In loving memory of my wonderful Mum

Diane Hall

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Molecular characterisation of the *day neutral flowering (dnf)* mutant in *Arabidopsis thaliana*

by

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Abstract

For many plants day length is critical for the control of flowering time, as the ability to respond to environmental signals is fundamental for the induction of flowering in optimal conditions. Arabidopsis is a facultative long day plant and as such flowers under both long and short days but sooner under long day conditions. To identify novel flowering mutants of the photoperiodic pathway a T-DNA tagged population of Arabidopsis was obtained from NASC (INRA Versailles lines) and screened for altered flowering times in long and short days. A novel flowering time mutant, termed *day neutral flowering* (*dnf*) was isolated as a result of this screen which displayed no short-day inhibition of flowering. The *dnf* mutant, therefore, flowers at the same time under both long and short day conditions.

Complementation of the *dnf* mutant was carried out by re-introducing the wild-type *DNF* gene under the control of its endogenous promoter. Further complementation was also achieved using N-terminal TAP-tag fusion proteins. This complementation confirmed that the *dnf* mutation is responsible for the observed phenotype.

The ability to perceive daylength in Arabidopsis is achieved by the coincidence of light with *(CONSTANS) CO* expression. This promotes the expression of *FLOWERING LOCUS T (FT)* which in turn leads to the promotion of flowering. Quantitative real-time PCR (qRT-PCR) has shown that the expression of both *CO* and *FT* is altered in the *dnf* mutant, which is indicative of its early flowering phenotype. Furthermore the expression of *GIGANTEA (GI)* was unaltered in *dnf*. These results position *DNF* upstream of *CO* and downstream of *GI* within this photoperiodic flowering pathway.

Crosses between *dnf* and the *co-2* mutant flowered at the same time as the *co-2* mutant and as such confirms that *DNF* lies on the photoperiodic flowering pathway. The fact that *dnf* flowers early in SD combined with the qRT-PCR analysis of *GI*, *CO* and *FT* suggest that *DNF* plays a role in the repression of *CO* in such photoperiods. The DNF protein is 141 amino acids in length and contains a predicted membrane spanning domain between residues 13 and 33 and a putative RING finger domain between residue 79 and 121.

This putative ring finger domain within *DNF* shows sequence similarity to both an E3 ligase and a PHD domain. Many E3 ubiquitin ligases have been shown to target specific proteins for degradation by the 26S proteosome whereas PHD domains have been implicated in the regulation of gene transcription through chromatin remodelling. The biochemical function of DNF is unknown, however one method to elucidate its role is to look at possible protein interactors. With this aim in mind we have decided to use an *in vivo* pull-down approach and have produced plants expressing a TAP tagged DNF protein for this purpose. However, due to time constraints pull down and MASS SPEC analysis was unable to be performed.

The results of EGFP expression assays revealed that DNF protein is localised in the cytoplasm when driven by its native promoter and in the cytoplasm and nucleus of plant cells when over-expressed from the strong P35S promoter.

The hypothesised role of *DNF* in the control of flowering will be discussed.

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Thanks should also go to the University of Warwick biological sciences department who took time to help obtain GFP images using the confocal microscope.

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Declaration

This thesis contains experiments performed by Dr Stephen Jackson and by Dr Karl Morris. All such experiments are included to present the project in its entirety and are clearly identified as been performed by Stephen Jackson or Karl Morris. None of the work in this thesis has been published or presented for another degree.

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List of abbreviations

BL	Blue Light
bp	Base Pair
CAB	CHLOROPHYLL A/B-PROTEIN
cBL	Continuous Blue Light
CDL	Critical day length
CO	CONSTANS
Col	Columbia
cRL	Continuous Red Light
DNA	DeoxyriboNucleic Acid
DNF	Day neutral Flowering
dNTPs	DeoxyNucleotide TriphosPhates
DUB	De-UBiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EE	Evening Element

ER	Endoplasmic Reticulum
FR	Far-Red Light
FT	FLOWERING LOCUS T
GAs	Gibberellin(s)
H	Histone
HDAC	Histone DeAcetylase
hr(s)	Hour(s)
kb	KiloBases
LD(s)	Long Day(s)
Ler	Landsberg erecta
LFY	LEAFY
ME	Morning Element
MS	Mass Spectrometry
NASC	Nottingham Arabidopsis Stock Centre
NLS	Nuclear Localisation Signal
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PHD	Plant Homeodomian
QTL	Quantitative Trait Locus/Loci
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RING	Really Interesting New Gene
RL	Red Light
RNA	RiboNucleic Acid
rpm	Revolutions Per Minute
RT	Reverse Transcriptase
SAM	Shoot Apical Meristem

SD(s)	Short Day(s)
SOC1	SUPRESSOR OF CONSTANS 1
TAP	Tandem Affinity Purification
Ub	UBiquitin
WL	White Light
WS	Wassilewskija
WT	Wild Type
w/v	Weight to Volume ratio
ZT	Zeitgeber time

Chapter 1

General Introduction

1.1Controlling flowering in Arabidopsis.

