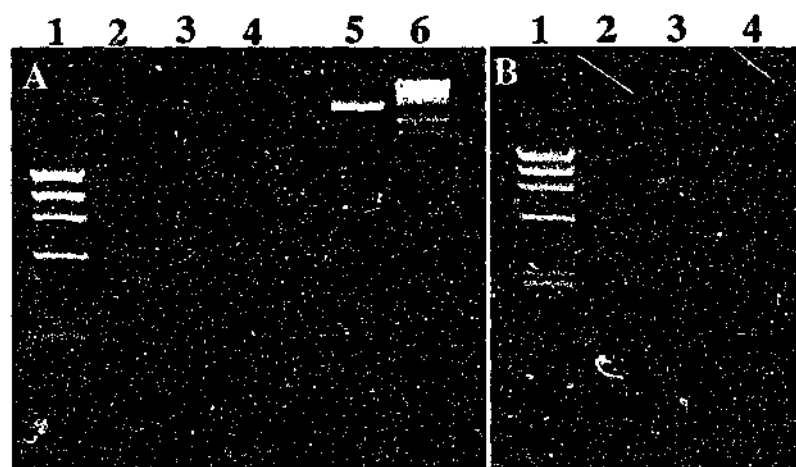

Figure 5.4

The cDNA subtraction products were initially amplified using PCR primers 1 and 2 (gel **A**), and subsequently re-amplified using nested PCR primers 1 and 2 (gel **B**). Lanes 1 and 8 in each gel contains ϕX174 *Hae*III markers. Lane 2 in each gel contains wild-type Pitztal cDNA subtracted against cDNA from mutant line 300. Lane 3 in each gel contains amplified unsubtracted wild-type Pitztal cDNA. Lane 4 in each gel contains skeletal muscle cDNA spiked with ϕX174 *Hae*III markers subtracted against unspiked skeletal muscle cDNA. Lane 5 in each gel contains unsubtracted skeletal muscle cDNA. Lane 6 in each gel contains control subtracted skeletal muscle cDNA. Lanes 7 and 9 in each gel contain negative and positive PCR controls respectively. Lane 10 in each gel contains λ *Bst*eII markers.

Analysis of the cDNA subtraction products

In an attempt to reduce the complexity of the banding pattern observed in the plant subtraction sample, the subtracted cDNA was re-amplified and then digested with the *Rsa*I restriction enzyme. It was believed that the banding pattern observed in the subtracted sample may be the result of an initial incomplete digestion of the template cDNA. Gel B in Figure 5.5 demonstrates the results of this analysis. The banding pattern of the digested sample of subtracted cDNA (lane 2 in gel B of Figure 5.5) did not exhibit a dramatic decrease in complexity, suggesting the subtracted sample does not contain a large number of partially digested *Rsa*I restriction fragments, although a few smaller fragments were observed.
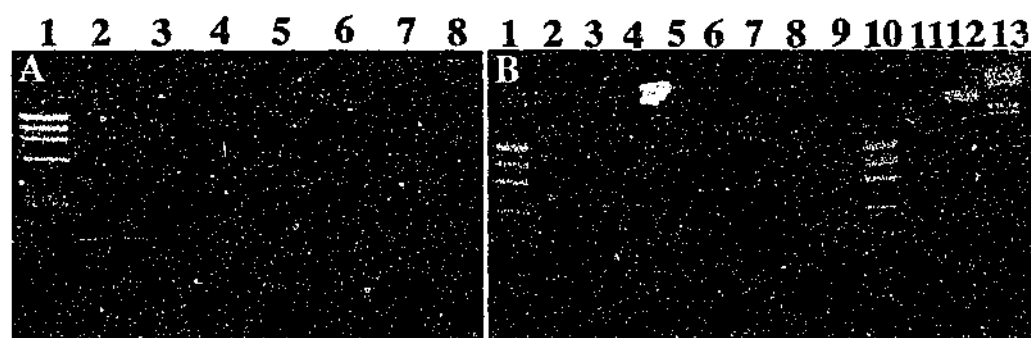

Figure 5.5

The re-amplified products of the plant subtraction sample are shown in gel **A** (lanes 2 and 3) compared with the re-amplified unsubtracted cDNA (lane 4). Lanes 1 and 6 contain φX174 *Hae*III and λ *Bst*EII markers respectively. Lane 5 contains a positive PCR control. Gel **B** shows the subtraction products digested with *Rsa*I (lane 2) compared to undigested subtraction products (lane 3) and unsubtracted cDNA (lane 4). Lane 1 of gel **B** contains φX174 *Hae*III markers.

Further PCR based analysis was used to determine if the large number of bands observed in the subtracted sample was due to the amplification of background, non-target, tester sequences present in the subtracted sample. Theoretically these sequences should not undergo significant amplification during PCR, as the adaptor sequences common to either end of these molecules form a double stranded region that inhibits binding of the primers to the adaptors. It was attempted to amplify any such sequences present in the subtracted cDNA sample by using only one type of primer, or nested primer, in the amplification reactions. Figure 5.6 displays the results of these amplifications (lanes 2 and 3 in gels A and B).

cDNA from the subtracted and unsubtracted experimental and control samples was amplified using the two different PCR or nested PCR primers as a PCR control. As no bands were detected in the PCR products when only one type of primer was used, it was concluded that the amplification of any non-target tester molecules had been efficiently suppressed. Therefore such sequences were not thought to constitute a large proportion of any of the background sequences present in the subtracted sample.

In an initial attempt to analyse the types of sequences obtained from the subtraction, three distinct bands present in the subtraction sample were purified separately and re-amplified using PCR primers 1 and 2. Figure 5.7 presents the results of this purification and re-amplification experiment. The bands highlighted in gel B of Figure 5.7 were again purified from the gel and two of these bands were used to probe RNA extracted from mutant and wild-type Pitztal plants.
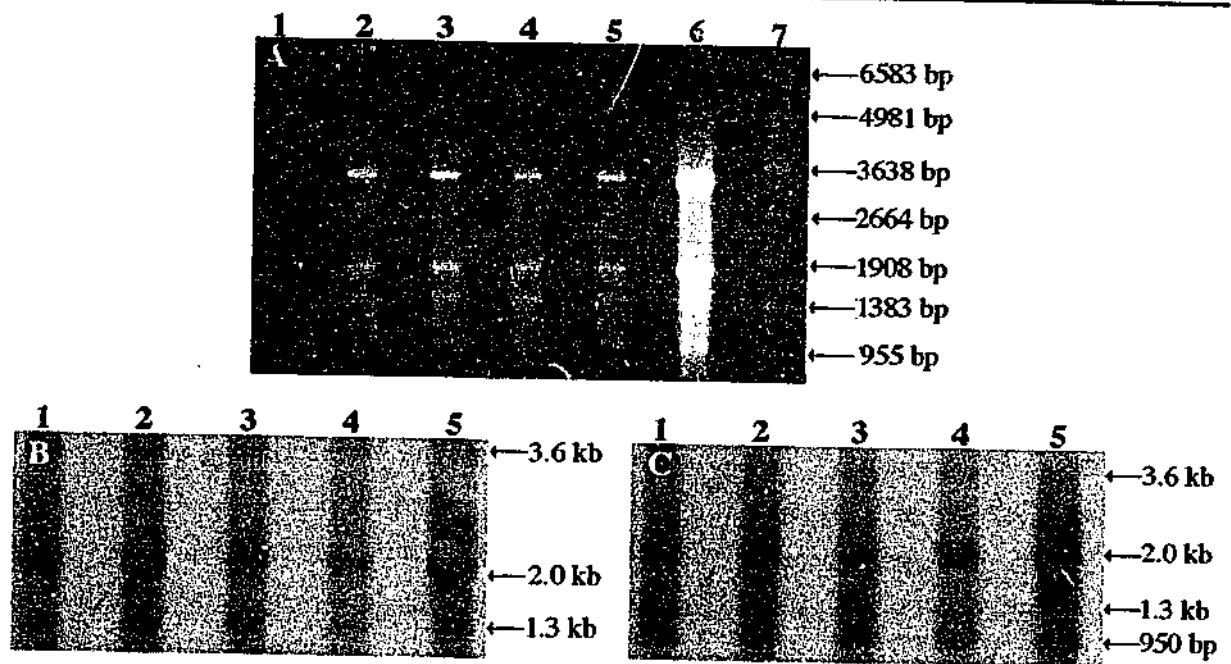

Figure 5.6

The results of PCR amplifications of the plant subtraction sample using only one type of primer (gel A), or nested primer (gel B). Lane 1 in gel A and lanes 1 and 10 in gel B contains ϕX174 *Hae*III markers. Lanes 2 and 3 of each gel contain the products of the amplification of subtracted cDNA using either PCR primer 1 or 2 respectively (A), or nested PCR primers 1 and 2 (B) respectively. Lane 4 in each gel contains subtracted cDNA amplified using both PCR primers 1 and 2 (A) or nested PCR primers 1 and 2 (B). Lane 5 in each gel contains amplified unsubtracted wild-type Pitztal cDNA. Lane 6 in each gel contains subtracted skeletal muscle cDNA amplified using only PCR primer 1 (A) or nested PCR primer 1 (B). Lane 7 in gel A and lane 8 in gel B contains unsubtracted skeletal muscle cDNA amplified. Lane 7 in gel B contains subtracted skeletal muscle cDNA amplified using both nested PCR primers. Lanes 9 and 12 in gel B contain negative and positive PCR controls respectively. Lane 13 in gel B contains λ *Hind*III markers.

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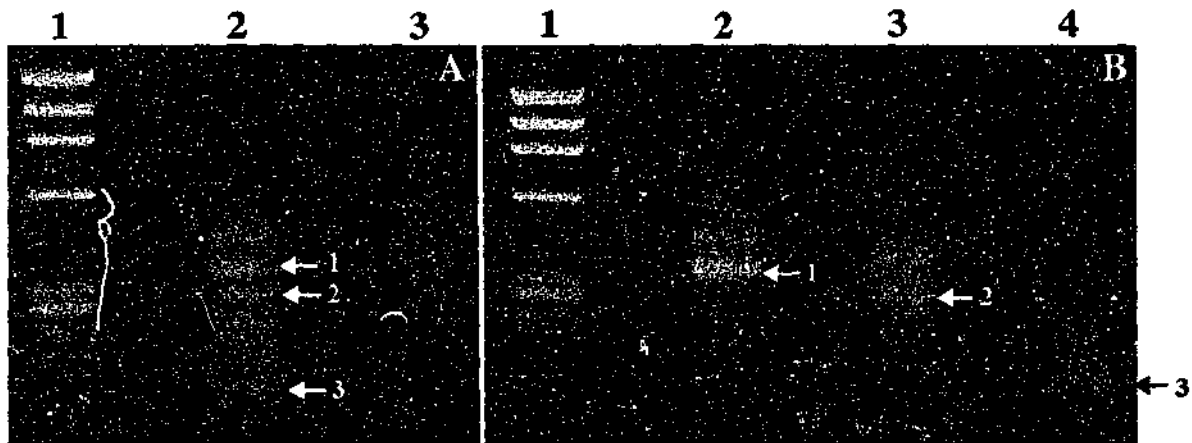
Figure 5.7

The three main bands distinguishable in the subtracted sample of cDNA, designated bands 1, 2 and 3, are shown in gel A (lane 2). Lane 1 in gel A contains φX174 *Hae*III markers and lane three contains unsubtracted cDNA. The results of the purification of these three bands are shown in lanes 2, 3 and 4 respectively of gel B. Lane 1 of gel B contains φX174 *Hae*III markers.

The RNA for this analysis was isolated from plants grown to the same developmental stages as those used for the subtraction experiments. Equal amounts of RNA from all five stages was then pooled together for the northern analysis. Presented in Figure 5.8 are the results of the northern blot analysis using band 1 (Filter B) or band 3 (Filter C) as probes. Only bands corresponding to ribosomal RNA were detected by these hybridisations. From this experiment it was not clear whether bands 1 and 3 represent very low abundance transcripts whose detection was masked by probe trapping in the ribosomal RNA, or if they represented high abundance ribosomal RNA sequences that had not been completely removed during the subtraction. In an attempt to assess whether there had been a significant reduction of non-target, tester sequences in the subtracted cDNA sample, a southern blot containing cDNA from subtracted and unsubtracted samples was probed with DNA from genes normally expressed at high abundance or low abundance in *Arabidopsis*. The genes chosen were a highly expressed *ubiquitin* gene and a lowly expressed gene potentially encoding for a transcription factor. Neither of these genes are believed to be directly involved in floral development in *Arabidopsis* plants, and as such were not expected to be enriched for in the subtracted cDNA. Figure 5.9 demonstrates that the abundance of both these genes was significantly reduced in the subtracted sample (lanes 7 and 8) compared to the unsubtracted cDNA (lanes 5 and 6). This suggests that a significant decrease in the abundance of non-target tester cDNA has occurred during the subtraction. Some slight expression of the *Ubiquitin* gene can be detected in the subtracted samples suggesting that some very highly abundant sequences may not be fully subtracted from this cDNA population.

Figure 5.8

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with either the purified cDNA band 1 (B) or band 3 (C) from the subtracted cDNA sample. Lanes 2 and 3 in gel A, and lanes 1 and 2 in panels B and C, contain wild-type Pitztal RNA. Lanes 4 and 5 in gel A, and lanes 3 and 4 in panels B and C, contain RNA extracted from mutant line 300. Lane 6 in gel A, and lane 5 in panels B and C, contain control total RNA from *Sporobolus stapfianus.* Lanes 1 and 7 in gel A contain RNA markers.
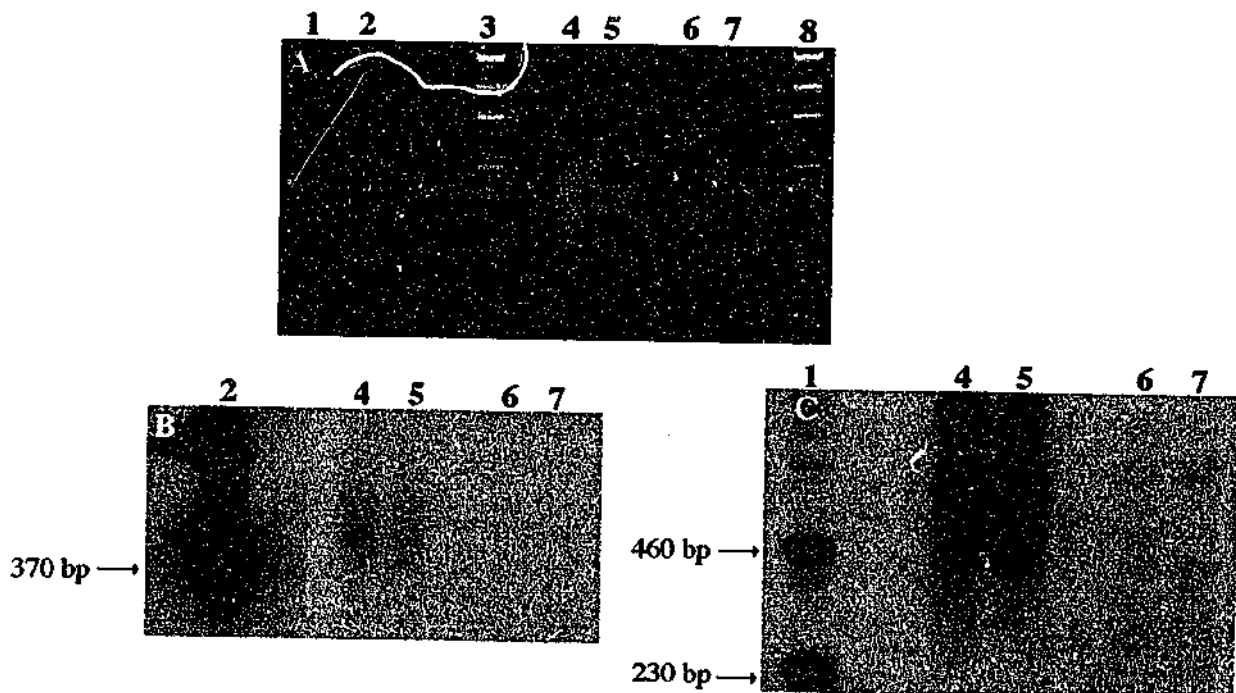
Figure 5.9

The ethidium bromide-stained gel (A) was blotted, and the filter hybridised with a gene encoding for a transcription factor (B), and then with the highly expressed *ubiquitin* gene (C). Lanes 1 and 2 contain positive control DNA for the *ubiquitin* gene and the putative transcription factor respectively. Lanes 3 and 8 contain φX174 *Hae*III markers. Lanes 4 and 5 contain unsubtracted cDNA samples and lanes 6 and 7 contain subtracted cDNA.

cDNA subtraction of in vitro grown mutant and wild-type Pitztal plants

The cDNA subtraction procedure was repeated in order to assess the reproducibility of the method. Plants grown *in vitro* were used in this new subtraction to help eliminate any potentially contaminating sources of RNA that may be present in soil-grown plants. RNA was again extracted from plants at the cotyledon, 2-leaf, 4-leaf, 6-leaf stage, and at the time of mutant flowering for both mutant and wild-type plants. mRNA, and subsequently cDNA, for both mutant and wild-type samples, was produced as per the initial subtraction procedure, and adaptors were ligated as described above for the appropriate populations of cDNA. For this subtraction, primers specific to a *ubiquitin* gene in several plant species were available for the ligation efficiency test. Figure 5.10 demonstrates the results of these tests for both the experimental samples and control skeletal muscle cDNA. The intensity of the PCR products amplified using two gene specific primers (lanes 10 and 11 in gel A and B) is similar to that of the products amplified using one gene specific primer and one PCR primer (lanes 2 to 9 in both gels) for both muscle and plant cDNA samples. This suggests that successful ligation of adaptors had occurred for both the plant and control samples.

The hybridisation and PCR steps of the cDNA subtraction were performed as described previously. Figure 5.11 demonstrates the results of this second subtraction. Again the subtraction of the spiked skeletal muscle cDNA subtracted against unspiked muscle cDNA was successful, as the only remaining bands in the subtracted sample (lanes 4 in each gel) correspond to those of the φX174 *Hae*III markers. However, the complexity of the banding pattern of the plant subtraction sample (lane 2 in each gel) was not significantly reduced from that of the unsubtracted cDNA sample (lane 3 in each gel). The hybridisation steps of this subtraction were therefore repeated once more. The PCR products of this second subtractive hybridisation again showed little difference in banding patterns between subtracted and unsubtracted cDNA samples. It was therefore decided that the products of the initial subtraction involving soil-grown plants would be used for the majority of further analysis.

Cloning of the cDNA subtraction samples

The subtraction sample was digested with *Rsa*I, to remove the adaptor sequences, and the resulting blunt ended fragments ligated into pBluescript that had been digested with *Hinc*II. The ligated vector and insert molecules were then transformed into *E. coli* utilising the TB transformation method (section 2.1.3) and a library of approximately 30,000 colonies, putatively containing inserts, was thus created. The resulting plasmid library could then be screened for sequences hybridising only to cDNA created from the wild-type Pitztal genome.
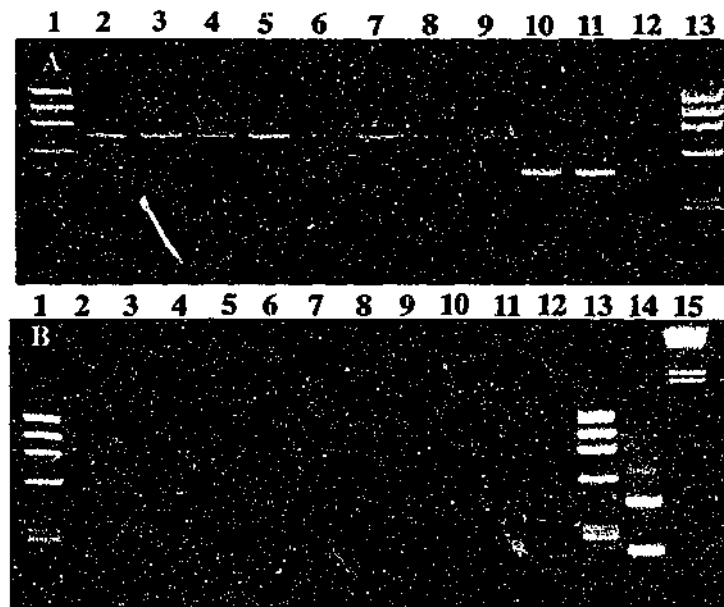
Figure 5.10

A ligation efficiency test performed for the control skeletal muscle tester cDNA (A) and the Pitztal tester cDNA (B), using primers specific for the *G3PDH* and *ubiquitin* genes respectively and PCR primers specific to the adaptor sequences. Lanes 1 and 13 in both gels contain φX174 *Hae*III markers. Samples ligated with either adaptor 1 (lanes 2 and 3 in each gel) or adaptor 2 (lanes 4 and 5 in each gel) or both adaptors (lanes 6, 7, 8, and 9 in each gel) were amplified with one gene specific primer and either primer 1 (lanes 2, 3, 5, and 6 in each gel) or primer 2 (lanes 3, 4, 7, and 8 in each gel). Lanes 10 and 11 in each gel contain samples ligated to both adaptors amplified using two gene specific primers. Lane 12 in each gel contains a negative PCR control. Lanes 14 and 15 in gel B contain genomic DNA amplified using *ubiquitin* gene primers and λ *Hind*III markers respectively.
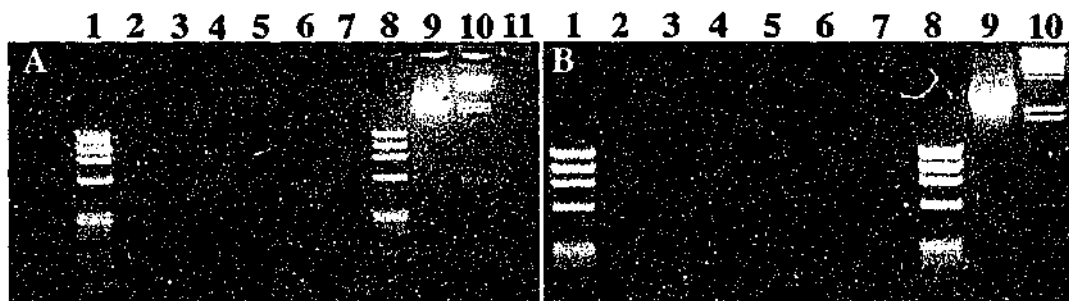
Figure 5.11

The cDNA subtraction products were initially amplified using PCR primers 1 and 2 (gel A) and subsequently re-amplified using nested PCR primers 1 and 2 (gel B). Lanes 1 and 8 in gels A and B contain φX174 *Hae*III markers. Lane 2 in these gels contains Pitztal cDNA subtracted against cDNA from mutant line 300. Lane 3 in each gel contains unsubtracted Pitztal cDNA. Lane 4 in these gels contains skeletal muscle cDNA spiked with φX174 *Hae*III markers subtracted against unspiked skeletal muscle cDNA. Lane 5 in each gel contains unsubtracted skeletal muscle cDNA. Lane 6 in each gel contains control subtracted skeletal muscle cDNA. Lanes 7 and 9 in these gels contain negative and positive PCR controls respectively. Lane 10 in each gel contains λ *Bste*II markers.

Several replica colony blot filters, representative of approximately 1600 colonies, were progressively screened separately with wild-type and mutant cDNA probes. The replica colony blot filters were left on fresh bacterial media plates for several hours before the DNA was hydrolysed and fixed to allow an even amount of bacterial growth to be present on each filter. The wild-type and mutant cDNA probes were also checked in a scintillation counter prior to screening, and their volumes adjusted to give approximately the same amount of counts per minute.

Following the primary screens, any colonies showing a stronger signal when hybridised to the wild-type probe (approximately 200 to 300 per primary screen), were re-screened with wild-type and mutant cDNA probes. These colonies were also screened with the subtracted cDNA sample from plants grown *in vitro*, to assess whether any of these colonies contained sequences strongly expressed in a subtracted sample. The DNA from several of the colonies isolated from the primary screens subsequently showed stronger signals with the wild-type cDNA probe following the secondary screen. Unexpectedly, some colonies gave stronger signals with the cDNA prepared from mutant mRNA, while another colony exhibited the strongest hybridisation to the subtracted cDNA sample.

To determine the types of sequences present in the colonies showing stronger hybridisation with either the mutant, wild-type cDNA, or subtraction cDNA probe, the insert DNA of several of the above colonies was sequenced (a complete summary of the types of sequences found in these clones is presented later in this chapter). Four of the ten colonies initially sequenced were found to contain sequences homologous to genes encoding for the light-harvesting, CAB proteins. This result suggested that a large proportion of colonies in the library may contain fragments of genes coding for these CAB proteins. In addition, hybridisation of a filter containing subtracted and unsubtracted cDNA showed that there was a significant enrichment of CAB gene sequences in the subtracted sample. It was therefore decided to pre-screen the colony library with a CAB gene sequence in an attempt to prevent the isolation of any further clones with similar insert sequences. The number of colonies detected by this pre-screen was not as numerous as predicted however, and therefore no further pre-screening was performed.

*Cloning of individual bands from the subtracted cDNA samples*

As it is difficult to ensure that the same amount of DNA template was present on the colony blots probed with either the mutant and wild-type cDNA, a new more definitive method was employed to firstly identify, and then clone, individual bands that appear to be enriched in the subtraction sample. This involved running the subtracted and unsubtracted samples from the hybridisations of cDNA from the plants grown in soil or *in vitro*, on a large acrylamide

gel. The resolution of bands achieved by running the samples on these gels makes the separation of individual bands relatively easy. For this analysis subtracted and unsubtracted samples were amplified as previously described, however the S^{35} isotope was also incorporated into the products of these reactions. The radioactive samples were then run, in parallel, on the sequencing gel along with radioactively labelled markers that were prepared as described in section 5.2.9 (Figure 5.12).

34 distinct cDNA bands were present in the subtracted samples (lanes 2, 4, 6 and 8), one of which appeared to be specific to the subtracted cDNA from plants grown *in vitro* (Band 33). The rest of the bands were either specific to the subtracted cDNA from soil-grown plants, or were common to the two types of subtracted samples. The differences in the banding pattern between subtracted and unsubtracted samples from soil-grown plants is clearly visible (lanes 2 to 5). The banding pattern of the subtracted and unsubtracted samples from the plants grown *in vitro* was quite similar, as had previously been observed (lanes 6 to 9).

The 34 bands labelled in Figure 5.12 were extracted from the gel as described in section 5.2.8. The bands were then re-amplified via two rounds of PCR using PCR primers 1 and 2, and subsequently nested PCR primers 1 and 2. In some instances several attempts were required before a product could be obtained, and several reactions resulted in the amplification of multiple bands. Once the majority of bands could be reproducibly amplified, the PCR products of these 34 bands were botted, and the filters hybridised with wild-type Pitztal or mutant cDNA in an attempt to detect sequences exhibiting stronger signals with the wild-type probe (Figures 5.13 (a) and 5.13 (b)).

The cDNA probes for this analysis were prepared from RNA harvested from plants at the same five developmental stages as those used for the subtractions (cotyledon, 2-leaf, 4-leaf, 6-leaf, mutant flowering). The radioactivity of these two probes was again checked and the volumes of the probes adjusted so that they both displayed similar counts per minute. Although this difference was more pronounced for several bands, the intensity of the signal detected was generally stronger following hybridisation with the wild-type probe (Figures 5.13a and 5.13b). Therefore all bands were chosen for further investigation. Initially several purified bands (2, 5, 6, 7, 10, 11, 13, 14, and 23) were used to screen the previously constructed plasmid library for positive clones. As this plasmid library was created using blunt-ended ligation of fragments however, several of the clones identified via this colony blot analysis contained multiple RsaI fragment inserts. Therefore the remaining bands were directly cloned into the pGEM-T™ cloning vector. Following the isolation of recombinant clones, five to six colonies putatively containing the appropriate insert, were selected for each band and the inserts of the plasmids sequenced. Not suprisingly the sequences of the inserts of these colonies for each band were all similar in size, however they represented sequences showing homology to several different types of genes.

205
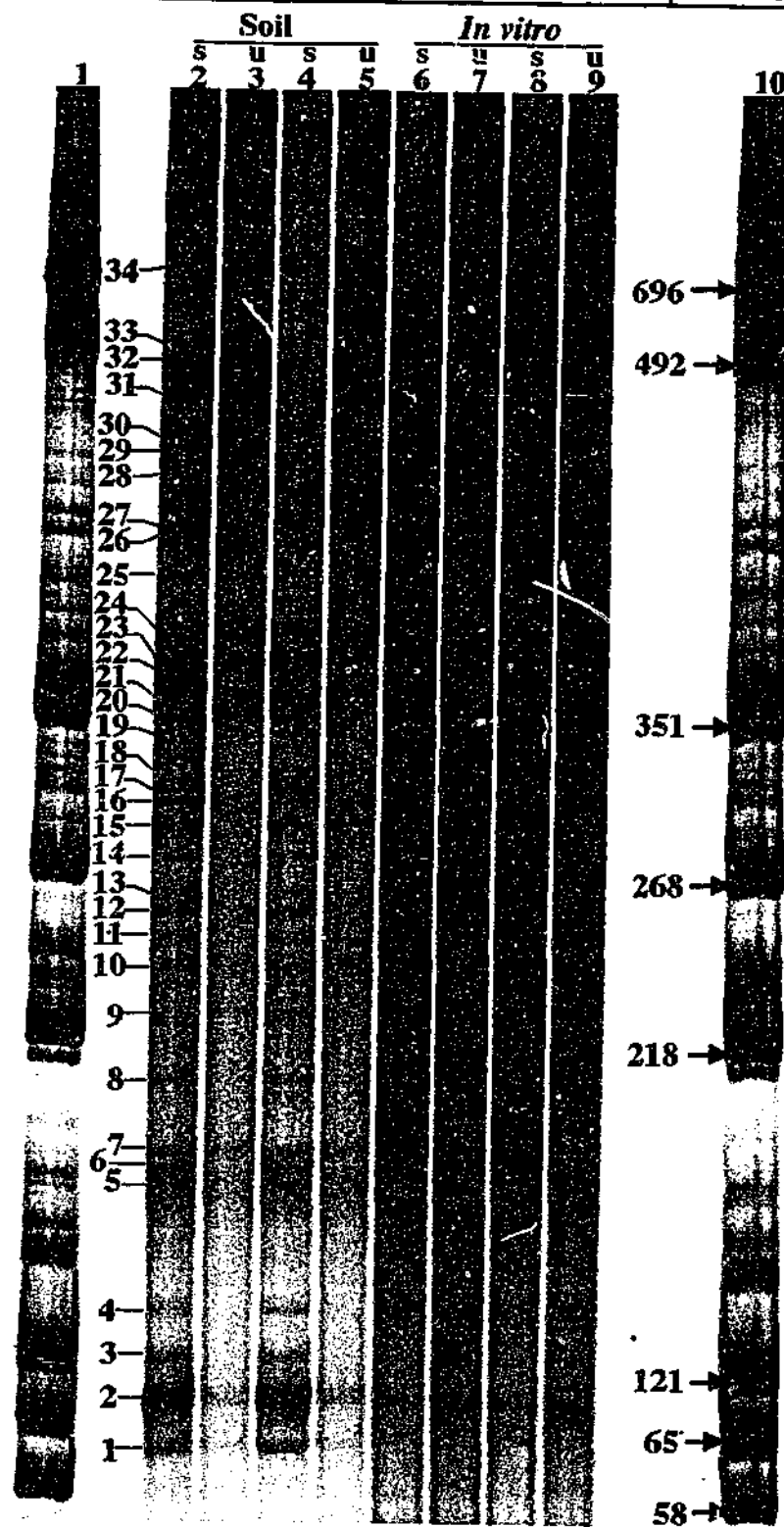
Figure 5.12

The PCR products of the cDNA subtractions were electrophoresed on a polyacrylamide gel in order to isolate and clone individual sequences. Lanes 2 and 4 contain the subtracted cDNA (s), and lanes 3 and 5 the unsubtracted cDNA (u), from the experiment that utilised RNA extracted from plants grown in soil. Lanes 6 and 8 contain subtracted cDNA (s), and lanes 7 and 9 contain unsubtracted cDNA, from the experiment that utilised RNA extracted from *in vitro*-grown plants. Lanes 1 and 10 contain the pUC119 vector digested with *ScRF*I and *Ssp*I enzymes and radioactively labelled with α-^{33}P-dATP.
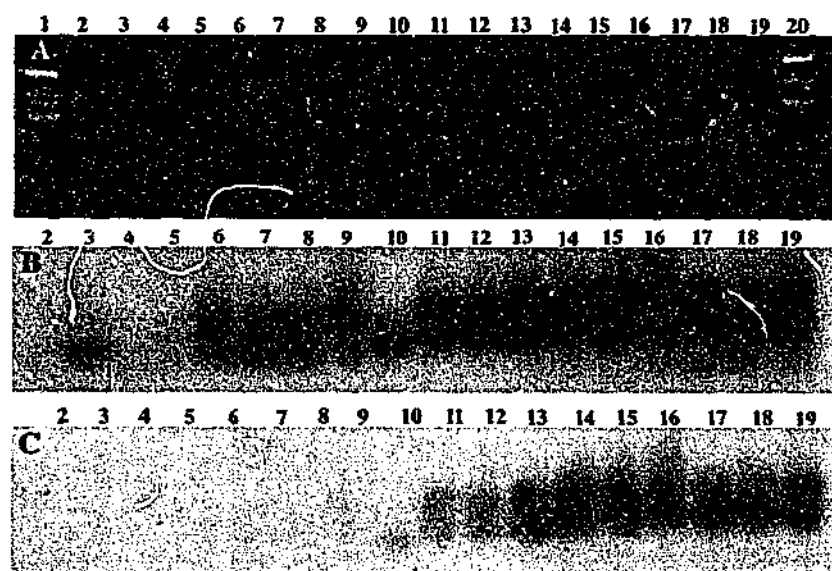
Figure 5.13(a)

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with wild-type Pitztal cDNA (B) or cDNA from mutant line 300 (C). Lanes 2 to 19 contain the PCR products amplified from the purified bands 1 to 18 from the cDNA subtraction sample. Lanes 1 and 20 contain ϕX174 *Hae*III markers.
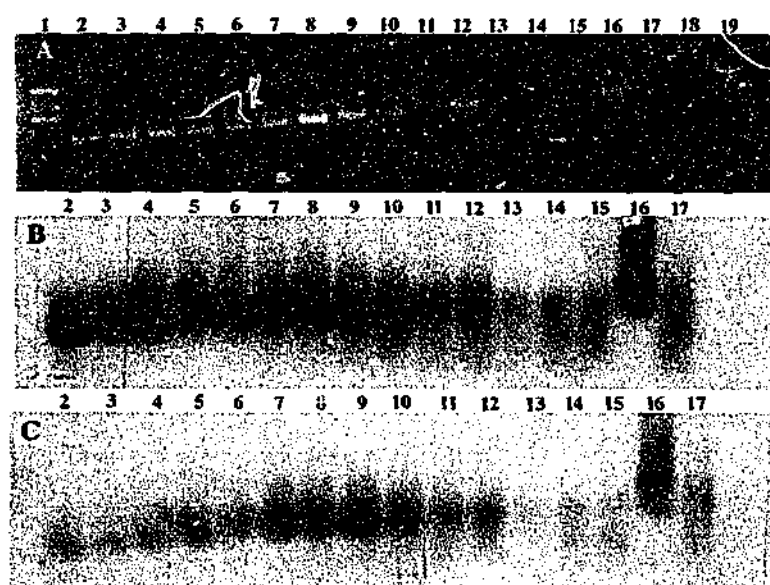
Figure 5.13(b)

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with wild-type Pitztal cDNA (B) or cDNA from mutant line 300 (C). Lanes 2 to 17 contain the PCR products amplified from the purified bands 19 to 34 from the cDNA subtraction sample. Lanes 1 and 19 contain ϕX174 *Hae*III markers and lane 18 contains a negative PCR control.

A summary of the types of sequences obtained in the cDNA subtractions is presented in subsequent pages of this chapter. This summary details the types of genes the sequences are homologous to, the number of clones in total showing homology to these genes and a physical map of the position of these clones within the gene. A brief description of the genes and their regulation is also described in this summary.

The most highly represented gene amongst the subtraction clones, with 20 in total, encodes for the chloroplast 23S ribosomal RNA protein. Indeed, several genes located on the plastid genomes are well represented amongst the subtraction clones, including several types of ribosomal proteins and mitochondrial ribosomal RNA genes. The genes with the largest range of family members represented amongst the subtraction population are those encoding for the light-harvesting CAB proteins of the chloroplast, with 17 clones showing homology to six different types of CAB genes from a number of species. A large number of clones showing homology to genes encoding for proteins involved in other downstream light reactions of photosynthesis were also identified. Such proteins were involved either directly or peripherally in the electron transport chain between PSII and PSI, or were structural proteins of the PSI and PSII reaction centres and surrounding molecules. Several of these genes are also located on the chloroplast genome.

Other genes highly represented amongst the subtraction sample are those encoding for the proteins involved in the dark reactions of photosynthesis. In particular, 15 clones showed homology to the four genes encoding for the small sub-unit polypeptides of the Ribulose-1,5-Bisphosphate carboxylase (Rubisco) enzyme. Clones showing homology to the large sub-unit of Rubisco, as well as a Rubisco activase gene, were also isolated. Several genes unrelated to photosynthesis or other light-regulated process were however also represented amongst the subtraction clones. These genes included those encoding for a putative heavy metal binding protein, translation elongation factors and proteins involved with the hormonal and nutrient regulation of the plant. Many of these genes are basic housekeeping genes and therefore the roles of these genes in the early-flowering mutant phenotype is difficult to assess.

The fact that many chloroplast-encoded genes, as well as genes involved with the photosynthetic reactions of the plant, were isolated from the subtraction suggested some disruption in the light-harvesting functions of the mutant line. The paler green phenotype of the 300 *fler* mutant line, particularly at earlier stages of development, was also indicative of some disruption in chlorophyll levels. Therefore to test whether this disruption may be in pigment molecules of the mutant, a chlorophyll assay for both mutant and wild-type plants was performed. Figure 5.14 shows the levels of chlorophyll a and b detected in non-flowering or flowering mutant and wild-type plants. The leaf phenotype at the relevant

stages of development of these plants is also displayed. Following this analysis no major difference in the levels of either chlorophyll a or b was observed between mutant and wild-type plants. There was slightly lower levels of both types of chlorophyll however, detected in the flowering mutant plants compared to flowering wild-type plants.

Several genes which show strong homology to genes in the database with no specified function, as well as genes showing little if any homology to any genes represented in the database, were also isolated. It was thought that these genes may represent novel genes having functions putatively related to the early-flowering phenotype of the *fler* mutant line 300.

In order to test that all the genes thus-far identified had been enriched in the subtracted samples a representative of each gene, or type of gene, was chosen to probe filters of subtracted and unsubtracted cDNA. The results of these hybridisation are also provided in the subtraction clone summary pages. The majority of the genes represented within the subtraction population did exhibit significant enrichment in the subtraction sample. Some genes, including several of the sequences whose functions were unknown, however showed little difference in their abundance between unsubtracted and subtracted cDNA. Such genes were therefore not chosen for further analysis.

Sequences representative of most types of genes found amongst the subtraction population, and showing a strong enrichment in the subtracted cDNA, were then used to probe Northern blots. These filters contained RNA extracted from mutant line 300 and wild-type Pitztal plants grown to the same five developmental stages as those used to isolate RNA from for the subtraction. Figures 5.15(a) to 5.15(s) present the results of these hybridisations.
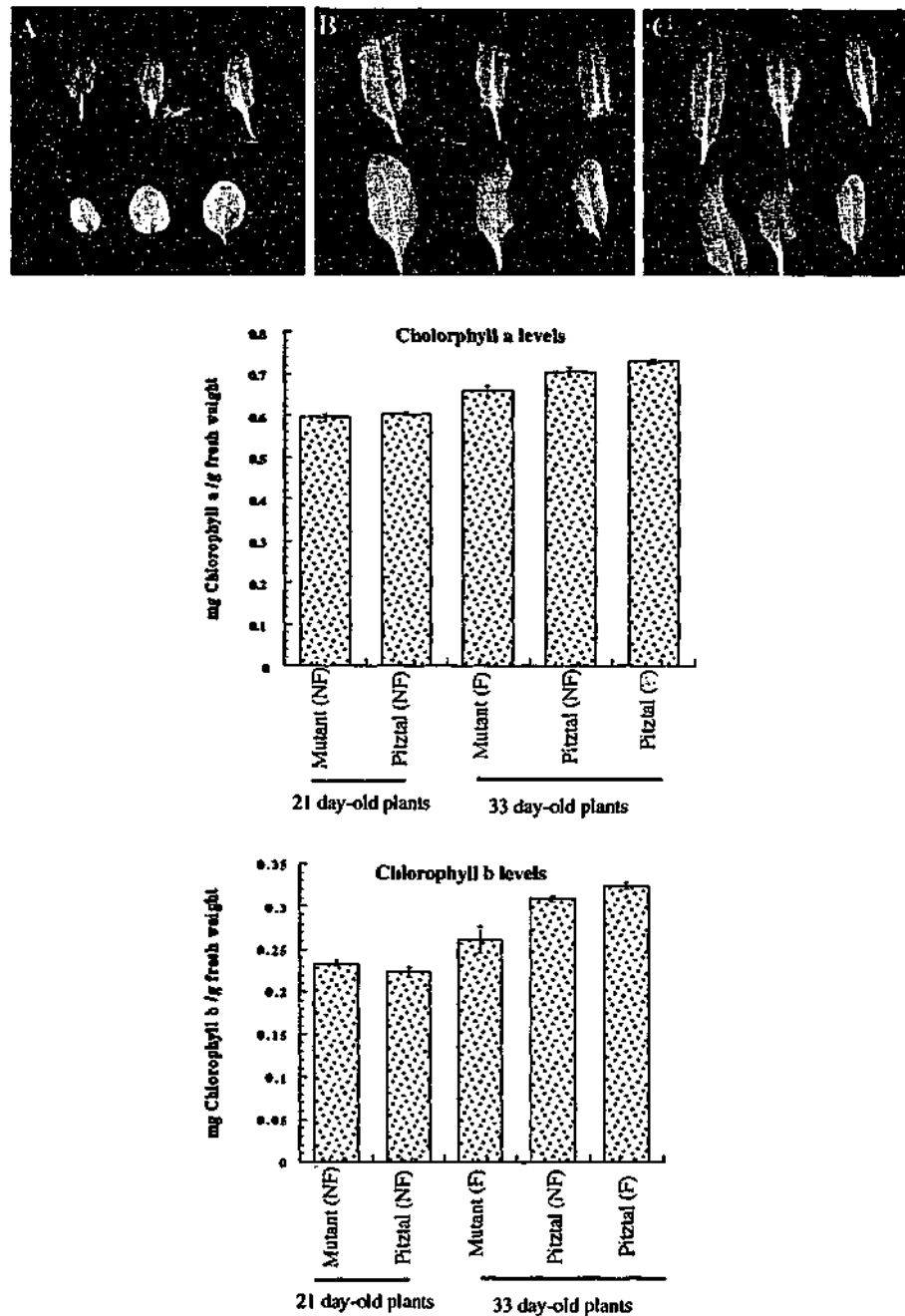
Figure 5.14

The leaf phenotype of mutant line 300 and wild-type Pitztal plants is shown in panels A, B and C. The top row of leaves in each panel are from wild-type Pitztal plants while the lower leaves were taken from mutant plants. Panel A shows leaves from non-flowering 21 day old plants. Panels B show leaves from 33 day old flowering mutant plants and 33 day old non-flowering Pitztal plants. Panel C displays leaves from 33 day old flowering mutant and 33 day old wild-type Pitztal plants. The chlorophyll a and b levels in leaves of mutant line and wild-type Pitztal plants at 21 days, or 33 days post-germination are shown in the histograms above. Chlorophyll levels were measured in non-flowering (NF) plants of the mutant line and wild-type Pitztal at the day 21 post-germination, or in flowering (F) mutant and wild-type plants as well as in non-flowering(NF) wild-type Pitztal plants at day 33 post-germination.

cDNA SUBTRACTION CLONE SUMMARY

The diagrams below show the regions of homology between sequences from the database and the various clones. The thicker line in each clone diagram represents the regions of the clone showing the homology to the gene of interest. The first number in the clone nomenclature refers to the number of the corresponding band in the subtracted cDNA sample that was used to identify or create the clone. Other clones with a prefix of mut, wt or sub followed by a number were isolated via screening the initial colony library with mutant, wild-type, and the *in vitro* subtraction cDNA. Colonies showing stronger hybridisation with the wild-type cDNA are referred to as wt clones, colonies hybridising more strongly with mutant or subtraction cDNA sample were labelled mut or sub clones respectively. The second number in all clone names refers to the number of the clone that has been sequenced.