1.1.1 The right time to flower

Arabidopsis is a facultative (long Day) LD plant thus flowers much earlier when grown under LD conditions (16hrs light) than when grown under SD conditions (8hrs light). The transition from a vegetative state to a reproductive (flowering) state is affected by both endogenous and environmental cues including light quantity and intensity, photoperiod, temperature and nutrition. Variations in these cues will dictate which of the different flowering pathways has the major influence on flowering. Plants thus have the ability to regulate the timing of flowering in order to take maximum advantage of the most favourable environmental conditions.

Before being able to respond to these cues plants must pass through a juvenile phase before the vegetative apical meristem can develop and give rise to an inflorescence meristem from which the floral meristem will develop. Different species have different lengths of juvenile phase and it is only after the juvenile phase has passed that the transition to flowering can be induced by the endogenous and environmental cues.

All ecotypes of Arabidopsis fall into either summer annuals, which are rapid flowering and complete their life cycle over a few short months in the summer, or winter annuals which germinate in the autumn grow as seedlings during winter then flower in the spring. Summer annuals have arisen from their winter annual ancestors.

1.1.2 **The pathways that control flowering in Arabidopsis**

There are several genetic pathways that control flowering in Arabidopsis. These pathways subsequently regulate the floral integrator genes which then signal the transition from vegetative to floral reproductive state. The floral integrator genes next activate downstream floral meristem identity genes to initiate flowering (Figure 1).

Many genes have been identified and positioned on these pathways through studying mutant plants and how they respond to different growth conditions such as light quality or quantity and temperature. The findings of these studies have lead to the conclusion that normally mutations of genes that are involved in the promotion of flowering are late flowering, whilst mutations of genes that repress flowering are early flowering (Koornneef *et al.*, 1998; Boss *et al.*, 2004).

The main pathways involved in the promotion of flowering are the photoperiodic, gibberellin, vernalisation, autonomous and ambient temperature pathways. These pathways lead to the promotion of flowering by responding to internal and external environmental cues. The key genes of these pathways and the roles the play in the flowering response will be discussed in the following sections.

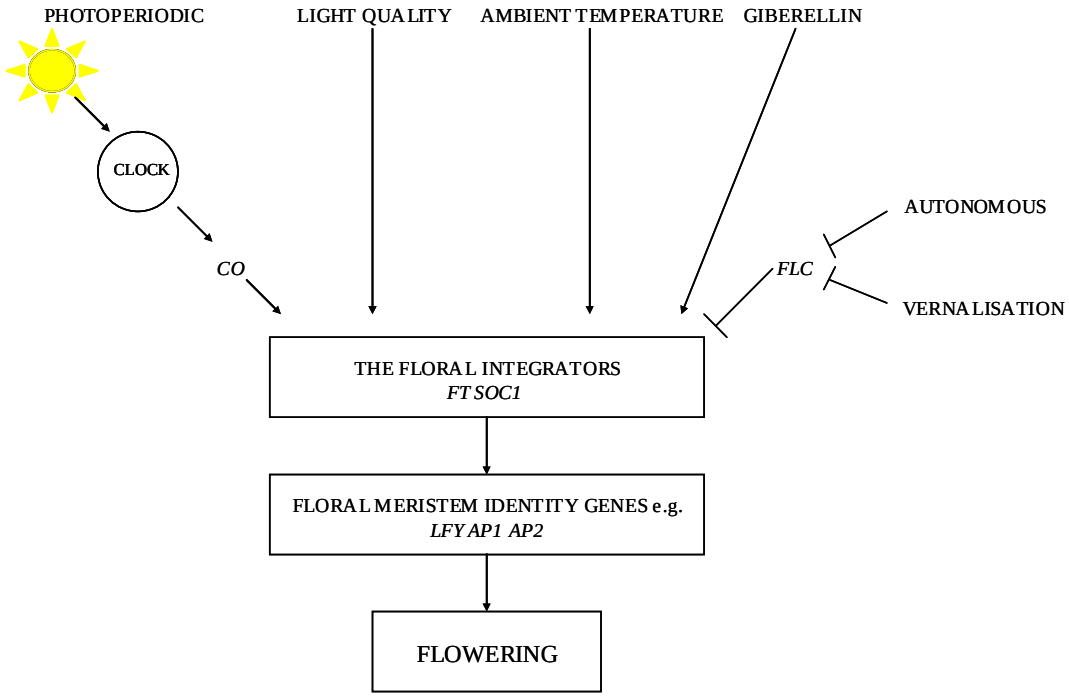


Figure 1. A simplified illustration of the pathways that control flowering in Arabidopsis.