NUCLEAR ENCODED RIBOSOMAL RNA GENES

Database matches to the *ATRRN18* (18S rRNA) gene from *Arabidopsis*

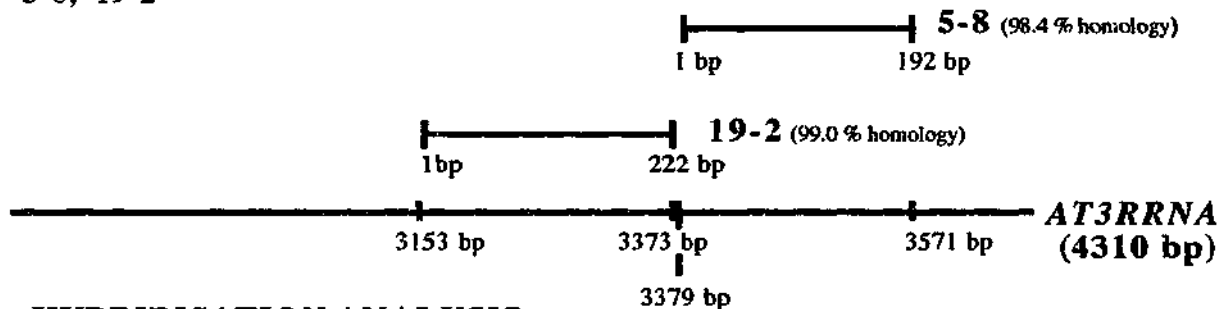(Unfried and Gruendler, 1990).

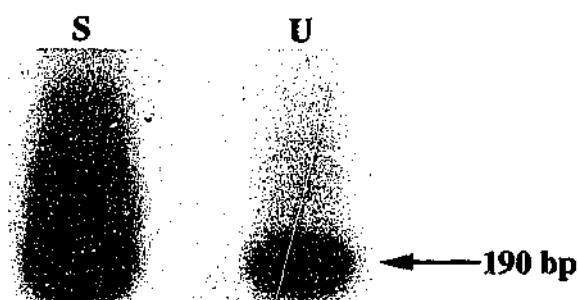**Clones (6)**
13-1,13-4,14-3, 14-4, 18-7, 29-1
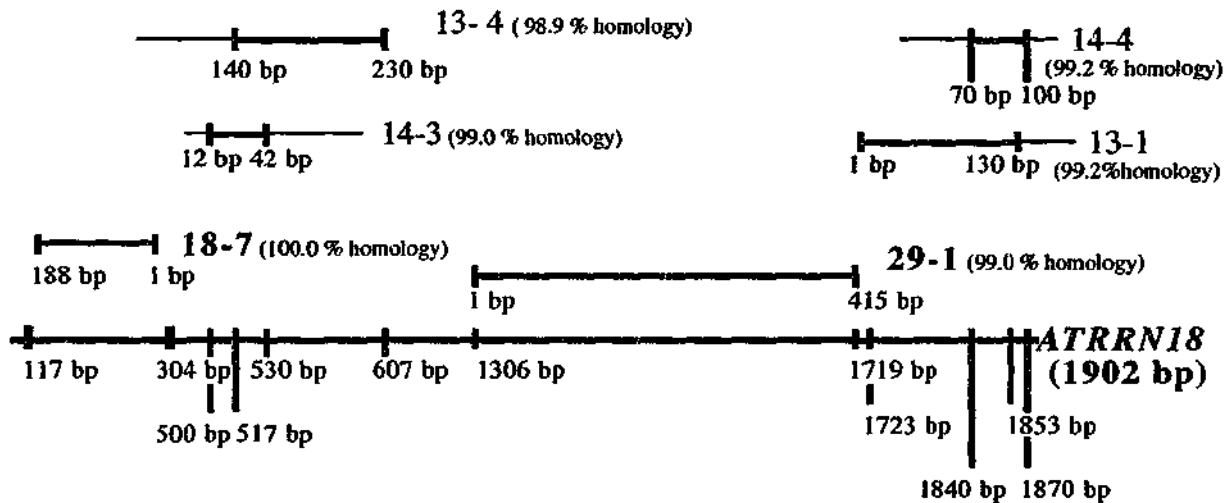
Database matches to the *AT3RRNA* (5.8S rRNA) gene from *Arabidopsis*.

Clones (2)
5-8, 19-2
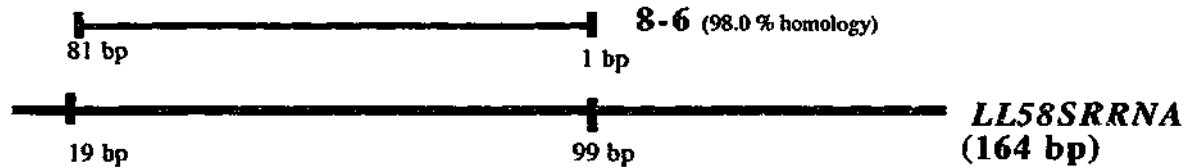

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 5-8. The autorad shown was exposed to the filter for 5 minutes at -70°C with an intensifying screen.

←190 bp

Database match to the *LL58SRRNA* (5.8S rRNA) gene fragment from *Lupinus luteus* (Yellow Lupin).

Clones (1);
8-6

```
|————————————————————————————|   8-6 (98.0 % homology)
81 bp                          1 bp
```

```
——————|—————————————————————————|——————————  LL58SRRNA
19 bp                          99 bp          (164 bp)
```
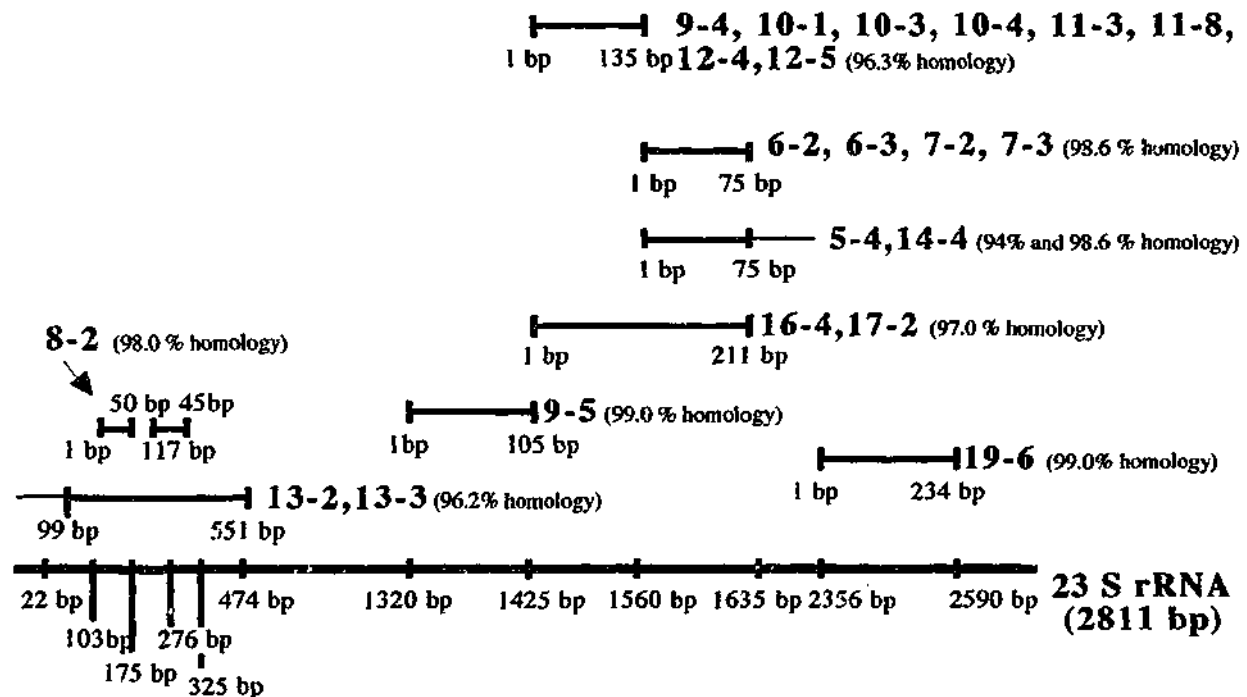
PLASTID ENCODED RIBOSOMAL RNA GENES

The complete chloroplast genome of several species has been sequenced and has been found to be very homologous to each other (Link and Langridge, 1984; Strittmatter and Kossel, 1984; ; Hiratsuka *et al.*, 1989; Shimada and Sugiura, 1991; Wakasugi *et al.*, 1994). The 23S, 4.5S and 5S rRNS transcripts are thought to be co-transcribed in Zea Mays (Strittmatter and Kossel, 1984). In addition the complete mitochondrial genome of *Arabidopsis* has now been sequenced and found to contain three rRNAs (18S, 26S, and 5S).

Database matches to the 23S chloroplast rRNA gene from *Alnus incana.*

Clones (21);
5-4, 6-2, 6-3, 7-2, 7-3, 8-2, 9-4, 9-5, 10-1, 10-3, 10-4, 11-3, 11-8, 12-4, 12-5, 13-2, 13-3, 14-4, 16-4,17-2, 19-6.

```
                                 |————|   9-4, 10-1, 10-3, 10-4, 11-3, 11-8,
                                1 bp  135 bp 12-4,12-5 (96.3% homology)
```

```
                                      |————|  6-2, 6-3, 7-2, 7-3 (98.6 % homology)
                                      1 bp  75 bp
```

```
                                      |————|——  5-4,14-4 (94% and 98.6 % homology)
                                      1 bp  75 bp
```

```
                                 |—————————|16-4,17-2 (97.0 % homology)
 8-2 (98.0 % homology)           1 bp       211 bp
        ↓
      50 bp 45bp
     |—|  |—|                |————|9-5 (99.0 % homology)
   1 bp  117 bp            1bp   105 bp
                                                   |————————|19-6 (99.0% homology)
 ——————|—————————| 13-2,13-3 (96.2% homology)    1 bp    234 bp
 99 bp        551 bp
```

```
——|—|—|—|————|————————|————————|————————|————————|————————|———  23 S rRNA
22 bp |  | | 474 bp  1320 bp  1425 bp 1560 bp 1635 bp 2356 bp  2590 bp  (2811 bp)
    103bp 276 bp
      175 bp 325 bp
```

HYBRIDISATION ANALYSIS;
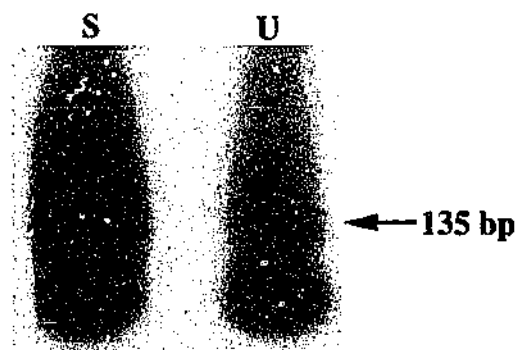
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 10-3. The autorad shown was exposed to the filter for 1.5 hours at -20°C.

←— 135 bp

<u>**Database matches to the 26S mitochondrial rRNA gene from the**</u>
<u>**mitochondrial genome, part A, from *Arabidopsis.***</u>
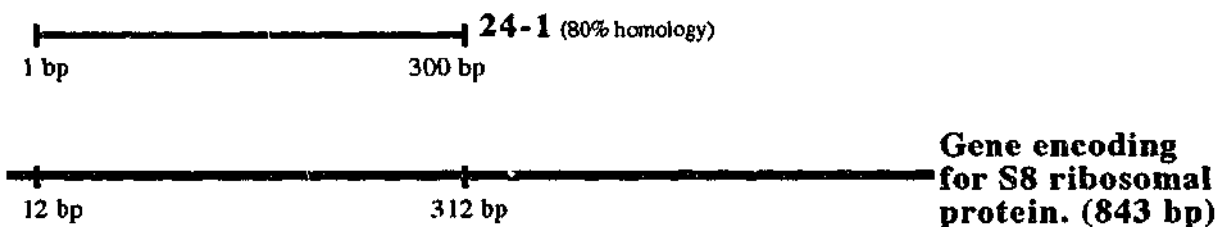
Clones (5);
sub1, 7-4, 9-3, 29-3, 29-5
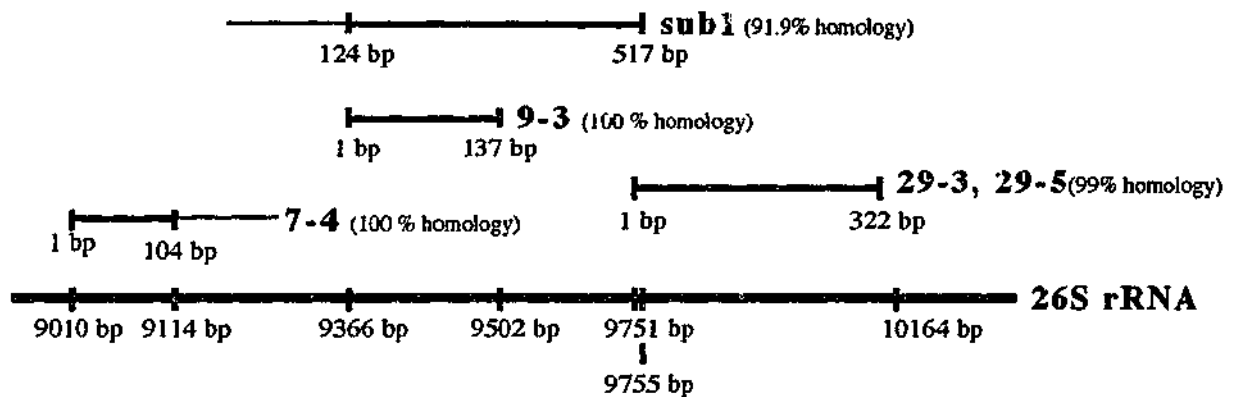
RIBOSOMAL PROTEINS

Genes encoding ribosomal proteins are found on both the chloroplast and mitochondrial genomes in plants. Ribosomal proteins have been found to be quite strongly expresed in meristematic regions of plants including floral meristems and root apices. In addition, ribosomal proteins have been found to be co-ordinately activated and their expression can be induced by plant hormones such as auxins and cytokinins (Gantt and Key, 1985; Kohler *et al.*, 1992).

<u>**Database match to the gene for the chloroplast encoded ribosomal protein**</u>
<u>**S8 from *Zea Mays.***</u>

Clone (1);
24-1

Database matches to the gene (CHBRRPL32) encoding for the chloroplast encoded ribosomal protein L32 from *Brassica Napus*.

Clones (8);
10-6, 11-6, 11-7, 12-2, 13-4, 18-5, 23-4, 23-5
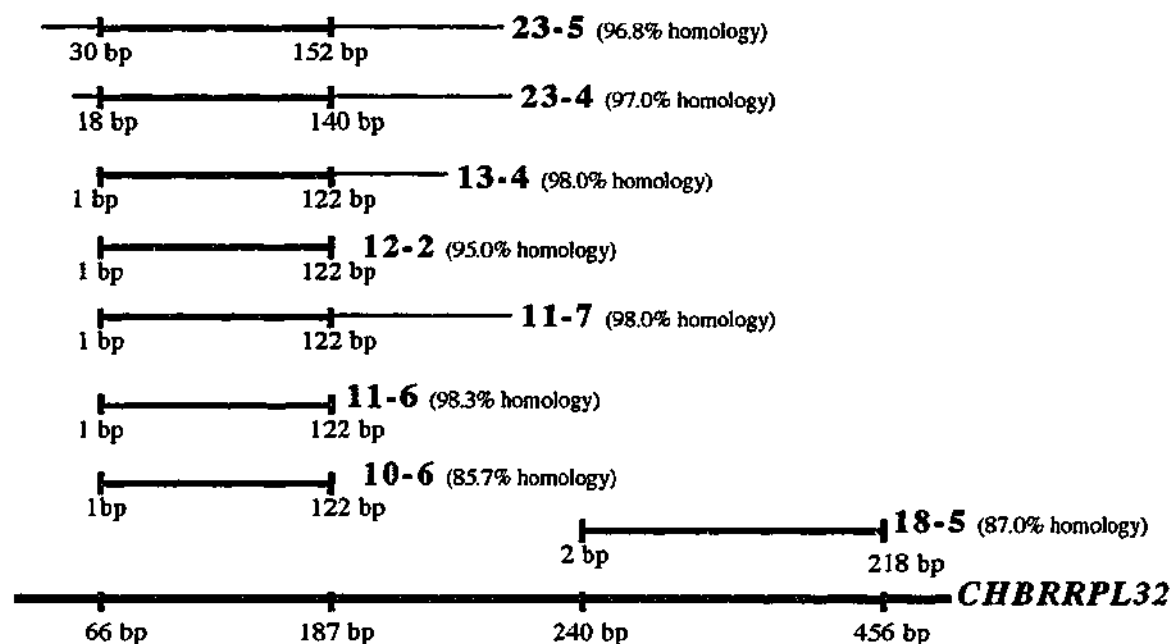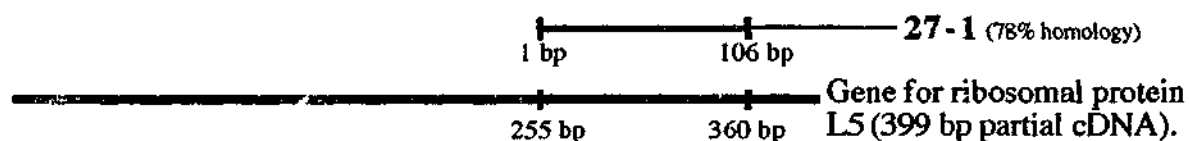

HYBRIDISATION ANALYSIS;
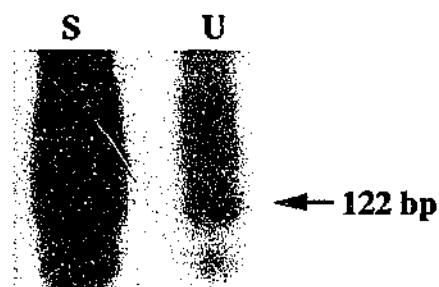
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 11-6. The autorad shown was exposed to the filter for 4 nights at -70°C with an intensifying screen.

Database match to the gene for the mitochondrial encoded ribosomal protein L5 from *Solanum melongena*.

Clone (1);
27-1

CHLOROPHYLL A/B BINDING GENES

Chlorophyll a/b binding proteins bind to chlorophyll molecules in the antenna light harvesting complexes of PSII and PSI (Lam *et al.*, 1984; Pichersky *et al.*, 1991). The different pigment protein complexes formed with different cab genes allows a range of wavelengths of red light to be absorbed by the chlorophyll molecules (White *et al.*, 1994). CAB proteins are encoded for by nuclear genes, synthesised in the cytoplasm and post-translationally transported into the chloroplast via a transit peptide. They are cleaved in the chloroplast to become mature proteins and subsequently associate with chlorophylls. In most higher plants, including *Arabidopsis*, there are thought to be approximately 10 CAB protein types (I-X) (Jensen *et al.*, 1992; Leutwiler *et al.*, 1986; McGrath *et al.*, 1992). All CABs show significant homology to each other within and across several species.

Database matches to two (*LHCP AB 140*, *LHCP AB 165*) of a small family of genes encoding *Arabidopsis* chlorophyll a/b binding proteins.

Clones (2);
8-3, 23-1

23-1 (99.4% homology)

105 bp   282 bp

LHCP AB 140
(1109 bp)

189 bp   368 bp

8-3 (99% homology)

1 bp   100 bp

LHCP AB 165
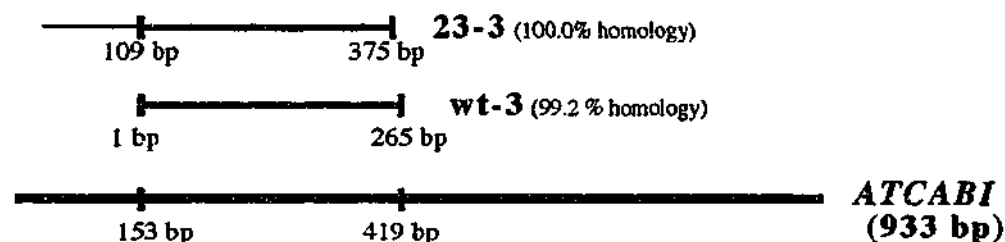**(999 bp)**

197 bp   296 bp
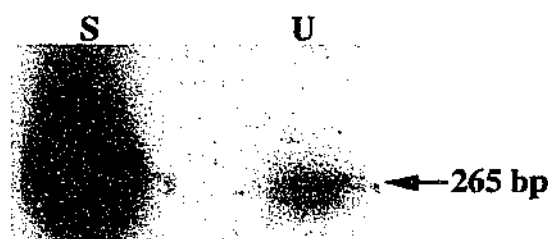
HYBRIDISATION ANALYSIS;

S   U
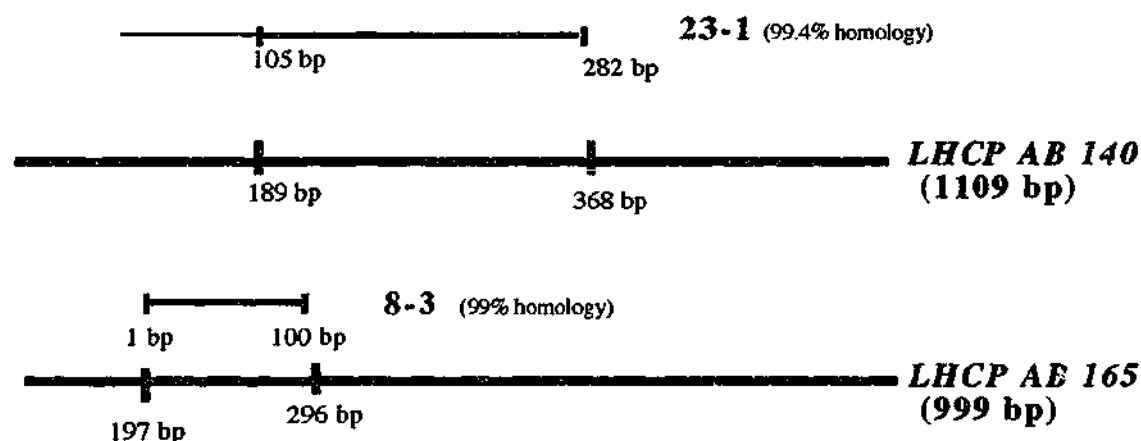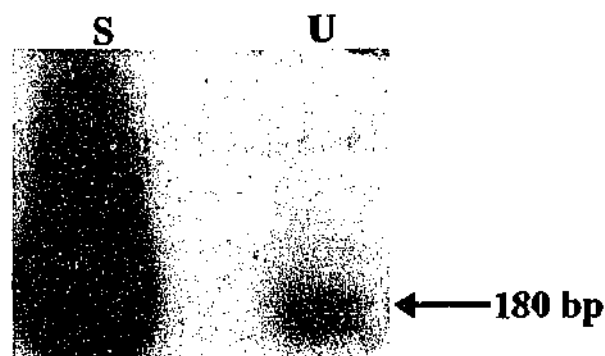
Subtracted (S) and unsubtracted (U) cDNA was probed with bases 105 to 284 of the insert sequence of clone 23-1. The autorad shown was exposed to the filter for 30 minutes at -70°C with an intensifying screen.

←— **180 bp**

215

Database matches to the gene (*ATCABI*) encoding for a CAB protein associated with PSI from *Arabidopsis.*
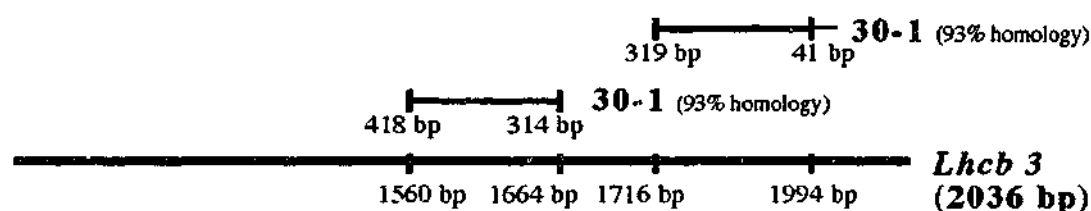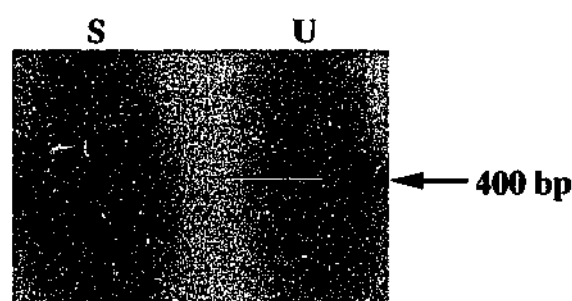
Clones (2);
wt-3, 23-3

23-3 (100.0% homology) — 109 bp, 375 bp

wt-3 (99.2 % homology) — 1 bp, 265 bp

ATCABI **(933 bp)** — 153 bp, 419 bp

HYBRIDISATION ANALYSIS;

S   U   ←265 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone wt-3. The autorad shown was exposed to the filter for 45 minutes at -70°C with an intensifying screen.

Database match to one of a small family of genes (*Lhcb 3*) encoding a type III CAB protein associated with PSII from *Brassica napus.* (Boivin *et al.*, 1993).

Clones (1);
30-1

30-1 (93% homology) — 319 bp, 41 bp

30-1 (93% homology) — 418 bp, 314 bp

Lhcb 3 **(2036 bp)** — 1560 bp, 1664 bp, 1716 bp, 1994 bp

HYBRIDISATION ANALYSIS;
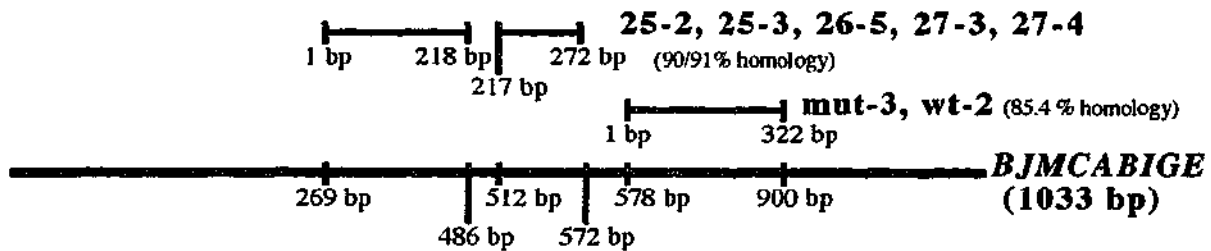

S   U   ← 400 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 30-1. The autorad shown was exposed to the filter for 2 hours at -20°C.
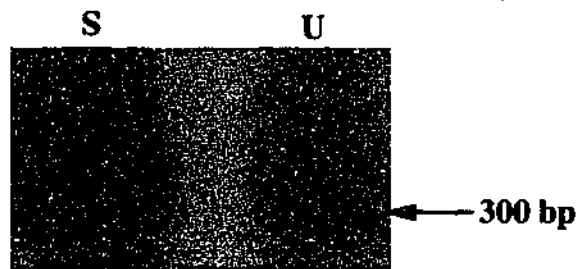
Database matches to a gene (*BJMCABIGE*) encoding for a CAB protein from *Brassica jubcea.*

Clones (7);
mut-3, wt-2, 25-2, 25-3, 26-5, 27-3, 27-4

25-2, 25-3, 26-5, 27-3, 27-4

1 bp   218 bp   272 bp   (90/91% homology)

217 bp

mut-3, wt-2 (85.4 % homology)

1 bp   322 bp

BJMCABIGE
(1033 bp)

269 bp   512 bp   578 bp   900 bp

486 bp   572 bp

HYBRIDISATION ANALYSIS;

S   U

◄─── **300 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 25-3 The autorad shown was exposed to the filter for 2 hours at -20ºC.
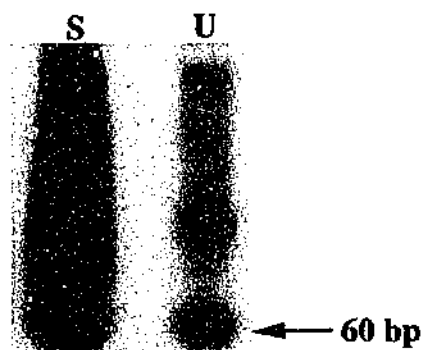
Database match to a gene (*Lhab*) encoding for a type II CAB protein associated with PSI from *Pisium sativum.*
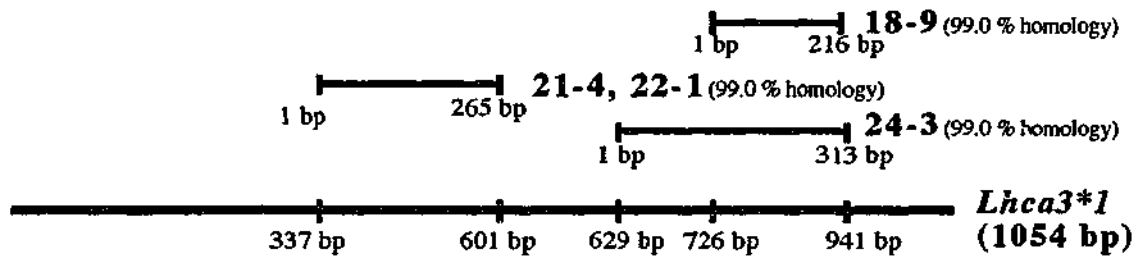
Clone (1);
5-4

5-4 (86% homology)

75 bp   133 bp

Lhab (**1014 bp**)

783 bp 841 bp

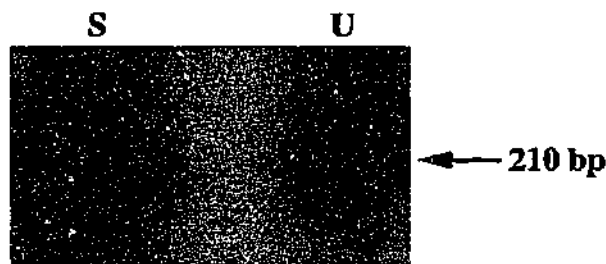## HYBRIDISATION ANALYSIS;

S   U

◄─── **60 bp**
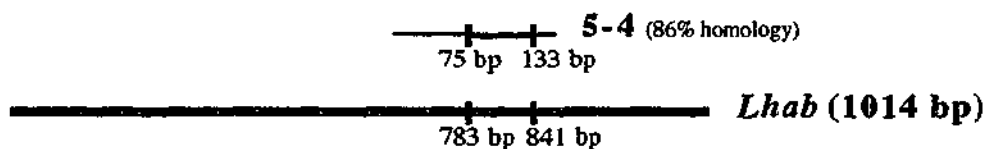
Subtracted (S) and unsubtracted (U) cDNA was probed with bases 75 to 133 of the insert sequence of clone 5-4 The autorad shown was exposed to the filter for 6 hours at-70ºC with an intensifying screen.

Database matches to a gene (*Lhca3*1*) encoding for a type III CAB protein associated with PSI from *Arabidopsis.*

Clones (4);
18-9, 21-4, 22-1, 24-3

18-9 (99.0 % homology)
1 bp   216 bp

21-4, 22-1 (99.0 % homology)
1 bp   265 bp

24-3 (99.0 % homology)
1 bp   313 bp

*Lhca3*1*
(1054 bp)
337 bp   601 bp   629 bp 726 bp   941 bp

HYBRIDISATION ANALYSIS;
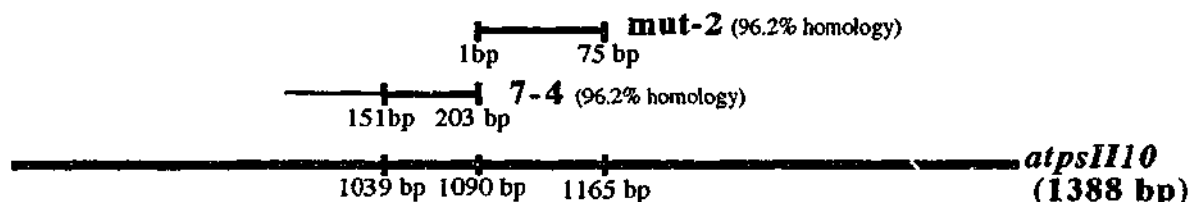
S   U

◄── 210 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 18-9. The autorad shown was exposed to the filter for 2 hours at -20ºC.
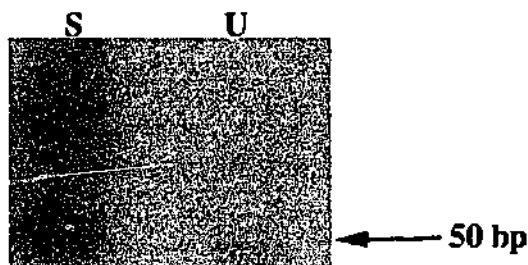
OTHER PHOTOSYNTHESIS RELATED GENES

Database matches to a gene (*atpsII10*) encoding for a 10kDa protein associated with PSII from *Arabidopsis.*

The transcription of this gene is strongly enhanced by light and only substantially expressed in stems and leaves (Gil-Gomez *et al.*, 1991).

Clones (2);
mut-2, 7-4

mut-2 (96.2% homology)
1bp   75 bp

7-4 (96.2% homology)
151bp   203 bp

atpsII10
(1388 bp)
1039 bp 1090 bp   1165 bp
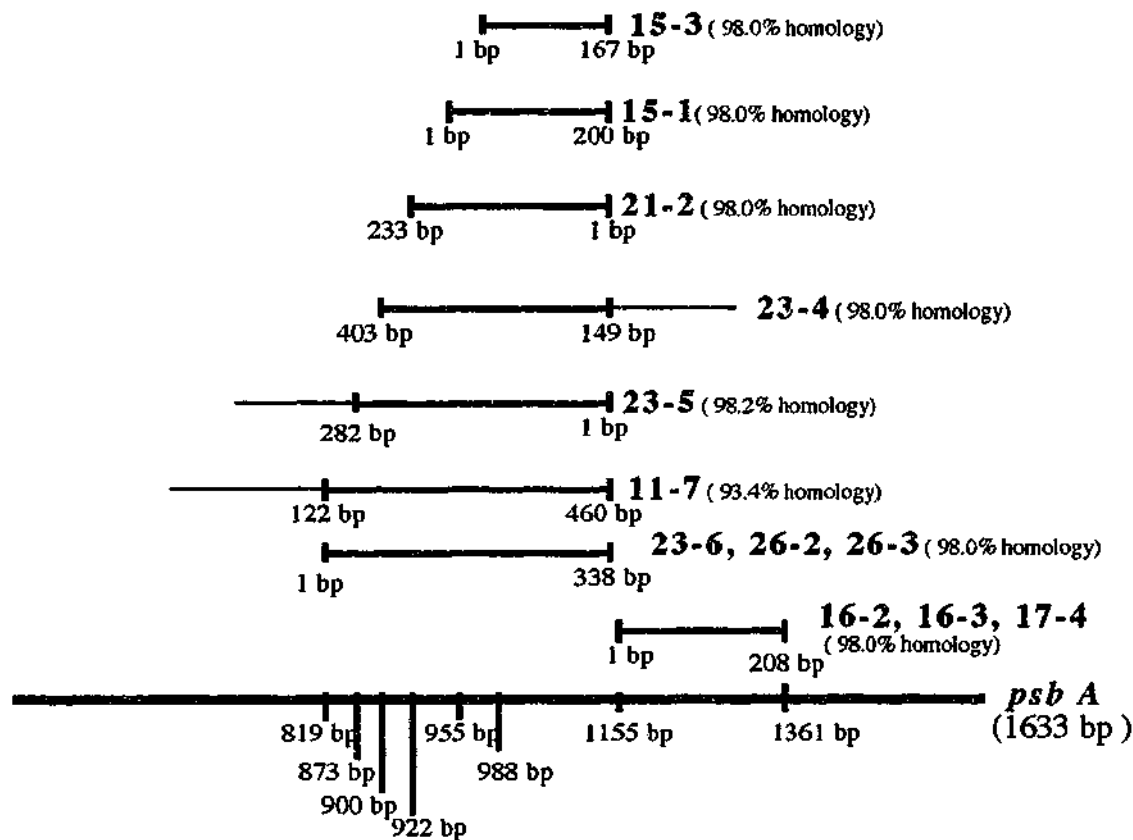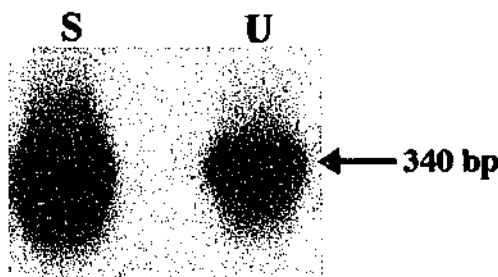
HYBRIDISATION ANALYSIS;

S   U

◄── 50 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with bases 151 to 203 of the insert sequence of clone 7-4 The autorad shown was exposed to the filter for 4 nights at -70ºC with an intensifying screen.

Database matches to the *psbA* gene from *B.napus.*

The *psb A* gene encodes for a precursor thylakoid membrane, protein of the D1 reaction centre of PSII (Mr 32,000) and is located on the chloroplast genome in a large single copy region (Spielmann and Stutz, 1983; Liere *et al.*, 1995). The *psb A* gene is characterised by its abundance in light-grown mature tissues and its rapid turnover rate (Zurawski *et al.*, 1982). Chlorophyll a avalability has also been found to alter the levels of the *psb A* gene product at the translational level (Qingfang and Vermaas, 1998).

Clones (13);
11-7, 15-1, 15-3, 16-2, 16-3, 17-4, 21-1, 21-2, 23-4, 23-5, 23-6, 26-2, 26-3
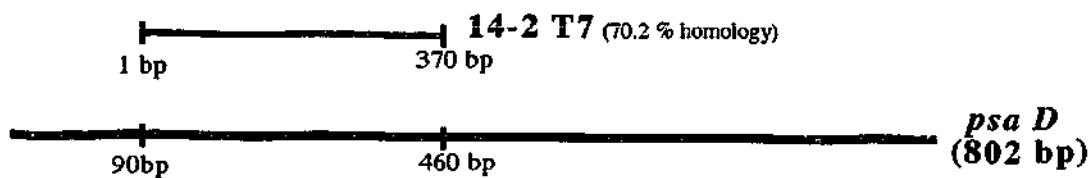

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 23-6. The autorad shown was exposed to the filter for 30 minutes at -70ºC with an intensifying screen.

219

Database match to a gene encoding for the *psa D* gene from *Lycopersicon esculentum* (Tomato).

The *psa D* gene is located in the nucleus and encodes for a peripheral protein of the reaction centre of PSI that is located on the reducing side of PSI. The main role of PSA D, along with the PSA C and PSA E proteins, is to form the reducing site of PSI on which Ferrodoxin (Fd) can dock and accept electrons from the electron transport chain (Chitnis, 1996). While this clone sequence shows homology to several *psaD* genes from several species the clone is most homologous to an *Arabidopsis* BAC that maps to chromosome four and may represent the *Arabidopsis* orthologue of *psa D*.
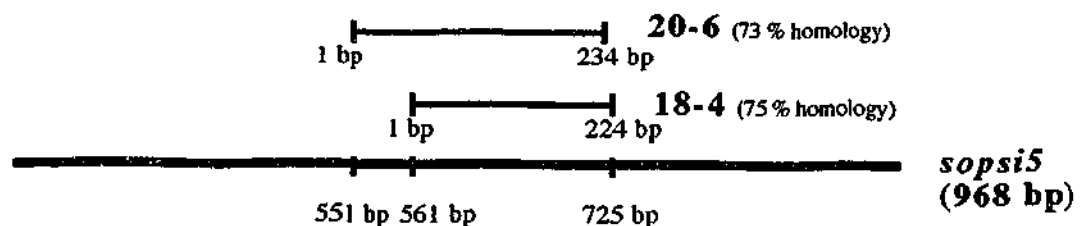
Clones (1);
14-2 (T7)

14-2 T7 (70.2 % homology)
1 bp   370 bp

90bp   460 bp
psa D
(802 bp)

HYBRIDISATION ANALYSIS;
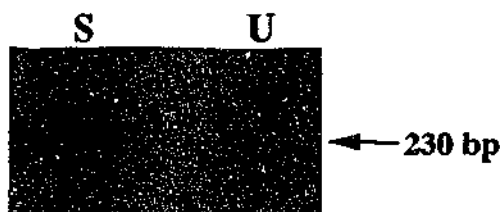
S   U

←— **370 bp**
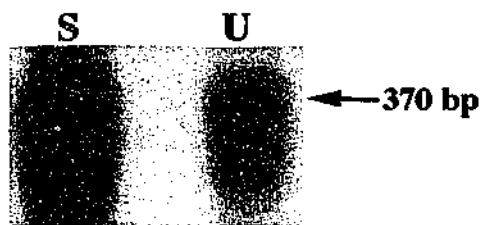
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 14-2 (T7). The autorad shown was exposed to the filter for 6 hours at-70ºC with an intensifying screen.

Database matches to a gene encoding for a precursor polypeptide for subunit IV of the PSI reaction centre from Spinach. (Stepphun *et al.*, 1988).

Clones (2);
18-4, 20-6

20-6 (73 % homology)
1 bp   234 bp

18-4 (75 % homology)
1 bp   224 bp

551 bp 561 bp   725 bp
sopsi5
(968 bp)

HYBRIDISATION ANALYSIS;
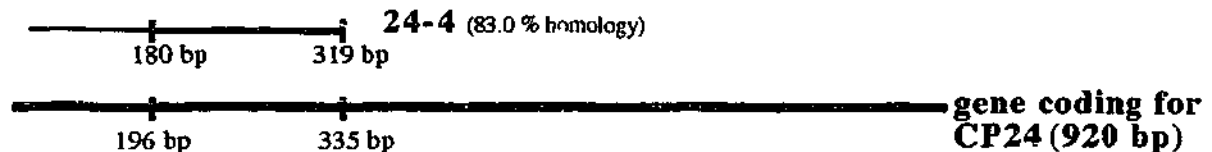
S   U

←— **230 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 20-6. The autorad shown was exposed to the filter for 2 hours at -20ºC.
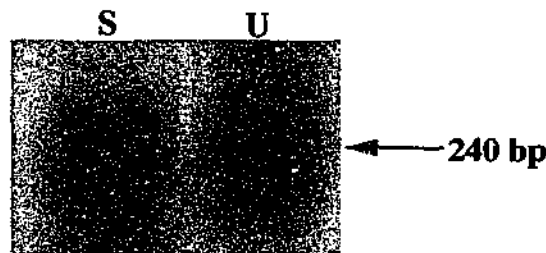
Database match for a gene encoding for a component of the PSII light harvesting complex (CP24) from *S.oleracea.*

This gene is located on the chloroplast and codes for the 20kDa CP24 protein, a component of the PSII light harvesting antenna complex. This clone sequence is also homologous (73%) to a nuclear encoded gene from *Arabidopsis*, COR15a, which contains 5' cis-acting elements that confer cold-, drought- and ABA-regulated induction of gene expression (Baker *et al.*, 1994).