1.1.3 Winter annuals

The flowering time of winter annuals is affected by both the length of the photoperiod and the requirement for vernalisation. Vernalisation is the promotion of flowering by a prolonged period of cold of several weeks e.g. over winter, thus leading to flowering in more favourable conditions (Chouard 1960). Two genes *FLC* (*FLOWERING LOCUS C*) and *FRI* (*FRIGIDA*) interact synergistically as floral repressors prior to vernalisation. FLC encodes a MADS-box transcription factor and loss of function of *flc* alleles causes early flowering (Michaels *et al.*, 1999). Expression of *FLC* is under the control of the autonomous pathway in which flowering is promoted with age and the vernalisation pathway where promotion of flowering occurs only after a period of prolonged cold. *FLC* lies at the point were the autonomous and vernalisation pathways converge and as such plays a dominant role in the control of flowering

(Figure 2) (Rouse *et al.*, 2002). FRI acts upstream of and is a positive regulator of *FLC* and encodes a protein that has not been shown to have any significant identity to other proteins of known function (Johanson *et al.*, 2000). *FRI* is also considered to be a major determinant of flowering time variation amongst Arabidopsis accessions specific to the vernalisation pathway (Shindo *et al.*, 2005).

1.1.4 Summer annuals

Summer-annual Arabidopsis ecotypes such as Lansberg erecta (*Ler*) and Columbia (*Col*) originate from temperate regions and exhibit a facultative long day response. Such ecotypes flower rapidly in long day photoperiods (LD; 16 h light, 8 h dark), resulting in the promotion of flowering in the spring and summer months.

As summer annuals they do not have a vernalisation requirement and have either a weak *FLC* allele or a non-functioning *FRI* alleles. It appears that such ecotypes have evolved on multiple occasions via disruption of *FLC* regulatory sequences by transposons and via deletions and frameshift mutations in *FRI* (Johnson *et al.*, 1994; Michaels *et al.*, 2001).

1.1.5 The Vernalisation Pathway

Vernalisation allows plants to delay flowering until after the passage of winter i.e (exposure to temperatures of approximately 1-10ºC for a prolonged period) when the more favourable conditions of spring have arrived (Simpson *et al.*, 2002). FLC is one of the best characterised floral repressors and along with FRI play central roles in this vernalisation response. The *FLC* gene encodes a MADSbox transcription factor and high levels of expression are associated with

late flowering in ecotypes that require vernalisation prior to flowering (Michaels *et al.,* 1999).

FLC functions as part of a large protein complex and suppresses flowering by repressing the expression of the floral integrators *FLOWERING LOCUS T (FT), SUPPRESOR OF OVEREXPRESSION OF CONSTANS* (*SOC1)* and *LEAFY (LFY)* (Nilsson *et al.,* 1998; Samach *et al.,* 2000; Hepworth *et al.,* 2002; Chris A. Helliwell 2006; Searle *et al.,* 2006).

FRI is predicted to contain two coiled-coil domains and is an upstream positive regulator of *FLC.* The absence of *FLC* has no effect on *FRI,* but in the absence of *FRI, FLC* is not expressed (Michaels *et al.,* 1999; Shindo *et al.,* 2005). However both *FRI* and *FLC* are required for the inhibition of flowering and loss of function of either of the two genes results in an early flowering phenotype (Michaels *et al.,* 2001).

FLC is a chromatin-regulated gene and as such can exist in three functional states, heterochromatin silenced state, repressed state and active state (Reyes 2006). Histones contain amino tails (mainly H3 and H4) that are able to be chemically modified by the addition of chemical groups or proteins. The addition of chemical groups occurs via acetlyation, phosphorylation or methylation and the addition of proteins via ubiqutination and SUMOylation. These modifications are proposed to provide information about the state of transcription of the gene and are able to be inherited epigenetically. Interactions between the DNA and the histone octomers can be altered by ATP-dependent chromatin remodelling enzymes. When *FLC* is in its active state it has been shown to be highly acetylated on the first intron of its promoter, along with trimethylation of the H3K4 histone (He *et al.,* 2005).

VERNALIZATION-INSENSITIVE 3 (VIN3) contains a PHD-domain protein and is another key regulator of the vernalisation pathway. *VIN3* represses *FLC* expression by reducing histone acetylation of the *FLC* gene. *VERNALIZATION 1* and *2 (VRN1 and VRN2)* are also required for the maintenance of *FLC* silencing. VRN1 encodes a protein of 341 residues comprising of two putative B3 DNA binding domains which enable it to bind to DNA and VRN2 encodes a nuclear-localised zinc finger protein (Levy *et al.,* 2002).In winter annuals the state of vernalisation is maintained through mitotic cell division by an epigenetic memory that is based upon histone modification (Bastow *et al.,* 2004; Sung *et al.,* 2004).