Clones (1);
24-4

24-4 (83.0 % homology)
180 bp   319 bp

196 bp   335 bp   **gene coding for CP24 (920 bp)**

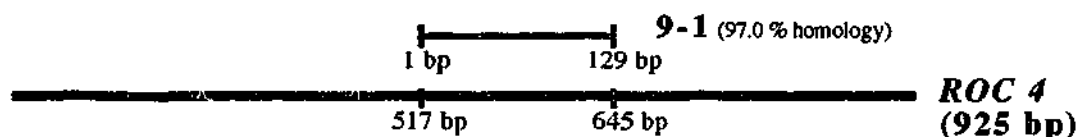## HYBRIDISATION ANALYSIS;

S   U

◄——— **240 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 24-4. The autorad shown was exposed to the filter for 45 minutes at -70°C with an intensifying screen.
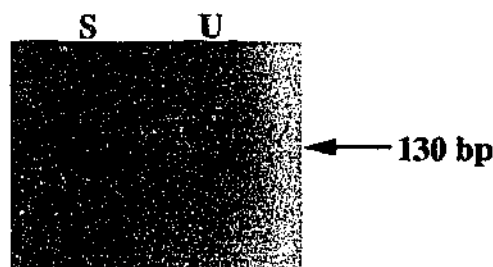
Database match for the *ROC4* gene from *Arabidopsis*

The *ROC4* gene is part of a small family of genes that code for Cyclophilin (CyP) proteins that have peptidyl-prolyl cis-trans isomerase (rotamase) activity. *ROC4* differs from the other five*ROC* genes (*ROC1, 2,3,5* and *6*) which code for cytosolic CyP proteins, as it codes for a chloroplast stromal protein and contains introns. Furthermore, unlike other CyP the *ROC4* gene product is only expressed in photosynthetic tissue and is strongly induced by light. The N-terminal of the *ROC4* product also contains properties similar to those of known chloroplast transit peptides (Lippuner *et al.*, 1994; Chou and Gasser, 1997)

Clones (1);
9-1

9-1 (97.0 % homology)
1 bp   129 bp

517 bp   645 bp   *ROC 4* **(925 bp)**

HYBRIDISATION ANALYSIS;

S   U

◄——— **130 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 9-1 The autorad shown was exposed to the filter overnight at -70°C with an intensifying screen.

Database match to the *DRT112* gene from *Arabidopsis.*

The *DRT* genes (*DRT111* and *DRT112*) code for a chloroplast targetted, recombination and DNA damage resistance, protein. When introduced into *E.coli* these genes, significantly increased resistance to UV light and chemical DNA-damaging reagents, and are highly homologous to the *Arabidopsis* plastocyanin genes. Plastocyanin is a member of the photosynthetic electron transport chain that transfers electrons to the oxidised P700 of PSI reaction centre from cytochrome f (Rother *et al.*, 1986; Vorst *et al.*,1988; Pang *et al.*, 1993).

Clones (1);
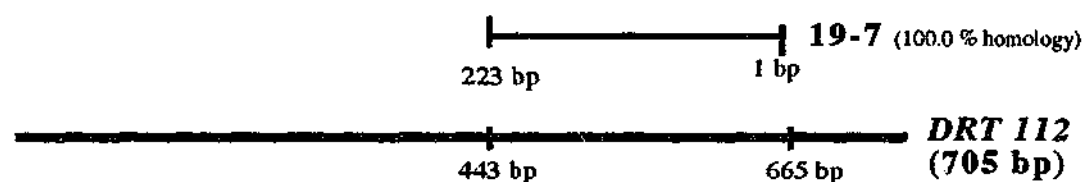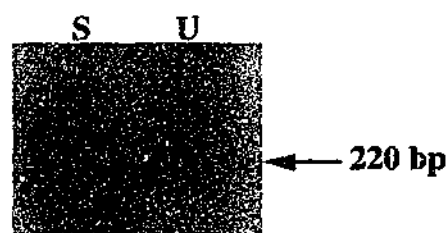19-7

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 19-7. The autorad shown was exposed to the filter overnight at -20ºC with an intensifying screen.

Database match to the geranylgeranyl reductase (hydrogenase) from *Arabidopsis.*

(This clone also shows some homology (75%) to the chlorophyll biosynthesis chlP gene of *Synechocystis sp*).
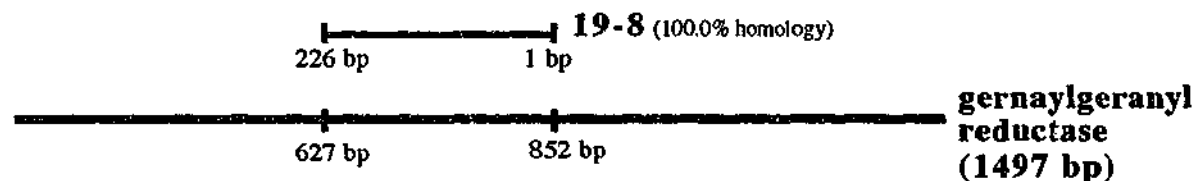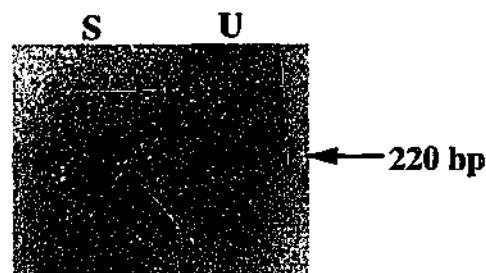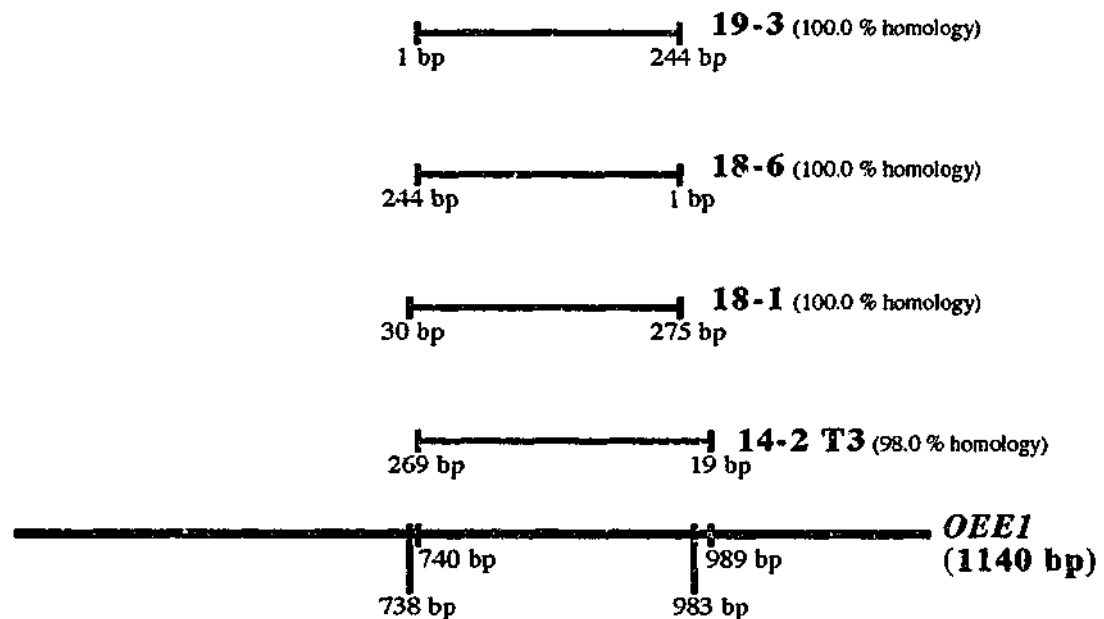
Clones (1);
19-8

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 19-8. The autorad shown was exposed to the filter for 45 minutes at -70ºC with an intensifying screen.
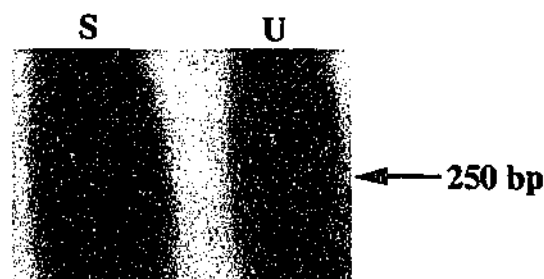
Database matches to the *OEE1* gene encoding a chloroplast protein of PSII from *Arabidopsis.*

This nuclear gene codes for a 33 KDa chloroplast protein that is involved in the oxygen evolving system of PSII. The mRNA levels of this gene are found to increase in light grown plants and be absent in etiolated seedlings. In addition, the light induced transcripts have been found to decrease to non-detectable or very low levels by a three day dark treatment. The RNA transcript levels of this gene were also found to be at their highest in immature leaves while the highest protein levels were found in mature leaves (Ko *et al.*, 1990; Palomares *et al.*, 1993).

Clones (4);
14-2, 18-1, 18-6, 19-3

19-3 (100.0 % homology)
1 bp — 244 bp

18-6 (100.0 % homology)
244 bp — 1 bp

18-1 (100.0 % homology)
30 bp — 275 bp

14-2 T3 (98.0 % homology)
269 bp — 19 bp

OEE1
(1140 bp)
740 bp   989 bp
738 bp   983 bp

HYBRIDISATION ANALYSIS;

S   U

◄——— 250 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 14-2(T3) The autorad shown was exposed to the filter for 20 minutes at -70°C with an intensifying screen.
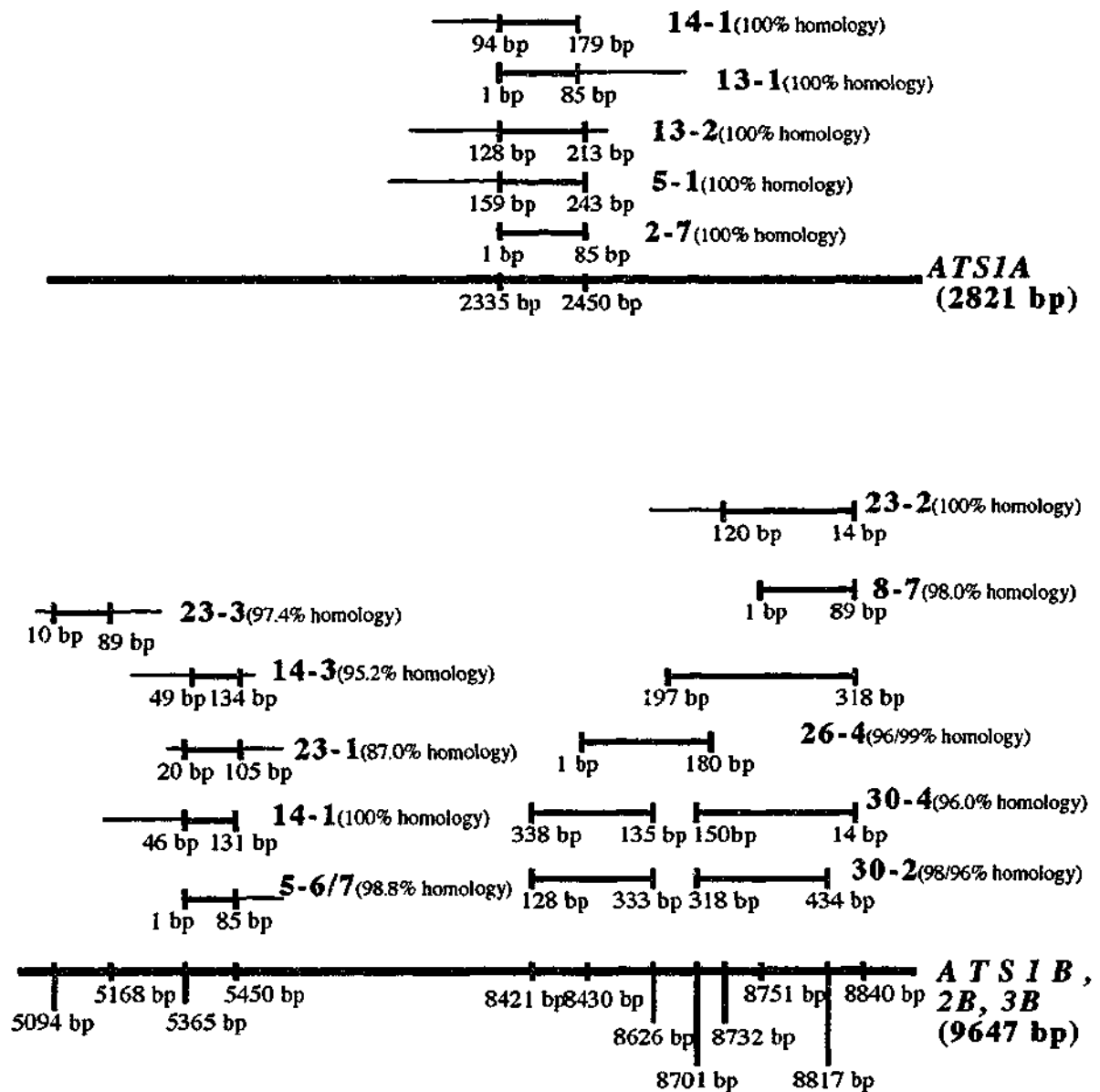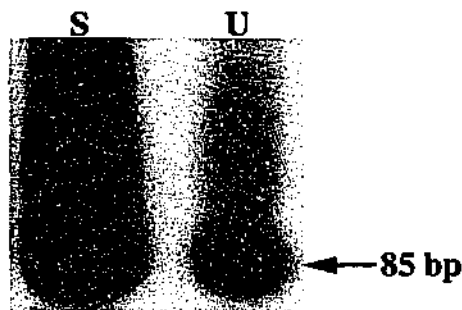
GENES INVOLVED IN THE PHOTOSYNTHETIC DARK REACTIONS

Database matches to the *ATS1A*, *ATS1B,2B* and *3B* genes from *Arabidopsis.*

These nuclear genes code for the small sub-unit polypeptides of the ribulose-1, 5-bisophate carboxylase (Rubisco) enzyme. The four genes are divided into two diverged subfamilies A and B. The three B subfamily genes are clustered on chromosome five and are much more similar to each other than *ATS1A* at both the nucleotide and protein level. The *ATS1A* gene map position is unknown (Krebbers *et al.*, 1988).

Clones (15);
2, 5-1, 5-7, 8-7, 13-1, 13-2, 14-1, 14-3, 14-4, 23-1, 23-2, 23-3, 26-4, 30-2, 30-4b

14-1 (100% homology)
94 bp   179 bp

13-1 (100% homology)
1 bp   85 bp

13-2 (100% homology)
128 bp   213 bp

5-1 (100% homology)
159 bp   243 bp

2-7 (100% homology)
1 bp   85 bp

ATS1A (2821 bp)
2335 bp   2450 bp

23-2 (100% homology)
120 bp   14 bp

8-7 (98.0% homology)
1 bp   89 bp

23-3 (97.4% homology)
10 bp   89 bp

14-3 (95.2% homology)
49 bp 134 bp

197 bp   318 bp

23-1 (87.0% homology)
20 bp   105 bp

26-4 (96/99% homology)
1 bp   180 bp

14-1 (100% homology)
46 bp   131 bp

30-4 (96.0% homology)
338 bp   135 bp 150bp   14 bp

5-6/7 (98.8% homology)
1 bp   85 bp

30-2 (98/96% homology)
128 bp   333 bp 318 bp   434 bp

ATS1B, 2B, 3B (9647 bp)
5094 bp   5168 bp   5450 bp   8421 bp 8430 bp   8751 bp   8840 bp
5365 bp   8626 bp   8732 bp
8701 bp   8817 bp

224

HYBRIDISATION ANALYSIS; *ATS1A*

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 2-7. The autorad shown was exposed to the filter for 6 hours at -70°C with an intensifying screen.

HYBRIDISATION ANALYSIS; *ATS1B, 2B, 3B*
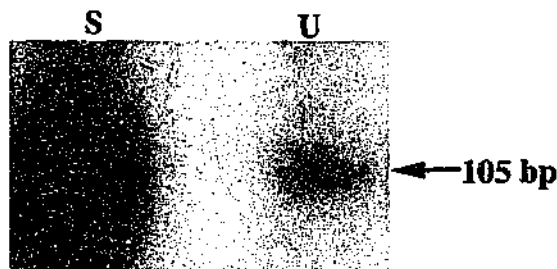
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone23-2. The autorad shown was exposed to the filter for 45 minutes at -70°C with an intensifying screen.

Database match to the *rbcL* gene from *Arabidopsis.*

This chloroplast gene encodes for the large subunit of the Rubisco enzyme. There is evidence that suppression of this genes expression at the transcriptional level occurs in non-green tissue such as roots (Isono *et al.*, 1997).
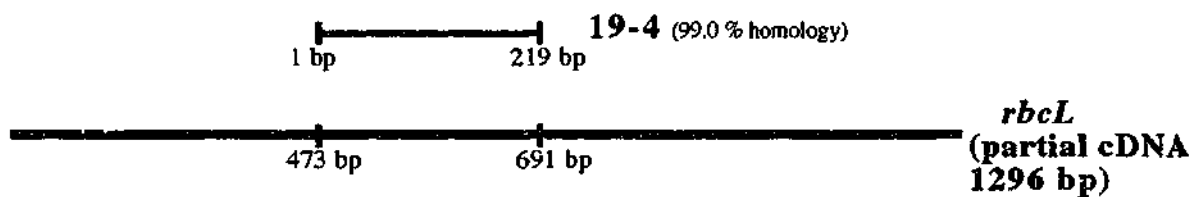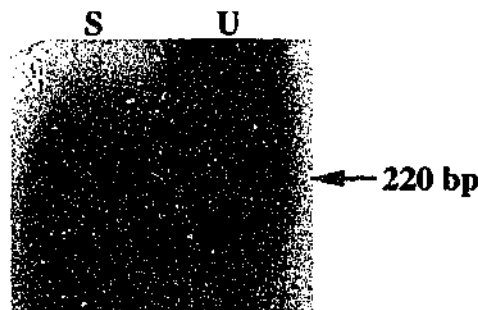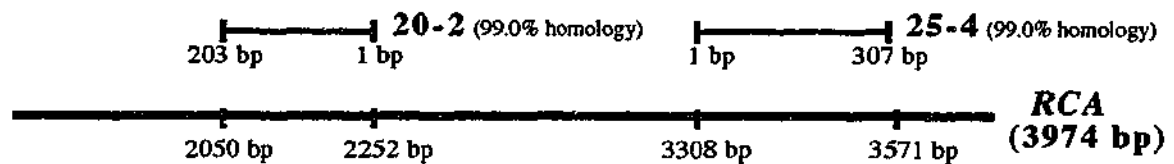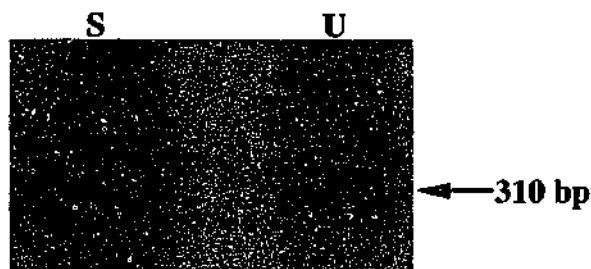
Clones (1);
19-4

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 19-4. The autorad shown was exposed to the filter for 3 hours at -70°C with an intensifying screen.

225

Database match to the *RCA* gene from *Arabidopsis.*

This nuclear gene codes for the Rubisco activase enzyme that catalyses the activation of Rubisco. The cDNA sequence shows consensus nucleotide binding sites consistant with an ATP requirement for Rubisco activity and a derived amino acid sequence common to chloroplast transit peptides (Werneke and Ogren, 1989).

Clones (2);
20-2, 25-4

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 25-4. The autorad shown was exposed to the filter for 30 minutes at -20°C.

Database matches to the ATPase subunit genes from *S.oleracea.*

This chloroplast gene codes for the beta and epsilon subunits of the cp coupling factor complex (ATPase) (Shinozaki *et al.*, 1983).
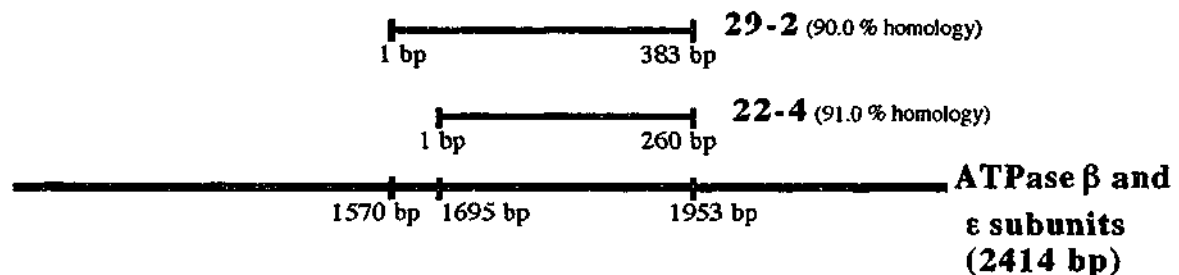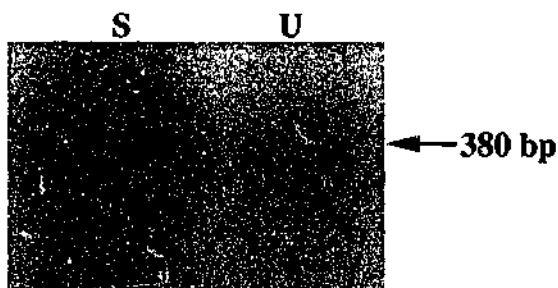
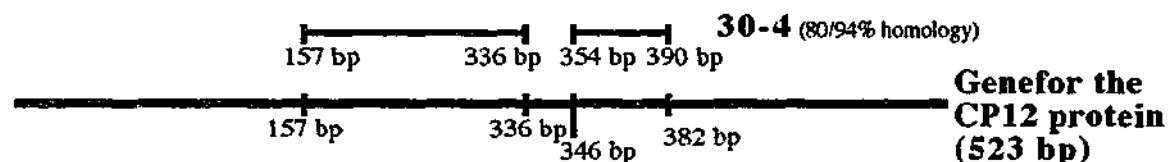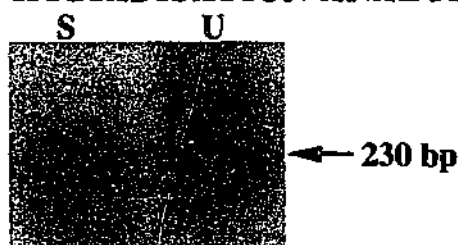**Clones (2);**
22-4, 29-2

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 29-2. The autorad shown was exposed to the filter overnight at -70°C with an intensifying screen.

226

Database match to a gene encoding for the CP12 protein from *S.oleracae*.

This clone is homologous to the gene coding for the Calvin cycle protein CP12, involved in GAPDH evolution from a range of species. The sequence this clone is most homologous to is an *Arabidopsis* chromosome II BAC (T08I13) which may contain the *Arabidopsis* orthologue of *cp12*.

Clones (1);
30-4

30-4 (80/94% homology)

157 bp   336 bp 354 bp 390 bp

Gene for the CP12 protein (523 bp)

157 bp   336 bp 346 bp 382 bp

HYBRIDISATION ANALYSIS;

S   U

◄— 230 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 30-4. The autorad shown was exposed to the filter overnight a t-20°C.

Database match to a gene encoding for a putative sugar transporter from *Prunus armeniaca*.

This clone was also homologous to a G-box binding factor from Dictostelium (59%) and a glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene from *Caenorhabditis briggsae* (62%) (Lee *et al.*, 1992; Schinitzler *et al.*, 1994).

Clones (1);
33-5

332 bp   141 bp   33-5 (79/71% homology)

179 bp   1 bp

Gene for a putative sugar transporter (2065 bp)

444 bp   624 bp 635 bp   802 bp

HYBRIDISATION ANALYSIS;

S   U

◄—330 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 33-5. The autorad shown was exposed to the filter for 3 nights at -70°C with an intensifying screen.

OTHER GENES

Database match to the *TGG1* gene from *Arabidopsis*.

This nuclear gene codes for a thioglucosidase glucohydrogenase (myrosinase) enzyme. Unlkie other Brassicae species *Arabidopsis* contains only three myrosinase genes (*TGG1*, *2, 3*). Two of these genes (*TGG1* and *2*) have recently been cloned and are located next to each other in an inverted manner. Both *TGG1* and *TGG2* are expressed in leaves, sepals, petals and gynoeciums. *TGG1* but not *TGG2* is also expressed in developing seeds (Xue *et al.*, 1992; Xue *et al.*, 1995; Zhou and Goldsbrough, 1995).

Clones (1);
15-2

15-2 (100 % homology)
1 bp   181 bp

TGG1
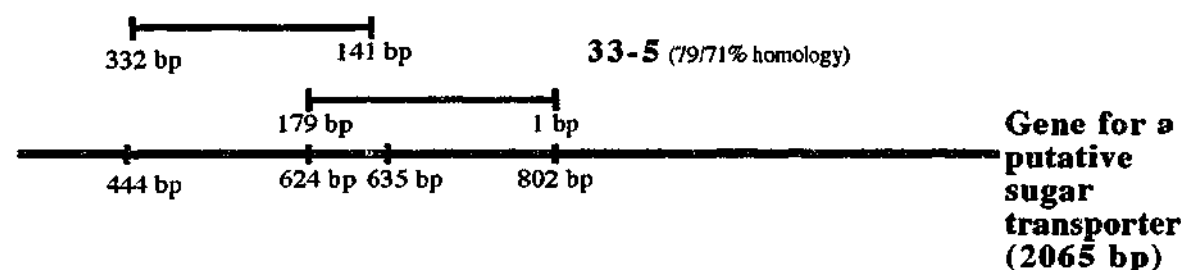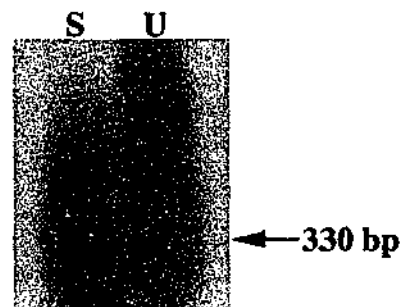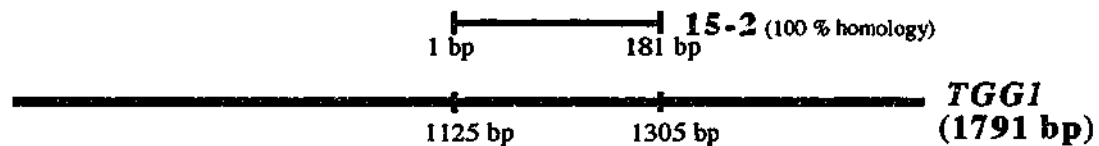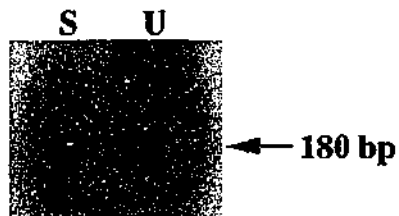(**1791 bp**)
1125 bp   1305 bp

HYBRIDISATION ANALYSIS;

S   U

◄— **180 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 15-2. The autorad shown was exposed to the filter for 3 hours at -70ºC with an intensifying screen.

Database match to the *ghmt1* gene from *F.pringlei*.

This gene codes for a glycine hydroxymethyltransferase. This clone also shows significant homology to genes encoding serine hydroxymethyl transferases. The SHMT enzymes appear to have both mitochondrial and nuclear forms (Kopriva and Bawe, 1995). The expression of the mitochondrial form is found only in leaves, while nuclear forms are expressed in both leaves and roots (Turner *et al.*, 1992).

Clones (1);
20-5

20-5 (83.0 % homology)
243 bp   1 bp

ghmt1
(**1791 bp**)
1090 bp   1331 bp
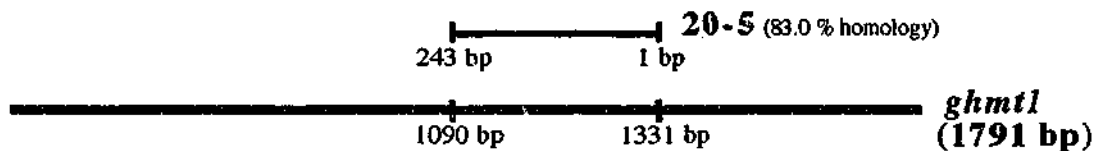
HYBRIDISATION ANALYSIS;

S   U

◄— **240 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 20-5. The autorad shown was exposed to the filter for 45 minutes at -70ºC with an intensifying screen.

228

Database match to the *MT2B* gene from *Arabidopsis.*

This gene is one of five in *Arabidopsis* that code for the heavy metal binding, cysteine-rich metallothionein proteins. The roles of these proteins in plants is not well understood, although it has been suggested that their metal binding functions may be required for the correct functioning of metal containing enzymes and transcription factors. The metallothionein genes of *Arabidopsis* can be divided into two differentially expressed groups (MT1 and MT2). *MT2a* and *MT2b* are more abundant in leaves, infloresences and roots that the *MT1* genes. *MT2b*, unlike *MT2a*, is however only weakly induced after exposure to copper. *MT2b* maps to chromosome 5 (Kawashima *et al.*, 1991; Zhou and Goldsbrough, 1995; Giritch *et al.*, 1998).

Clones (1);
20-5

5-1 (97.7% homology)
134 bp   4 bp

705 bp   835 bp

MT2b
(1611 bp)

HYBRIDISATION ANALYSIS;

S   U

← 130 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with bases 4 to 134 of the insert sequence of clone 5-1 The autorad shown was exposed to the filter for 3 nights at -70°C with an intensifying screen.

Database match to the *A1, A2* and *A3* genes from *Arabidopsis.*

These genes are part of small family of genes divided into 2 subfamilies (A4 and A1,2 and 3) that code for a translation elongation factor 1-alpha (Axelos *et al.*, 1988).

Clones (2);
5-6, 5-7

5-6/7 (97.7% homology)
158 bp   98 bp

735 bp   795 bp

A1, 2 and 3
(6022 bp)

HYBRIDISATION ANALYSIS;

S   U

← 60 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with bases 98 to 158 of the insert sequence of clone 5-7. The autorad shown was exposed to the filter for 5 minutes at -70°C with an intensifying screen.
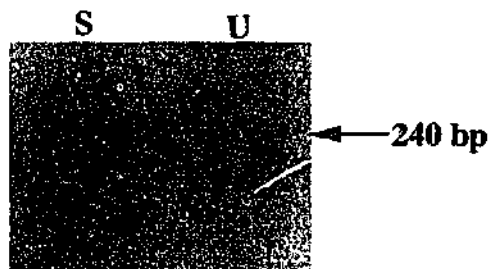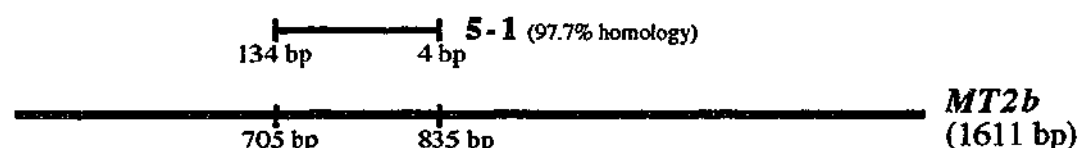
Database match to the *nit1* gene from *Arabidopsis*.

This gene codes for one of three *Arabidopsis* nitrilases (NIT1,2 and 3) (Zhou *et al.*, 1996a; Zhou *et al.*, 1996b). The role of nitrilases is thought to be in the conversion of indole-3-acetonitrile to indole-3-acetic acid. The *nit1* gene has been found to be expressed throughout development, while the *nit2* gene expression is barley detectable in young rosettes, and strongly expressed in bolting and flowering plants as well as during fruit development (Bartling *et al.*, 1992; Bartel and Fink, 1994).

Clones (1);
22-2
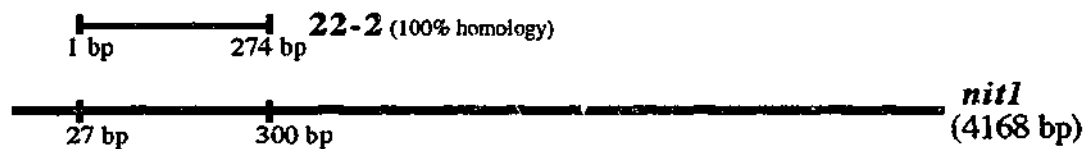
22-2 (100% homology)

1 bp   274 bp

27 bp   300 bp
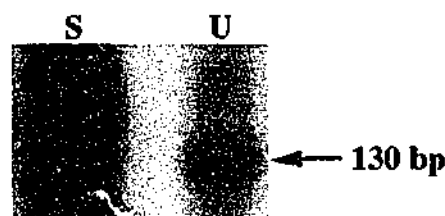
nit1
(4168 bp)

HYBRIDISATION ANALYSIS;

S   U

←——270 bp
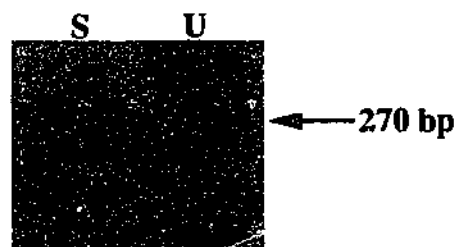
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 22-2. The autorad shown was exposed to the filter for 3 nights at -70°C with an intensifying screen.

Database matches to the *NIA 1* gene from *Arabidopsis*.

The *NIA1* and *NIA2* genes code for functional nitrase reductase proteins in *Arabidopsis* (Cheng *et al.*, 1988). *nia2* mutants still however show 10% wild-type activity of nitrate reductase and grow normally with nitrate as the sole source of nitrogen. In contrast *nia1* and *nia2* double mutants have only 0.5% of wild-type nitrate reductase shoot activity (Crawford *et al.*, 1988; Wilkonson and Crawford, 1993). The *NIA1* gene maps to chromosome 1.

Clones (2);
33-1, 33-6
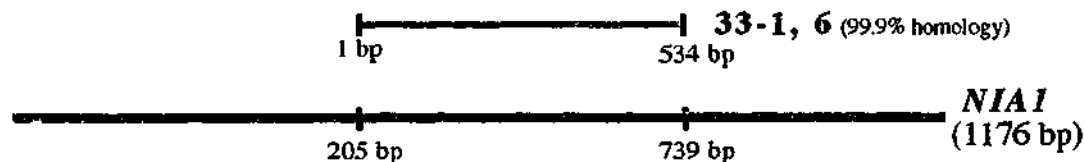
33-1, 6 (99.9% homology)
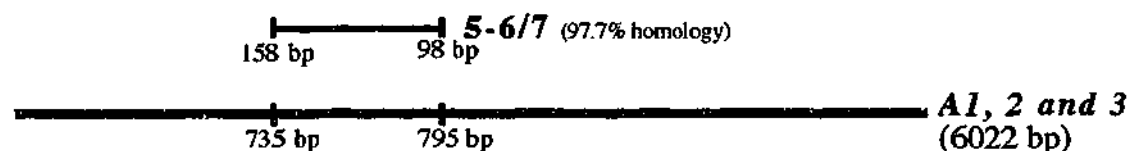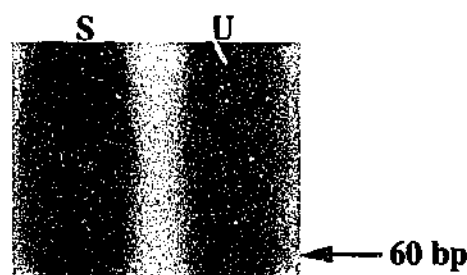
1 bp   534 bp

205 bp   739 bp

NIA1
(1176 bp)

HYBRIDISATION ANALYSIS;

S   U

←—— 530 bp
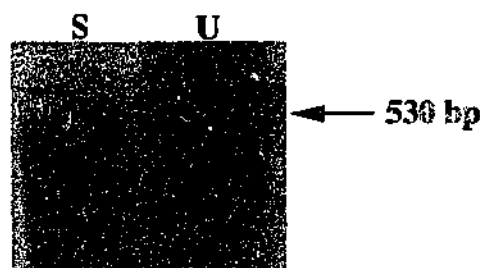
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 33-1. The autorad shown was exposed to the filter overnight at -70°C with an intensifying screen.

DATABASE SEQUENCES OF UNKNOWN FUNCTION.

Database matches to a sequence from *Homo sapiens* (clone hRPK.642).

These clones are only 68% homologous to this clone only over a region covering half of the clones (~100bp).

Clones (3);
17-3, 17-5, 17-7

17-3 (68.0 % homology)
2 bp   65 bp

17-5,7 (68.0 % homology)
212 bp   149 bp

hRPK.642
150765 bp   150828 bp   **(172,084 bp)**

HYBRIDISATION ANALYSIS;

S   U

← **200 bp**

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 17-5. The autorad shown was exposed to the filter overnight at -70ºC with an intensifying screen.

Database match to a BAC (BACF27L4) from *Arabidopsis* that maps to chromosome II.

Clones (1);
20-3
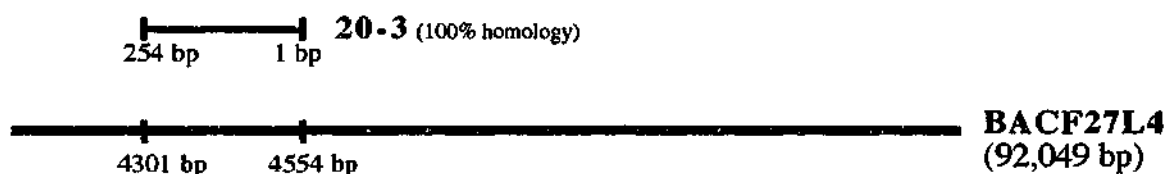
20-3 (100% homology)
254 bp   1 bp

BACF27L4
4301 bp   4554 bp   **(92,049 bp)**

HYBRIDISATION ANALYSIS;

S   U

←**250 bp**
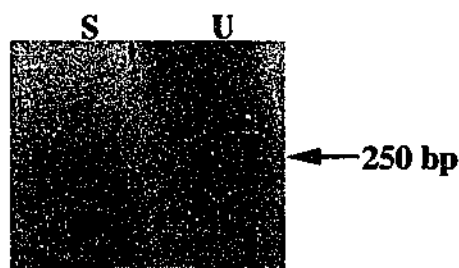
Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 20-3. The autorad shown was exposed to the filter for 30 minutes at -70ºC with an intensifying screen.

## Database match to a BAC (BACF21B7) from *Arabidopsis* that maps to chromosome I

Clones (1);
20-4

```
    |————————————|  20-4 (97.0% homology)
   1 bp         246 bp
```

```
━━━━━━━|━━━━━━━━━|━━━━━━━━━━━━━━━━━━━━━━  BACF21B7
     38179 bp   38424 bp                  (101,158 bp)
```

HYBRIDISATION ANALYSIS;

◄———250 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 20-4. The autorad shown was exposed to the filter for 30 minutes at -70°Cwith an intensifying screen.

Database match to a BAC (BACT419) from *Arabidopsis* that maps to chromosome IV.

Clones (1);
33-4

```
   |———|  |————————|  33-4 (98/100% homology)
  529 bp 460 bp 460 bp    1 bp
```

```
━━━━━|━━|━|━━━━━━━━|━━━━━━━━━━━━━━━━━  BACT419
   30643 bp   31101 bp   31560 bp       (98,913 bp)
        30712 bp
```

HYBRIDISATION ANALYSIS;

◄———530 bp

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 33-4. The autorad shown was exposed to the filter overnight at-20°C.

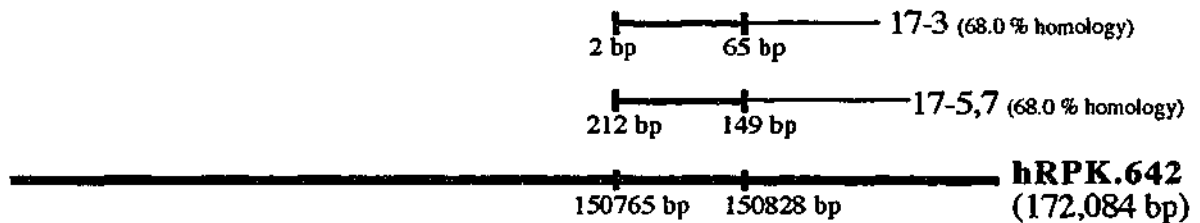*Clones 6-1 and 33-2 show little homology to any sequences in the database at the DNA or protein level.

HYBRIDISATION ANALYSIS;

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 6-1 The autorad shown was exposed to the filter for 1.5 hours at -70°C with an intensifying screen.
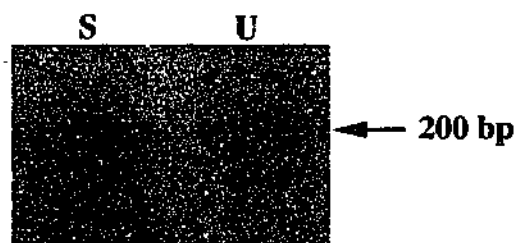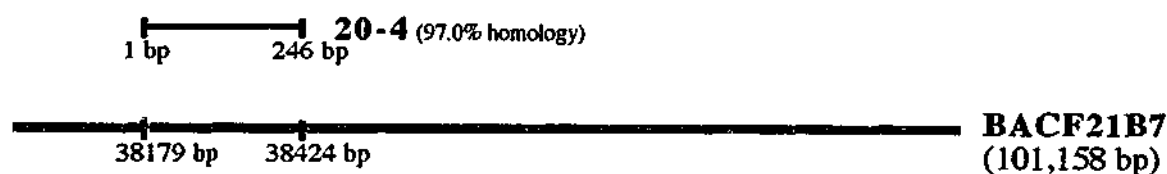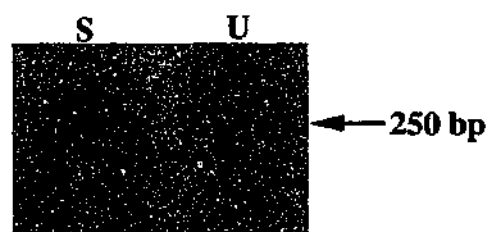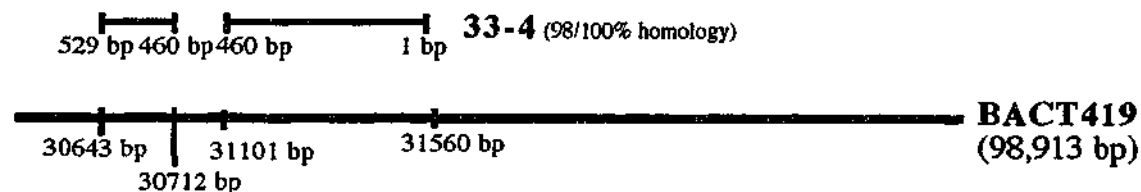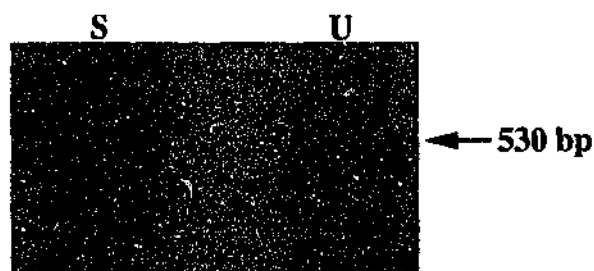
HYBRIDISATION ANALYSIS;
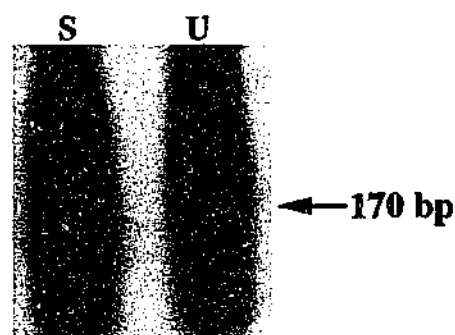

Subtracted (S) and unsubtracted (U) cDNA was probed with the insert sequence of clone 33-2. The autorad shown was exposed to the filter overnight at -70°C with an intensifying screen.
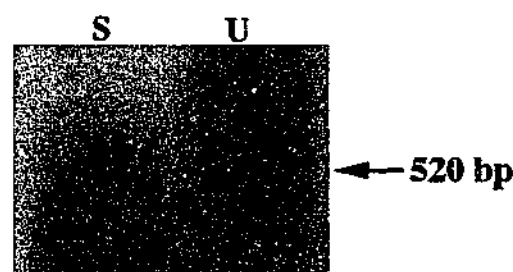
Northern blot analysis

The first gene analysed by Northern blot analysis showed homology to a type III *CAB* gene associated with PSI. Only slight differences in the expression of this gene were detected between mutant and wild-type Pitztal RNA, however unexpectedly for most developmental stages there was stronger expression of this gene in the mutant plants (Figure 5.15(a)). This trend was also noted when such filters were probed with several other genes. These included another *CAB* gene (Figure 5.15(d)), a gene encoding for a D1 reaction centre protein (Figure 5.15 (l)), a gene encoding for a glycine hydroxymethyletransferase protein from *F. pringlei* (Figure 5.15 (m)), and genes encoding for the small sub-unit of the Rubisco enzyme (Figure 5.15(n)). Often these differences were not consistent over all stages of development however, with some stages giving similar levels of expression between mutant and wild-type. The expression of the *LHCP AB CAB* gene (Figure 5.15(d)), and the gene encoding for the D1 reaction centre (Figure 5.15(l)), was for example, higher at all stages of development in the mutant plants compared to the wild-type plants, except for the cotyledon stage. The type III *CAB* gene also showed weaker expression in the mutant line at the cotyledon stage as well as at the 6-leaf stage of development (Figure 5.15(a)). The gene encoding for the glycine hydroxymethyltransferase also showed slightly less expression in the mutant lines at the cotyledon and 2-leaf stages of development (Figure 5.15 (m)). The genes encoding for the small sub-unit of the Rubisco enzyme showed stronger expression in the wild-type Pitztal plants at all stages of development except for the 4-leaf stage when plants were harvested at 4 pm. In addition, differences in the temporal peak of expression throughout the day, between mutant and wild-type plants, was noted for all of these genes. The peak expression for the two *CAB* genes was for example, was found to be at 12 noon (lane 4 in figure 5.15 (a) and 5.15(d)) in the mutant lines, however the peak expression in wild-type plants was found to be either slightly earlier or later than this.