Prior to vernalisation flowering is repressed by high levels of *FLC* which is maintained by *FRI*. During vernalisation *FLC* is repressed by *VIN3* before *VRN1* and *VRN2* cause the dimethylation of K9 and K27 of histone 3 (Bastow *et al.,* 2004). This ensures that *FLC* expression remains at a stably repressed state when plants are returned to warm temperature and persists until the chromatin is reset during meiosis.

EARLY FLOWERING (ELF) genes *ELF7-8* and *VERNALIZATION INDEPENDENCE (VIP)* genes *VIP3-6* have also been shown to be positive regulators of *FLC* expression.

ELF7, *ELF8* (He *et al.,* 2004), *VIP4* (Zhang *et al.,* 2002), *VIP5* and *VIP6* (Oh *et al.,* 2004) are Arabidopsis homologues of components of the yeast RNA polymerase II associated factor 1 (PAF1) complex.

The Arabidopsis RNA PAF1-like complex is composed of *ELF7 ELF8,VIP3,4,5* and *6*, and a putative histone methyltransferase called *EARLY FLOWERING IN SHORT DAYS* (*EFS*). This complex plays an active role in chromatin modification by recruiting methyltransferases that can transfer methyl

groups to the H3-K4 (histone 3 at lysine 4). In eukaryotes, the presence of these methylations has been associated with genes in an active state (Schubeler *et al.*, 2004). VIP3 protein is composed of Trp-Asp (WD) repeated motifs which are protein-protein interactors and is believed to a play a role in the assembly of the PAF1 complex (Zhang *et al.*, 2003).

1.1.6 The Autonomous pathway

The autonomous pathway functions independently of environmental factors and involves at least seven genes *FCA, FPA, FY, FVE,* (these genes do not have complete names) *FLOWERING LOCUS D (FLD), LUMINIDEPENDENS* (*LD*) and *FLOWERING LOCUS K (FLK). FLK* has only recently been identified whilst the other genes have been known for some time, suggesting that there may be other genes still unidentified. (Simpson *et al.*, 2002) Mutants of these genes are late flowering in both LD and SD, a phenotype that can be overcome by vernalisation (Simpson *et al.*, 2002). The genes of the autonomous pathway act to limit the expression of *FLC* and when mutated, the levels of *FLC* mRNA are elevated (Boss *et al.*, 2004). In addition the genes have been demonstrated to act independently each having distinct functions (Mouradov *et al.*, 2002).

FCA, FPA, FY and *FLK* encode proteins with RNA binding or RNA processing factors and as such may be involved in the regulation of *FLC* mRNA. The predicted FCA protein contains an RNA binding motif and a WW domain, which is thought to be involved in protein-protein interactions and has been shown to be able to bind RNA *in vitro* (Macknight *et al.*, 1997). FCA controls the expression of *FLC* and is able to self regulate its own expression by cleavage and polyadenylation of its own third intron, a process which requires the WW

domain and both functions require FY (Quesada *et al*., 2003). FY binds to the WW domain of FCA, and is involved in the processing of the 3' end of transcripts (Simpson *et al*., 2003). The FCA/FY interaction is required for the down regulation of the floral repressor *FLC*. If the complex is impaired this leads to an increase in abundance of *FLC* (Simpson *et al*., 2003).

FVE and *FLD* both encode homologs of components of histone deacetylase (HDAC) complexes (He *et al.,* 2003; Ausilon *et al.,* 2004; Kim *et al.,* 2004). Such complexes remove acetyl groups from histone tails, causing regulation of DNA at a transcriptional level by blocking the sites of transcription factors. Both FVE and FLD are required for normal deacetlylation of H3 and/or H4 of the *FLC* gene (He *et al.,* 2003; Ausilon *et al.,* 2004).

Chromatin immunoprecipitation was used to look at the acetlyation state of *FLC* in both *fve* and *fld* mutants and in both cases *FLC* was shown to be hyperacetylated. This hyperacetylation is not seen in other autonomous pathway mutants indicating that both FVE and FLD are components of the HDAC complex and as such are involved in *FLC* repression by histone deacetylation (He *et al.,* 2003; Henderson *et al.,* 2004).

LD encodes a homeodomain protein (Lee *et al.,* 1994; Aukerman *et al.,* 1999) many homeodomain proteins have been shown to interact with RNA acting as transcriptional repressors (Damante *et al.,* 1996). Although the RNA target of *LD* is unclear it is possible that *LD*, like other autonomous pathway genes, also acts as an RNA interactor.