As predicted, the majority of the other genes showed lower expression in the mutant line than in wild-type Pitztal plants at most stages of development (Figures 5.15 (b, c, e, f, g, h, i, j, k, o, p, r)), although these differences were generally not large. In addition, the decreased expression of these genes amongst the mutant line RNA population was not consistent over all stages of development except for the expression of a type II CAB protein whose expression was continually lower in mutant plants compared with wild-type Pitztal plants (Figure 5.15(e)). Furthermore, in cases in which more than one sequence was detected following hybridisation (Figure 5.15 (b, c, e, g, h and p)) the patterns of these differences were not always similar amongst these sequences. It should however be noted that only one or two of these bands may be representative of the sequences of interest. Several of these genes that exhibit weaker expression in mutant plants, also appear to have slight temporal and developmental disruptions in their expression in the mutant line compared to wild-type Pitztal. Expression of the *RCA* (*Rubisco activase*) gene for example,

(Figure 5.15 (i)) peaked at 12 noon in the mutant line, whereas the strongest expression of this gene was noted at 9 am in wild-type Pitztal.

As mentioned previously, several sequences represented in the subtracted sample could not be assigned any putative function. These sequences were thought to be of particular interest as they may represent previously uncharacterised genes that are important to the mutant phenotype. Three of these clones, 20-4, 33-2 and 33-4 were subsequently used to probe Northern blot filters. Only one such sequence, that contained within clone 33-4 however, was found to be significantly enriched in the subtraction sample. No signal could be detected when filters were probed with the sequence from clone 33-2 and the insert sequence from clone 20-4 gave an unusual pattern of expression. Lower expression of the 20-4 sequence was found at the cotyledon, 2-leaf, and mutant flowering stages of development, with the expression levels at the later stage being almost half that of the expression levels in wild-type Pitztal plants at the same developmental stage. Dramatically higher expression of this sequence however, was found in the mutant plants at the four-leaf stage of development across all time points throughout the day.

When Northern blot filters were probed with the sequence from clone 33-4 a dramatic differential expression pattern was noted. No signal was detected at any stage of development in the mutant plants, whereas reasonably strong expression was noted in wild-type Pitztal plants. The expression of this gene appeared to peak at the 2-leaf stage of development in Pitztal and subsequently decrease as the plants age. Stronger expression of this gene was also noted at earlier times throughout the day. This sequence was analysed in detail, as the extreme difference in expression of this gene between mutant and wild-type Pitztal cDNA may be directly linked to the early-flowering phenotype of the mutant lines. The results of this analysis are presented in Chapter 6 of this study.
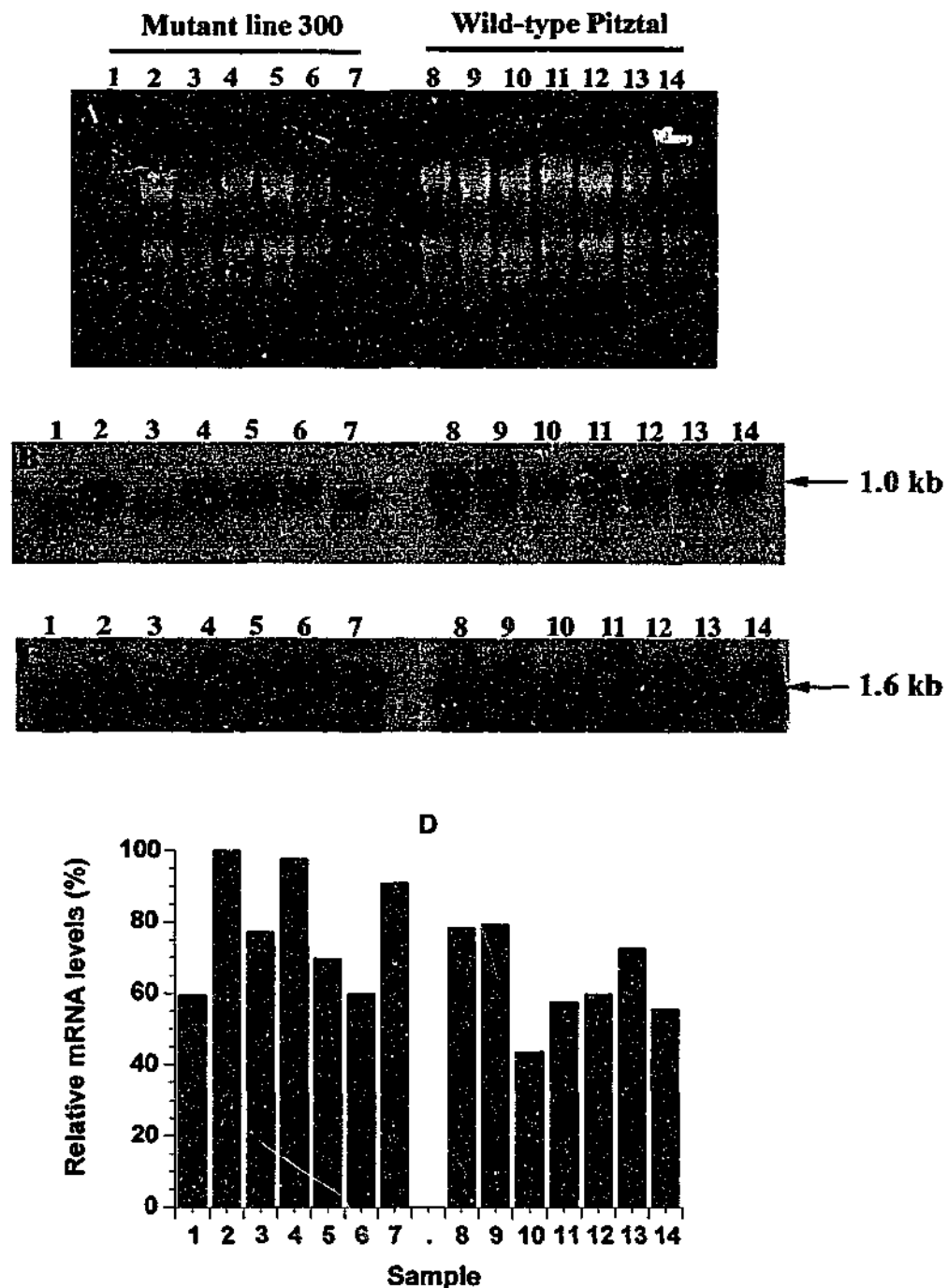
Figure 5.15 (a);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 18-9 (B). This sequence shows homology to a gene coding for a type III CAB protein associated with PSI. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

*The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (b);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 10-3 (B). This sequence shows homology to a chloroplast gene coding for the 23S rRNA protein from *Alnus incana*. This sequence is believed to be most similar to the 2.7 kb transcript detected (band1). The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal 4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

*The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (c);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 7-4 (B). This sequence shows homology to a gene coding for a 10 kDa protein associated with PSI. This sequence was believed to be most homologous to the 1.2 kb transcript detected (band 2). The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal 4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4 p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

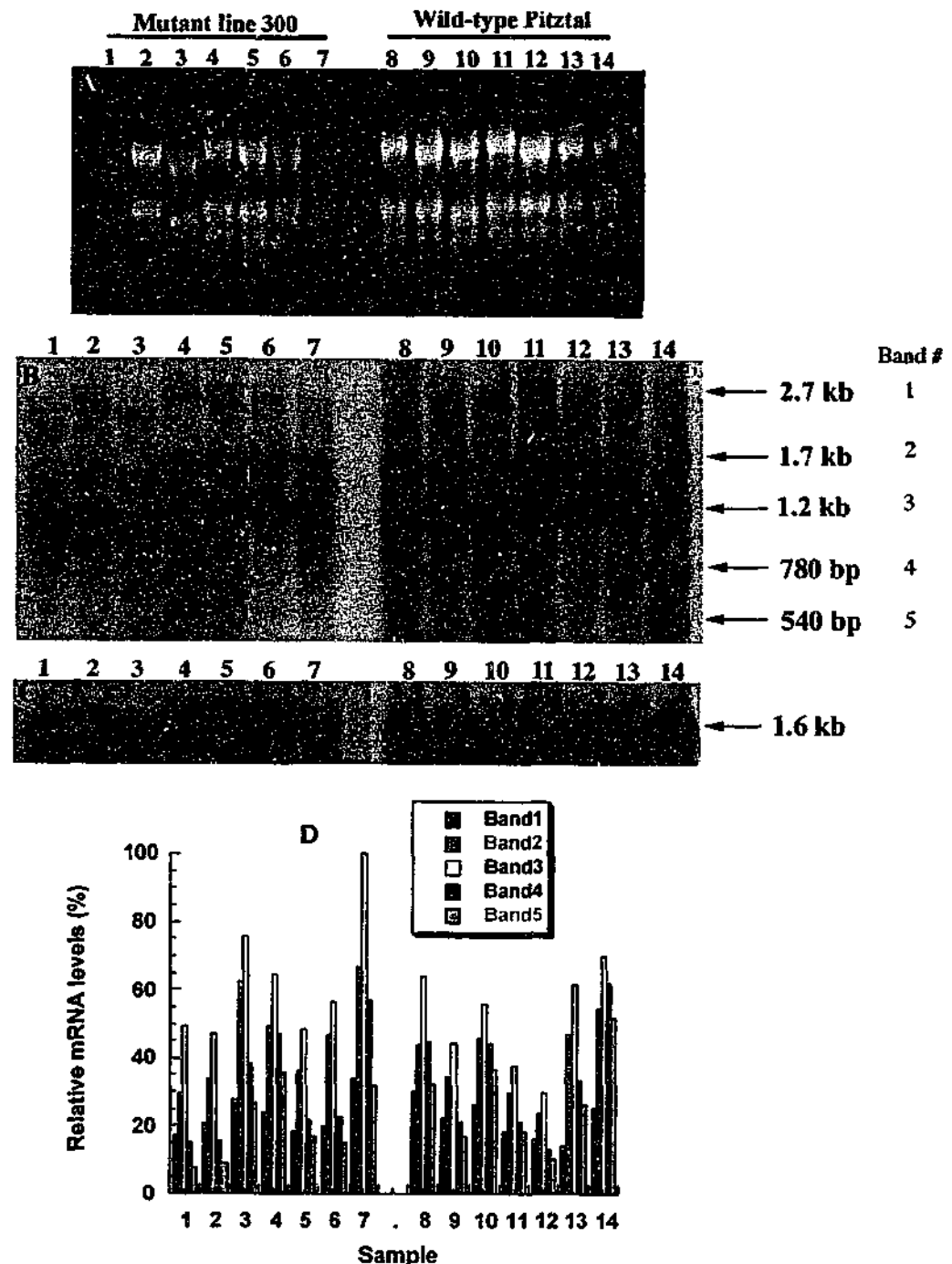* The time of harvesting of the plants used for the RNA extractions are shown in brackets

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Figure 5.15 (d);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 8-3 (B). This sequence shows homology to a gene coding for a LHCP AB 165 CAB protein. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (f);

The ethidium bromide-stained gel (**A**) was blotted and the filter hybridised with the insert sequence from clone 19-7 (**B**). This sequence shows homology to a gene coding for a recombination and DNA damage resisitance protein. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

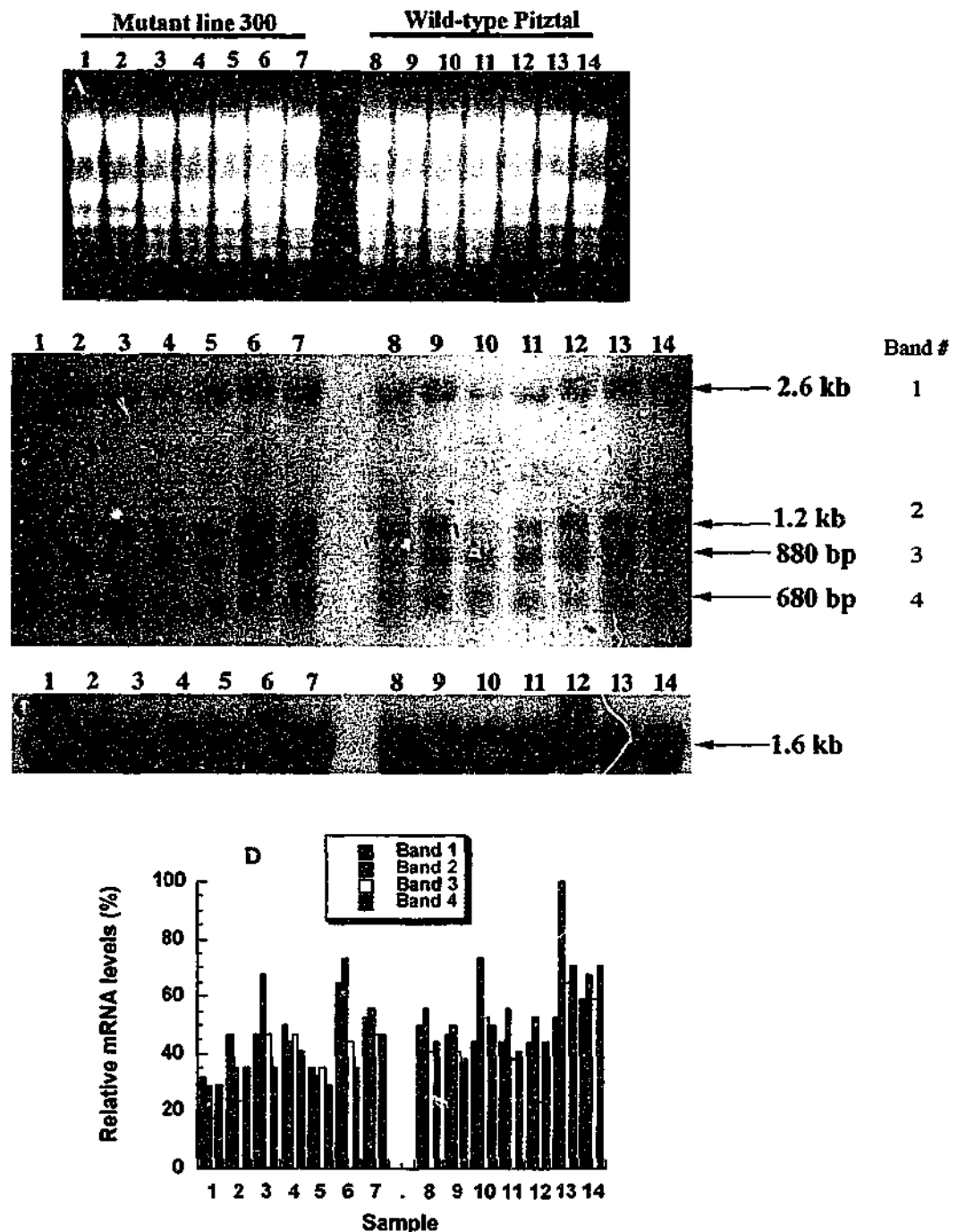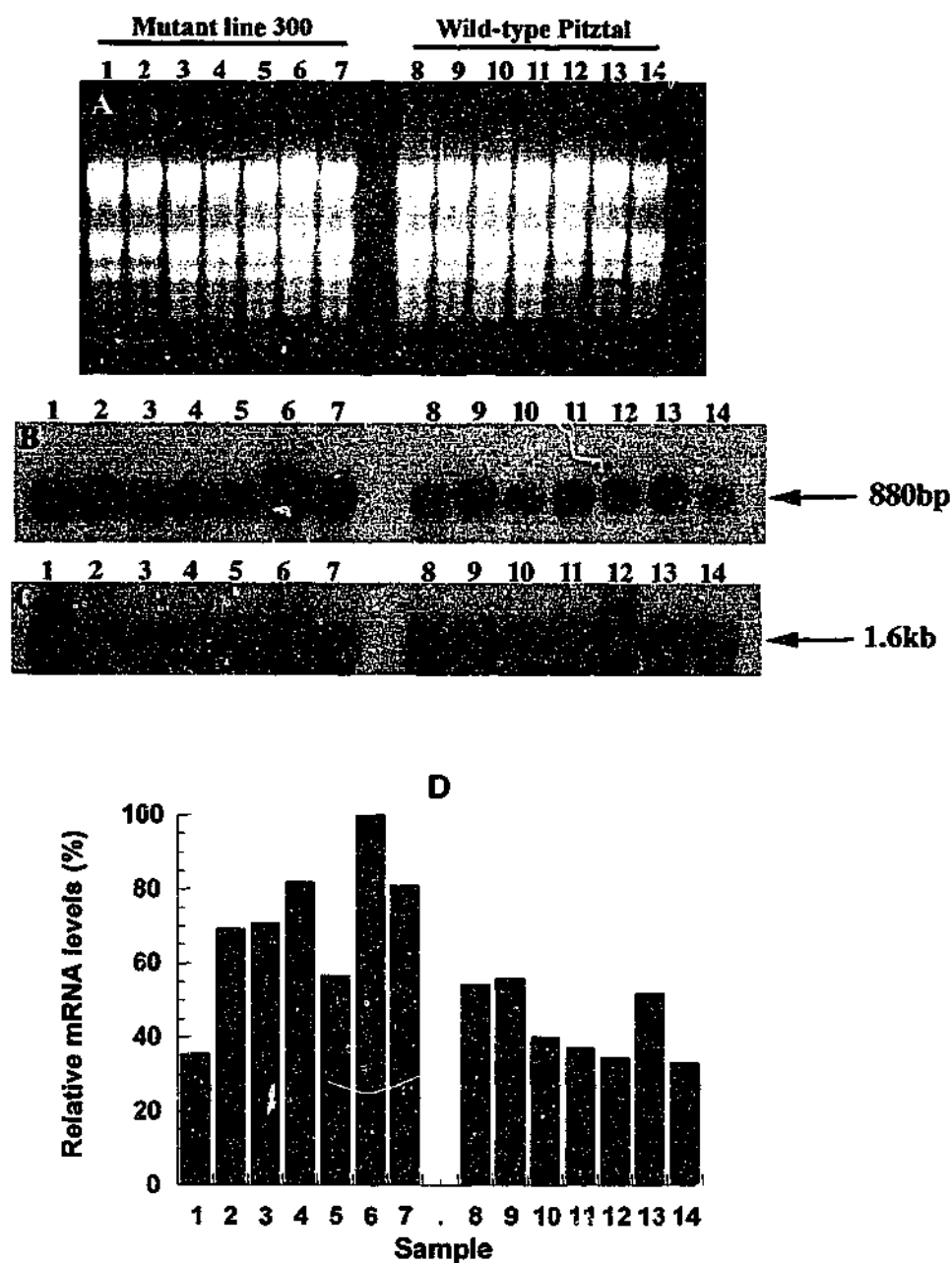* The time of harvesting of the plants used for the RNA extraction are shown in brackets
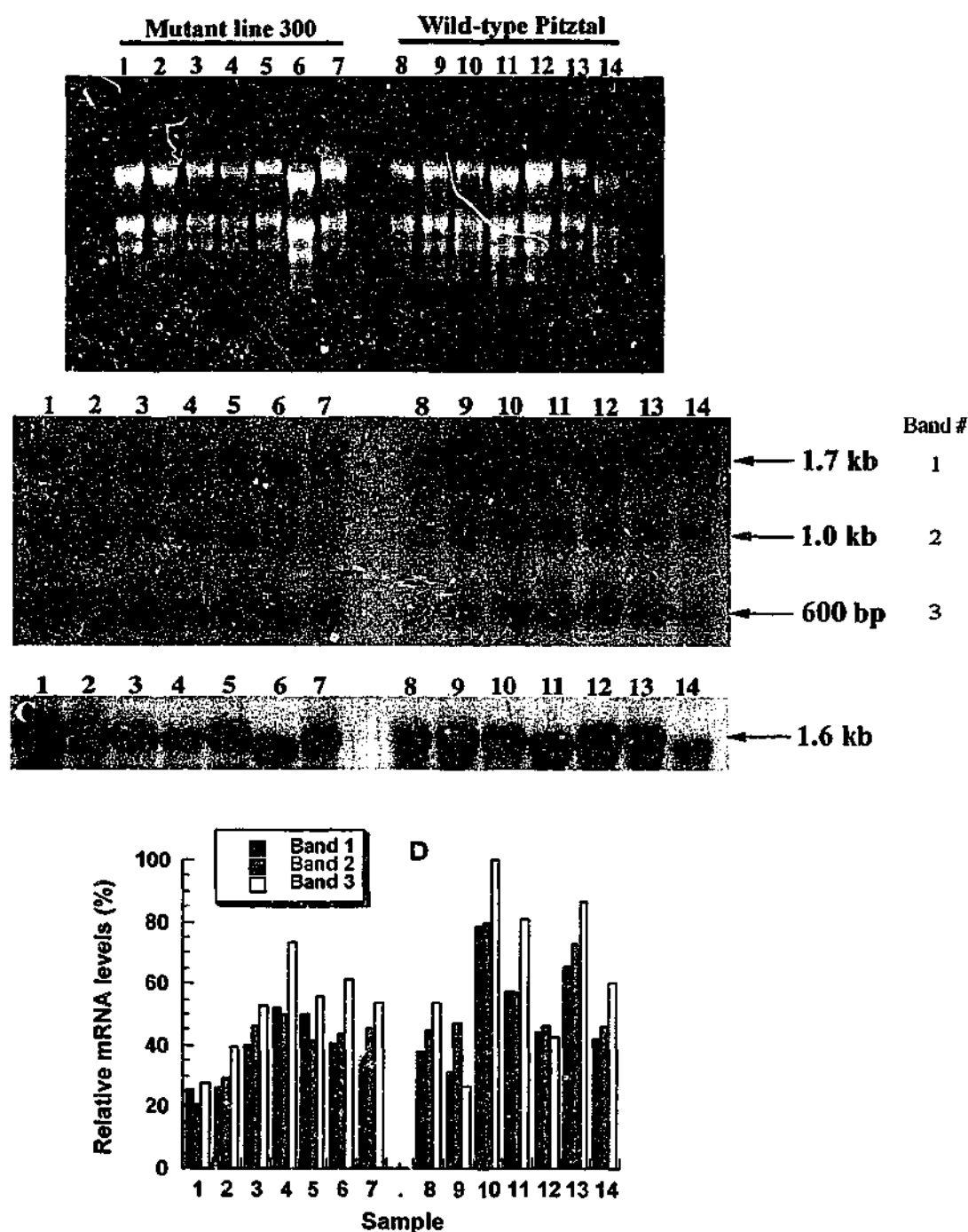
Figure 5.15 (g);

The ethidium bromide-stained gel (**A**) was blotted and the filter hybridised with the insert sequence from clone 11-6 (**B**). This sequence shows homology to a gene coding for the L32 chloroplast ribosomal protein from *Brassica napus*. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (e);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 5-4 (B). This sequence shows homology to a gene coding for a type II CAB protein associated with PSI from *Pisium sativum*. This sequence is believed to be most homologous to the 1.2 kb transcript detected (band 3). The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal 4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (h);

The ethidium bromide-stained gel (**A**) was blotted and the filter hybridised with the insert sequence from clone 5-1 (**B**). This sequence shows homology to a gene coding for the small sub-unit polypeptide of the Rubisco enzyme. This sequence is believed to be most homologous to the 720 bp transcript detected (band 2). The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal 4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

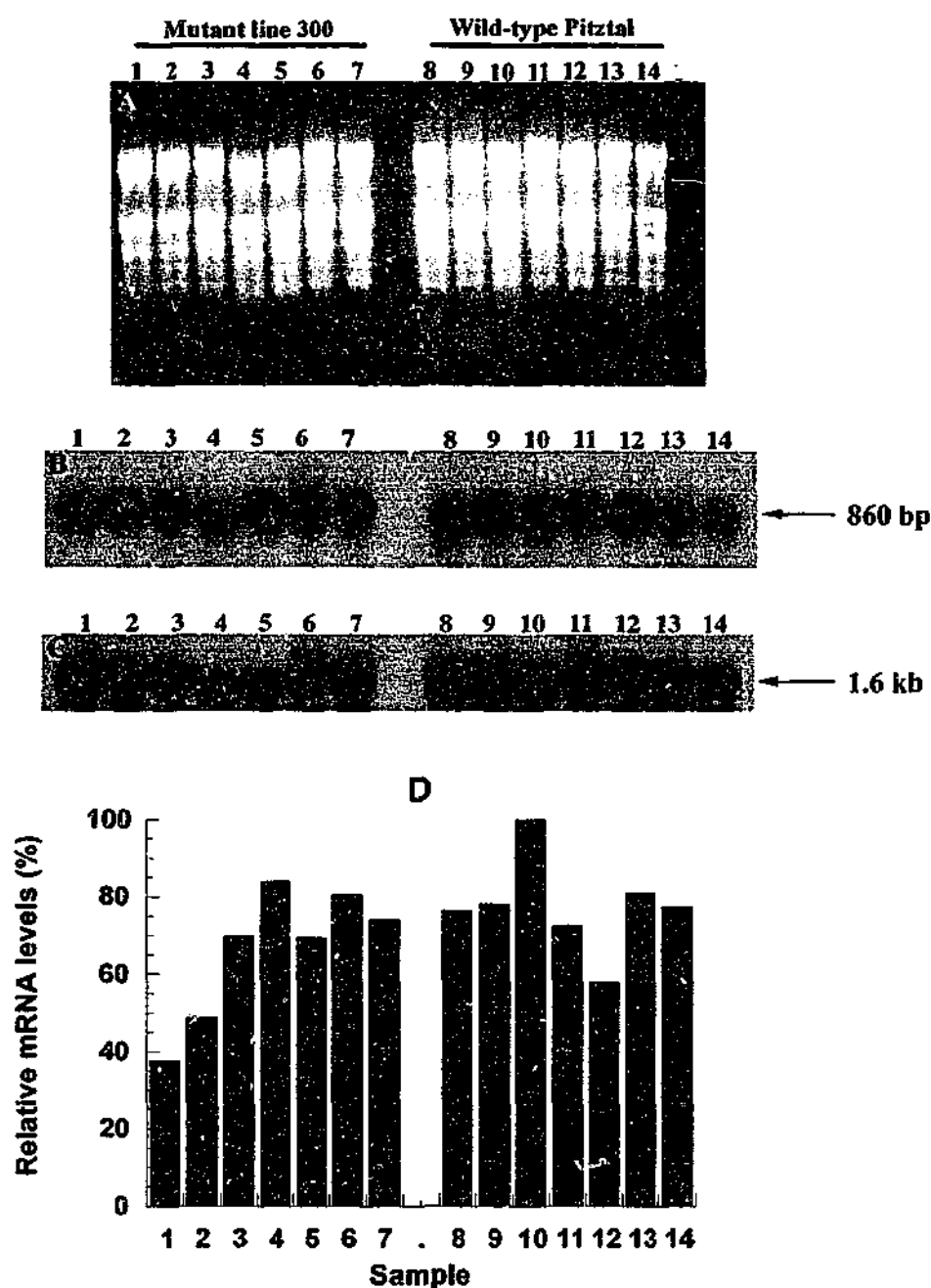* The time of harvesting of the plants used for the RNA extractions are shown in brackets

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Figure 5.15 (i);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 25-4 (B). This sequence shows homology to a gene coding for a Rubisco activase enzyme. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (j);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 9-1 (B). This sequence shows homology to the *ROC4* gene coding for a cyclophilin protein. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

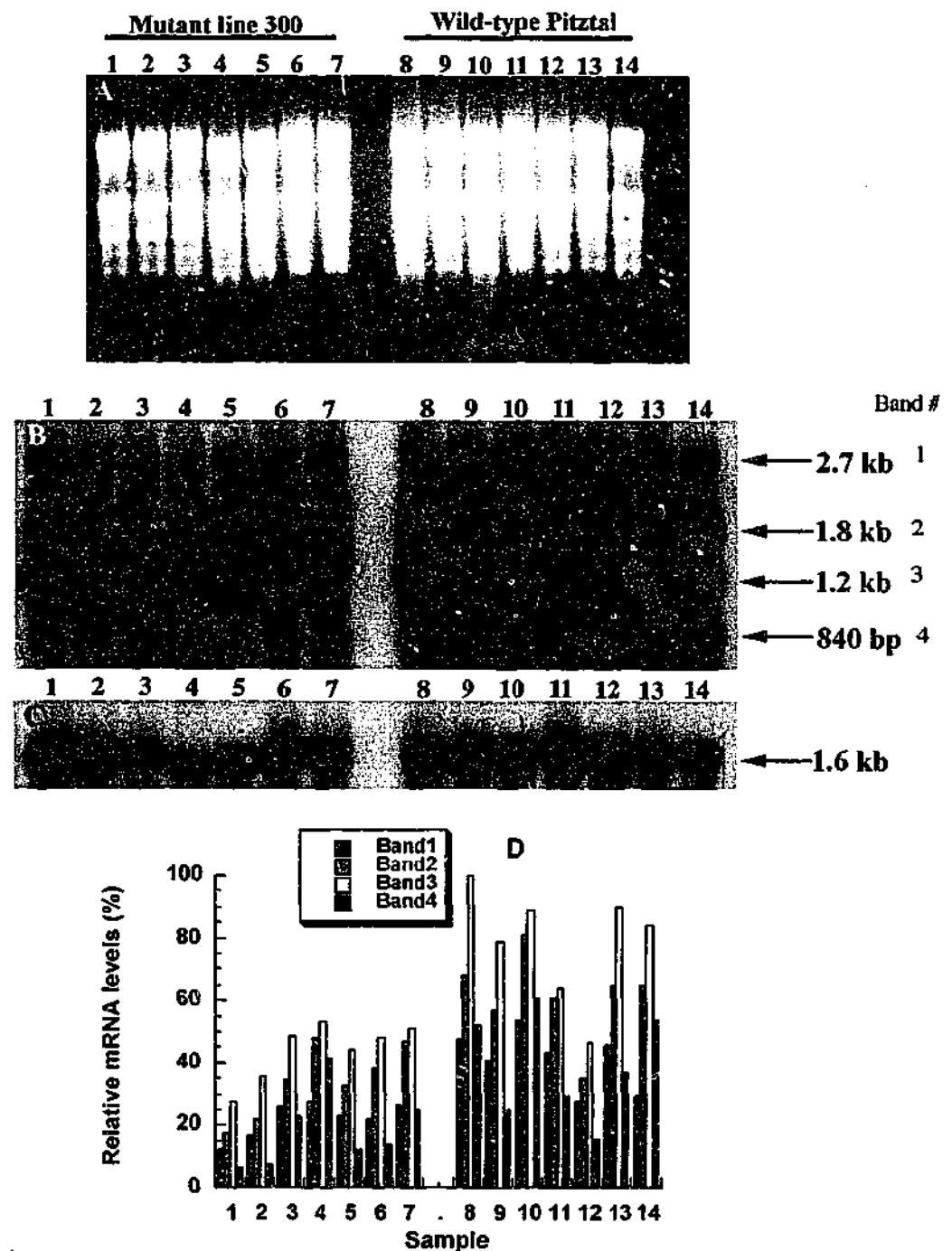*The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (k);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 5-7 (B). This sequence shows homology to a gene coding for a translation elongation factor protein. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon

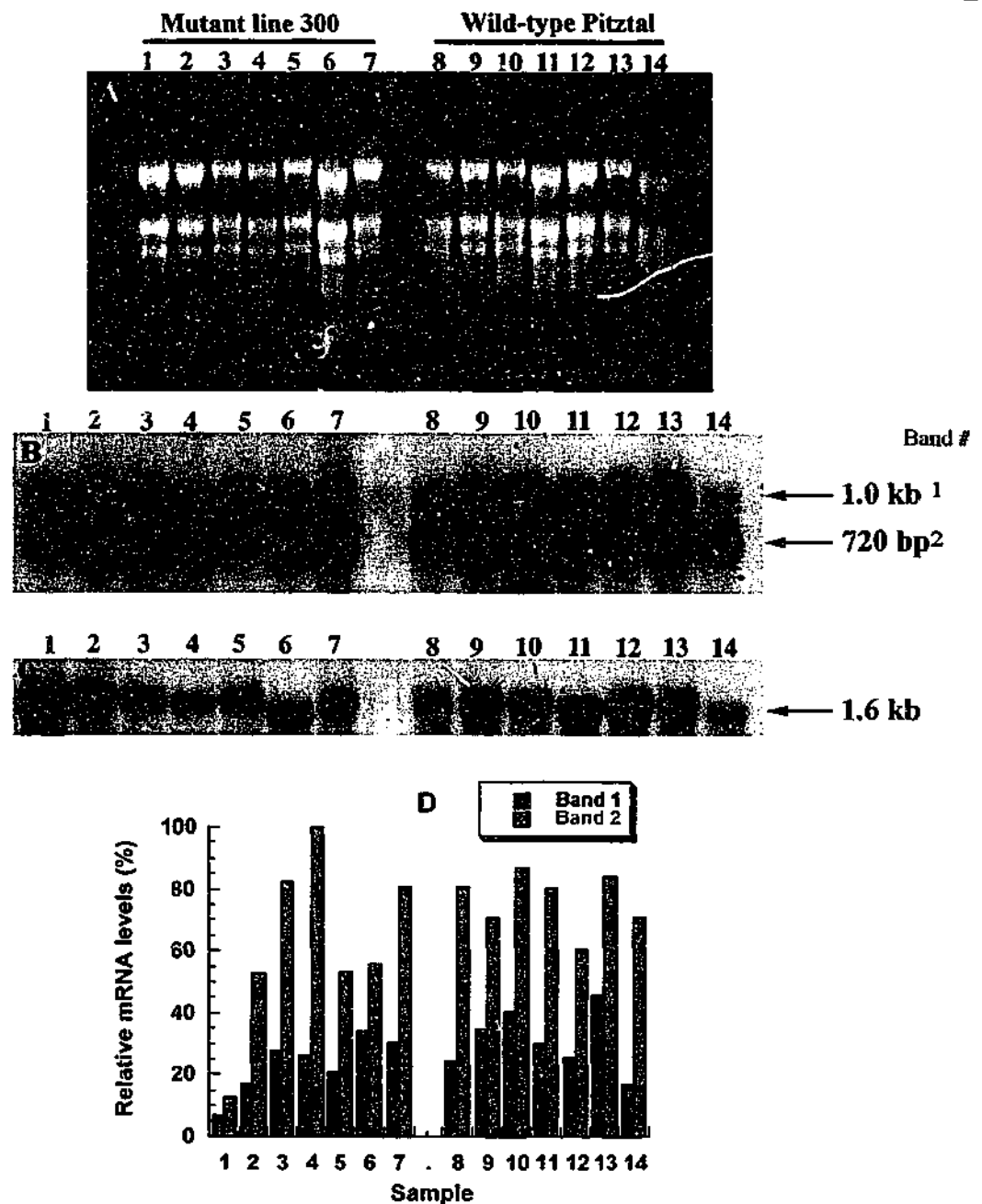* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (I);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 26-3 (B). This sequence shows homology to a gene coding for the D1 reaction centre, thylakoid membrane protein of PSII. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

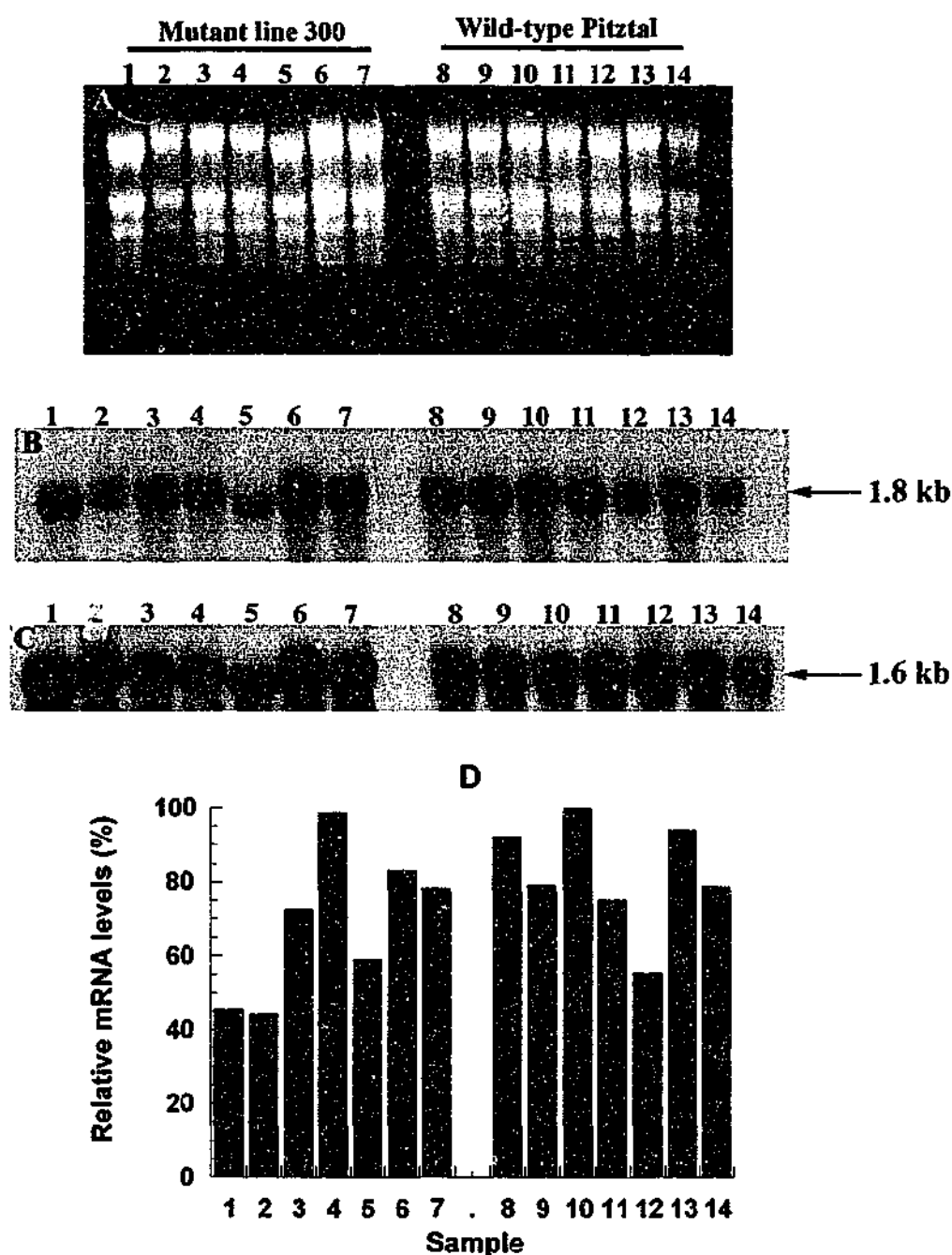* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (m);

The ethidium bromide-stained gel (**A**) was blotted and the filter hybridised with the insert sequence from clone 20-5 (**B**). This sequence shows homology to a gene coding for a glycine hydroxymethyltransferase protein from *F.pringlei*. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets

Figure 5.15 (n);

The ethidium bromide-stained gel (**A**) was blotted and the filter hybridised with the insert sequence from clone 26-4 (**B**). This sequence shows homology to a gene coding for a B type small sub-unit of Rubisco enzyme. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

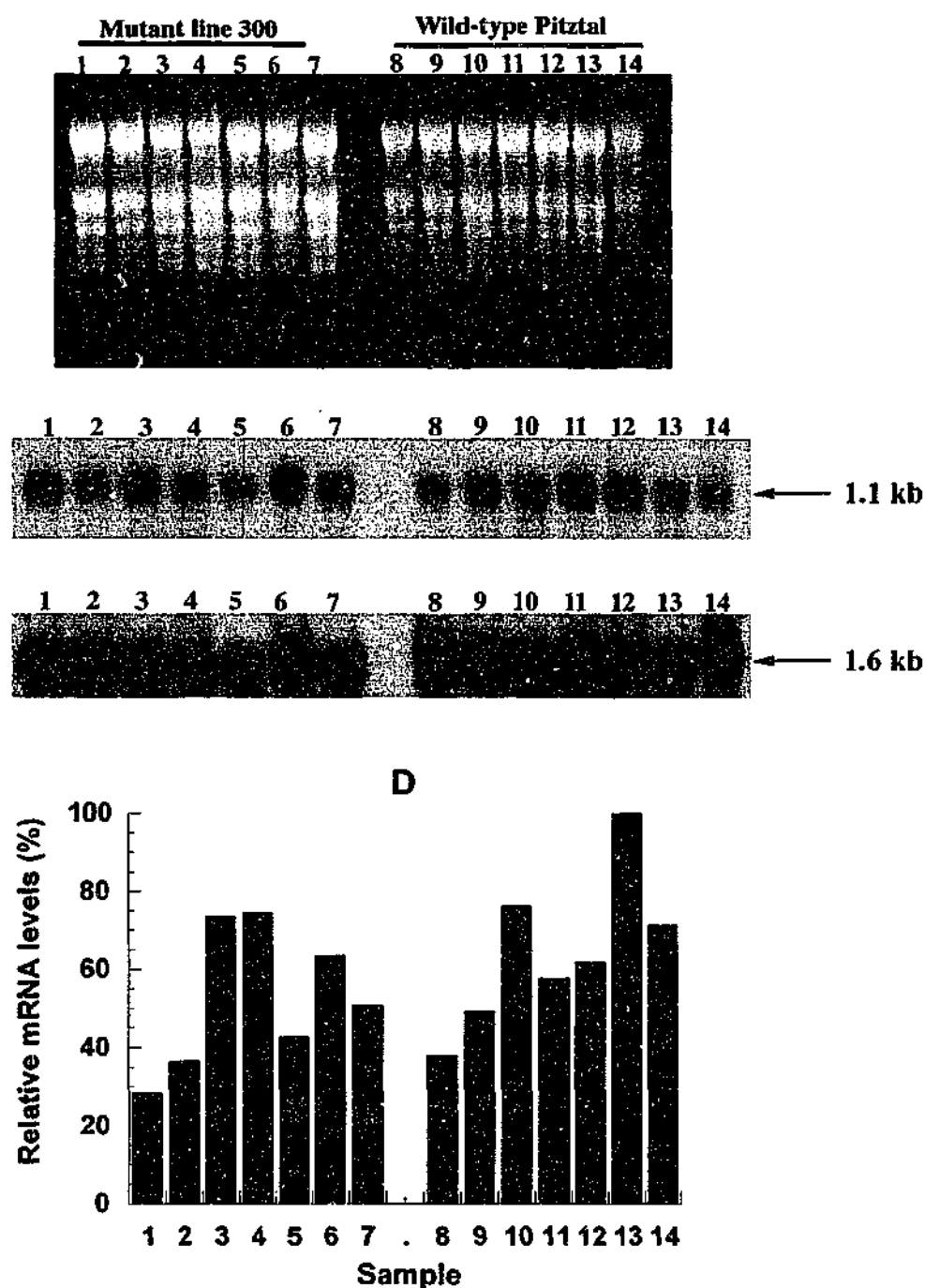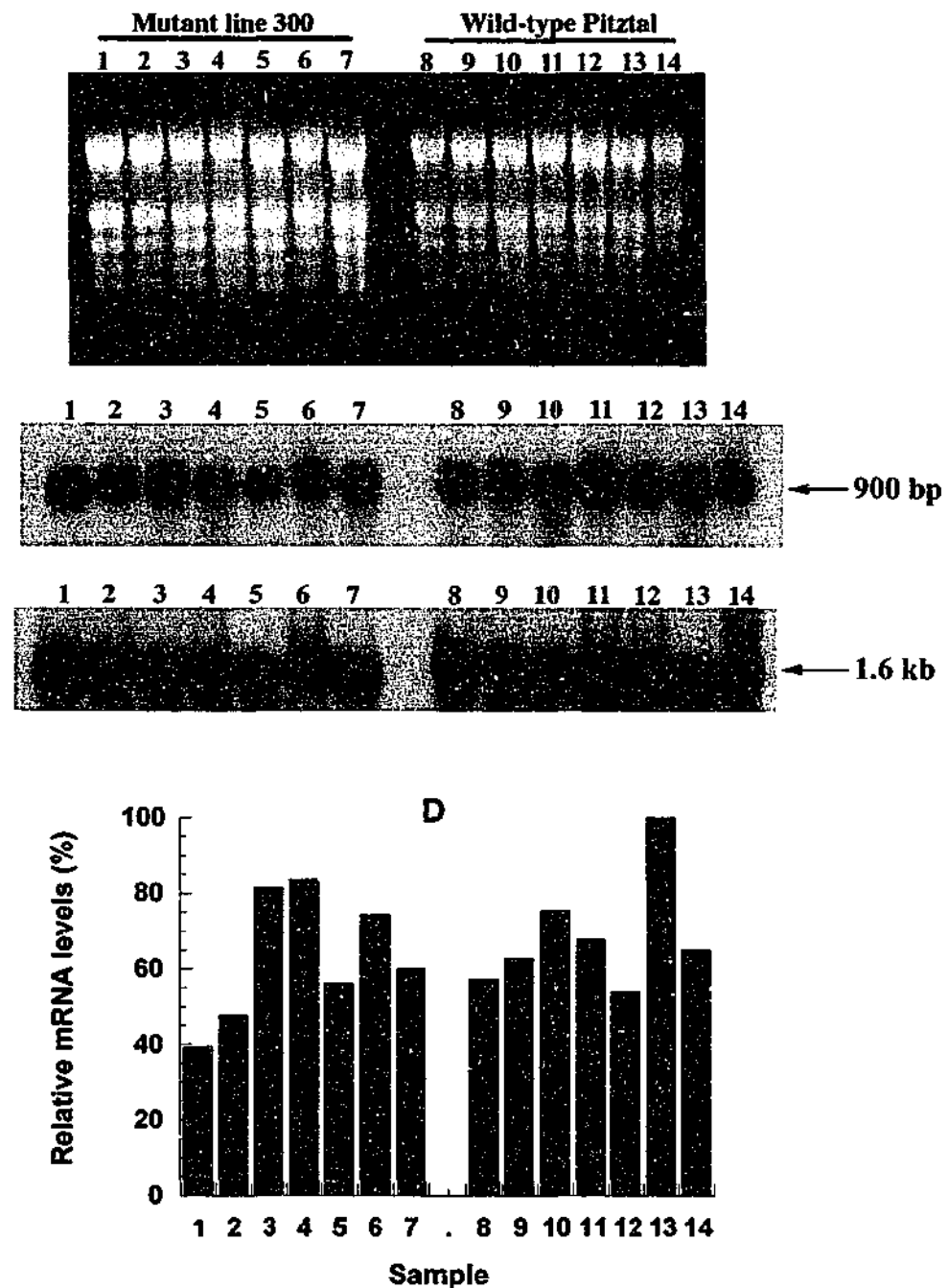* The time of harvesting of the plants used for the RNA extractions are shown in brackets.

Figure 5.15 (o);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 15-2 (B). This sequence shows homology to a gene coding for a thioglucosidase glucohydrogenase (myrosinase) enzyme. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

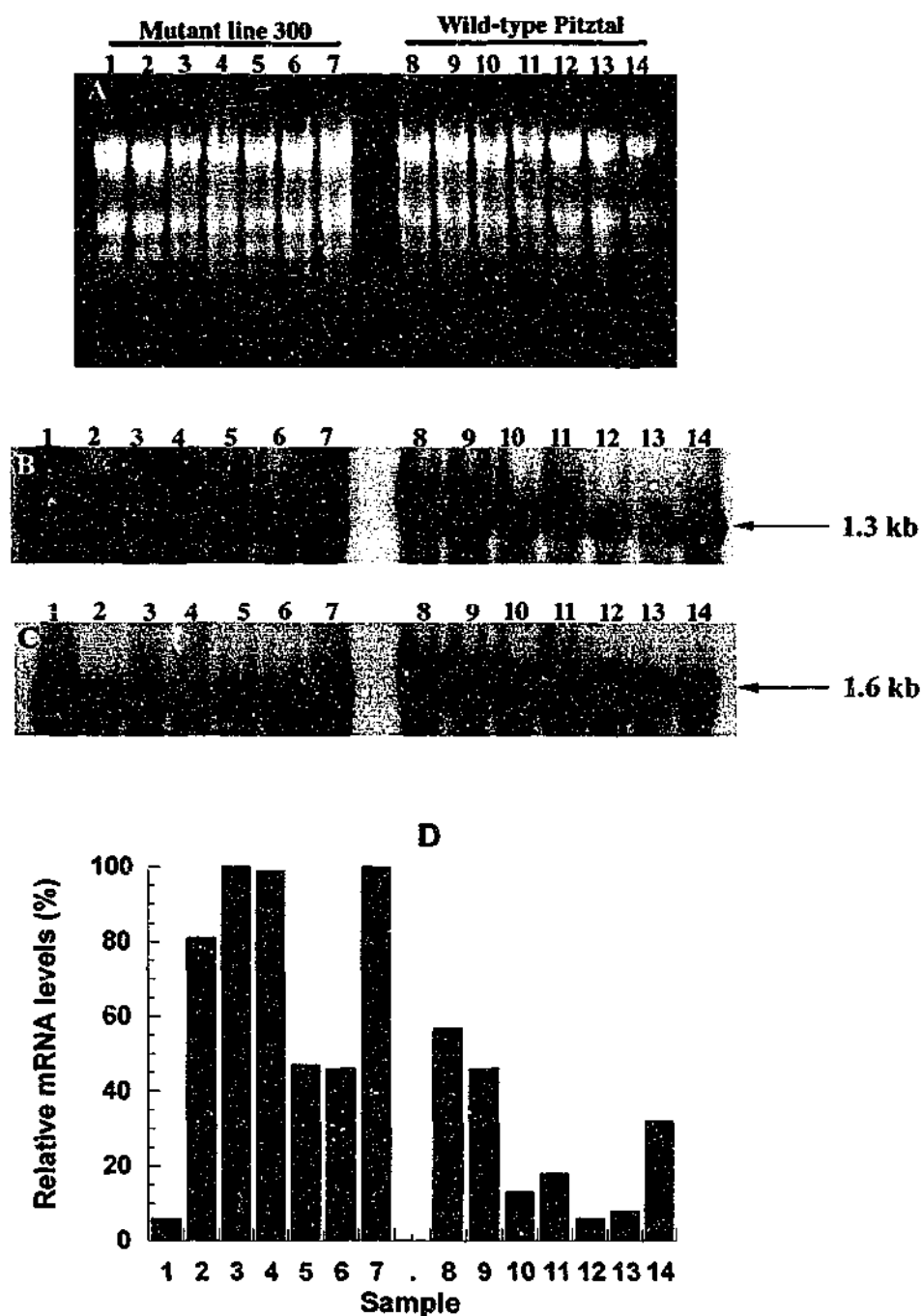| Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal 4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets.

Figure 5.15 (p);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 29-2 (B). This sequence shows homology to chloroplast gene coding for the beat and epsilon sub-units of an ATPase. This sequence is believed to be most homologous to the 2.5 kb transcript detected (band 1). The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets.

Figure 5.15 (q);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 20-4 (B). This sequence shows homology to BACF21B7 that maps to chromosome one. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

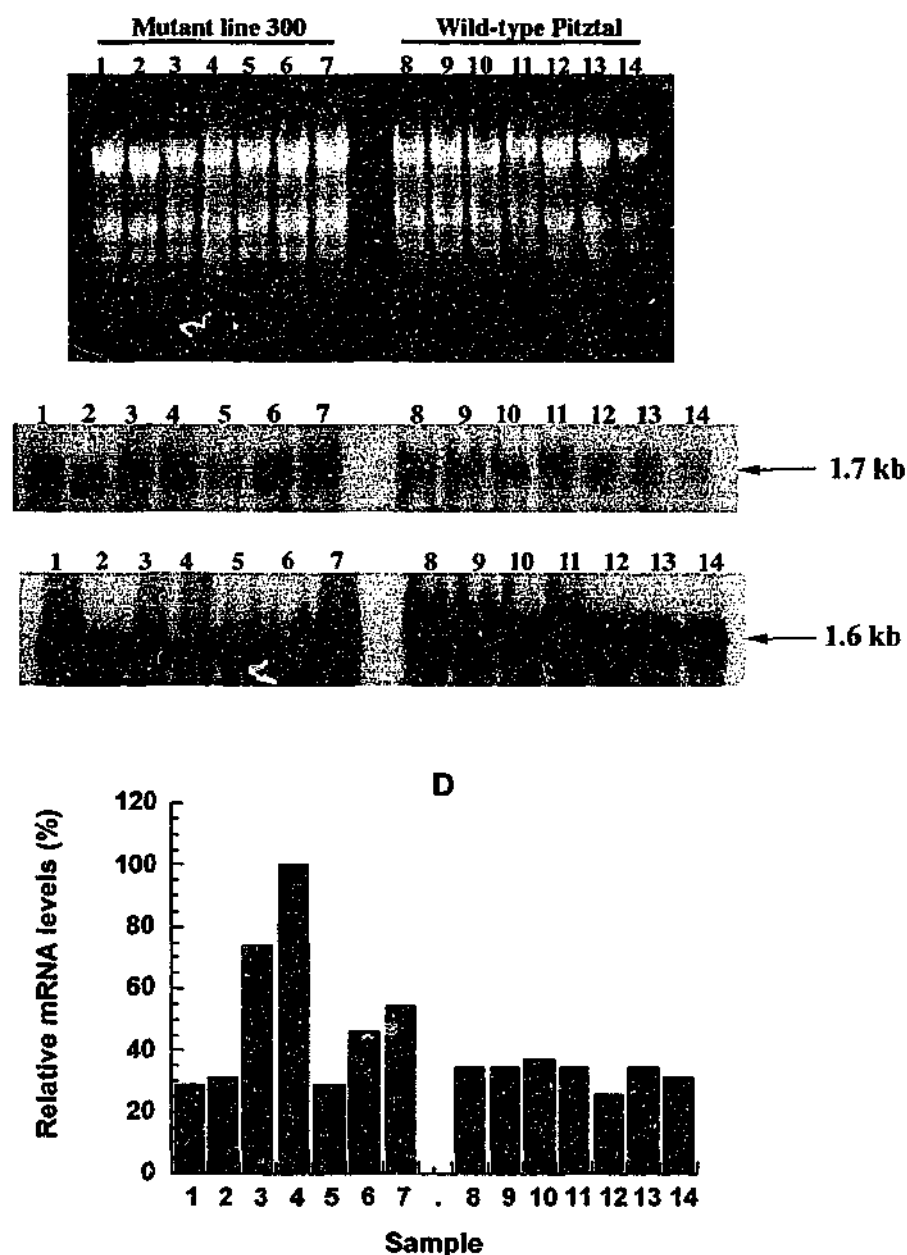| Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 a.m.)	10	Wild-type Pitztal4-leaf (9 a.m.)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4p.m.)	12	Wild-type Pitztal 4-leaf (4 p.m.)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

* The time of harvesting of the plants used for the RNA extractions are shown in brackets.

Figure 5.15 (r);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 18-4 (B). This sequence shows homology to a gene coding for a precursorpolypeptide for sub-unit IV of the PSI reaction centre. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 am)	10	Wild-type Pitztal4-leaf (9 am)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4 pm)	12	Wild-type Pitztal 4-leaf (4 pm)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

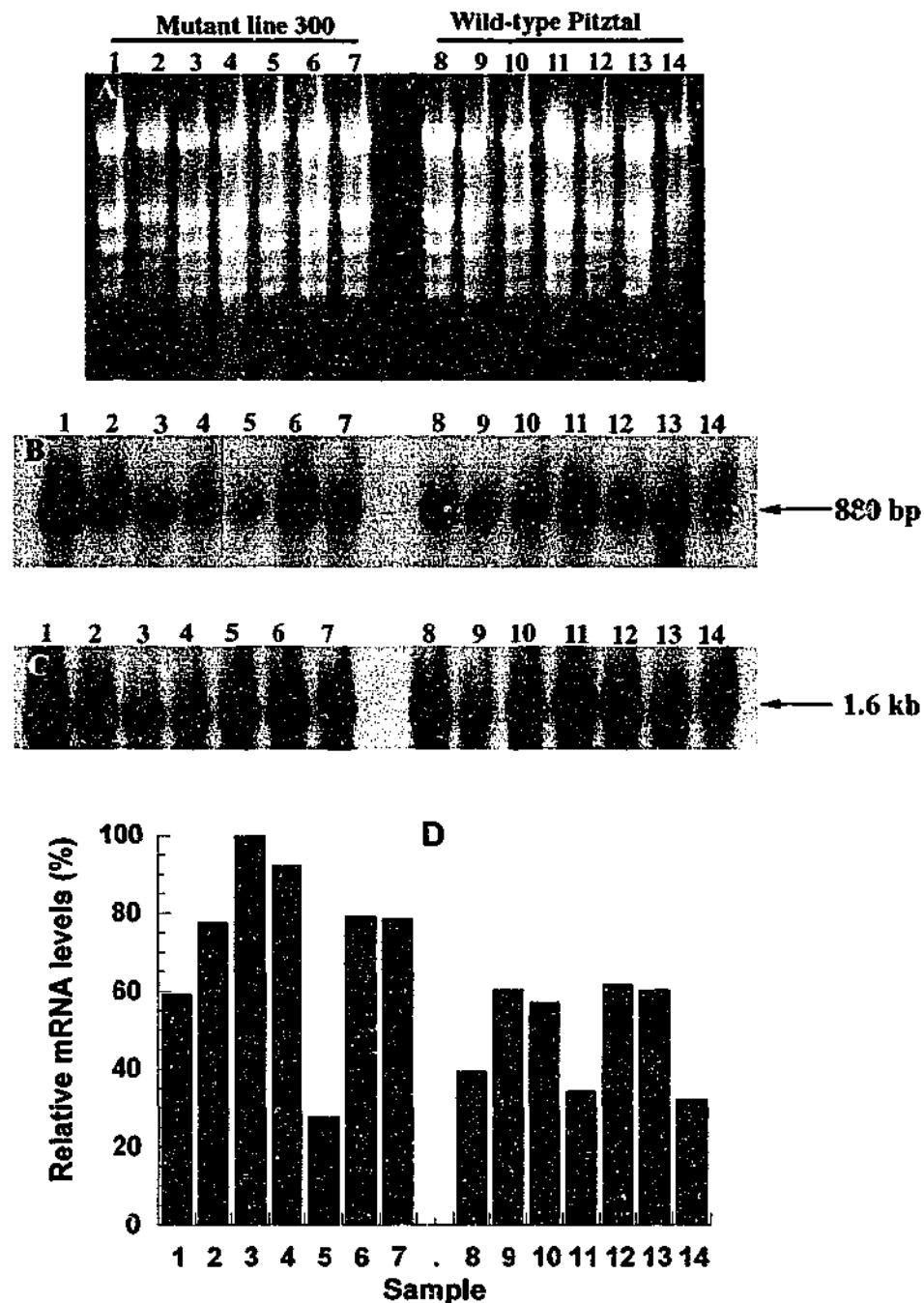* The time of harvesting of the plants used for the RNA extractions are shown in brackets.

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Figure 5.15 (s);

The ethidium bromide-stained gel (A) was blotted and the filter hybridised with the insert sequence from clone 33-4 (B). This sequence shows homology to BACT419 which maps to chromosome IV. The filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1	Mutant line 300 cotyledon (12 noon)	8	Wild-type Pitztal cotyledon (12 noon)
2	Mutant line 300 2-leaf (12 noon)	9	Wild-type Pitztal 2-leaf (12 noon)
3	Mutant line 300 4-leaf (9 am)	10	Wild-type Pitztal 4-leaf (9 am)
4	Mutant line 300 4-leaf (12 noon)	11	Wild-type Pitztal 4-leaf (12 noon)
5	Mutant line 300 4-leaf (4 pm)	12	Wild-type Pitztal 4-leaf (4 pm)
6	Mutant line 300 6-leaf (12 noon)	13	Wild-type Pitztal 6-leaf (12 noon)
7	Mutant line 300 flowering (12 noon)	14	Wild-type Pitztal mutant flowering (12 noon)

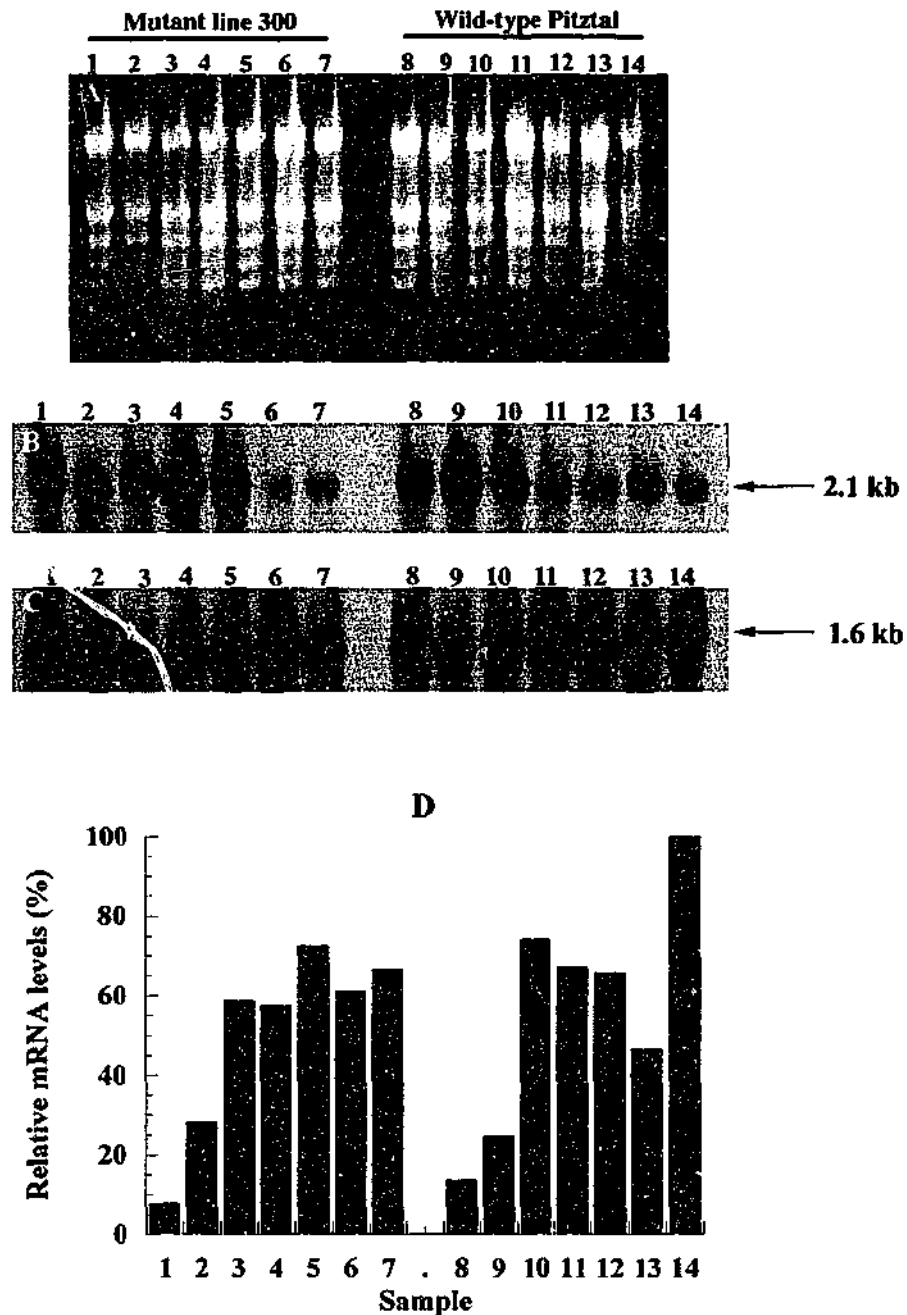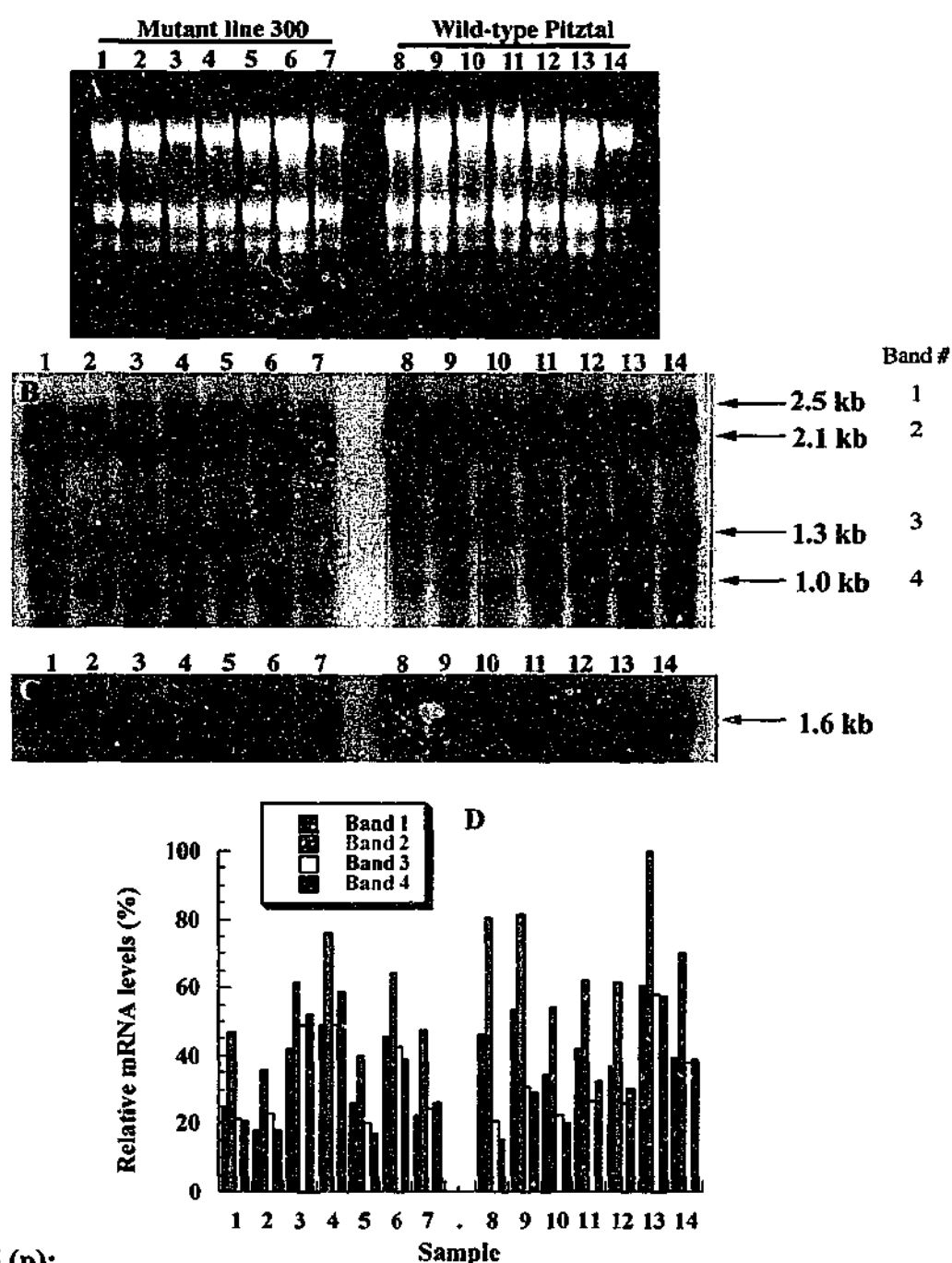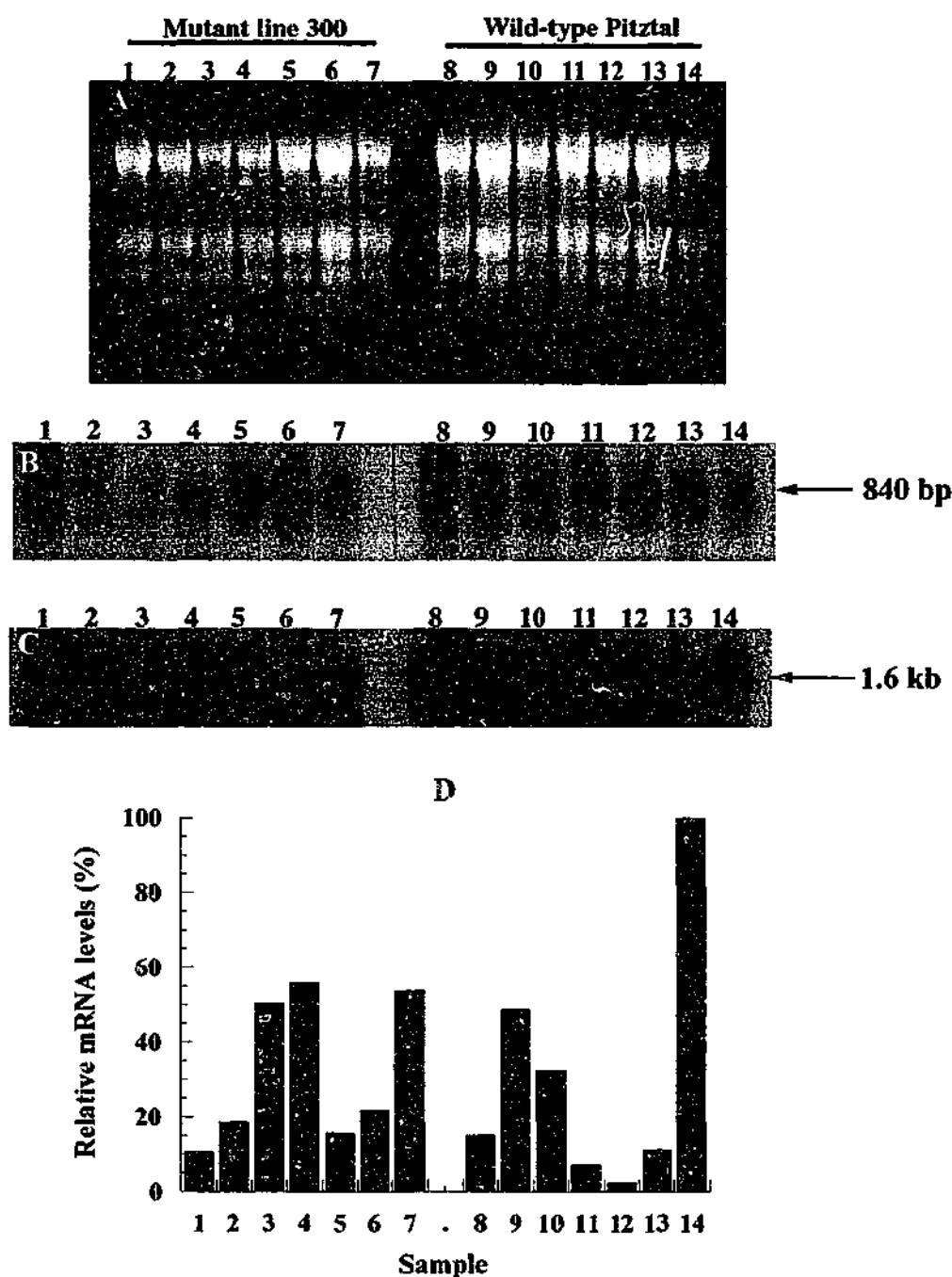* The time of harvesting of the plants used for the RNA extractions are shown in brackets.

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5.4 Discussion

The cDNA subtraction method has been used to successfully isolate at least one gene showing an extreme difference in its expression between the early-flowering *fler* lines and wild-type Pitztal plants. Genes exhibiting minor differences in their temporal and developmental expression patterns between mutant and wild-type plants were also isolated in this subtraction.

The types of genes enriched in the subtraction procedure included those coding for nuclear and plastid ribosomal RNA or plastid encoded ribosomal proteins. A large proportion of the other sequences represented in the subtracted sample show homology to genes involved with light-harvesting, or the subsequent signal transduction of light signals necessary for the physiological development of the plant. These transcripts encode light-harvesting CAB proteins, and other structural proteins of the PSI and PSII reaction centres. Genes coding for proteins associated with the electron transport chain between the two photosystems were also isolated. Other such transcripts included those encoding proteins involved in chlorophyll biosynthesis or proteins involved with the dark reactions of photosynthesis (such as Rubisco and Calvin cycle proteins).

While a few of the fragments isolated showed little enrichment in the subtracted cDNA sample compared to unsubtracted cDNA, the majority showed significant enrichment in the subtraction sample. It is not clear why, in the majority of cases, Northern blot analysis comparing expression in mutant and wild-type plants did not reveal significant differences in the expression profiles of the transcript fragments isolated from the subtraction. It is unlikely that all of the cDNA fragments isolated represent abundant background non-target sequences that are present in both the mutant and wild-type cDNA populations and were not completely removed via the subtraction technique. This is supported by the observation that the amplification of these molecules, which are expected to have the same adaptor sequence at either end, was effectively suppressed in the PCR steps In addition, the abundance of both lowly expressed and highly abundant control sequences was effectively reduced amongst the subtraction products (Figure 5.9).

It is possible that the extraction of RNA used in the subtraction from flowering mutant plants and from non-flowering wild-type Pitztal plants at the equivalent time, may have allowed enrichment of sequences that are down-regulated only at the time of flowering. In addition, the slightly paler phenotype of the *fler* mutant line 300 may help to explain the presence in the subtraction sample of several photosynthesis related genes, such as CAB transcripts that may be down regulated in the 300 mutant plants. It should however be noted that only slight differences in the levels of chlorophyll a and b were detected between 21 or 33 day old *fler*

lines and wild-type Pitztal plants. Furthermore, Northern analysis may not be able to distinguish between a number of different transcripts from a multi-gene family, making it difficult to determine if there is differential expression in mutant and wild-type plants of specific members of these gene families. Northern analysis has shown that several transcripts isolated did display fluctuations in their expression profiles throughout the day and at various developmental stages. Therefore, although all plants were harvested at similar times of the day, and at similar developmental stages, slight variations in the harvesting time of mutant and wild-type plants may have lead to significant differences in the mRNA populations used for the subtraction.

It is worth noting that amongst the 116 fragments characterised only 41 separate genes were represented. This suggests that a limited number of genes were enriched in the subtraction procedure. While Northern analysis does not provide a clear and obvious reason for the enrichment of the majority of these transcripts, it is never the less interesting that the role of circadian rhythm and phytochrome interactions in influencing both the expression of several of the genes isolated from the subtraction, as well as flowering time in a number of plants species has been well documented (reviewed in King, 1984; Coupland, 1997). The biologically active P_{fr} form of phytochrome induces transcription of both the nuclear encoded genes for the small sub-unit of Rubisco and the plastid encoded gene for the large sub-unit, as well as a gene encoding for a Rubisco activase (*RCA*) (Pilgram and McClung, 1993). The Rubisco enzyme catalyses the carboxylation of Ribulose-1,5-Bisphosphate (RuBP) in the CO_2 fixation reaction of photosynthesis, while the Rubisco activase enzyme promotes the disassociation of inhibitory RuBP and RuBP analogues molecules from the Rubisco enzyme (Pilgram and McClung, 1993). The P_{fr}-mediated induction of Rubisco and Rubisco activase is greatest at dawn in plants grown in light-dark cycles, a time at which induction of the photosynthetic machinery is vital (Pilgram and McClung, 1993). This suggests that it is the detection of light signals by photoreceptors, as well as the existing circadian rhythms in the plants that act together to optimise the timing of induction of these genes.

The fact that the interaction between photoreceptors and circadian rhythms has been shown to be crucial for photoperiodic regulation of flowering in a number of plants (Lumsden, 1991; Coupland, 1997), suggests that there may be some difference in these processes occurring between the *fler* mutants and wild-type plants. It has been demonstrated for example, that the promotion of flowering time by the perception of long days is only effective if such inductive photoperiods are given at the correct phase of the clock (Hamner and Bonner, 1983; reviewed in King, 1984). Physiological observations of mutants with disruptions to some components of their circadian rhythms, such as the *toc1-1*, *elf-3*, *lhy*, and *ccal* mutants, have further implicated circadian rhythms as a major contributor to altering flowering times in plants in response to photoperiod (Onouchi and Coupland, 1998; Somers

et al., 1998). In addition, some transgenic plants containing antisense copies of the *Lhca4 CAB* gene of *Arabidopsis* displayed a late-flowering phenotype (Zhang *et al.*, 1997). This flowering phenotype was not however directly correlated with a significant depletion in the Lhca4 protein, as those plants flowering latest had only a moderate decrease in Lhca4 levels. In contrast, a decrease in expression of a type II *CAB* gene isolated in the subtraction cDNA appears to be correlated with the early-flowering phenotype of the mutant line. Two other CAB genes isolated in the subtraction sample were however shown to have elevated levels of expression at several developmental stages in the mutant plants. It is also interesting to note that a slight difference in the chlorophyll levels was seen between flowering Pitztal and *fler* mutant line 300 plants suggesting that there is some small disruption in at least one light regulated process in this *fler* mutant.

It has also long been recognised that *CAB* gene expression is induced via phytochrome-mediated perception of light and that this expression cycles with a periodicity of 24 hours in plants (Tavaldorakai *et al.*, 1989; Paulson and Bogorad, 1988; Nagy *et al*., 1988). Indeed, the promoters of *CAB* genes have been used in reporter genes constructs to examine disruptions in the circadian rhythms of several *Arabidopsis* mutants (Somers *et al.*, 1998). The role of different phytochromes in the photoinduction of several *CAB* genes has also recently been investigated utilising *phyA* and *phyB* single and double null mutants. It was found that *PHYB* could photoreversibly induce *CAB* expression in low fluence red light, while *PHYA* could photoirreversibly induce *CAB* expression in very low light. Furthermore, *CAB* genes could still be photoreversibly induced in the *phya phyb* double mutants, suggesting that other phytochrome genes can also respond to the light environment to induce *CAB* gene expression (Hamazoto *et al.*, 1997).

The circadian rhythm and phytochrome-mediated availability of CAB proteins to act in the light harvesting complexes of PSI and PSII may potentially affect the expression of downstream photosynthesis related genes, such as genes coding for structural proteins of the PSI and II reaction centres, as well as those encoding proteins involved in the electron transport chain between the two photosystems. The regulation of chlorophyll biosynthesis and genes dependent on chlorophyll a availability such as *psbA* (He and Vermaas, 1998), is also light dependent as the P_{fr} form of phytochrome promotes the photoconversion of protochlorophyllide to chlorophyllide a and subsequently to chlorophyll a (Fujita and Hase., 1998).

Although a firm conclusion cannot be resolved from this analysis it is possible that the minor differences in the expression of these genes that were noted, are related to the mutation causal to the early-flowering phenotype of the *fler* mutant line. This would suggest that the *fler* mutation is disrupting circadian and phytochrome response within the mutant line, and

therefore also the expression of the aforementioned downstream genes, and that this disruption in the phytochrome and circadian interactions is also affecting the flowering time of the mutant lines.

It was noted that the expression of what are believed to be *Arabidopsis* orthologues of a chloroplast gene encoding the L32 ribosomal protein in *Brassica napus*, was generally lower in mutant line 300 compared to wild-type Pitztal throughout development (Figure 5.15g). The expression of these proteins can be induced by plants hormones and is strongest in meristematic regions of the plant (Gantt and Key, 1985: Kohler *et al.*, 1992; Stafstrom and Sussex, 1992). It is unlikely that a decrease in the activity of these ribosomal proteins in a floral meristematic region would cause an acceleration in development as a decrease in the expression of a ribosomal protein has been correlated with dormancy in the axillary buds of pea (Stafstrom and Sussex, 1992). These ribosomal proteins also appear to be quite crucial for the correct functioning of mitochondrial and chloroplast gene expression and function. The *maternal distorted leaf* (*mdl*) mutant of *Arabidopsis*, which contains major disruptions in two mitochondrial ribosomal protein genes for example, grows poorly, has distorted leaves, aborted floral organs and a large number of abnormal and non-functional mitochondria (Sakamoto *et al.*, 1996). It therefore would not be expected that major differences in the expression of genes encoding for ribosomal proteins would be detected within the mutant lines which appear to show no visible growth rate abnormalities.

Several other types of genes were also isolated from the subtraction that did not appear to be directly involved with any light-dependent processes. These included genes coding for a thioglucohydrogenase enzyme, a glycine or serine hydroxymethyltransferase, a metallothionien protein, a translation elongation factor, a nitrilase protein and a nitrate reductase gene. Of these genes, only those encoding for the metallothionien protein, the glycine hydroxylmethyltransferase, or the translation elongation factor subsequently showed a significant enrichment in the subtraction sample. Little difference in the expression of two of these genes, the glycine hydroxylmethyltranferase and the translation elongation factor, between mutant and wild-type plants was however noted. It is therefore unclear as to why genes such as these might be present in the subtracted sample.

The isolation of several genes within the subtraction population for which no function has as yet been assigned was of particular interest. These genes included three that displayed homology to BAC sequences, and two which exhibited no homology to genes within the databases. It was hoped that these remaining uncharacterised genes may have a novel involvement in controlling the flowering time of *Arabidopsis*. All five of these sequences showed some enrichment in the subtracted sample, although for sequences contained within clones 6-1, 20-3 and 33-2 this increase in abundance was small. Three of the gene

fragments (contained within clones 20-4, 33-2 and 33-4) were used to probe Northern blot filters of mutant and wild-type Pitztal RNA. The gene from clone 20-4 however exhibited only a slightly disrupted pattern of expression in the mutant line compared to wild-type Pitztal plants. When Northern blot filters containing 20µg of total RNA per sample were repeatedly probed with the gene fragment contained in clone 33-2, no signal was detected in either wild-type or mutant line RNA samples. The expression of this gene is thought to be quite low and therefore Northern blot analysis using purified mRNA may be required to detect the transcript. The presence of such a low abundance sequences within the subtraction population does provide some evidence that equalisation of low and high abundance sequences has taken place during the hybridisation steps of the subtraction.

When the gene sequence contained within clone 33-4 was used to probe Northern blots of mutant and wild-type RNA a dramatic difference in its expression was noted. No expression of this gene was able to be detected in any of the developmental stages of mutant line 300 that were examined. This sequence was expressed throughout wild-type Pitztal development however, although the expression at the cotyledon stage was quite low. Following the appearance of the first two leaves in wild-type Pitztal plants there was a large increase in the abundance of this transcript and the expression of this gene appeared to only gradually decrease as the plants became older. Peak temporal expression of this gene appeared to be at earlier times of the day.

The dramatically different expression of this gene between mutant and wild-type RNA populations is suggestive of the expression of this gene being closely linked to the early-flowering phenotype of the mutant line 300. Alternatively, the lack of expression of this gene may be attributable to an alteration in the expression of an upstream regulatory gene in the mutant lines and it may play no direct role in floral development. The BAC to which the insert sequence in clone 33-4 shows homology, maps to chromosome 4. However, this BAC sequence appears to be sufficiently distal from the nga8 marker (see Chapter 4) to make it unlikely that this gene represents the *fler* mutation causing the early-flowering phenotype of the mutants. While the lack of expression of this gene appears to correlate with early-flowering, further analysis is required to determine the role of this gene in either directly or indirectly repressing floral induction in wild-type Pitztal plants. This gene was therefore analysed in greater detail via sequence and Northern blot analysis and the results of this expression study, as well as a discussion of the potential role of this gene in the flowering response is presented in chapter 6.

Chapter 6

Analysis of the regulation and expression of the *FRP33-4* gene

6.1 Introduction

The cDNA subtraction technique, discussed in Chapter 5, was undertaken to isolate cDNA sequences that are differentially expressed between wild-type Pitztal plants and the early-flowering *fler* mutant line 300. Several sequences were isolated that exhibited slight differences in either their temporal expression throughout the day or during plant development. As these sequences only exhibited small variations in their expression patterns between mutant and wild-type plants, and the fact that the majority of these transcripts encoded 'house-keeping' genes, it is not clear that their expression is affected by the major *FLER* gene that is causal to the early-flowering phenotype mutation in the mutant line 300.

Unlike the majority of other sequences isolated during the subtraction procedure, one transcript showed a major difference in the pattern of expression between mutant and control plants, with expression below detectable levels in the mutant lines, while strong and relatively constant expression is detected in wild-type. Further investigation of this gene was undertaken to determine whether the lack of expression of this transcript in the mutant line is a result of an upstream mutation in the *FLER* gene and directly related to the early-flowering phenotype. The gene sequence from clone 33-4 has been tentatively assigned the name *FRP33-4* (*F*loral *R*epressor, clone *33-4*), and shows almost 100% homology to a gene located on BACT419 that maps to chromosome 4. Although this BAC sequence was isolated from the Columbia ecotype, analysis of the gene, its predicted protein sequence, and the 5' regulatory region, was carried out to obtain information relating to the function of this gene in wild-type Pitztal. A comprehensive study of the expression pattern of the gene via Northern analysis was also undertaken.

The expression pattern of the recently cloned *FLC* gene (Michaels and Amasino, 1999; Sheldon *et al.*, 1999) in the early-flowering mutant lines, was also examined. This gene is believed to be one of the most crucial in conferring the repression of flowering observed in late-flowering ecotypes of *Arabidopsis*. Indeed, it is now thought that the levels of the *FLC* gene product within the apical cells of *Arabidopsis* plants is directly proportional to the extension in time taken to flower (Michaels and Amasino, 1999). In light of the results of these Northern blot analyses, a model for the putative role of the *FRP33-4* gene in an *FRI/FLC* mediated floral repression pathway is discussed in this Chapter.

6.2 Methods

The RNA used for all Northern blot experiments was isolated from leaf tissue of plants at the various developmental stages Probes used in the Northern blot analysis were the cDNA insert from clone 33-4, and the cDNA sequence of the *FLC* gene which was obtained from Dr. E. Dennis, Division of Plant Industry, CSIRO, Black Mountain, Canberra. The signal intensities for each gene in the Northern blot analysis were quantified relative to those of *ubiquitin* using the ImageQuant software. Sequence analysis was performed using the Australian National Genome Information Service (ANGIS) (http://www.mel1.angis.org.au).

6.3 Results

Analysis of the coding sequence and 5' regulatory region of the FRP33-4 gene

The insert sequence from clone 33-4 showed homology to the coding sequence of a gene represented by bases 29,487 to 31,361 of BACT419 that has been assigned to YAC CIC4A7. This Columbia ecotype genomic DNA BAC sequence maps to approximately 17cM on the Lister and Dean RI map of chromosome IV, near the *HY4* gene. The structural gene contains three introns that comprise approximately 700 bases, and has a total length of 1.9 kb. Figure 6.1 displays the sequence of this gene as well as the nucleic acid sequence of clone 33-4. The clone sequence showed 100% identity to this gene from the end of the last intron to the stop codon, as well as over approximately 200 base pairs of 3' untranslated region. One base pair difference exists between the Pitztal clone 33-4 and the Columbia BAC gene in the penultimate exon at position 1253 which results in an altered amino acid residue (alanine compared with glutamic acid) at position 308 of the putative Columbia gene product. It is unclear at this stage as to whether this represents a genuine difference in sequences between these two ecotypes or is the result of a sequencing/PCR error. Interestingly, the BAC sequence also contained genes on either side of this gene that show 75 and 80% identity to the insert sequence of clone 33-4. Both of these genes are in the inverted orientation to the central gene and may have resulted from a duplication of the one ancestral sequence.