The *FLK* gene encodes a RNA binding protein and three KH motifs which are one of the most common RNA binding proteins found in Arabidopsis. In the *flk* mutant *FLC* mRNA levels are increased whilst the levels of *FT* and *SOC1* were reduced (Lim *et al*., 2004).

The exact mechanism by which the autonomous pathway functions is relatively unknown, but it is possibly related to the developmental age of the plant (Simpson *et al.*, 2002). The genes involved in the autonomous pathway appear to be part of protein complexes that are involved in histone modification and RNA processing and as such act to limit the accumulation of *FLC* mRNA.

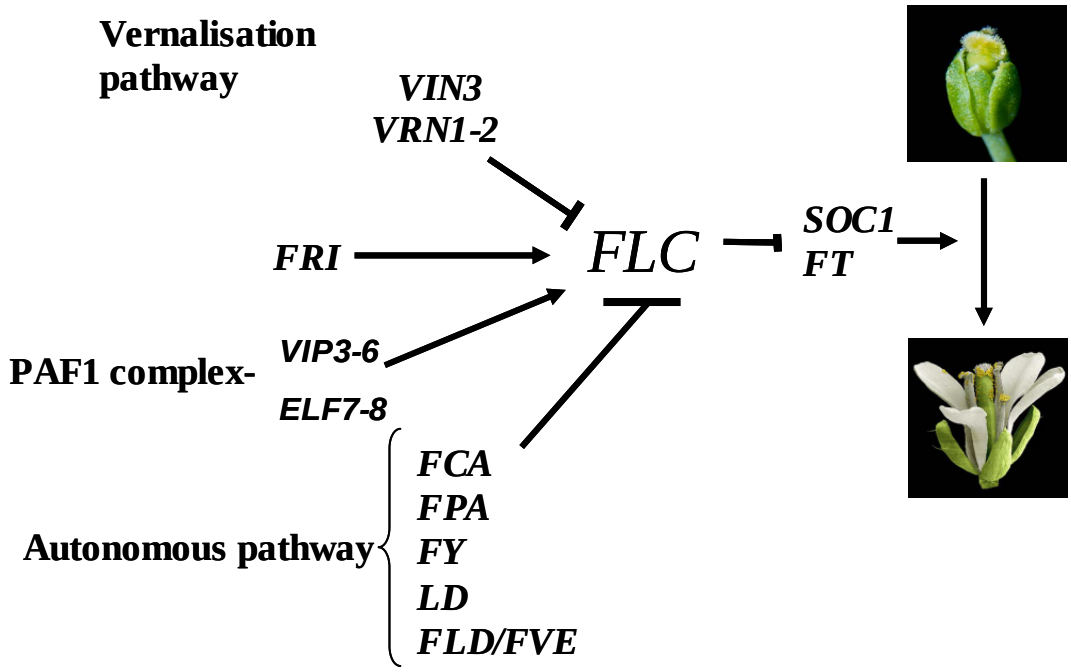


Figure 2. Regulation of the floral repressor *FLC.* *FRI* and the PAF1 complex promote the expression of *FLC.* The vernalization and autonomous pathways converge in the regulation of the expression of *FLC.*

1.1.7 The Giberellin GA Pathway.

Gibberellins (GA's) are plant hormones that act at all stages in the plant life cycle. They promote flowering, seed development, hypocotyl elongation/germination, root/stem/fruit development and phase transition. In addition, they have recently been shown to inhibit cytokinin action and have been implicated in meristem function (Greenboim-Wainberg *et al.,* 2005). The GA pathway has a strong effect on flowering under short day conditions (Moon *et al.,* 2003). The study of GA mutants such as *ga1-3* which are defective in GA biosynthesis has demonstrated that GAs are involved in flowering and mutants such as these are unable to flower in SD (Blázquez *et al.,* 1999). In Arabidopsis GA signalling regulates *LFY* expression and in *ga1-3* mutants there is no induction of the *LFY* promoter in SD. The *LFY* promoter has been shown to contain specific *cis* elements that are essential for GAs to promote flowering

(Blázquez 2000) but it is still unclear how signals from the LD photoperiodic promotion and GA pathways are integrated in the control of *LFY* expression. *GAMYB* genes act directly in the GA pathway (Gubler *et al.,* 2002). One of the Arabidopsis *GAMYB* transcription factor (AtMYB33) binds to an 8bp motif in the *LFY* promoter and this occurs both in vivo and in vitro. When 6bp of this motif were removed the majority of the binding capacity was lost (Blázquez *et al.,* 2000; Gocal *et al.,* 2001). *SOC1* also integrates signalling from the vernalisation and GA pathways, the expression of *SOC1* is regulated by GA and over-expression of *SOC1* can rescue the late flowering *ga1-3* (Moon *et al.*, 2003).

1.1.8 The Ambient temperature Pathway

The ambient temperature at which the plants are grown also has an effect on floral transition. In Arabidopsis the transition to flowering is delayed by lower temperatures (Thingnaes *et al.,* 2003). Most late-flowering mutants such as *co-2, gi-3, ga1-3* and *fwa-2* flower earlier in higher temperatures (Blázquez *et al.,* 2003). To date there is no known sensor for temperature in plants, however our understanding of the role that temperature plays on flowering time has been increased by work on the autonomous mutants such as *fve-1* and *fca-1*. These two are late flowering mutants but don't flower earlier at higher temperatures indicating this maybe due to the loss of ability to perceive or communicate the difference in temperature change (Blázquez *et al.,* 2003). It is worth noting, however, that the *fve-1* and *fca-1* mutants contain high levels of the floral repressor *FLC* and so it may be that this increased level of *FLC* leads to increased suppression of FT therefore maintaining the late flowering phenotype in increased temperatures (Bastow *et al.,* 2004).

Blazquez also looked at the effect on temperature of the *cry-2* mutant and found that at 23ºC plants showed a delay in flowering, a response that was greatly enhanced at 16ºC. At 23ºC PHYTOCHROME A (phyA) functions redundantly with CRYPTOCHROME 1 (cry2) to promote flowering, but phyA does not have any effect on flowering at 16ºC hence the effect of the *cry-2* mutation is greater at this temperature (Blázquez *et al.,* 2003).

1.1.9 Photoreceptors

Photoreceptors are light-sensitive proteins that are involved in the sensing and responding to light. To date several classes of photoreceptors have been described in plants, phototrophins (PHOT) cryptochromes (CRY), and phytochromes (PHY). Phototropins and Cryptochromes are blue light receptors and as such absorb blue/violet light (320-500nm). Phototropins have been shown to be involved in the mediation of stomatal opening, chloroplast relocation and phototropism (Briggs *et al.,* 2001; Lin 2002). Two phototrophins have been characterized in Arabidopsis PHOT1 PHOT2. Each phototrophin contain a serine/threonine kinase domain and two very similar LIGHT, OXYGEN OR VOLTAGE (LOV1 and LOV2) domains each of which binds a molecule of flavin mononucleotide (FMN). The phototrophins undergo autophosphorylation which is blue light dependent thus indicating that they act as blue light photoreceptors (Christie *et al.,* 1999; Sakai *et al.,* 2001).

Cryptochromes are flavoproteins and act with photorophins in the mediation of stem elongation, leaf expansion, regulation of gene expression and entrainment of the circadian clock (Cashmore *et al.,* 1999 52; Lin 2002 196). Cryptochrome 1 (*CRY1*) has been identified as having significant sequence homology to prokaryotic DNA photolyases but without photolase activity. It has also been

shown to contained a FAD domain and C-terminal extension not found in the photolyases (Ahmad *et al.,* 1993). *CRY2* is very similar to *CRY1* but without the C-terminal extension.

CRY1 and *CRY2* are members of the cryptochrome gene family found in Arabidopsis. CRY1 protein has been shown to respond to higher fluence rates of blue light and is more stable than CRY2 (Lin *et al.,* 1998). *CRY2* is believed to be the major blue light photoreceptor involved in Arabidopsis flowering. However *cry1cry2* double mutants flower earlier in blue light than do single mutant suggesting that both play a role in floral promotion (Mockler *et al.,* 1999).

Phytochromes are homodimers that exist in two interconvertable forms (Pr and Pfr). When red light is absorbed by Pr it is converted to Pfr, which is converted to Pr after absorbing far red light. In Arabidopsis five *PHY* genes have been identified (*PHYA-PHYE*) (Sharrock *et al.,* 1989; Clack *et al.,* 1994). These different phytochromes play different roles in controlling various aspects of plant development. *PHYA* encodes a well characterised apoprotein, plays a dominant role in seedling etiolation, is photolabile (degraded rapidly when exposed to light) and is required for normal day length perception (Johnson *et al.,* 1994; Weller *et al.,* 2001). *PHYB-E* are relatively more photostable, they also encode apoproteins but of lower abundance. The protein sequence of PhyB and PhyD in Arabidopsis have been demonstrated to be about 80% identical and are more related to PhyE than to PhyA or PhyC (Mathews *et al.,* 1997). In Arabidopsis PhyB, D and E promote early flowering in response to low-red to far-red ratios (low R:FR) (Halliday *et al.,* 2003).

1.1.9.1 Photoreceptors and Flowering.

In white light (natural conditions) PhyB, PhyA, Cry1 and Cry2 mediated pathways will be stimulated at the same time. PhyA detects far-red and Cry1/ Cry2 blue light promoting the stability of CO protein. Mutant analysis has suggested that far-red light acting through PhyA and blue light acting through Cry1 and Cry2, may act to antagonize the repression of flowering mediated by PhyB (Mockler et al., 2003).