Approximately 2 kb corresponding to the 5' regulatory region of the *FRP33-4* gene from the Columbia database sequence was analysed in an attempt to gain information about possible regulatory mechanisms controlling the expression of this gene in *Arabidopsis*. Figure 6.2 displays the results of this analysis. Apart from the basic transcriptional regulatory CAAT and TATA boxes, several putative elements potentially involved in light-induced regulation are present. These elements are found in *CAB* genes, genes coding for the small sub-unit of Rubisco and Rubisco activase genes (Green *et al.*, 1987; Giuliano *et al.*, 1988; Ha and An,

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   1  ATGGGTTCATGCAGTCTTCAGCTTCCACTCATCAATCTTGCGGACAAAACCCTAGAACCAGGAAGCTCAAAGTGGGCTGAAGTGA
  89  GGAGTGATGTCCGTAAAGCTCTCGAAGACTTCGGTTGTTTCGAAGCTTCATATGATAAAGTGTCATTGGAGCTTCAGGAATCAAT
 177  TATGAAAACCATGGAAGAGCTTTTCGCATTGCCAGTTGAGACCAAACAACGAAACGTGTGCCCCAAACCTTACGTTGGATATTTG
 265  AATCATAATAATCTCTCCGAGAGTTTGGGGATAAGTAATGCTAATATTTTGGAGAACATCAACGAATTTACTCAACAACTATGGC
 353  CTCATGGTGACGGTAACGAGAATATCAGGTGAAAACTCATATGTTTTTACTATTGTTTAGTAAAAATCATGTTAATCTTTTTTCT
 441  CTAAAAAGTAAAATGTTAAATTCACTCTCAAATCGTATGAAACCAATGAACTGAAGACAAAAAAAAAAAATTATCTGAAAACAAA
 529  TTTTCTGCATATTCATTTAATTTGTATTTTCTTGTTTTCATATGAATCCTCACACTTTTGTTTTTGGTTGAAAACAGTAAAAACGA
 617  TCCAGTTGTTTGCGGAGAAGTTAGTGCAAATAGATGTGATGGTGAGAAGAATGGTAATGGAGAGCTTTGGAATAGAAAAATACAT
 705  TGATGACCATCTAAAGTCTACGGCGTATGCGACTGATGAAGTATATTGCACCACCTGAGGGTGATGCTAATACTACTGTTGACGA
 793  TAATGCTGATCTTCTTGCTAAGCTTAATATTGTTGGTGTTGAACCTAATGTTGGTGTTAAAGTTAATGCCGATATTAGTGATGAT
 881  GTTAATGCTAATGCTAGTGTTAATGCTGGTGTTGGTGCTAATGTTAACGCTGATACCGGTGTTAATGATAATCTTAATGTTGATG
 969  CTAATGTTGCCGTCGGTGGTGGCGTTAATGCTAATACTGATCTTGGTGTTGGTGTTAATGTCAATTCTAATGTTGCTGTGAATGC
1057  AAAACTGGTGGTGATGATGTTGAGGCTAATGATGATAATGAGGAAAAGAAGTTGGGTTTACCTTGTCATACTGATAAAAACCTTT
1145  TCACAGTACTTTTTCAACATGAAATTGAAGGTTTGGAGGTAAAGACCAAAGATGAGAAGTGGATCAGAGTGAAACCATCCCCGAA
                                           ||||||||||||||||||||||||||||||||||||||||||||
 530                                          GATGAGAAGTGGATCAGAGTGAAACCATCCCCGAA


1233  TACTTTCATTGTTATTGCCGGAGATTCCCTATGTGTAAGTTTTTTCATTTCGTTTTTTACTTTCTATGGCACACAAATATTAACC
      ||||||||||||||||||||||| |||||||||||||||
 495  TACTTTCATTGTTATTGCCGAAGATTCCCTATGTG


1321  TTATTCATTCTTCAAGGTTCATACTAGTCTAGTTCGGTCATGTGGCAAACAGTCTCGAAAAGTCGAGCTAAGGGTCTTTGGCCTT
1409  TACAAAAATTACATATGTGCATGGATCTATAACTTATTTTGATTAGAGGCATATATAACTCAAAATTTAAATAAATAATATAGAC
1497  AACCCATTTCTCTAGCAATCACTTTATAATGCTTTAACTTCATACCAAATCTCAATTCTTATGATTTTATTTGCTCTTATCACTT
1585  CAATGTTGGTCGATGCACTACTATTGTTTTCTCGTACATAATCTTCTTGTTCATAATCATTTACTTGGTGATGATGTAGGCTCTT
                                                                                     |||||||
 460                                                                                 GCTCTT


1673  ATGAATGGTAGAATCAGGGCTCCGTATCACCGAGTAAGAGTGACCGAGAAAAAGAGGACAAGATACACAGCAGCAATTTTCACGT
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 454  ATGAATGGTAGAATCAGGGCTCCGTATCACCGAGTAAGAGTGACCGAGAAAAAGAGGACAAGATACACAGCAGCAATTTTCACGT


1761  GTCCAAAACCGGACTATGTCATAGAGGCACCAAAAGAACTTGTGGACGAGAAGCATCCACGTCTCTTCAGACCTTTTGATTACCG
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 369  GTCCAAAACCGGACTATGTCATAGATTCACCAAAAGAACTTGTGGACGAGAAGCATCCACGTCTCTTCAGACCTTTTGATTACCG


1849  CGACTTGTTTACATTCTATCACTCAGAAGCTGGTCGCAAAATTCAATATACTCTTCAAGCTTATTGTGCCGTCTCCGAAGCATAA
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 284  CGACTTGTTTACATTCTATCACTCAGAAGCTGGTCGCAAAATTCAATATACTCTTCAAGCTTATTGTGCCGTCTCCGAAGCATAA


      TTCAATGTGTGCGCTTGATTCCGAGTTTCGACAATGTTTGGTGATTTCAAGTTATCAAAAACCATGTGGTATACGAAATAAGGGC
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 199  TTCAATGTGTGCGCTTGATTCCGAGTTTCGACAATGTTTGGTGATTTCAAGTTATCAAAAACCATGTGGTATACGAAATAAGGGC


      TTTTGATTAGTTATGTTATTCTGTTTGTGTGATAAGTATGTTTTATGGAGGTCCAACTTCTTTGTGTTGTATGAAAATCAATAAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
 114  TTTTGATTAGTTATGTTATTCTGTTTGTGTGATAAGTATGTTTTATGGAGGTCCAACTTCTTTGTGTTGTATGAAAATCAATAAAG


      TATGTGTGCATGCATGCACATGCACATGTACAATAATG
      ||||||||||||||||||||||||||||||||||
  29  TATGTGTGCATGCATGCACATGCACATGT
```

Figure 6.1

Figure showing identity of the cDNA sequence from clone 33-4 (lower sequence) to the genomic sequence represented by bases 29,487 to 31,361 of BACT419 (upper sequence) that maps to chromosome four. Bases 1 to 460 of clone 33-4 show 100% identity to this gene sequence, while bases 461 to 530 show 98% identity to the gene. The three introns of this gene are shaded in grey and the translation start and stop codon of this gene are highlighted in green and red respectively.

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2086  CCCAAAACTC  TGGTGAAGAT  TATGACAAAG  ATATCCATGA  AAGCATTGCG  AATACACGTT
2026  TCTCAGTTTG  GTCTGAATCG  GCAACTCGAA  AAGCTCTTCC  ATGGCTTCAA  CAACAGAGTT
1966  CTTAAGCTTT  ACAGACAATA  TATGTCAAAA  GAAGCTTCGA  AACAACCGCA  GTCCTCTAGA
1906  AGCTTCCTGG  TTTTAGGGTT  TGGTTAGAGA  AATCGATAAC  CGGGTCCGGG  AGCTGAAAAG
1866  ATATAGAACG  AGGAAGAAGA  GAATCTGAAT  CCATTATTTT  GATTCTGAGA  ATTTTGAGGT
1806  TTTTTTTTTG  CTTTATGATT  TTGGCCTAAA  TAATTAAAAG  AATATTTGTA  TGGAAGAATT
1746  CTGTAATTAG  AAGTTAGTAT  ATGCGTTTAC  GGATGGTACG  TTGCTATTAT  GCGTAGATGT
1686  TTATACATTT  AATAAATTAT  GCAATATTCG  CGTTTGATAA  AGATTTATCT  CTTTATCCAA
1626  TAAAGAGTTA  AAATATCAAA  GAGTTAAAGA  GATAAAAAAA  AAAAAGTTTA  GAATTGAGGA
1566  AAAGAGATAA  AATATATATA  ACCGTGTGAT  CATCTTAAAA  TAAAATGTCT  ACATTTTTAC
1506  TTACCTAGCT  AACCACTGTT  ACAACCCCAT  GTTGATAATG  TCTCTTGTAT  TACGGCATAT
1446  GTTAATTTTA  ATAAAACAAT  TGAATAATTT  CTTAAATTAC  ATAATACAAG  AAGAAGAAAA
1386  ACTAAATAAT  GACTGACTAT_AGAACAAAAT  TTGTTTAAAC  TTTAAACAAT  GATTTTCAAT
1326  TTTCTCTTTC  GTTCTTAGTT  GAGATACCTC  TTCAATTCTC  TGCAGGTATG  TTATGTAAAA
1266  ATATGTTTTA  GACTTTTAGT  TAACAAAAAG  AAAAAGAAAC  AAAGAAGACA  TGAGCCTTGG
1206  GCTAAAAAGC  AAACATTAAA  AGTTCTGGGT  CAGTTCTAAT  ATATAGATCA  CGTCCTTTAT
1146  GCGATCATGC  TTTAAAGGCC  GATCACTGTT  TCTTACATTC  CTAAGTCGGC  ACTTCTTCAC
1086  TTTTTCCTAC  GACATGAATT  GAGAATTGAA  GACCAAACGC  CAAAAGACAT  TTTATTCCTC
1026  TAATTGTCTC  ATTTTATCAT  TTTTTTGGGC  TATTCAAGAA  TATATTATAG  CAAACGTACA
 966  AGTCAAAAAC  TTGATCGATC  GTCTCGTATT  TCTGTTATAC  ATTGTGATAA  AAAAAACTTA
 906  ACAATACCTA  AACTGATTAA  TCATCTTCCA  CCTGCAATGT  TATCATATTA  CAGAGACGAC
 846  GTGTATGCAG  CAGGTAAAAC  AAAAGCTCTG  ATCCAAAGTA  ATGGCCAAAT  GATACAGTTG
 766  GATCCAACCG  AGATAAGTAA  AAAAAGATTC  TATCTTTAAA  ATGGGACCTC  TCTCCTTTTT
 706  CTCTTCATTA  TTGCTATGGT  TCAAAACGTA  CAATCATCGG  CCTCGCTCGG  AATTTAAATC
 646  ATATACAATA  TGCTTACTAA  GTATTATTCA  CGTATAAGTA  ACAATATGCA  ACTAAAGGTT
 586  TTGGGCCACG  AATTACTTAC  AATAAAAAAC  TTTTGTAAAC  TCAAGTTCCA  AATGTTTAGT
 526  AATATAAATT  CACATTTTAT  CTCAAAAAAA  AAGAGAAAAA  GAGAAAACTT  TTTATCAAAA
 466  GAGAAAAGTT  AACTTAAGTT  ATAACTCGTT  GAGTTGTTCT  TCATCTTGTA  CAACAATAAT
 406  GCCCACACAT  CGTATGGATT  GATATTTTCT  ATGATGAAAA  TAAACGATTC  ACCAACTTTC
 346  CTTTTTTTGT  ATATAAAATG  TGAACTTTTA  ATAGTATATA  CCAACTTTCC  TTCCTCTGTC
 286  TATTATTATT  AAGCATGTGT  TTGCTTAAAT  TAAGCAAAGC  GACAAAAAAA  AAACTTAATA
 226  CAATCACTTG  TGAATAATTT  CTCTATAAAA  TGGGGACCCT  TCACTATTCT  TACTCACACA
 166  GAAGAGAAAA  ATCTCTAGAG  CTAGCAAAGT  AAAAACAATT  AATATAACAG  AAAGTCCAAA
 106  GGTAATTTTC  TTATGCGTTT  CGAATGTTTT  TTTTTCTTAT_TAATTATTGC  TACTATCTGC
  46  TAACATCTGC  AAACGTTTTT  CGATTACACC  AAAAAGGGAA  AGAAGAATG
```

Site name	Consensus	Information/Reference
TATA box 3 ·	TATTAT	Putative eukaryote TATA box (Shirsat *et al.*,1989)
CAAT box 1	CAAT	Putative eukaryotic CAAT box (Shirsat *et al.*,1989)
MADS binding site	CC(W), NNGG	Putative binding site for the MADS box gene*AGL1* (Huang et al., 1996)
CCA1ATLHCB1	AAMAATCT	Putative binding site for the CCA1 protein (Wang *et al.*,1997)
E box	CANNTG	Common positive regulatory element (Stalberg *et al.*, 1996)
GT consensus	GRWAAW	Binding site of the nuclear factor GT-1, found in several light regulated genes (Ou-Lee *et al.*, 1988)
GATAmotif/I box	GATA/GATAA	Common light regulatory elements (Terzaghi and Cashmore, 1995)
Pyrimidine box	TYCTTTTYC	Element found in several genes regulated by GA (Huang *et al.*, 1990)
Myb pzm	CCWACC	Recognition site for some plant Myb proteins (Grotewold *et al.*, 1994)
CircadianleLHC	CAANNNNATC	Element conferring circadian mRNA oscillations in *CAB* genes (Piechulla *et al.*, 1998)
LtrecoreatCOR15	CCGAC (Reverse)	Low temperature responsive element (Jiang and Singh, 1996)
AMYBOX 1	TAACARA	Binding site of endosperm specific factors (Huang *et al.*, 1990)
Boxiintpatpb	ATAGAA	Recognition site for plastid RNA polymerase (Kapoor and Sugiura, 1999)
Sif Box	ATGGTA	Element down regulating promoter activity of *r*-protein genes (Lagrange *et al.*, 1993)

Figure 6.2

Several regulatory elements were identified within a 2100 bp region upstream of the start codon of the gene contained on BACT19 to which clone 33-4 showed homology. (M = A or C, N = A, G, T or C, R =A or G, W =A or T, Y = C or T)

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1988; reviewed in Terzaghi and Cashmore 1995; Piechulla *et al.*, 1998), and include the GT consensus sites (GT-1 sites), and GATA and I-box motifs (Terzaghi and Cashmore, 1995). GT-1 sites are believed to be binding sites for the nuclear DNA binding factor GT-1 and are found to be necessary, although not sufficient, for the transcription of several light-regulated genes (Le Gourrierec *et al.*, 1999; Gilmartin *et al.*, 1990). GATA and I-boxes are common regulatory elements which are thought to be binding sites for several different factors involved in conferring high levels of expression of genes in response to light (Gilmartin *et al.*, 1990; Terzaghi and Cashmore, 1995). It is believed that a combination of these different elements is required for the transcriptional regulation of genes by light, and that the presence of several light regulatory regions within a promoter allows the gene to respond to the various light fluences detected via different photoreceptors (Gilmartin *et al.*, 1990). The presence of multiple GT sites, GATA boxes, and I boxes suggests that the *FRP33-4* gene may be regulated by light.

Previously, analyses of the cDNA subtraction products have suggested the possibility that some disruption of phytochrome and circadian rhythm interactions may be occurring in the mutant lines. Interestingly, the regulatory region of the Columbia version of the *FRP33-4* gene also contains an element found in the promoter region of several *CAB* genes from tomato and *Arabidopsis*, that confers oscillations in their mRNA levels in response to the circadian rhythm (Piechulla *et al.*, 1998). In addition, the 5' region of the Columbia gene also contains a putative binding site for the CCA-1 (Circadian Clock Associated -1) protein. The CCA-1 protein has been found to bind to a region of the *Lhcb1*3* regulatory sequence that is necessary for its regulation by phytochrome, and is thought to be a key element in the phytochrome signal transduction pathway leading to *CAB* transcription (Wang *et al.*, 1997). The presence of these elements within the 5' region of the Columbia gene suggest expression of this gene may also be influenced by circadian rhythm mediated process. The binding of CCA-1 is mediated by a region in its N-terminus that has homology to the DNA binding domain of Myb proteins. As the promoter region in the Columbia database sequence also contains a recognition site for some plant Myb proteins (Grotewald *et al.*, 1994) it is also possible that the expression of the Columbia *FRP33-4* gene is regulated by other Myb, or Myb-like proteins.

The 5' region of the Columbia *FRP33-4* gene also contains two elements found in the regulatory region of a gene encoding the enzyme α-amylase. This enzyme is required for the breakdown of the starchy endosperm into metabolisable sugars necessary for germination and early-seedling growth. It has been demonstrated that the expression of the α-amylase gene is stimulated by endogenous GA that induces the production of factors that bind to specific regions of the α-amylase gene promoter (Ou-Lee *et al.*, 1988; Huang *et al.*, 1990). The Columbia gene contains both a regulatory element sequence found in α-amylase genes that is necessary for the binding of endosperm specific factors, as well as two putative

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pyrimidine boxes thought to be involved in the GA-mediated regulation of such genes. This pyrimidine box has also been found in several other GA inducible genes such as the β-glucanase and carboxypeptidase genes (Ou-Lee *et al.*, 1988). Therefore, the expression of the Columbia gene may also potentially be regulated by GA, perhaps by a similar mechanism to that observed for the α-amylase genes.

Two other sequences found in the promoter region of the BAC gene are homologous to the BoxI element from the promoters of plastid genes, and Sif box elements. The Sif box was found in the promoter region of the *rps1* gene coding for the ribosomal protein S1, and is believed to act as a negative element down-regulating the promoter activity of this gene (Lagrange *et al.*, 1993). The BoxI element is thought to be involved in the influencing the activity of non-consensus type II promoters that are recognised by a nuclear encoded, DNA dependant, RNA polymerase that transcribes plastid genes (Kapoor and Suigiura, 1999).

Another putative regulatory element found in the reverse orientation in the 5' region of the BAC gene shows homology to low temperature response elements (LTRE's) found in the *cor15* gene of *Arabidopsis* and the *BN115* gene of *Brassica napus*. The AP2 domain, DNA-binding CBF1 (*C*-repeat/dehydration responsive element *B*inding *F*actor 1) protein interacts with these elements and mediate the cold-induced expression of such genes (Baker *et al.*, 1994; Jiang and Singh, 1996; Stockinger *et al.*, 1997). A further element found in the 5' region of the Columbia gene shows similarity to the predicted consensus binding site for the *AGL1* and (*AGAMOUS LIKE 1*) MADS box genes from *Arabidopsis* (Huang *et al.*, 1996). The *AGL1* gene is expressed in the gynoecium and ovules of the flower during the later stages of flower development (Ma *et al.*, 1991; Flanagan *et al* 1996).

Analysis of the protein sequence of the FRP33-4 gene

The translated protein sequence of the Pitztal *FRP33-4* gene fragment from clone 33-4 shows 99.08% identity to the hypothetical Columbia *FRP33-4* gene product (Figure 6.3). The protein sequence of the gene found on the Columbia ecotype BAC may encode an oxidoreductase as database searches for homologous sequences showed matches to a number of oxidase genes. The homology to these proteins is low however, with the highest identity at the amino acid level, 36%, being observed between the Columbia *FRP33-4* gene product and hyoscyamine 6-dioxygenase over a stretch of 65 amino acids. In addition, the C-terminal end of the BAC protein shows the most homology to these sequences. The BAC protein also shares approximately 21% to 27% similarity, over the entire protein sequence, to GA-20-oxidase genes isolated from plants such as *Arabidopsis, Pisum sativum, Phaseolus vulgaris,* and *Oryza sativa*.

```
  1  MGSCSLQLPLINLADKTLEPGSSKWAEVRSDVRKALEDFGCFEASYDKVS

 51  LELQESIMKTMEELFALPVETKQRNVCPKPYVGYLNHNNLSESLGISNAN

101  ILENINEFTQQLWPHGDGNENISKTIQLFAEKLVEIDVMVRRMVMESFGI

151  EKYIDDHLKSTAYATDELNIVGVEPNVGVKVNADISDDVNANASVNAGVG

201  ANVNADTGVNDNLNVDANVAVGGGVNANTDLGVGVNVNSNVAVNAKTGGD

251  DVEANDDNEEKKLGLPCHTDKNLFTVLFQHEIEGLEVKTKDEKWIRVKPS
                                            |||||||||||
  1  ..............................DEKWIRVKPS

301  PNTFIVIAGDSLCALMNGRIRAPYHRVRVTEKKRTRYTAAIFTCPKPDYV
     |||||||:|||||||||||||||||||||||||||||||||||||||||
 11  PNTFIVAEGDSLCALMNGRIRAPYHRVRVTEKKRTRYTAAIFTCPKPDYV

351  IEAPKELVDEKHPRLFRPFDYRDLFTFYHSEAGRKIQYTLQAYCAVSEA
     ||||||||||||||||||||||||||||||||||||||||||||||||
 61  IEAPKELVDEKHPRLFRPFDYRDLFTFYHSEAGRKIQYTLGAYCAVSE.
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Figure 6.3

An alignment of the putative gene product encoded by the insert from the Pitztal clone 33-4 (lower sequence) and the Columbia *FRP33-4* gene from BACT419 (upper sequence).

Figure 6.4 presents an alignment of these GA-20-oxidase amino acid sequences with that encoded by the BAC gene. The GA-20-oxidase proteins shown in this figure share between 70% to 90% identity with each other, however they only share between 20 to 28% identity at the amino acid level to the BAC gene product. The highest level of identity, 28%, was observed between the Columbia BAC gene product and one of the three GA-20-oxidase sequences isolated from *Arabidopsis* (YAP169). The Columbia *FRP33-4* gene product also shows little homology to two conserved regions of amino acids found in the GA-20-oxidase proteins. The first of these consensus sequences, NYYPXCXXP (residues 230 to 239 in the At2310 protein sequence), is thought to be involved in the binding of the GA-20-oxidases to the co-substrate 2-oxoglutarate. The second sequence, LPWKET (amino acids 148 to 153 in the At2310 protein sequence), is thought to be important in binding of the GA substrate (Xu *et al.*, 1995; Wu *et al.*, 1996).

The BAC gene product does however contain three conserved residues, two histidines and an aspartate residue (at positions 247, 249, and 303 in the At2310 sequence) which may be involved with binding of the Fe^{2+} ions by the GA-20-oxidase enzymes (Garcia-Martinez *et al.*, 1997). In addition, the Columbia BAC gene product appears to contain several conserved amino acid motifs that are found in either all, or at least one class of other dioxygenases. These dioxygenases include flavone 3β-hydroxylases, ACC oxidases and

```
Bacpep  MGSCSLQLPL  INLADKTLEP  GSSKWAEVRS  DVRKALEDFG  C E  YDKVS  LELQESIMKT  60
At2310  .....  VSF  VTTSPEEEDK  ..PK....LG  LG..NIQTP   I NP M NL   AN........  39
At2353  .....  IL   TTTSPAEKEH  EPK.....QD  LEK.DQTSP   I NP   NL   SQ........  41
YAP169  .....  TE   IATVPQIFSE  NKT.....KE  DSS.......  I D K  NQH  SHH.......  36
U50333  ..........  MSMVVQQEQE  ..........  .........V  V D AV SG   TE........  33
U58830  .....  KVL  SS..MLFAPP  NAN.....ES  FMN.EQKQC   DNTS   PLQI  TN........  39
U70530  .....  ID   MTNIQTMSQP  QKH.....HH  QDNKEDEAP   V D   RH   LN........  43

Bacpep  MEEL AL VE  T Q.RNVC K  PY GYLNHNN  LSESLGISNA  NILENIN FT  QQLWPHGDG   119
At2310          D   .  IN L   D       QN  L  D.PSSTL  D S LIS   K          97
At2353          E  .  IDIP   N   E  SS  QD ....TL  E P VIA   T          95
YAP169    Q  V  H  . TD  Q   Q    AG  F  GDSCLAS  E T  SK A  T      IT   95
U50333  I S     AE  S G VA E   E A  VGA  GAE.....RS  SVV Q G   ER          78
U58830   SE     H  . CLTP K   E  F  KA  F  DDPKSIS  N CSK NH   K          98
U70530  L K     D   .CMN      V       RG  F  GDPVATM  E A M G   Q          101

Bacpep  EN SKTIQLF   EKLVEIDVM  VRRMVMESFG  IE YIDDHLK  STAYATDELN  IVGVEPNVGV  179
At2310   S SEE  SD   EYTSR  D  M  S       L  S  SV           T              156
At2353   V SES  AD   RL ES  D  M . AG  K   P  SC           T              154
YAP169   VDES LSR   YLH  S K  A . AC  K   W  SS                         154
U50333   G EAA LEE   RC     T  L  GE   R  SGAR .RTA                       136
U58830   VDNK  AQ   KLV  E C  MQ. S        I  HC   N I                    157
U70530   DAN  SH   SY  D   E  V . TQ       T  HC                         160

Bacpep  KVNA I D..  DVNANASVNA  GV ANVNADT  VNDNLNVDA  NVAVGGGVNA  NTDLGVG N.  236
At2310  R CD M R..   KS O  FCD  A HG .QP   K   E CE    S    K        L    K.. 212
At2353  Q N N G..   RT O  FSD  T QE .EQ   K  D         S S K      L    N. 210
YAP169  K PEEKIH.   OT K  FVEK  KM DGY.ED   K  E A    NT   K        M    E. 211
U50333  RY SAGDEEG  EEG GE LVR  K  AEHGRRL  E  SR HE   R  EL   V  E   IVG 196
U58830  RY AD   ...  CRT E  FVH  IM ED .RQ   I    K         M  G      M    G. 212
U70530  Q  AEEKS..   TI K  LCH  T  QE .EQ   R  D  D    M  G      M    G. 216

Bacpep  .....VNSNV  AV AKTGGDD  VEANDDNEEK  K LPC T K  NLF V  HE  IE    KTK  291
At2310  ..  D        E         I K             H   M       .E 269
At2353  ..  D  G     E         QT      H      H   M       . 267
YAP169  ..  R  K    DS    E     KQ  E   A          Q   G      . 268
U50333  DR H  R     QR         A QR LD             H   G  E WA. 255
U58830  ..KE        G  V        KN      AF          Q   E    L . 269
U70530  ..KAC       E        R   QK       Q  G      . 273

Bacpep  EK IRVK S   NT I IA   S  LC    IR  APY VR TE  K RT YTA I   T  P  Y   351
At2310   R R       K          D        SE      K R       328
At2353   Q  R          K        IF         M        K      326
YAP169   K  P      H                T       ERE  TE     GE   327
U50333  GR RA R R   G  V     A R         TAP R       EM T   314
U58830  GI H VV KE  D            MF       D IV       NE   338
U70530   E H N  F   N                 TT        G   333

Bacpep  EA K     EK  H ..  LERP   DYRD FT ..  Y SE GRKIQ  YTLQAYCAVS  EA......  399
At2310  T R   L SI  TS.  R    S F      N  Q   D TK.  ..PI....  377
At2353  K  SDILEKM  KT..  K      S F     VN   DS N VITM  NNPI....  378
YAP169  K  E  NGV  KSGE K     S F      N   DE  I KNR  RSF.....  380
U50333  R  E    DH  H ..  V    RA       R F  Q  D NHH  RHLQPTIY  370
U58830  T    I KE  N ..  K N   PS      ER   D  M QEK  TT......  378
U70530  S  S   DL  T ..  L      P      K   E TN QQK  R.......  381
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Figure 6.4

The amino acid sequence of the gene contained on BacT419 that shows homology to clone 33-4 (Bacpep) was aligned with the protein sequences of three GA 20-oxidases from *Arabidopsis* (At2310, At2353, and YAP169; Phillips *et al.*, 1995), as well as with GA 20-oxidases from *O. sativa* (U50333, unpublished), *P. sativum* (U58830; Lester *et al.*, 1996) and *P. vulgaris* (U70530; Garcia-Martinez *et al.*, 1997). Identical amino acids are highlighted in black, similar residues are shaded in grey. Consensus sequences and conserved residues found in GA-20-oxidase sequences are underlined.

hycoscyamine 6β-hydroxylase (Garcia-Martinez *et al.*, 1997). The majority of the amino acids conserved between the BAC gene and these other dioxygenases were observed in the Carboxyl terminus rather than the amino terminus of the proteins (Figure 6.4). Interestingly, it is the C-terminal region of these proteins that has been suggested to be the region that confers substrate specificity to the different classes of dioxygenase proteins (Garcia-Martinez *et al.*, 1997). From these observations it appears that while the BAC gene and the Pitztal version of this gene, the *FRP33-4* gene, may share some homology and hence similar functions to oxidase genes in general, it is unclear as to which class of oxidoreductase enzymes this gene may belong.

Southern Blot analysis of the FRP33-4 gene

DNA extracted from wild-type Columbia, L. *erecta*, San-Feliu-2, Pitztal, and *fler* mutant line 300 and 260 plants was digested with either *Eco*RI or *Hind*III, or with both restriction enzymes before being probed with the *FRP33-4* gene fragment from clone 33-4 (Figure 6.5). The Columbia BAC sequence indicates that the *FRP33-4* probe should detect a single band of approximately 4kb in the *Eco*RI digests of the Columbia DNA. Assuming complete digestion, the presence of several other bands in these digests suggests there are four or less sequences within the *Arabidopsis* genome that are related to the *FRP33-4* gene. Two bands would be expected to be detected by the probe for both the *Hind*III and *Eco*RI/*Hind*III double digests corresponding to sizes of approximately 1100bp and 3070bp, or 1100bp and 488bp, respectively. The presence of extra bands in these digest supports the suggestion that other related genes are being detected by the *FRP33-4* Columbia probe. Some of the likely candidates for these related sequences include the two genes flanking the Columbia *FRP33-4* gene which share approximately 75% to 80% identity with the *FRP33-4* gene. Other possibilities include the three or more GA-20-oxidase loci present in the *Arabidopsis* genome (Phillips *et al.*, 1995) although they only share approximately 40-50% identity at the nucleotide level with the Columbia *FRP33-4* gene.

It is interesting to note that while there are similar numbers of bands detected in all four ecotypes tested, the size of several of these bands is different between the early and late-flowering ecotypes, particularly in the L. *erecta* ecotype, and the banding pattern for the two late-flowering ecotypes, Pitztal and San-Feliu-2 is almost identical. These observations suggest that some significant difference in the RFLP pattern of these genes exist between early- or late-flowering ecotypes. The two mutant lines examined however show a similar banding pattern to that of the late ecotypes suggesting no large structural disruptions of the *FRP33-4* gene have occurred within the mutant plants.
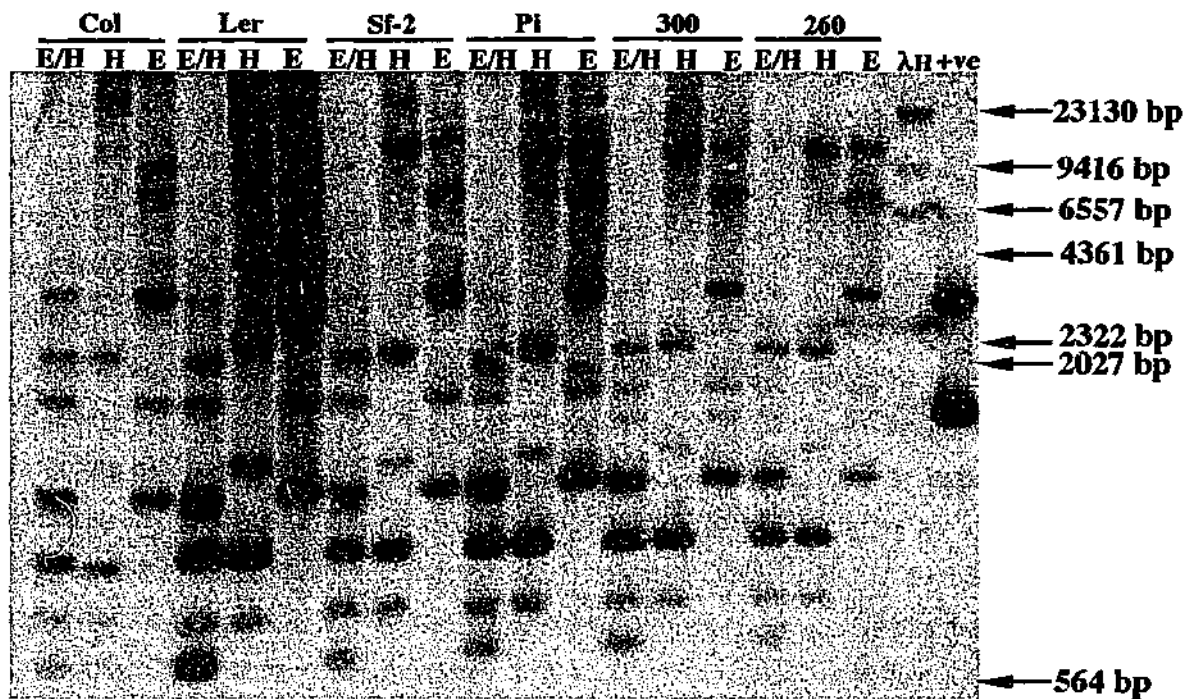
Figure 6.5

Genomic DNA isolated from wild-type Columbia, L. *erecta*, San-Feliu-2, Pitztal and early-flowering mutant lines 300 and 260 was digested with either *Eco*RI, *Hind*III or both restriction enzymes before being hybridised with the *FRP33-4* gene fragment from clone 33-4.

Northern blot analysis of the FRP33-4 gene

The expression profile of the *FRP33-4* gene was examined via Northern Blot analysis, the results of which are presented in Figures 6.6 to 6.12. The expression of the *FRP33-4* gene is found to be very low in all of the nine *fler* mutant lines (Figure 6.6 and Figure 6.7) and the two early-flowering ecotypes, Columbia and L. *erecta* , (Figure 6.6), at the four and six leaf stages of development of plants grown in continuous light. In comparison, there is strong expression of the gene in the two late flowering ecotypes, San-Feliu-2 and Pitztal, at similar stages of development (Figure 6.6 and 6.7) when grown in the same light conditions. The expression of *FRP33-4* was also found to decrease slightly throughout development of wild-type Pitztal plants with a lower level of expression evident in flowering Pitztal plants (Figure 6.7). Figure 6.7 also demonstrates that a three week vernalisation treatment of germinating Pitztal seedlings results in a dramatic increase in the expression of this gene in the treated plants. In addition, and in contrast to the expression of this gene in non-vernalised Pitztal plants, flowering vernalised Pitztal plants display the highest abundance of the *FRP33-4* transcript.

Figure 6.8 displays the expression profile of the *FRP33-4* gene in wild-type Columbia and Pitztal plants grown in either short (8 hours light) or long days (16 hours light) and harvested at various stages of development. Little if any transcript is detected in wild-type Columbia plants grown in either short or long days. In wild-type Pitztal plants, short days appears to markedly decrease expression of the *FRP33-4* while long day photoperiods appear to induce its expression. This effect is particularly noticeable when plants that have been grown in short days up to the 8-to-10 leaf stages of development are transferred into long day conditions for 2-to-7 days, and then harvested at either the 10-to-12 leaf stage or the 14-to-16 leaf stage. This change in photoperiodic growth conditions results in a distinct induction of the *FRP33-4* transcript (lanes 9, 10, 11, and 12 in Figure 6.8), although these levels never reach those observed in plants grown continually in long day conditions (lanes 13, 14, and 15 in Figure 6.8). In contrast, a large decrease in the abundance of the *FRP33-4* transcript occurs when plants that are grown in long days up to the 8-to-10 leaf stage of development are subsequently transferred to short day conditions for 1-to-7 days and then harvested at 8-to-10, 12 or 14 leaf stage of development (lanes 10, 11 and 12 in Figure 6.9). From these observations it appears as if the short-day induced inhibition of transcription of the *FRP33-4* gene is of equal or greater magnitude than the induction of expression that resulted from the transfer of short day grown plants into long day conditions.

The expression level of the *FRP33-4* gene in the F1 plants of a cross between Pitztal and L. *erecta* or Columbia ecotype plants grown in continuous light was also examined, and found to be at similar or at higher levels, than that observed in Pitztal plants at the same developmental stages (Figure 6.9). Although slightly higher *FRP33-4* transcript levels are found in Columbia plants compared to L. *erecta* plants, the expression levels in the F1 plants from a cross of Pitztal with L. *erecta* appear to be distinctly higher than those observed in the F1 plants from a cross of Pitztal and Columbia (Figure 6.9).

In order to try and correlate the lack of expression of the *FRP33-4* gene with the early-flowering phenotype of the mutant line 300, the abundance of the *FRP33-4* transcript in the F3 progeny lines of early and late flowering F2 plants from a cross of mutant line 300 and two different late-flowering ecotypes was measured. Figure 6.10 displays the results of this analysis for the F3 progeny lines of a cross between mutant line 300 and the San-Feliu-2 ecotype. The expression pattern of the F3 progeny lines from a cross of wild-type Pitztal plants and the mutant line 300 are shown in Figure 6.11. Little if any transcript is detected in all the F3 progeny lines created from early-flowering F2 plants of both these crosses. Strong expression of the *FRP33-4* gene however, is evident in F3 lines descended from late-flowering F2 plants. These results therefore suggest that the early-flowering mutant phenotype is linked with a lack of expression of the *FRP33-4* gene. It is also interesting to note that quite strong expression of *FRP33-4* is evident in F3 progeny lines created from F2 plants of those crosses that flowered at an intermediate number of leaves (42 leaves for line

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41 in the 300 X Sf-2 cross, and 15 leaves for line 11 in the 300 X Pitztal cross), and that high expression of the *FRP33-4* gene occurs in the F1 plants of mutant line 300 crossed to Pitztal (lane 3 in Figure 6.11). This suggests that a threshold level of *FRP33-4* expression correlates well with delayed flowering but not necessarily with the extent of the delay.

As a distinct induction of the *FRP33-4* gene was noted in vernalised wild-type Pitztal plants, and an inhibition of this expression was noted in Pitztal plants grown in short day conditions, the transcript levels of the *FRP33-4* gene in similarly treated mutants lines was also examined. Neither a three week vernalisation treatment nor growing the mutant plants in short day conditions was however found to alter the lack of expression of the *FRP33-4* gene in mutant line 300 plants (Figure 6.12). This result therefore suggests that there is a constitutive loss of transcription of the *FRP33-4* transcript in the mutant lines, rather than a qualitative difference in the expression patterns of this gene between the mutant lines and wild-type Pitztal under different growth conditions.
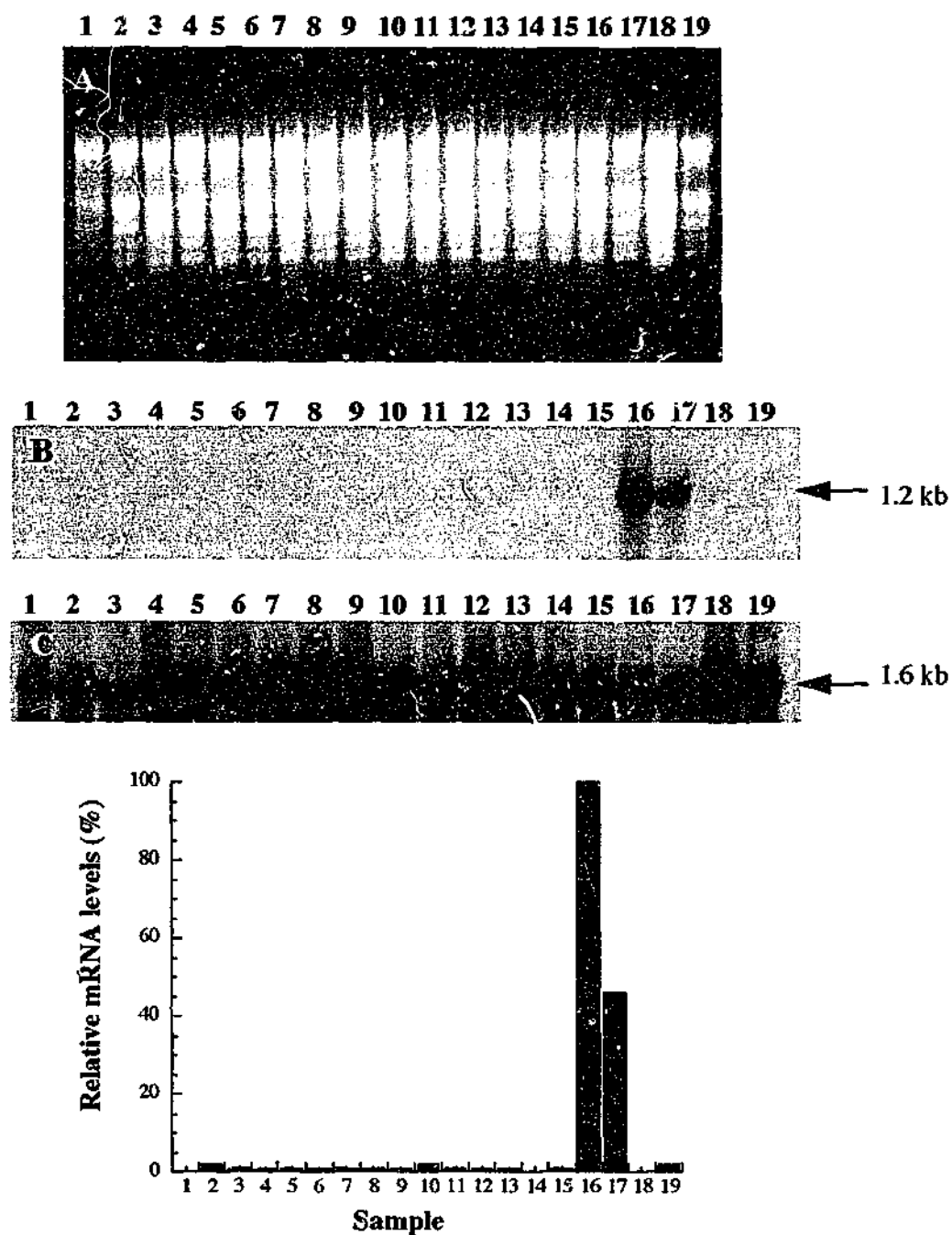
Figure 6.6

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1.	Mutant line 220 4-leaf	11.	Wild-type L .*erecta* 6-leaf
2.	Mutant line 220 6-leaf	12.	Wild-type L .*erecta* flowering
3.	Mutant line 230 4-leaf	13.	Wild-type Columbia 4-leaf
4.	Mutant line 230 6-leaf	14.	Wild-type Columbia 6-leaf
5.	Mutant line 240 4-leaf	15.	Wild-type Columbia flowering
6.	Mutant line 240 6-leaf	16.	Wild-type Pitztal 4-leaf
7.	Mutant line 250 4-leaf	17.	Wild-type Pitztal 6-leaf
8.	Mutant line 250 6-leaf	18.	Mutant line 300 4-leaf
9.	Mutant line 260 4-leaf	19.	Mutant line 260 6-leaf
10.	Wild-type L. *erecta* 4-leaf		

* All plants were grown in continuous light and harvested at 12 noon for RNA extraction .

Figure 6.7

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert
sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from
Arabidopsis (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1.	Mutant line 270 4-leaf	11.	Wild-type Pitztal 4-leaf
2.	Mutant line 270 6-leaf	12.	Wild-type Pitztal 6-leaf
3.	Mutant line 280 4-leaf	13.	Wild-type Pitztal 12-leaf
4.	Mutant line 280 6-leaf	14.	Wild-type Pitztal 18-20-leaf
5.	Mutant line 290 4-leaf	15.	Wild-type Pitztal 30-40-leaf
6.	Mutant line 290 6-leaf	16.	Wild-type Pitztal flowering
7.	Mutant line 300 4-leaf	17.	Vernalised wild-type Pitztal 4-leaf
8.	Mutant line 300 6-leaf	18.	Vernalised wild-type Pitztal 6-leaf
9.	Wild-type San-Feliu-2 4-leaf	19.	Vernalised wild-type Pitztal flowering
10.	Wild-type San-Feliu-2 6-leaf		

* All plants were grown in continuous light and harvested at 12 noon for RNA extraction.

Figure 6.8

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert

sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from

Arabidopsis (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample	RNA*
1.	Wild-type Columbia 2-leaf (SD)	10.	Wild-type Pitztal 12-14-leaf (SD)
2.	Wild-type Columbia 6-leaf (SD)	11.	Wild-type Pitztal 10-12-leaf (SD to LD)
3.	Wild-type Columbia 10-12-leaf (SD)	12.	Wild-type Pitztal 14-16-leaf (SD to LD)
4.	Wild-type Columbia 4-leaf (LD)	13.	Wild-type Pitztal 4-leaf (LD)
5.	Wild-type Columbia 6-leaf (LD)	14.	Wild-type Pitztal 6-leaf (LD)
6.	Wild-type Columbia flowering (LD)	15.	Wild-type Pitztal 12-leaf (LD)
7.	Wild-type Pitztal 2-leaf (SD)	16.	Wild-type Pitztal 18-20-leaf (LD)
8.	Wild-type Pitztal 6-leaf (SD)	17.	Mutant line 300 4-leaf
9.	Wild-type Pitztal 8-10-leaf (SD)		

* The light conditions in which the plants were grown are detailed in brackets; SD= Short days and LD=Long days. All plants were harvested at 12 noon for RNA extractions.