In the *phyB* mutant increased CO protein was observed both in red light and early in the morning, therefore PhyB destabilises CO protein in these coditions (Valverde *et al.,* 2004). Furthermore plants that express CO mRNA at a constant level (35S::CO) only accumulate high levels of the CO protein towards the end of the day (Valverde *et al.*, 2004).

From these experiments it has been determined that there is a gradual shift in the balance of different photoreceptor pathways throughout the day such that PhyB promotes the degradation of CO in the morning and around 12 hours later this effect is antagonized by the effects of Cry1/Cry2 and PhyA, thus allowing the accumulation of CO protein in the light (Figure.4). In addition PhyB mRNA has been shown to peak in the morning whereas that of PhyA and Cry1/Cry2 peak later in the day (Toth *et al.,* 2001; Valverde *et al.,* 2004).

Mutations in the PhyB photoreceptor lead to a dramatic early flowering in both LD and SD suggesting that it plays a role in both photoperiods (Halliday *et al.,* 2003). PhyB is known to be a major player in the shade avoidance response where far red light signals the presence of neighboring plants and potential competition for light. Under these conditions numerous physiological responses occur such as hypocotyl elongation and floral promotion, thus allowing the plants to obtain more enriched light conditions and avoiding the far red enriched light

of the shade. In addition PhyD and PhyE have overlapping role with that of PhyB and are also responsible for that shade avoidance response but not to the same degree as that of PhyB (Devlin *et al.,* 1998; Devlin 2002).

These shade avoidance responses occur independently of photoperiod and recent work has identified PHYTOCHROME AND FLOWERING TIME 1(PFT1) to be required for phyB regulation of flowering in response to R/FR ratio (Cerdán *et al.,* 2003). The *phyB* mutant has elevated levels of *FT* under both photoperiods thus demonstrating consistency with its early flowering phenotype. The Chory laboratory found that the *pft1* and the *pft1/phyB* double mutant had reduced levels of *FT* mRNA Therefore PFT1 is also required for these high levels of *FT* transcripts (Halliday *et al.,* 1994; Cerdán *et al.,* 2003).They also looked at the levels of *CO* mRNA in these mutants, as CO is a key component of the photoperiodic pathway and is directly responsible for the activation of *FT*. They found that when *phyB* mutants were grown in LD the level of *CO* mRNA was increased, but in the *pft1* mutant the *CO* mRNA levels were lower than that of WT. This suggests that PFT1 may function upstream of CO. However when the mutants were grown under SD the same effect on *CO* was not detected even though flowering time was altered in the mutants. In addition, in *CO*-overexpressing lines the *pft1* mutant did not have an effect on the flowering time or *FT* mRNA levels (Cerdán *et al.,* 2003). The level of the floral integrator *SOC1* mRNA levels was also examined in the *phyB and pft1* mutants and no correlation between the mutants and the level of *SOC1* mRNA was found. This lack of correlation between the flowering time of the mutants and the level of expression of *CO* and *SOC1* indicates that phyB regulates *FT* mRNA levels in a process that is independent of *CO or SOC1*. This provides a mechanism by which PhyB can bypass the other floral pathways in the control of flowering (Cerdán *et al.,* 2003).

In addition to light quality small changes in temperature can have a large effect on flowering time of photoreceptor mutants (Franklin *et al.,* 2003; Halliday *et al.,* 2003) The *phyB* mutant has frequently been reported as having such an effect. As previously described this mutant flowers early in both LD and SD at 22°C to 24°C, however when the temperature is lowered to 16°C this early flowering phenotype is abolished and the mutant flowers around the same time as WT. These observations demonstrated that the control of flowering by PhyB is regulated by temperature (Halliday *et al.,* 1994). The Halliday lab grew WT plants at 16°C in shaded light conditions (lower R:FR ratio) and the plants demonstrated an early flowering response, thus suggesting that other phytochromes were able to fully compensate when PhyB activity is lost. Further analysis found that PhyE and to a lesser extent PhyD are two such phytochromes. The *phyE* and *phyD* mutants flowered earlier than WT in SD at 16° and both exhibited an increased level of *FT* mRNA. From these experiments it appears that PhyB, PhyE and PhyD are involved in the negative regulation of flowering and that PhyB effect is predominant at higher temperatures whilst PhyE and PhyD are predominant at lower temperatures (Franklin *et al.,* 2003; Halliday *et al.,* 2003).

1.1.10 Circadian Clock

To try to explain how photoperiod regulates flowering (Bünning 1936) proposed the external coincidence model. This model proposed the existence of an endogenous rhythm in photoperiodic photosensitivity during which the night phase is sensitive to light whilst the day phase is photoinsensitive. By the 1960's two models had been proposed, the internal and the external coincidence models reviewed by (Thomas *et al.,* 1997). The external coincidence model is a refinement of Bunning's original model and proposes that it is the coincidence of light during the day (external stimulus) reacting with the internal rhythm of sensitivity to light that leads to the promotion of flowering.