Figure 6.9

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert

sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from

Arabidopsis (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1.	Wild-type L *.erecta* 6-leaf (CL)	11.	Wild-type Pitztal 12-leaf (LD to SD)
2.	Pitztal X L *.erecta* F1 6-leaf (CL)	12.	Wild-type Pitztal 14-leaf (LD to SD)
3.	Wild-type Columbia 6-leaf	13.	Wild-type Pitztal 10-12-leaf (SD to LD)
4.	Pitztal X Columbia F1 6-leaf (CL)	14.	Wild-type Pitztal 12-14-leaf (SD to LD))
5.	Wild-type Pitztal 4-leaf (LD)	15.	Wild-type Pitztal 2-leaf (SD)
6.	Wild-type Pitztal 6-leaf (LD)	16.	Wild-type Pitztal 6-leaf (SD)
7.	Wild-type Pitztal 12-leaf (LD	17.	Wild-type Pitztal 8-10-leaf (SD)
8.	Wild-type Pitztal 14-16-leaf (LD)	18.	Wild-type Pitztal 12-leaf (SD)
9.	Wild-type Pitztal 18-20-leaf (LD	19.	Mutant line 300 4-leaf (CL)
10.	Wild-type Pitztal 8-leaf (LD to SD)		

* The light conditions in which the plants were grown is detailed in brackets. SD=Short days, LD= Long days and CL= Continuous
light. All plants were harvested at 12 noon for RNA extractions.

Figure 6.10

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert

sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from

Arabidopsis (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample #	RNA*
1.	300 X Sf-2,# 6 4 leaf (8)	10.	300 X Sf-2,# 32, 4-leaf (96)
2.	300 X Sf-2,# 41 4 leaf (42)	11.	Mutant line 300 4-leaf
3.	300 X Sf-2,# 40A 4 leaf (6)	12.	Mutant line 300 6-leaf
4.	300 X Sf-2,# 49A 4 leaf (6)	13.	Wild-type Pitztal 4-leaf
5.	300 X Sf-2,# 20A 4 leaf (6)	14.	Wild-type Pitztal 6-leaf
6.	300 X Sf-2,# 9 4 leaf (98)	15.	Wild-type San Feliu-2 4-leaf
7.	300 X Sf-2,# 42 4 leaf (156)	16.	Wild-type San Feliu-2 6-leaf
8.	300 X Sf-2,# 49 4 leaf (150)	17.	Wild-type San Feliu-2 flowering
9.	300 X Sf-2,# 33 4 leaf (70)		

* All plants were grown in continuous light and plants were harvested at 12 noon for RNA extractions. Plant number refers to
the F2 plant from which these F3 plants were derived. The number in brackets is the rosette leaf number at the time of
flowering of the F2 parental plants. Sf-2 =San-Feliu-2

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Figure 6.11

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert
sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from
Arabidopsis (**C**) in order to quantify the relative mRNA levels (**D**).

Sample	RNA*	Sample	RNA*
1.	Mutant line 300 6-leaf	9.	300 X Pi, # 23 4-leaf (9)
2.	Wild-type Pitztal 6-leaf	10.	300 X Pi, # 11 4-leaf (15)
3.	300 X Pitztal 6-leaf, F1	11.	300 X Pi, # 14 4-leaf (50)
4.	Wild-type Columbia 6-leaf	12.	300 X Pi, # 4 4-leaf (66)
5.	300 X Pi, # 8 4 leaf (7)	13.	300 X Pi, # 5 4-leaf (70)
6.	300 X Pi, # 9 4 leaf (6)	14.	300 X Pi, # 13 4-leaf (88)
7.	300 X Pi, # 19 4 leaf (8)	15.	300 X Pi, # 22 4-leaf (48)
8.	300 X Pi, # 15 4 leaf (10)		

* All plants were grown in continuous light and harvested at 12 noon for RNA extractions. Plant number refers to the F2 plant from
which these F3 plants were derived. The number in brackets is the rosette leaf number at flowering of the F2 parental plants. Pi =
Pitztal.
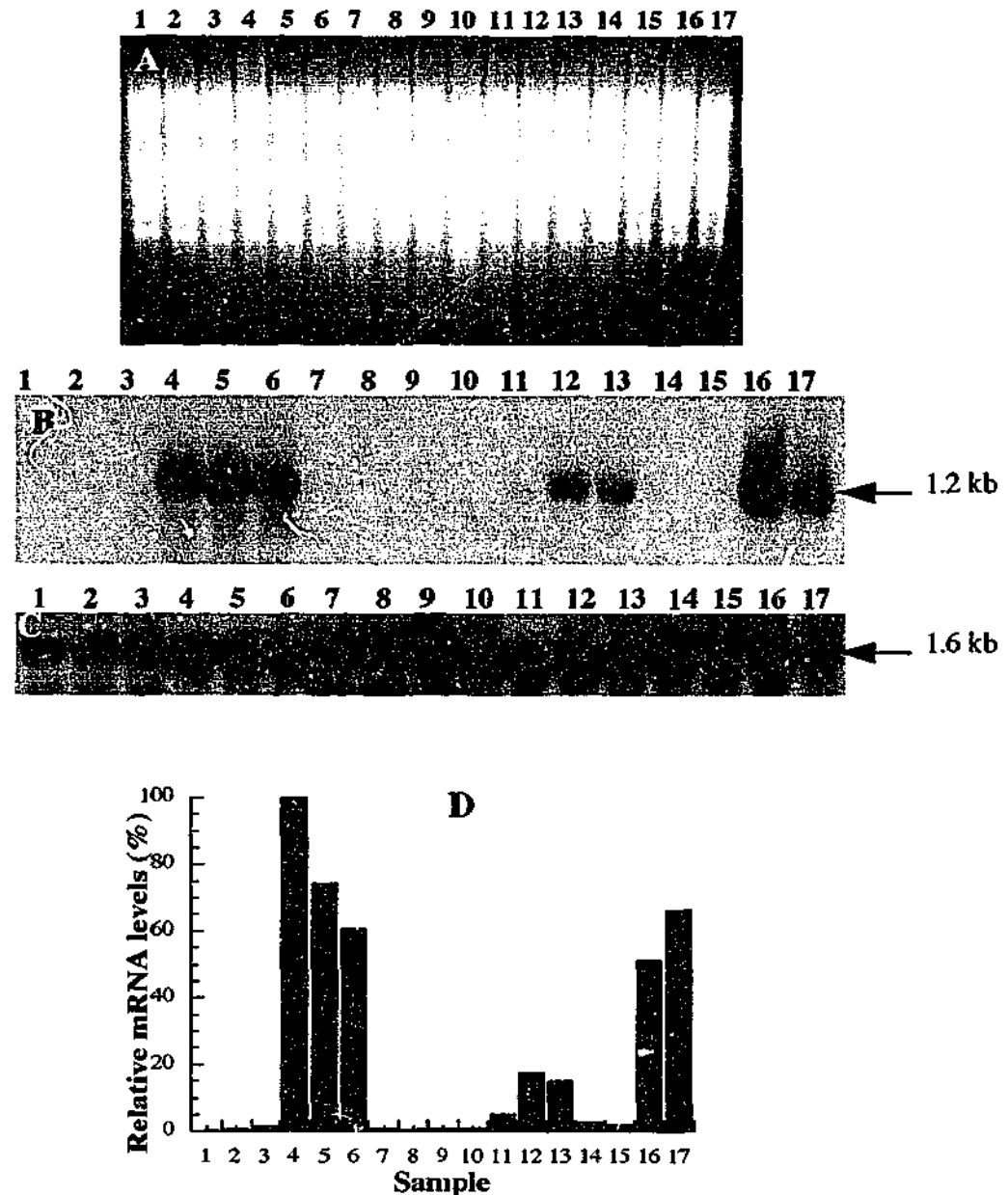
Figure 6.12

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the insert

sequence of clone 33-4 (**B**). This filter was then hybridised with the *ubiquitin* gene from

Arabidopsis (**C**) in order to quantify the relative mRNA levels (**D**).

Sample#	RNA*	Sample#	RNA*
1.	Vernalised mutant line 300 4-leaf (CL)	10.	Mutant line 300 12-14-leaf (SD)
2.	Vernalised mutant line 300, 6-leaf (CL)	11.	Wild-type Pitztal 2-leaf (SD)
3.	Vernalised mutant line 300, flowering (CL)	12.	Wild-type Pitztal 6-leaf (SD)
4.	Vernalised wild-type Pitztal, 4-leaf (CL)	13.	Wild-type Pitztal 8-10-leaf (SD)
5.	Vernalised wild-type Pitztal, 6-leaf (CL)	14.	Wild-type Pitztal,12-14-leaf (SD)
6.	Vernalised wild-type Pitztal, flowering (CL)	15.	Mutant line 300 6-leaf(LD)
7.	Mutant line 300 4-leaf (SD)	16.	Wild-type Pitztal 6-leaf (LD)
8.	Mutant line 300 6-leaf (SD)	17.	Mutant line 300 X Pi 6-leaf, F1
9.	Mutant line 300 8-10-leaf (SD)		

* The light conditions in which the plants were grown are detailed in brackets, SD=Short days, LD= Long days, and CL= Continuous
light. All plants were harvested at 12 noon for RNA extractions.

Expression of the FLC gene in the mutant lines and wild-type plants

The expression profile of the flowering-time gene *FLC* in the early-flowering mutant lines and wild-type Pitztal plants was also examined. As is the case for the *FRP33-4* gene there are low levels of expression of the *FLC* gene in all of the *fler* mutant lines and strong expression of *FLC* is detected in wild-type Pitztal plants throughout development (Figures 6.13 and 6.14). The levels of *FLC* transcripts, particularly in mutant lines 260 and 290 however, are at detectable levels, whereas it was difficult to detect any appreciable levels of *FRP33-4* expression in any of the mutant lines. As previously observed very low expression of the *FLC* gene was noted in the early-flowering Columbia and L. *erecta* ecotypes (Michaels and Amasino, 1999; Sheldon *et al.*, 1999) (Figure 6.13).

In contrast to the observations for the *FRP33-4* gene, the expression of the *FLC* gene decreases in Pitztal plants given a three week vernalisation treatment, as has also been previously reported (Sheldon *et al.*, 1999) (Figure 6.14). In addition, unlike the *FRP33-4* gene, little induction of the *FLC* transcript was noted in Pitztal plants transferred from short to long day conditions (lanes 9, 10,11 and 12 in Figure 6.15). interestingly there does appear to be slightly stronger expression of this gene in Pitztal plants grown in long days compared to short days at early developmental stages (lanes 13 and 14 in Figure 6.15). However, little difference in the transcript levels of *FLC* was noted in Columbia plants grown in short or long day conditions (Figure 6.15).

Similarly to the *FRP33-4* expression profile, the lower expression of the *FLC* gene in the mutant lines also appears to correlate with the early-flowering phenotype of the F3 progeny lines from a cross of mutant line 300 with the late-flowering San-Feliu-2 ecotype (Figure 6.16). However in most cases the level of *FLC* expression appears to correlate more closely than that of *FRP33-4* with the extent of the delay in flowering, as a lower level of expression of the *FLC* gene was detected in F3 progeny lines created from an F2 plant with an intermediate number of leaves (lane 2) compared to those F3 lines descending from late flowering parents (lanes 6, 7, 8, 9, 10). This result is expected as the *FLC* gene product is predicted to exert its effects in a dose dependant manner (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The correlation between reduced expression of the *FLC* gene and earlier flowering in the mutant plants therefore indicates that the mutation in the *FLER* gene that results in low *FRP33-4* expression may also be responsible for the altered *FLC* expression in these lines. The different response to vernalisation and light conditions of the *FRP33-4* and *FLC* genes however suggests that these genes may act in two separate flowering pathways downstream of such a gene.
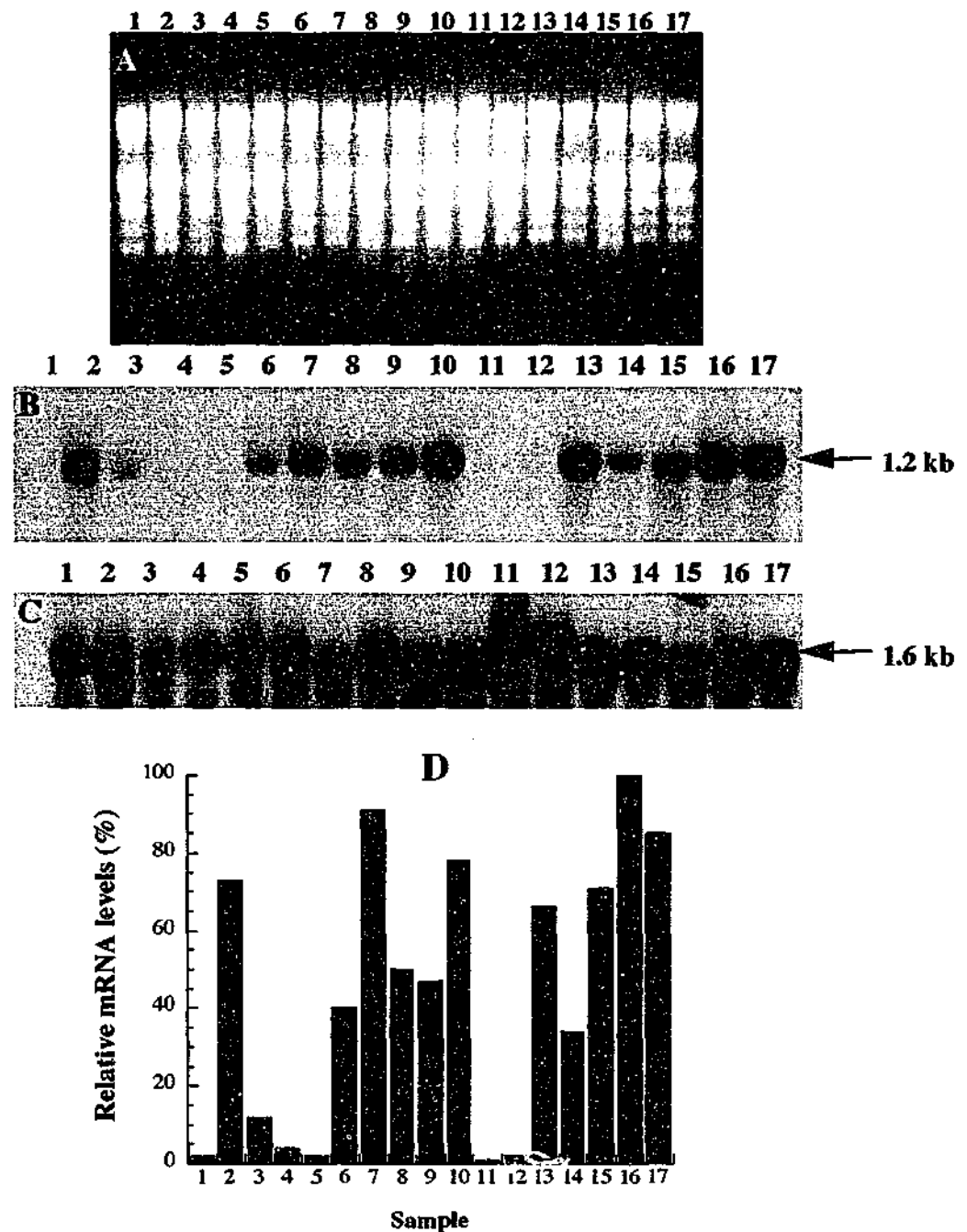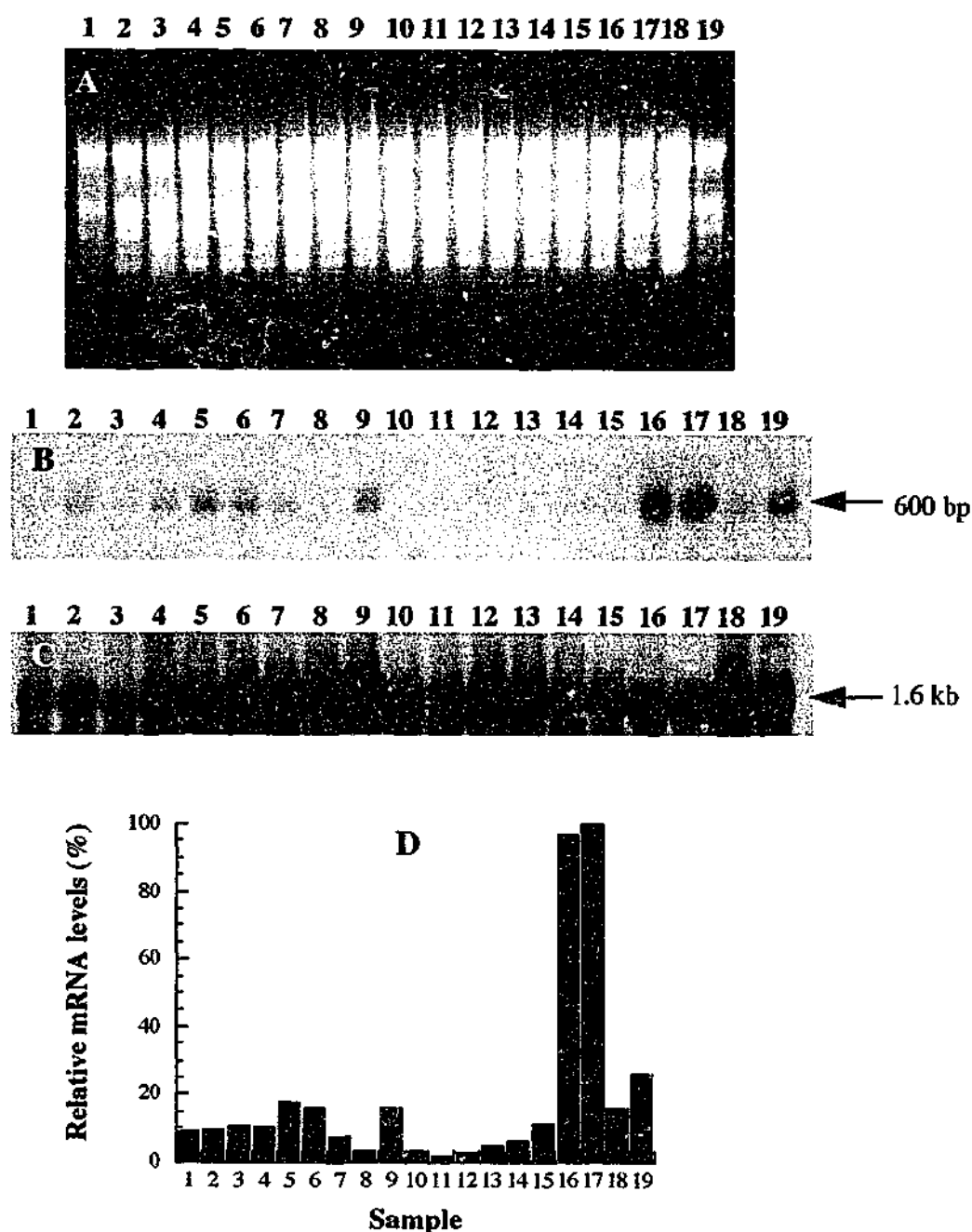
Figure 6.13

The ethidium bromide stained gel (A) was blotted and the filter hybridised with the *FLC* cDNA sequence (B). This filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1.	Mutant line 220 4-leaf	11.	Wild-type L .*erecta* 6-leaf
2.	Mutant line 220 6-leaf	12.	Wild-type L .*erecta* flowering
3.	Mutant line 230 4-leaf	13.	Wild-type Columbia 4-leaf
4.	Mutant line 230 6-leaf	14.	Wild-type Columbia 6-leaf
5.	Mutant line 240 4-leaf	15.	Wild-type Columbia flowering
6.	Mutant line 240 6-leaf	16.	Wild-type Pitztal 4-leaf
7.	Mutant line 250 4-leaf	17.	Wild-type Pitztal 6-leaf
8.	Mutant line 250 6-leaf	18.	Mutant line 300 4-leaf
9.	Mutant line 260 4-leaf	19.	Mutant line 260 6-leaf
10.	Wild-type L. *erecta* 4-leaf		

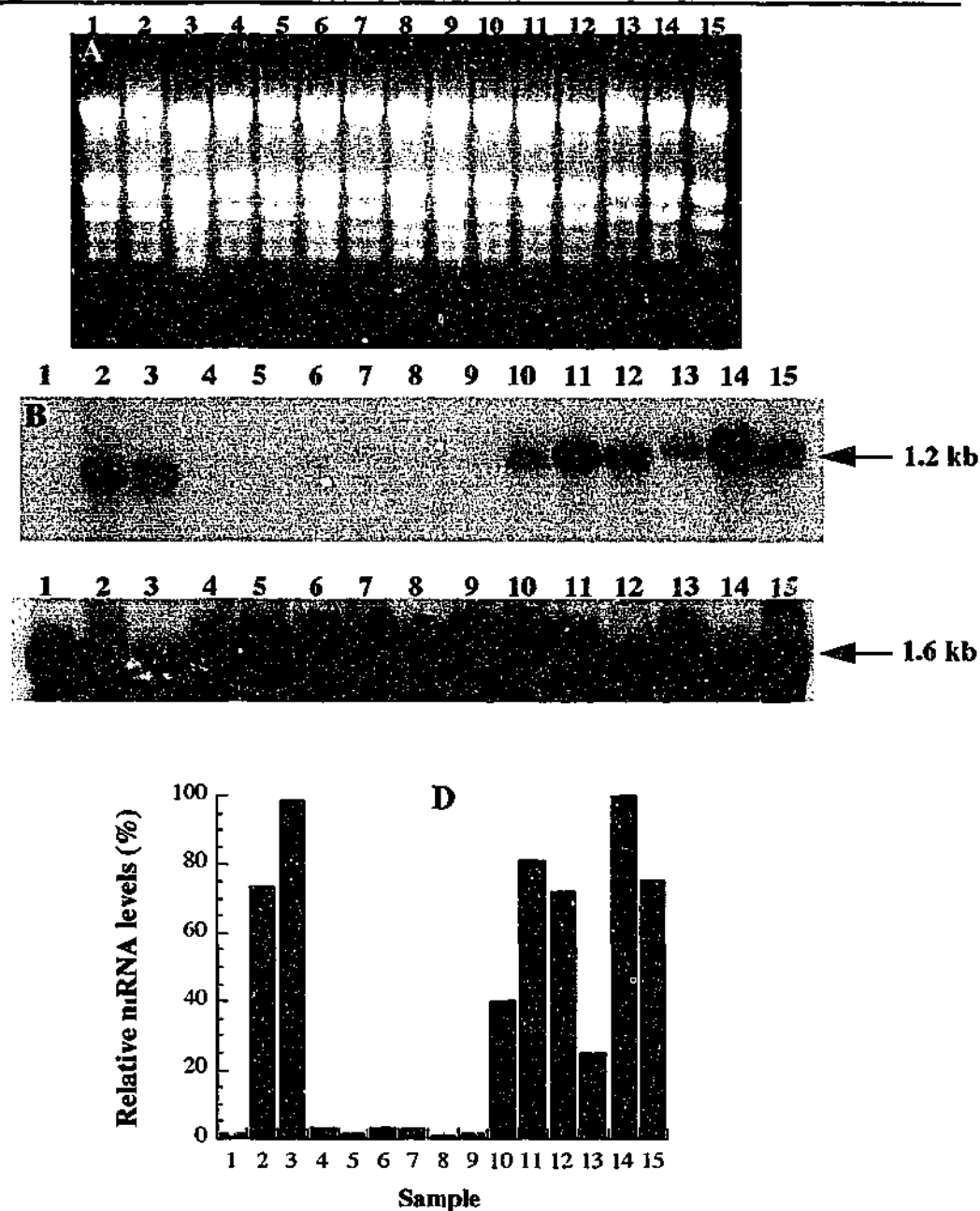* All plants were grown in continuous light and harvested at 12 noon for RNA extraction .

Figure 6.14

The ethidium bromide stained gel (A) was blotted and the filter hybridised with the *FLC* cDNA sequence (B). This filter was then hybridised with the *ubiquitin* gene from*Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1.	Mutant line 270 4-leaf	11.	Wild-type Pitztal 4-leaf
2.	Mutant line 270 6-leaf	12.	Wild-type Pitztal 6-leaf
3.	Mutant line 280 4-leaf	13.	Wild-type Pitztal 12-leaf
4.	Mutant line 280 6-leaf	14.	Wild-type Pitztal 18-20-leaf
5.	Mutant line 290 4-leaf	15.	Wild-type Pitztal 30-40-leaf
6.	Mutant line 290 6-leaf	16.	Wild-type Pitztal flowering
7.	Mutant line 300 4-leaf	17.	Vernalised wild-type Pitztal 4-leaf
8.	Mutant line 300 6-leaf	18.	Vernalised wild-type Pitztal 6-leaf
9.	Wild-type San-Feliu-2 4-leaf	19.	Vernalised wild-type Pitztal flowering
10.	Wild-type San-Feliu-2 6-leaf		

* All plants were grown in continuous light and harvested at 12 noon for RNA extraction.

Figure 6.15

The ethidium bromide stained gel (**A**) was blotted and the filter hybridised with the *FLC* cDNA sequence (**B**). This filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (**C**) in order to quantify the relative mRNA levels (**D**).

Sample #	RNA*	Sample	RNA*
1.	Wild-type Columbia 2-leaf (SD)	10.	Wild-type Pitztal 12-14-leaf (SD)
2.	Wild-type Columbia 6-leaf (SD)	11.	Wild-type Pitztal 10-12-leaf (SDto LD)
3.	Wild-type Columbia 10-12-leaf (SD)	12.	Wild-type Pitztal 14-16-leaf (SD to LD)
4.	Wild-type Columbia 4-leaf (LD)	13.	Wild-type Pitztal 4-leaf(LD)
5.	Wild-type Columbia 6-leaf (LD)	14.	Wild-type Pitztal 6-leaf (LD)
6.	Wild-type Columbia flowering (LD)	15.	Wild-type Pitztal 12-leaf(LD)
7.	Wild-type Pitztal 2-leaf (SD)	16.	Wild-type Pitztal 18-20-leaf (LD)
8.	Wild-type Pitztal 6-leaf (SD)	17.	Mutant line 300 4-leaf
9.	Wild-type Pitztal 8-10-leaf (SD)		

* The light conditions in which the plants were grown are detailed in brackets; SD= Short days and LD=Long days. All plants were harvested at 12 noon for RNA extractions.

Figure 6.16

The ethidium bromide stained gel (A) was blotted and the filter hybridised with the *FLC* cDNA sequence (B). This filter was then hybridised with the *ubiquitin* gene from *Arabidopsis* (C) in order to quantify the relative mRNA levels (D).

Sample #	RNA*	Sample #	RNA*
1.	300 X Sf-2,# 6 4 leaf (8)	10.	300 X Sf-2,# 32, 4-leaf (96)
2.	300 X Sf-2,# 41 4 leaf (42)	11.	Mutant line 300 4-leaf
3.	300 X Sf-2,# 40A 4 leaf (6)	12.	Mutant line 300 6-leaf
4.	300 X Sf-2,# 49A 4 leaf (6)	13.	Wild-type Pitztal 4-leaf
5.	300 X Sf-2,# 20A 4 leaf (6)	14.	Wild-type Pitztal 6-leaf
6.	300 X Sf-2,# 9 4 leaf (98)	15.	Wild-type San Feliu-2 4-leaf
7.	300 X Sf-2,# 42 4 leaf (156)	16.	Wild-type San Feliu-2 6-leaf
8.	300 X Sf-2,# 49 4 leaf (150)	17.	Wild-type San Feliu-2 flowering
9.	300 X Sf-2,# 33 4 leaf (70)		

* All plants were grown in continuous light and plants were harvested at 12 noon for RNA extractions. Plant number refers to the F2 plant from which these F3 plants were derived. The number in brackets is the rosette leaf number at the time of flowering of the F2 parental plants. Sf-2 =San-Feliu-2

6.4 Discussion

An analysis of the 5' regulatory region of the *FRP33-4* gene in the Columbia ecotype was conducted to obtain information regarding the types of mechanisms which may be regulating this gene. Such analysis has revealed the presence of numerous sequences within this 5' region that are similar to those believed to be associated with the light regulated induction of several genes (Ou-Lee *et al.*, 1988; Gilmartin *et al.*, 1990; Terzaghi and Cashmore, 1995; Piechulla *et al.*, 1998; Le Gourrierec *et al.*, 1999). This observation, as well as the fact that the transcript levels of the *FRP33-4* gene in the Pitztal ecotype are elevated by exposure of the plant to long day conditions and repressed by short day conditions, suggest that the expression of the *FRP33-4* gene is regulated by light. However it is yet to be determined if the differences in *FRP33-4* expression between the Pitztal and Columbia ecotypes is the result of differences in the 5' regulatory regions of *FRP33-4* or in the activity of regulatory DNA-binding transcription factors present in each ecotype. A direct comparison of the 5' regulatory regions of the Pitztal and Columbia genes following the isolation of the genomic sequence from the Pitztal ecotype is required and may provide some indication whether any of the proposed regulatory mechanisms suggested by the examination of the Columbia 5' regulatory region (discussed below) are worthy of further investigation.

A putative low temperature response element similar to those found in cold-regulated genes of *Arabidopsis* (Baker *et al.*, 1994; Jiang and Singh, 1996) was also detected in the promoter region of the Columbia version of the *FRP33-4* gene. This element in the *FRP33-4* 5' regulatory region may be associated with the dramatic induction of the transcript levels observed in Pitztal plants that had been vernalised for three weeks at the germination stage. The maintenance of the high levels of transcription of *FRP33-4* throughout the development of the vernalised plants also suggests that the cold treatment may cause a prolonged alteration in the regulation of expression of this gene in such plants. It is however still unclear as to whether the induction of the *FRP33-4* gene by vernalisation of seedlings influences the early flowering phenotype of the resulting plants as elevated levels of *FRP33-4* correlate with later flowering times in unvernalised plants. In addition, it remains to be determined if this vernalisation response operates in the Columbia ecotype as the expression of the *FRP33-4* gene was not examined in vernalised early-flowering ecotype plants.

Another element of interest found in the Columbia *FRP33-4* gene promotory region is that showing homology to an element that is responsible for the regulation of a chloroplast ribosomal protein (r-protein) gene. This element is thought to be involved in down-regulating the expression of this nuclear gene in response to chloroplast-nucleus signals, possibly via an interaction with a chloroplast signal factor. The synchronised expression of several other nuclear and plastid encoded genes are also thought to be regulated by a putative

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plastid factor that allows the nuclear genes to respond to the functional state of the chloroplast (Lopez-Juez *et al.*, 1998). The *cue* (CAB protein underexpressed) mutants of *Arabidopsis* for example, are believed to be disrupted in the coordinated regulation of nuclear encoded proteins by the plastid factor and hence show altered expression of CAB genes (Lopez-Juez *et al.*, 1998). The presence of an element similar to that involved in the regulation of the expression of a nuclear gene by a plastid factor indicates that the *FRP33-4* gene may be regulated by a system associated with coordinating expression of nuclear and plastid encoded genes. This suggests it may be worthwhile investigating whether the lack of transcription of the *FRP33-4* gene in the mutant lines is linked to a disruption in the nuclear-chloroplast signalling process similar to that seen in the cue mutants. In this context it is interesting that several other transcripts, such as those coding for *CAB* or chloroplast ribosomal proteins, that are also thought to be involved in phytochrome and plastid mediated signalling processes, were enriched during the cDNA subtraction procedure.

The promoter region of the Columbia version of the *FRP33-4* gene also contains an element similar to that which binds the circadian clock associated-1 (CCA-1) protein and another element conferring circadian mRNA oscillations in *CAB* genes (Wang *et al.*, 1997; Piechulla *et al.*, 1998). The *CCA-1* gene codes for a myb-related transcription factor that is associated with phytochrome and circadian rhythm regulation of genes, perhaps at the point of integration of the circadian clock and phytochrome signalling pathways (Green and Tobin, 1999). In addition, the expression of the *CCA-1* gene is itself transiently induced by phytochrome and oscillates with a circadian rhythm. It has also been demonstrated that constitutive expression of the *CCA-1* gene in transgenic plants abolishes circadian oscillations of several genes, including CAB genes, and results in delayed flowering of plants. These results suggested that the CCA-1 protein may be involved in part of a feedback loop in *Arabidopsis* that is closely associated with the circadian clock (Wang *et al.*, 1997; Wang and Tobin, 1998). Interestingly, disruptions in the *LHY* gene, the product of which shares 60% similarity to the CCA-1 protein at the amino acid level, causes flowering to occur independently of photoperiod, again indicating that disruption in circadian rhythm dependant genes can significantly disrupt flowering responses (Wang *et al.*, 1997; Green and Tobin, 1999). The presence of a putative binding site for the CCA-1 protein in the Columbia version of the *FRP33-4* gene's promoter suggests that expression of this gene may be regulated by phytochrome and circadian rhythms. Furthermore, the decreased expression of the *FRP33-4* gene in the mutant lines may indicate a disruption in CCA-1 regulation in these plants. The suggestion from these analyses that the *fler* lines do contain a disruption in a phytochrome signal transduction pathway and its interaction with circadian rhythms, may also help to explain the isolation of several other transcripts whose expression is influenced by these processes during the cDNA subtraction experiment. However experiments to determine if *FRP33-4* is under circadian rhythm regulation in the Pitztal ecotype are yet to be conducted.

The role of GA in controlling the flowering time of plants has been well documented and it has been suggested that GAs may help to mediate floral evocation via their activation of the *LEAFY* promoter, particularly in short days (Blazquez *et al.*, 1998). Furthermore, vernalisation treatments have been proposed to promote flowering time by the demethylation of genes involved with GA biosynthesis. It also been proposed by Sheldon *et al.* 1999 that the *FLC* gene may mediate its inhibition of flowering by either directly or indirectly decreasing the levels of GA in the apex of the plant, that may in turn decrease the activity of genes such as *LEAFY* that are involved in floral development. Interestingly the *FRP33-4* gene has some similarities to GA-2-oxidase and GA-20-oxidase genes from several different species that are involved in both the regulation and biosynthesis of bioactive GA's. The GA-2-oxidases metabolise the ent-kaurene synthetase gene product which catalyses the first committed step of the GA biosynthetic pathway (Phillips *et al.*, 1995). The transcription of these GA-2-oxidase genes increased when GA was given exogenously to the *ga1-2* mutants supporting their role in GA metabolism (Thomas *et al.*, 1999). The GA-20-oxidase genes are believed to be involved in the synthesis of bioactive GAs by catalysing successive oxidation steps in the GA biosynthesis pathway (Phillips *et al.*, 1995). In addition, as the transcript levels of all three GA-20-oxidase genes are decreased in *ga1-2* mutants fed with exogenous GA_3, it is believed that GA regulation may in part be controlled through the down regulation of the GA-20-oxidase genes in a feed-back control mechanism. Furthermore, the activity of GA-20-oxidases has been shown to increase in long days, and the flowering response of spinach plants in long days is thought to be associated with the elevated levels of these enzymes (Phillips *et al.*, 1995; Wu *et al.*, 1996). It is also likely that several GA biosynthesis regulatory steps not regulated by GA-20-oxidases exist within *Arabidopsis* plants to affect the flowering time and other developmental processes. This is suggested by the observations that *ga4* and *ga5* mutants of *Arabidopsis* that contain mutations in the GA biosynthetic enzymes 3β-hydroxylase and GA-20 oxidase respectively, still contain some bioactive GAs that support bolting of the plants (Coles *et al.*, 1999).

While the Columbia version of the FRP33-4 protein does share some conserved amino acids, common to GA-20-oxidases, that are responsible for the binding of Fe^+ ion by these enzymes (Xu *et al.*, 1995), it lacks some regions that are strongly conserved amongst these peptides, and shows only limited homology to the N-terminus of these proteins (Garcia-Martinez *et al.*, 1997). It is therefore unlikely that the FRP33-4 protein interacts with the same compounds as the GA-20-oxidase genes, or has a role that is completely similar to that predicted for the GA-20-oxidases isolated thus-far. It is however plausible that the *FRP33-4* gene may be involved in some GA-mediated processes, as the *fler* mutation which appears to affect *FRP33-4* expression, also results in plants showing some similarities to transgenic plants with altered expression of GA-20 oxidases which are affected in hypocotyl elongation and epinasty, chlorophyll content and flowering time (Coles *et al.*, 1999).

The role of *FRP33-4* in GA-mediated processes is further supported by the presence of two pyrimidine boxes in the 5' regulatory region of *FRP33-4*, similar to those known to be associated with the transcriptional activity of GA-regulated genes (Ou-Lee *et al.* 1988). It has been postulated that interactions of a GA inducible protein factor with these pyrimidine boxes may be important in the down regulation of GA regulated genes in a feedback mechanism (Huang *et al.*, 1990).

Recent studies have shown that GA stimulates various developmental growth responses by antagonising the negative regulators of GA-mediated processes, GAI (<u>GA</u> INSENSITIVE) and *RGA* (<u>R</u>EPRESSOR OF GA1-3). The *SPY* gene is also thought to have a similar role to *GAI* and *RGA* in inhibiting GA mediated process, although it is believed *GAI* and *RGA* act upstream of *SPY* in a GA signalling pathway (Harberd *et al.*, 1998). The presence of a binding site for GA induced protein factors in the promoter region of the Columbia *FRP33-4* gene could indicate a role of this gene with similarities to that of the *GAI* and *RGA* genes. In support of this, an increase in the expression of *FRP33-4* does correlates with delayed flowering. However, if this gene did have a direct role in repressing the GA-mediated acceleration of flowering it would be expected that floral inductive conditions, such as vernalisation and long days should reduce rather than increase levels of *FRP33-4* expression.

If the *fler* mutation is affecting flowering time via GA responses it could be expected that it may influence *FLC* activity as the late-flowering phenotype of *FLC* overexpression mutants can be partially overcome by exogenous GA application (Sheldon *et al.*, 1999). However it should be noted that GA does not affect *FLC* transcript levels and is thought to act downstream of *FLC* to promote flowering (Sheldon *et al.*, 1999). The expression of the *FLC* transcripts within the early-flowering mutant lines was examined and low levels of the *FLC* transcripts were observed in the mutant lines suggesting that the *fler* mutation occurs upstream of *FLC*. It is interesting to note that the Columbia *FRP33-4* gene contains a potential binding site for the *AGL1* MADS box protein in the promoter region, and that the *FLC* gene contains a MADS domain that is similar in the M-I-K region to the *AGL14* gene (Sheldon *et al.*, 1999). However at this stage neither the Pitztal FRP33-4 5' regulatory region nor the consensus sequence of the DNA binding site of *FLC* is known, and it is therefore unclear as to whether *FLC* may interact with this *AGL1* binding site in the *FRP33-4* gene. While the low *FLC* transcript levels within the mutants suggests a disruption in the *FLC*-mediated floral repression pathway, the *FRP33-4* gene may not be regulated directly by the *FLC* gene in such a process, as conditions that induced transcription of the *FRP33-4* gene such as longer photoperiods or vernalisation, either did not dramatically affect *FLC* levels or actually decreased its transcription.

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Genetic analysis has suggested that the major difference in the flowering time of late and early-flowering ecotype is due to the activity of the *FRI* and *FLC* genes that act synergistically to repress flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999), probably via blocking the activity of some floral promotory substance. As *FLC* gene expression is often lower in conditions that promote flowering, such as after a vernalisation treatment, and it levels are lowest in the apex of the plants after the transition to flowering, it is now believed that it is the quantitative levels of the *FLC* gene that act to control the lateness to flower in the late-flowering ecotype (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The *FRI* gene is believed to be in control of promoting the activity of the *FLC* gene. (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). From previous genetic analysis it is thought that the *FLER* gene may be upstream of the flowering *FRI* /*FLC* repression pathway, however we have no direct evidence to indicate whether the *fler* mutation acts directly on *FRI* or *FLC* or indirectly via another pathway. While the potential role that *FRP33-4* may have in floral development is not yet clear, the linkage of the early-flowering phenotype of the Pitztal mutants with a lack of expression of this gene suggests that if *FRP33-4* does have a role it would be likely to act in the inhibition of floral induction. Pending the results from overexpression and antisense studies we have proposed a model (Figure 6.17 a, b, c, d and e) which hypothesises that *FRP33-4* acts downstream of the *FLER* mutation which causes early-flowering. In this model, it is proposed that *FLC* activity is affected indirectly by the *fler* mutation causing a removal of inhibition of the autonomous flowering pathway.

In the current model of flowering control it is thought that the activity of a GA-mediated promotion of flowering within the plant apex is one of the most important factors controlling the switch for the plant to start flowering. It is also proposed that multiple floral repression and promotory pathways interact to balance the GA levels and the subsequent physiological consequences of GA action within the apex (Sheldon *et al.*, 1999, Michaels and Amasino, 1999). Sheldon *et al.* (1999) and Michaels and Amasino, (1999) have also proposed that the genes involved in the autonomous flowering pathway may act to repress *FLC* levels, and thus indirectly increase GA levels, as *FLC* is thought to delay flowering by reducing the levels of active GAs present in the apex. It has been further postulated that the major role of the *LD* and *FLD* genes is to promote flowering via their inhibition of the *FLC* gene. Autonomous flowering pathway genes may however also have other roles in promoting flowering, perhaps by directly promoting GA mediated processes. It is unclear as to whether genes involved in the long day promotory pathway are involved in modulating *FLC* levels (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

In the model presented here it is proposed that *FLER* acts via *FRP33-4* to delay flowering by an inhibition of the activity of genes involved in an autonomous floral promotory pathway

(Figure 6.17 a, b, c, d and e) and thereby indirectly promoting FLC activity and decreasing GA levels. It is unclear as to whether this repression of the autonomous pathway is mediated by an enhancement of EMF activity which is also thought to antagonise the effects of floral promotory pathways (Yang *et al.*, 1995a). The removal of the *FRP33-4*-mediated inhibition of the autonomous pathway genes, which exert their effects via both a direct promotion of floral activity and by an inhibition of *FLC* activity, in the *fler* mutant lines may explain the early-flowering phenotype and low levels of *FLC* expression (Figure 6.17d).

The flowering time phenotype of late-flowering ecotypes grown in long day conditions is thought to be as a regulated by a balance between a promotion of flowering via genes in the long day floral promotory pathway, and the repression of GA levels mediated by *FLC*. As the *FRP33-4* genes expression is slightly elevated in these conditions, particularly at earlier stages of development, perhaps via light regulatory elements in its promoter region, the plant may again be reducing the activity of genes within the autonomous floral promotory pathway and relying more on genes within the long day promotory pathway to promote flowering (Figure 6.17a). Unlike vernalisation signals, or genes within the vernalisation dependant pathway, that can actively decrease *FLC* activity, it is thought that genes within the long day pathway cannot dramatically inhibit the activity of *FLC* (Sanda and Amasino, 1996b). Therefore, the model would predict that while long days can promote flowering to a certain extent in late-flowering ecotypes, the activity of *FLC* is not markedly decreased by such conditions resulting in plants that still flower quite late (Figure 6.17a). When late-flowering ecotypes are placed in short day conditions, a further short-day-induced floral repression pathway may be activated (Martinez-Zapater *et al.*, 1994), that along with the active *FLC* gene product are thought to result in plants with low levels of GA, that are extremely late-flowering (Figure 6.17b). The model suggests that short day do not induce an increase in *FLER* activity (Figure 6.17b) and results in the lower levels of *FRP33-4* expression observed in Northern blot analysis.

When late-flowering ecotypes are vernalised it is believed that a vernalisation dependant floral promotory pathway is activated that causes an increase in GA biosynthesis possibly via a change in the methylation state of the relevant genes, that in turn results in earlier flowering plants (Dennis *et al.*, 1996). Vernalisation may also act to decrease *FLC* levels directly, as observed in Northern blot analysis of vernalised Pitztal plants. This repression of an *FLC*-mediated inhibitory action on GA levels within the plants may also contribute to the very early-flowering phenotype of vernalised plants (Michaels and Amasino, 1999). The increase in transcription of the *FRP33-4* gene in these conditions, presumably by cold-regulated factors, may result in the down regulation of the autonomous floral promotory genes which the plant no longer utilise as the promotion of flowering is now occurring via the vernalisation dependant pathway (Figure 6.17c). While the model suggests that a decrease in the activity of the autonomous flowering genes via increased *FRP33-4* activity would

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potentially act to indirectly increase *FLC* levels within the plant, it is proposed that FLC levels remain low due to the inhibition of its activity by the vernalisation signal (Figure 6.17c)

In early flowering ecotypes it is believed that the low levels of an active *FLC* gene product, possibly via mutations in the *FRI* gene that normally induces the activity of the *FLC* gene, results in a lack of *FLC* mediated inhibition of the GA levels within the apex in these plants causing flowering to occur when plants are competent (Figure 6.17e). The model proposes that the *FLER* and *FRP33-4* floral regulatory pathway is non-functional in these ecotypes. The decreased expression of the *FRP33-4* gene within these ecotypes may therefore result in decreased repression of genes within the autonomous floral promotory pathway, which in turn may contribute to the decreased expression of the *FLC* transcripts observed. In long day conditions these plants can presumably use both the long day and autonomous flowering pathways to promote early flowering (Figure 6.17e). In short days, these ecotypes may flower later due to the activation of the short day repression pathway and the lack of induction of the long day dependent floral promotory pathway.

Despite the fact that *FLC* transcript levels are low in the mutant lines, and that *FRP33-4* levels were found to be low in early-flowering ecotype lines which lack *FRI* and in some cases *FLC* activity, *FRP33-4* is not thought to be directly downstream in an *FRI/FLC* mediated floral repression pathway, as the response of the *FLC* and *FRP33-4* genes at the transcriptional level to vernalisation and long day conditions is contrary. The model depicted in Figure 6.17(a-e) instead hypothesises that the *FRP33-4* gene is involved with a novel late ecotype specific floral repression process that may involve an inhibition of genes in the autonomous floral promotory pathway. Implications of this model suggest that the accelerated flowering capability of early-flowering ecotypes may result from mutations in the *FLER* gene that not only affects FLC activity but also inactivates the FRP33-4 mediated repression of the autonomous flowering pathway.
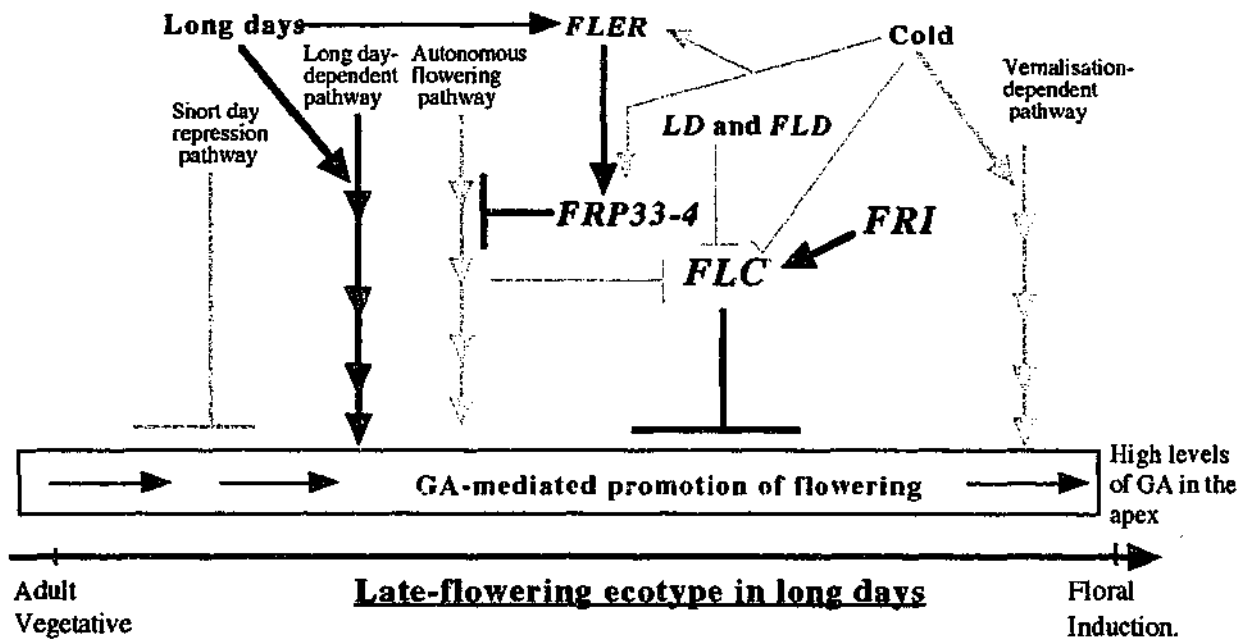
Figure 6.17a

Putative pathways conferring either a promotion or repression of flowering, by increasing or decreasing levels of a GA-mediated flowering signal, are depicted for late-flowering ecotypes grown in long days. Any process activated by long days are shown in green, and those constitutively active are shown in black, while any processes either directly or indirectly repressed,or that are inactive in these conditions, are shown in grey. Such ecotypes utilise the long day pathways to flowering as the autonomous flowering pathway is repressed by the activity of *FRP33-4*, however the plants still flower late as a repression of flowering by *FLC* is maintained in these conditions.
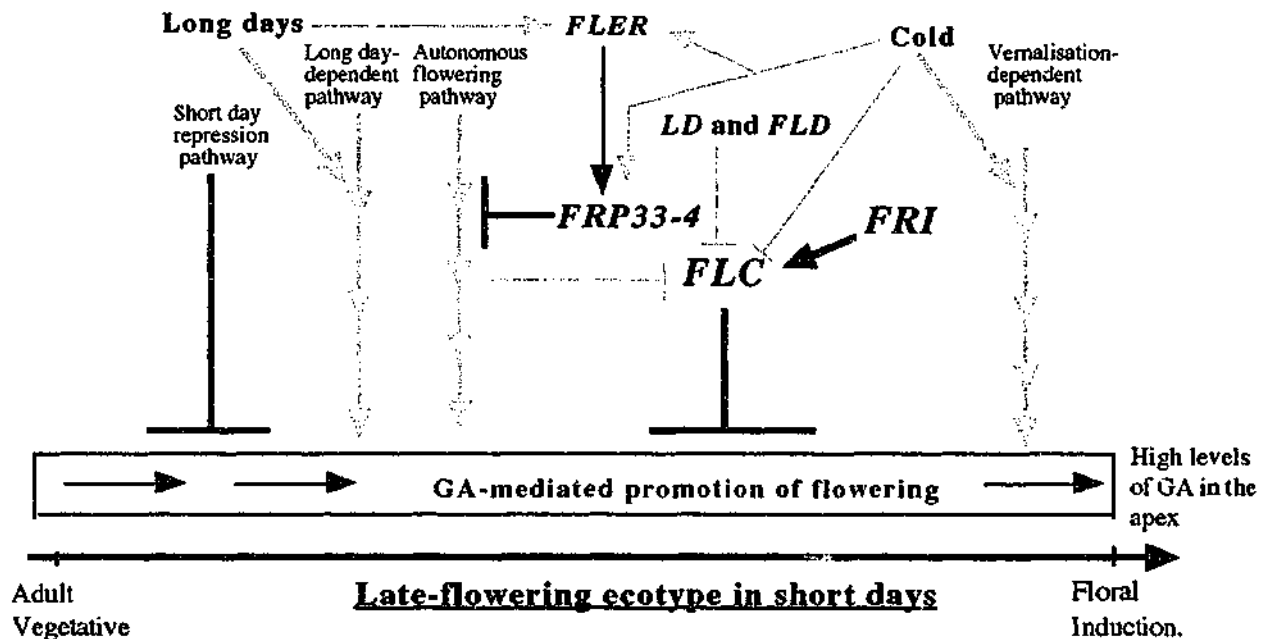
Figure 6.17b

Putative pathways conferring either a promotion or repression of flowering by increasing or decreasing levels of a GA-mediated flowering signal are depicted for late-flowering ecotypes grown in short days. Any processes activated by short days are shown in red, and those constitutively active are shown in black, while any processes either directly or indirectly repressed, or that are inactive in these conditions are shown in grey. Late-flowering ecotypes are extremely late-flowering in short days due to the activation of the short day repression pathway that may act in combination with the floral repression mediated by *FLC*.

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Late-flowering ecotype following vernalisation

Adult Vegetative — Floral Induction.
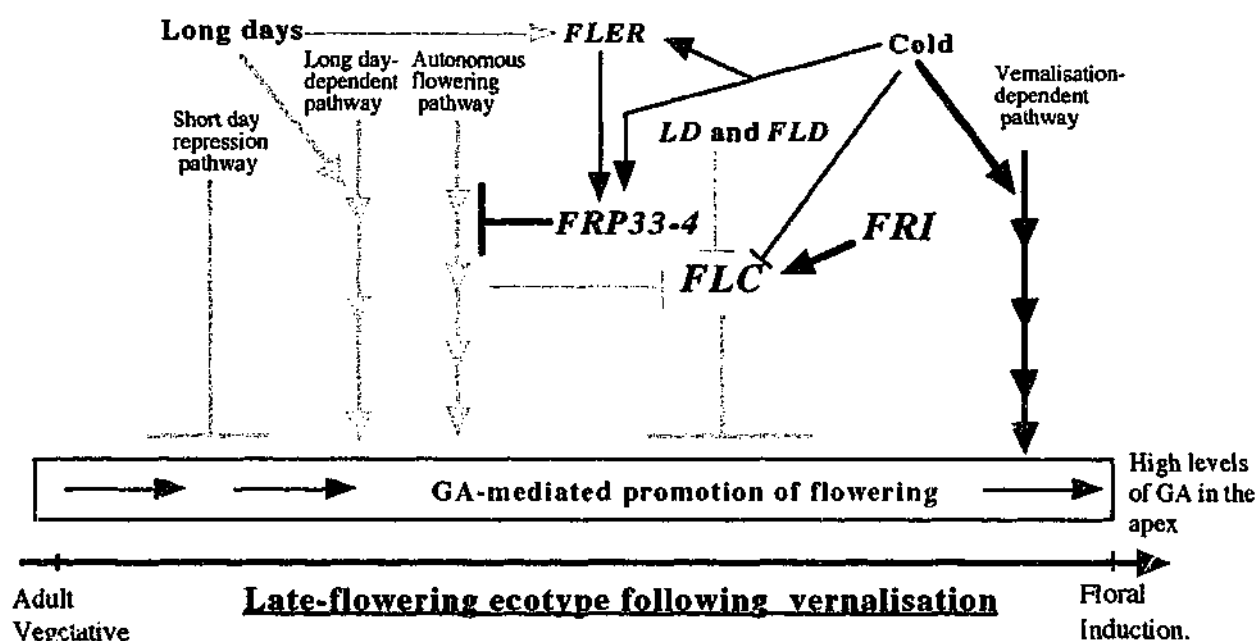
Figure 6.17c
Putative pathways conferring either a promotion or repression of flowering by increasing or dereasing levels of a GA-mediated flowering signal are depicted for vernalised late-flowering ecotype plants. Any process activated by vernalisation are shown in blue, and those constitutively active are shown in black, while any processes either directly or indirectly repressed, or that are inactive in these conditions are shown in grey. Late-flowering ecotypes may be early-flowering following vernalisation due to a repression of the autonomous pathway by the activity of *FRP33-4*, making the plant reliant on a vernalisation-dependent flowering pathway in conjunction with a repression of *FLC* activity by the cold treatment.

Early-flowering mutant lines in long days

Adult Vegetative — Floral Induction.
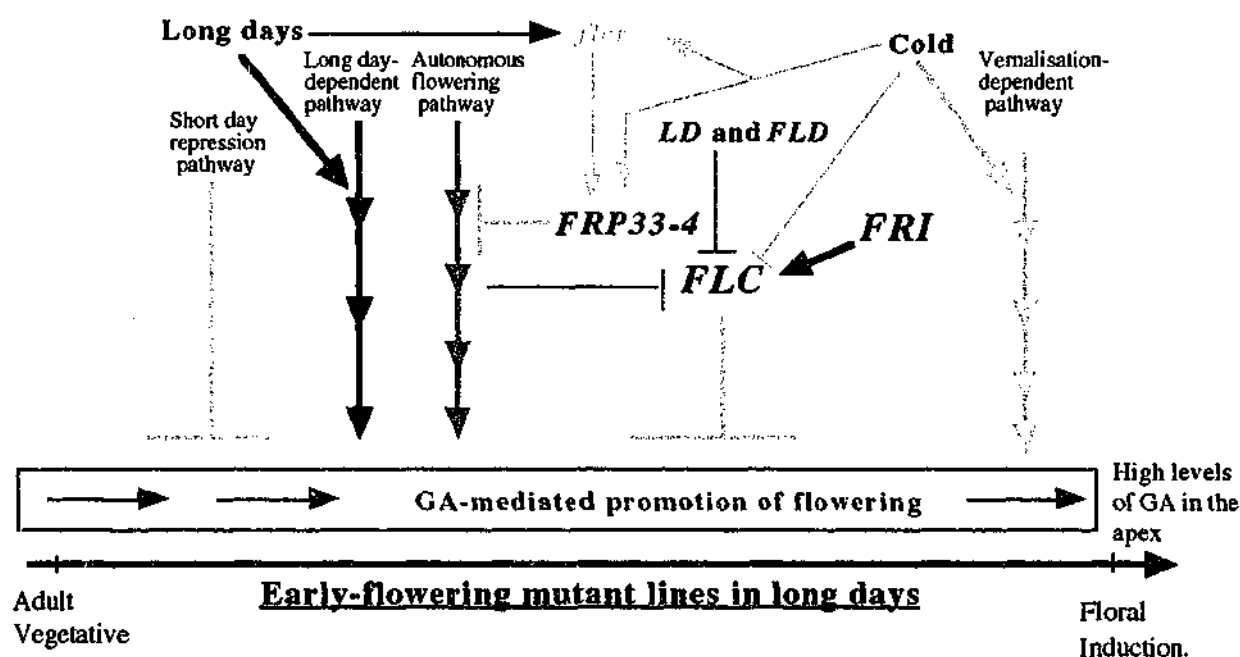
Figure 6.17d
Putative pathways conferring either a promotion or repression of flowering by increasing or decreasing the levels of a GA-mediated flowering signal are depicted for early-flowering mutant lines grown in long days. Any process activated by these mutations are shown in purple, or those constitutively active are shown in black, while any processes either directly or indirectly repressed, or are that inactive in these conditions are shown in grey. The mutant lines may flower early due to a mutation in *FLER* gene that results in a lack of activation of the *FRP33-4* gene. The lack of activity of the *FRP33-4* gene may therefore result in a decrease in the activity of *FLC*, as genes in the autonomous flowering pathway are no longer repressed.

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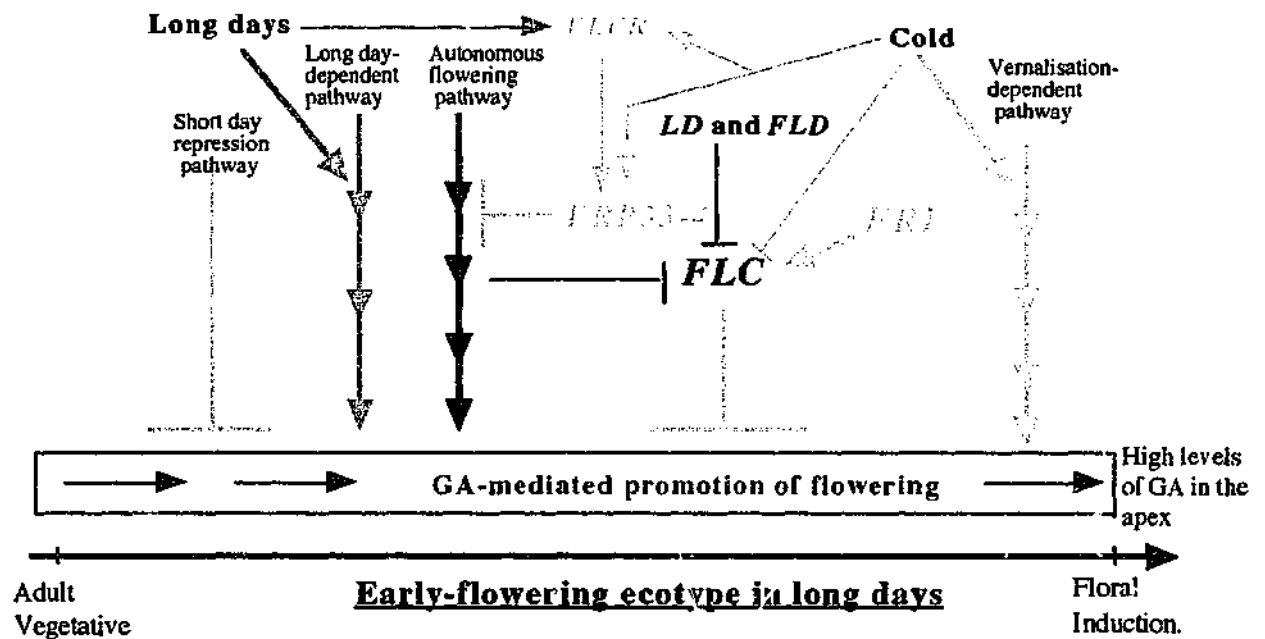
Figure 6.17e
Putative pathways conferring either a promotion or repression of flowering by increasing or decreasing the levels of a GA-mediated flowering signal are depicted for early-flowering ecotypes lines grown in long days. Any processes active in these ecotypes in long days are shown in orange, and those constitutively active are shown in black, while any processes either directly or indirectly repressed, or are that inactive in these conditions are shown in grey. Early-flowering ecotypes may flower early due to defective copies of the *FRI* gene that causes decreased activity of the *FLC* gene. Low expression of the *FRP33-4* gene is also detected in these lines and is believed to be due to the mutation in the *FLER* gene.

Chapter 7

Final Discussion

Creation of novel early-flowering mutants in the late-flowering Pitztal ecotype background was undertaken to identify, and eventually isolate, genes involved in the repression of floral induction in *Arabidopsis*. The dominant and semi-dominant, *FRI* or *FLC* genes are believed to be primarily responsible for conferring the late-flowering phenotype of Pitztal plants (Lee *et al.*, 1994). The early-flowering plants created in the Pitztal background in this study represent mutants with disruptions in a late-ecotype specific, floral repression pathway, probably associated with both the *FRI* and *FLC* genes.

Complementation tests have revealed that it is likely that all of the radiation-induced mutant lines used for the majority of this analysis are allelic, and therefore carry mutations in the same gene. These mutants are referred to as the *fler* (*f*loral *l*ate *e*cotype *r*epressor) mutant lines. The similarity in the responses of all the *fler* lines to photoperiod and vernalisation again indicates that the same mutation is present in all these lines. The fact that these lines still display a significant delay in their flowering times in response to short days, and little promotion of flowering following cold treatment, further suggests that the mutations causal to the early-flowering phenotype may disrupt a constitutive floral repression pathway or vernalisation requirement, rather than one that is dependent on the perception of photoperiod. These phenotypes are consistent with the theory that there may be an alteration in the activity of the *FRI* or *FLC* genes within the *fler* lines.

As mentioned previously the presence or absence of active alleles of the *FRI* and *FLC* genes are believed to be the major cause of the differences in floral induction times between late and early-flowering ecotypes (Burn *et al.*, 1993b; Lee *et al.*, 1994). This is borne out by the observation that introduction of an introgressed *FRI* allele in the Columbia ecotype results in late-flowering plants. L. *erecta* plants however, require both an introgressed late-flowering ecotype *FRI* allele and introgressed *FLC* alleles to display a similar late-flowering phenotype (Lee *et al.*, 1994). This indicates that these early-flowering ecotypes differ in the ability of their *FLC* alleles to respond to the *FRI* gene. More recently it has been shown that the protein product of the *FLC* gene does not differ in the Columbia and L. *erecta* ecotypes (Sheldon *et al.*, 2000). This may indicate that the differences in *FLC* activity between these ecotypes may be due to differences in the regulatory region of *FLC* (Sheldon *et al.*, 2000). Early-flowering phenotypes of the F1 progeny was observed in both the crosses of the *fler*

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mutant lines to Columbia and to L. *erecta* plants. This strongly suggests that the *fler* mutation is not in the *FLC* gene as delayed-flowering progeny would have been expected in the Columbia crosses if the F1 plants contained one copy of the dominant Piztal *FRI* allele from the mutant background, and one copy of a functional allele of the semi-dominant *FLC* locus from the Columbia parent. In addition, none of the F1 plants from backcrosses of these F1 progeny to the early-flowering ecotypes or mutant lines, showed any delay in their flowering, further supporting the above conclusion. In other crosses it has previously been demonstrated that, in the growth conditions used in this study, a single copy of both the *FRI* and *FLC* alleles is sufficient to significantly delay flowering. The F1 progeny from a cross of wild-type Pitztal with Columbia or L. *erecta* for example, all flowered very late. Further evidence to indicate *fler* is not allelic to *FLC* comes from the microsatellite marker analysis which suggested that the *FLER* gene is linked to the nga8 marker on chromosome 4, whereas the *FLC* locus maps to chromosome 5 (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

The linkage of the *fler* mutation with the region of chromosome 4 containing the *FRI* gene, along with several other observation initially indicated that the *FLER* gene may indeed be allelic to the *FRI* gene. The flowering times of the F1 progeny from crosses of the mutant lines to wild-type Pitztal and other late-flowering ecotypes for example, suggests that a dominant locus like *FRI* is disrupted in the *fler* lines. The absence of late-flowering F1 progeny from a cross of all the *fler* mutant lines with L. *erecta* plants containing an introgressed copy of a San-Feliu-2 version of the *FRI* gene however, indicates that the mutant phenotype is not complemented by the introduction of a late-flowering version of the *FRI* gene. The presence of a small proportion of later-flowering plants, in not only the F2 progeny of these crosses, but also in the F2 progeny of the control crosses of the mutant lines with L. *erecta*, further indicates that these plants are not delayed solely due to the presence of a late-flowering allele of the *FRI* gene. The presence of these later-flowering F2 plants also reinforces the proposal that the *fler* lines are not disrupted in the *FLC* locus, as no such delayed flowering plants would be expected if this was the case. Instead, delayed flowering in some of F2 plants may result from the segregation in some F2 progeny, of genes within the Pitztal background of the mutant lines and the L.*erecta* ecotype, that individually have relatively minor but cumulative effects in delaying flowering time.

The presence of 'minor effect' flowering time genes in the *fler* background which can act to delay or promote flowering is supported by several observations. The flowering time distributions of the F2 progeny from crosses of the mutant with Pitztal for example, were often skewed toward earlier flowering, and always contained some plants with intermediate flowering times. Furthermore, several intermediate flowering plants were also detected amongst the F1 progeny of the mutant lines backcrossed to either Pitztal or the parental *fler* line. In addition, several of the F1 progeny in the allelism analysis flowered at intermediate

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times suggesting that some lines may contain 'minor effect' genes that can interact to produce a small yet noticeable delay in flowering time. It should be noted that a large variation in the flowering times of wild-type Pitztal plants has been observed, indicating that the Pitztal background of the *fler* mutants is indeed quite polymorphic with respect to such 'minor effect' genes. Therefore, both the natural variation present in the Pitztal ecotype, as well as a possible additional variation due to the mutation process, are likely to be contributing to the variability in the flowering times seen in such analyses.

Alternatively, it may be suggested that the *fler* mutation is epistatic to the *FRI/FLC* repression system in the L. *erecta* (*FRI*) and L. *erecta* crosses, and that only in the absence of *fler* can the delayed-flowering phenotypes of some F2 plants be detected. This latter theory appears unlikely however, as it would mean that the *fler* mutation would be behaving differently when in early-flowering or late-flowering backgrounds, as this mutation appears to be completely recessive to the late-flowering phenotype of the Pitztal, Stockholm and San-Feliu-2 ecotypes. Transforming a late-ecotype version of the *FRI* and *FLC* genes into the mutant lines in an attempt to complement the early-flowering phenotype should determine whether the *FLER* gene is allelic to either the *FLC* or the *FRI* gene. If these experiments support the conclusion that *FLER* is not allelic to either locus, it may be interesting to then check for decreased expression, at either the RNA or protein level, of the recently cloned *FRI* gene within the *fler* lines, as well as confirm that there are no structural abnormalities in this gene between wild type Pitztal and the mutant lines.

The apparent failure to obtain *FRI* or *FLC* mutants may suggest the presence of genes within the Pitztal genome that have partially redundant functions with the *FRI* or *FLC* loci. If such genes can at least partially compensate for defects within the *FRI/FLC*-mediated floral repression system to cause delayed flowering, the selection of only the earliest flowering M2 plants following mutagenesis may have negated the possibility of detecting *fri* and/or *flc* mutants. The presence of genes with potentially similar functions to *FLC* has also been suggested following the cloning of genes such as SVP that encodes a MADS domain protein involved in floral repression (Hartmann *et al.*, 2000). The selection of only early-flowering M2 plants may also help to explain the suggested allelic nature of the mutation in all the lines. Selection of intermediate flowering M2 plants or selection under different light conditions for example, may have resulted in the isolation of other mutations that alter flowering time.

The fact that the intermediate flowering F1 plants in the allelism analysis were often not found in both reciprocal crosses, may further indicate that some 'minor effect' flowering time genes present in the *fler* lines may be inherited via the maternal genome. The presence of such maternally inherited genes able to affect flowering time is also suggested by the observations that the F1 and F2 progeny from crosses in which mutant line 300 is the female parent and wild-type Pitztal is the male, flowered, on average, earlier than the cross in which

the mutant was the male. It is not clear whether these 'minor effect' genes represent natural variation within the mutant lines or were introduced during the radiation treatment. The unusual leaf morphology of some of the *fler* mutant lines suggests some additional defects within these lines which may indirectly affect their flowering time were introduced during the mutation process. The pale green leaf colour of three of the mutant lines for example, suggests that these lines potentially contain defects in their biosynthesis of chlorophyll, a process that affects the photosynthetic output of plants and therefore the floral induction process (King and Bagnall, 1996). Such a disruption however, could be expected to delay, rather than promote floral induction in the mutant lines.

Several genes isolated following the cDNA subtraction procedure are suggestive of a defect in the normal functioning of some light-regulated photosynthetic process in line 300. These included plastid-encoded ribosomal protein genes which can be induced by phytohormones, show strong expression in the meristematic regions of the plant, and are known to be crucial for the correct functioning of the chloroplasts or mitochondria (Stafstrom and Sussex, 1992). Other transcripts isolated included those coding for chlorophyll a and b binding proteins, structural proteins of the chloroplast reaction centres, proteins involved with the electron transport chain between the two photosystems, and proteins involved with the dark reactions of photosynthesis. All of these genes have been shown to be controlled either directly or indirectly by phytochrome activity or circadian clock regulated processes (Stafstrom and Sussex, 1992; Pilgram and McClung, 1993; Hamazoto *et al.*, 1997; Fujita and Hase 1998; Nagy *et al.*, 1998). It is unclear at this stage whether the preponderance of light-regulated genes in the subtraction sample does reflect some alteration in the circadian responses and phytochrome regulation within the mutant lines, or is an artefact. Variations in the relative abundance of some transcripts in the experimental samples used in the subtraction procedure may result from differences in the rate of development between wild-type and mutant plants. Some minor variations could also be expected due to the time taken to concurrently harvest all the plant tissue at equivalent stages in the diurnal cycle.

Despite the enrichment of light-regulated sequences within the subtraction samples, Northern blot analyses did not reveal any obvious major differences in their expression pattern between mutant and wild-type Pitztal plants. It is interesting that for some of these genes however, there are some small differences in the developmental expression patterns of the various transcripts detected, between mutant and Pitztal plants. It is also interesting to note that several of the phytochrome mutants, as well as those affected in the circadian regulated processes, also show some alterations in their flowering times. *phyA* and *phyB* mutants, for example, display slightly later- or earlier-flowering respectively, independently of the photoperiod such plants are exposed to (Devlin *et al.*, 1998; Whitelam *et al.*, 1998). Furthermore, *toc1-1* and *elf3* mutants, disrupted in their circadian responses, display an almost day-length neutral flowering response (Onouchi and Coupland, 1998; Somers *et al.*,

1998). A possible explanation, for the minor, yet significant influences phytochrome-or circadian rhythm-mediated processes may have on the flowering times of the *fler* lines, may lie in the role these factors play in the proposed long day-dependent, floral induction pathway. *PHYB*, for example, may be involved with the repression of the floral promotory activity of such a pathway, as it has been suggested that the levels of protein product of the long day-induced *CONSTANS* flowering time gene may be elevated in early-flowering *phyB* mutants (Putterill *et al.*, 1995). Therefore, disruptions in phytochrome biosynthesis and/or its regulation, may cause a partial de-repression of such a pathway in the mutant lines, and may contribute to their early-flowering phenotype. At least for line 300, these alterations may also explain the pale green colouring of these plants, the slightly decreased levels of chlorophyll detected in these lines, and potentially the altered expression of many light-regulated genes involved in the photosynthetic process. If such a repression of the long day-dependent pathway via phytochrome occurs in short day conditions in wild-type plants, minor defects in this process may also help to explain the smaller delay in flowering seen in the *fler* mutants compared to wild-type plants grown in these conditions.

Disruptions in these process are not thought to be primarily responsible however, for the major effect on the flowering time of the mutant lines. It is possible that altered expression of phytochrome-and circadian rhythm-regulated genes within the *fler* lines may primarily be due to the removal of the activity of some overall floral inhibitory pathway in these mutants. The lack of activity of the *FRI/FLC* mediated repression pathway (Burn *et al.*, 1993; Lee *et al.*, 1993) in the mutant plants, may for example alter the spatial and temporal expression patterns of some light-regulated genes and mediate an accelerated flowering response.

Despite the aforementioned uncertainties as to the nature of the *fler* mutation, a gene (*FRP33-4*) displaying a profound difference in transcript levels between mutants and wild-type plants has been isolated from the cDNA subtraction procedure. It is interesting to note, that the Columbia version of this *FRP33-4* gene contains several putative light-regulatory elements within its promoter region. Whether the lack of expression of *FRP33-4* is mediated by a disruption in light-regulated processes within the mutant lines is yet to be determined. It is more likely that the radiation-induced disruption in the putative *FLER* locus, believed to be responsible for the early-flowering phenotype of the *fler* lines is causing the lack of expression of *FRP33-4* in the mutants. It is also interesting to note that if indeed the *FLER* locus, mutations in which may be epistatic to *FRI/F* activity, is influencing the expression of the *FRP33-4* gene, then the inability to detect transcripts of the *FRP33-4* gene in early-flowering ecotypes may result from the predicted lack of an *FRI/FLC*-mediated floral repression system within such plants (Lee and Amasino, 1995).

While the role of the *FRP33-4* gene in floral induction is unclear at this stage, expression profiles indicate a tight correlation between a lack of expression of the *FRP33-4* transcript and early-flowering of the mutant lines. This analysis also correlates relatively strong expression of this transcript with delayed flowering in the F2 progeny of crosses of mutant line 300 with two naturally occurring late-flowering ecotypes. In addition, the higher levels of this *FRP33-4* transcript appear to be present during the earlier development of the late-flowering Pitztal ecotype, and to some extent decrease as the plant ages, a profile consistent with a gene involved in a floral repression process. Experiments are now currently under way to more to definitively correlate a lack of expression of this transcript with early-flowering in naturally occurring early-flowering ecotypes, as well as to produce transgenic plants containing overexpression and antisense expression constructs of this gene.

The *FRP33-4* gene does not appear to be regulated in the same manner as the *FLC* gene, as transcript accumulation profiles of the *FLC* and *FRP33-4* genes show opposite responses in wild-type Pitztal plants when given vernalisation treatments, or subjected to changes in photoperiodic conditions. Therefore in the proposed model of the floral induction process in late-flowering ecotypes of *Arabidopsis*, *FRP33-4* has been given a putative role in the repression of the activity of genes within the autonomous flowering pathway, which themselves have been proposed to act to repress the effects of the flowering time gene, *FLC* (Sanda and Amasino, 1996; Sheldon *et al.*, 1999). This model predicts that the lack of expression of the *FRP33-4* gene in the mutants may explain the low levels of expression of the *FLC* transcript noted in the *fler* lines, as this gene is presumably constitutively repressed by the genes in the autonomous floral promotory pathway in these plants. In support of this repression process, Sheldon *et al.* (1999), noted that there was increased expression of the *FLC* transcript in L. *erecta* and Wassilewskija plants that contained mutations within the autonomous flowering pathway (*fca*, *fve*, and *ld*), while plants with defects in the long day dependent pathway (*gi*, *co*, and *fha*) displayed no such increase (Sheldon *et al.*, 1999).

As an important future direction for this current study it would therefore be interesting to examine the RNA and protein expression profiles of several of the genes within either the autonomous, or long day-dependent, flowering pathways in the *fler* mutant lines. If the model proposed in this study is correct, expression of genes within the constitutive flowering pathway may be expected to be increased within the mutant lines compared to wild-type Pitztal plants, particularly at earlier stages of development. Little change in the expression of the genes within the long day-dependent flowering pathways may however be expected in the *fler* mutant lines, as the *FLER*-mediated induction of *FRP33-4* in wild-type Pitztal plants is not predicted to significantly affect the expression of these genes. Conversely it may also be interesting to determine if there is any change in the expression levels of *FRP33-4* transcripts in the autonomous flowering pathway mutants.

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Levels of the *FRP33-4* gene were found to increase and stay relatively strong in vernalised Pitztal plants, suggesting that the lack of *FRP33-4* expression in the *fler* mutant lines may contribute to the lack of a vernalisation response observed in these plants grown in either long or short day conditions. This lack of a response would be predicted by the model proposed in chapter 6 of this study, as mutant plants would show little *FRP33-4*-mediated repression of genes within the autonomous flowering pathway. Such plants would no longer be required to use the vernalisation dependent flowering pathway, that can normally override the flowering pathways in wild-type Pitztal plants to cause very early-flowering even in non-inductive conditions. The vernalised mutant lines would therefore flower at similar times to unvernalised plants, and show no induction of expression of the *FRP33-4* gene transcript. Since vernalisation signals are believed to be perceived at the apex while light signals are thought to be detected within leaves, it may also be interesting to examine the tissue-specific expression of *FRP33-4*, given the differing levels of induction of *FRP33-4* in vernalised plants compared to those exposed to changes in photoperiod.

The flowering pathway model proposed in this study is based on the proposal that one of the main factors controlling the timing of flowering in *Arabidopsis*, is the levels of active gibberellins (GAs) within the plant, and in particular within the apical meristem (Michaels and Amasino , 1999; Sheldon *et al.*, 1999). It is therefore interesting to note that the promoter region of the Columbia version of the *FRP33-4* gene (*FRP33-4* [Col.]), contains elements important in the regulation of several GA responsive genes (Huang *et al.*, 1990). In addition, the coding sequence of the Columbia version of this gene shows some homology to genes coding for the GA biosynthesis and the regulatory enzymes, GA-20-oxidases. Furthermore, some of the *fler* mutants which have altered expression of the *FRP33-4* gene also have some of the characteristics of plants overexpressing GA-20-oxidases (Coles *et al.*, 1999), such as alterations in hypocotyl length, leaf epinasty, chlorophyll content, and flowering time. The presence of GA responsive elements in the promoter of the *FRP33-4* (Col.) locus, as well as the fact that GA-20-oxidase enzymes are thought to be involved in a GA metabolism feedback control mechanism (Phillips *et al.*, 1995; Wu *et al.*, 1996), also suggests that *FRP33-4* function may influence GA-mediated processes such as flowering. The model proposed in this study suggests that GA activity may be influenced via the *FRP33-4*-mediated repression of genes within the autonomous flowering pathway which normally act to increase GA levels. The model also suggests that *FRP33-4* may have a role similar to *FLC* in floral repression, which is thought to involve regulation of either GA production, or the plants response to this hormone, as mutants overexpressing *FLC* transcripts required longer and more frequent applications of exogenous GA to promote their flowering than other late-flowering mutants, such as *fca* (Sheldon *et al.*, 1999).

To test the speculative roles of the *FRP33-4* and *FLER* genes in regulating GA responses, it may be worthwhile analysing the responses of the mutant lines to different GA treatments, as well as assessing the pattern of expression and activity of different bioactive GAs within the mutant and wild-type Pitztal plants. In addition, it may be useful to analyse the expression at both the protein and RNA level of some of the genes involved in the biosynthesis of active GAs, such as GA-20-oxidases, in these plants.

In conclusion, this present study has identified novel early-flowering mutants of the late-flowering Pitztal ecotype of *Arabidopsis* which are believed to cause disruption within the *FRI/FLC*-mediated floral repression pathway. The exact role the *FLER* and *FRP33-4* genes play in conferring the early-flowering phenotype of the mutant lines is yet to be determined, however they are believed to be important factors in influencing flowering time in this species. Future analysis of the function of these genes, will yield information regarding their modes of action that may provide some insight into the complicated process of floral induction in *Arabidopsis*.

Addendum: Future Research Directions

The studies conducted here evoke a number of questions which remain to be answered. One question relates to the molecular nature of the primary lesion resulting in the early-flowering phenotype of the mutant lines. Another question concerns the biological function of the protein encoded by *FRP33-4* and the role it may play in controlling flowering time. In addition, while the results suggest that the primary lesion affecting flowering time is also responsible for the absence of the *FRP33-4* transcript in mutant lines, the nature of the interaction between the primary mutation and the regulation of expression of *FRP33-4* requires further investigation.

The recent cloning and characterisation of *FRI* (Johanson *et al.*, 2000[1]) and *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999) has clearly indicated that these loci are distinct from *FRP33-4*. Initial experiments in determining the role of *FRP33-4* should focus on the ability, or otherwise, of this Pitztal gene to affect flowering time in either long days, short days or both. Over-expression using a constitutive promoter to drive the *FRP33-4* gene in both early flowering ecotypes and in mutant lines may provide clear evidence of a role for *FRP33-4* in regulating flowering time. However, it is possible that additional factors, whose expression has also been affected in mutant plants, are required in order for the mutant phenotype to be rescued. These additional factors may also not be present in early-flowering ecotypes. For this reason co-suppression experiments to alter the expression of *FRP33-4* in wild-type Pitztal plants, and hopefully produce early-flowering transgenics, should also be performed. In these experiments, the possibility of gene redundancy needs to be considered.

The homology of the Columbia version of FRP33-4 to proteins in the database suggest that this gene product may be a dioxygenase. These proteins comprise a large class of Fe-containing enzymes in plants that can act on a variety of substrates (Coles *et al.*, 1999). The lack of detectable levels of the *FRP33-4* transcript in the Columbia ecotype raises the possibility that this early-flowering ecotype sequence represents a non-functional copy of the gene. Therefore, determination of the complete Pitztal *FRP33-4* coding sequence may provide additional clues to the possible function of the gene product. The putative Columbia FRP33-4 protein has only limited homology to the dioxygenase, gibberellin C-20 oxidase which catalyses the removal of carbon-20 on the GA skeleton in the latter stages of biosynthesis of active GAs (Phillips *et al.*, 1995). In addition, the conserved regions thought to be involved in substrate binding by GA-20 oxidases are not present in Columbia FRP33-4 (Xu *et al.*, 1995: Wu *et al.*, 1996). This indicates that the Columbia version of *FRP33-4* does not have a role completely identical to these

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

dioxygenases. Although the various oxidases which act on GA only have similar levels of homology to each other as to any plant dioxygenase, motif searches on these enzymes may prove useful in order to help determine if any candidate substrate binding sites are present in the FRP33-4 sequence.

Assuming flowering time is found to be affected in transgenic plants with altered *FRP33-4* expression, investigation of the biological function of the *FRP33-4* would be warranted. A number of experiments utilising these transgenics could be undertaken to investigate the role of *FRP33-4* in plant development, as well as to determine its potential role in GA metabolism. These include an examination of the effect of exogenous GA application on the flowering time in these lines, as well as measurements of the concentration of endogenous GAs including GA$_4$, the major biologically active GA in *Arabidopsis* in such plants (Evans, 1999). If necessary, an attempt could be made to extend the studies to the ability of FRP33-4 to modify the biological activity of GAs *in vitro*. Crosses with various GA mutants may also be informative. The effect of environmental factors such as day-length and vernalisation on flowering time in the transgenics could also be ascertained, along with an examination of the effect of altered *FRP33-4* expression on transcript levels of other known flowering time genes. Such experiments may help to support or refute the model presented in chapter 6. Further analysis of the role of *FRP33-4* should include an analysis of the Pitztal regulatory region controlling expression of *FRP33-4*. An examination of *FRP33-4* spatial and temporal expression patterns using reporter gene constructs and *in situ* hybridisation, for example, may provide additional information as to a potential function of the *FRP33-4* gene product. Furthermore, an analysis of the primary sequence of the Pitztal 5' regulatory region may indicate whether it is worthwhile investigating if *FRP33-4* is under the regulation of phytochrome and circadian rhythm.

If over-expression or co-suppression of *FRP33-4* is found not to alter flowering time in transgenic plants then future experiments should focus on an investigation of the primary lesion responsible for the early-flowering phenotype of the mutant lines. While experiments have revealed that the *fler* mutants do not express *FLC*, there are several lines of evidence which suggest that *FLC* does not represent the primary lesion in the mutant plants. Firstly, introduction of *FLC*[Col], either through crosses with Columbia or with L. *erecta* plants introgressed with *FLC*[Col], does not restore late flowering. It should be noted that the possibility that *FLC*[Col] has a dysfunctional regulatory region is unlikely as *FRI*[Sf-2] introgressed into the Columbia ecotype causes very late flowering (Lee and Amasino, 1995). Secondly, the mapping experiments described in chapter 4 indicate that the mutation conferring early-flowering is linked to chromosome 4 and not chromosome 5 where *FLC* maps. The model of the repression of flowering in *Arabidopsis* proposes that *FLC* expression is regulated by *FRI* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). This suggests that the *fler* mutation affects *FRI* activity either directly or indirectly. Little is currently known about the mechanism of regulation of *FRI* or about the mechanism whereby

FRI regulates *FLC* activity, hence the primary lesion in the *fler* mutants could conceivably be at the *FRI* locus or upstream or downstream in the *FRI* repression pathway. However, in Chapter 4 it was demonstrated that introduction of *FRI*[Sf-2] using an introgressed line of L. *erecta* does not restore late-flowering in mutant plants, suggesting that the lesion is not at the *FRI* locus itself nor upstream of *FRI*, but rather may act downstream of *FRI* preventing *FLC* and *FRP33-4* transcription. It would therefore be interesting to examine the expression of *FRP33-4* is seen in the L. *erecta* [Sf-2] line.

Following the entry of both the *FRI* and *FLC* sequences in the database, direct molecular techniques can be utilised to investigate the nature of the primary defect in the *fler* mutants. Mutant plants could be transformed for example, with constitutively expressed *FRI* to test the above hypothesis that *FRI* is not the primary lesion, along with direct comparisons at the molecular level of the *FLC* and *FRI* loci in wild-type and mutant Pitztal plants using PCR and sequencing techniques. These experiments, along with analyses of *FRI* expression in wild-type Pitztal plants and mutant plants, as well as in the F1 from the mutant crossed with L. *erecta* (*FRI*[Sf-2]) using Northern or RT-PCR techniques, should conclusively determine whether or not there are lesions in the genes encoding the FRI or FLC proteins in the *fler* mutants.

Mapping experiments have linked the *fler* mutation to chromosome 4 near the nga8 microsatellite marker. If *FRI* has been effectively eliminated as a candidate, additional mapping experiments could be undertaken in an attempt to localise the mutation sufficiently to allow complementation experiments to be performed.

As there is evidence that the expression of both *FLC* and *FRP33-4* is affected by the primary lesion in the *fler* lines, an alternative strategy to determine the primary lesion in the mutants may involve working back upstream from the promoters of these two genes. While there are no indications as to how many steps are involved, an investigation of the regulatory mechanisms driving *FRP33-4* and *FLC* expression using techniques such as the yeast one-hybrid system, may eventually identify the mutation responsible for the early-flowering mutant phenotype.

In the long-term, microarray analysis using probes from mutant and wild-type plants may provide further useful information on alterations in the expression profiles of a number of genes in the mutant plants and lead to a much broader understanding of the genetic control of flowering in *Arabidopsis*.

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