The internal coincidence model was proposed by (Shindo *et al.,* 2005). They describe that the lights only role is to entrain the circadian clock and that two internal rhythms are in phase under inductive photoperiods leading to the promotion of flowering. When the photoperiods are non-inductive these two rhythms are out of phase and plants are not induced to flower. With the advent of molecular biology, studies have shown that the photoperiodic promotion pathway involves a complex set of interactions between specific photoreceptors and the circadian clock which in turn controls the expression of flowering time genes that promote flowering (Suarez-Lopez *et al.*, 2001).

Plants activities are co-ordinated under the control of the circadian clock, which is an endogenous timing mechanism running at a natural 24 hour light dark cycle. The clock is entrained by both light and temperature but other factors such as nutrient availability also have an effect. The rhythm of the clock persists even in the absence of such inputs such that they can still be observed even when plants are grown in constant light/dark conditions (Figure 3.).

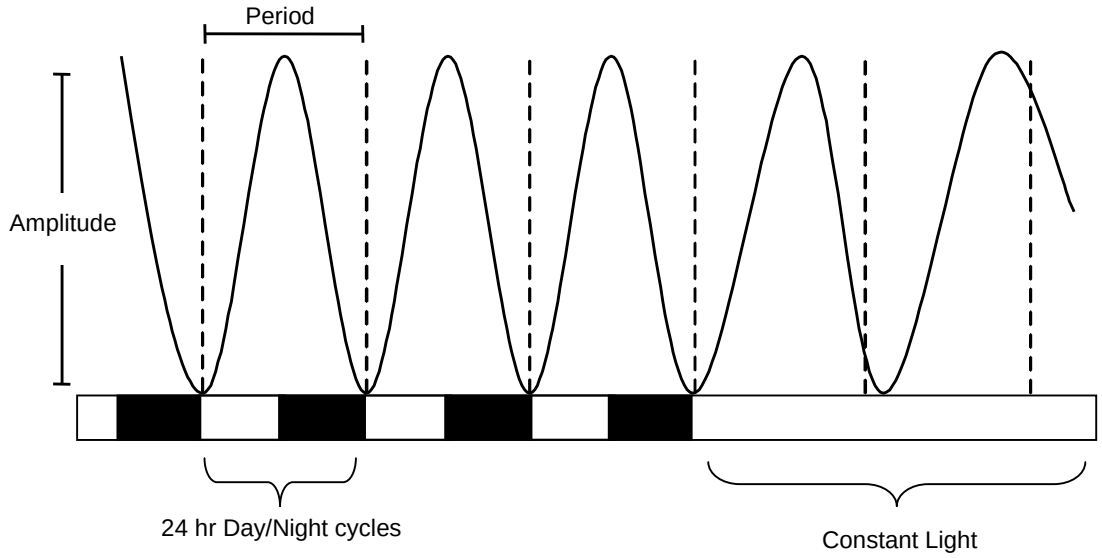


Figure 3. Illustration of circadian rhythm. Bar below shows light dark cycles followed by continuous light.

1.1.10.1. Model of the Clock

The rhythms of gene expression rely on feedback loops and over recent years these often complex networks have been examined in some detail in Arabidopsis. Circadian clocks exist in many organisms and generate the oscillating rhythms with a period of approximately 24 hrs. These oscillations are entrained by external stimulus such as light and temperature. In the absence of external stimulus the oscillations still persist but the length of the period alters as resetting of the clock via light/dark transition no longer occurs (Figure 4).

LHY, *CCA1* and *TOC1* are three genes that have been shown to be central components of one of the Arabidopsis feedback loops (Figure 5.) (Hicks *et al.*, 1996; Somers 1999). *CCA1* was first identified after analysis of the promoter of *LIGHT HARVESTING CHLOROPHYLL A/B-PROTEIN II (LHCB2* also referred to as *CAB*). Cab expression is induced by light and is under the control of the circadian clock (Carre *et al.*, 1995). *CAB2* is expressed rhythmically as an output from the clock and as such *CAB2* can be linked to the reporter gene luciferase

(luc) (cab2::luc) to aid measurements of circadian rhythms and isolate mutants of the circadian clock (Millar *et al.,* 1992; Bognar *et al.,* 1999)

By analysing the rhythm of expression of cab2::luc in the *cry1 cry2* double mutant Devlin and Kay found no alteration in period amplitude or length therefore illustrating that plant cryptochromes do not form an integral part of the Arabidopsis circadian clock (Figure:4) (Devlin *et al.,* 2000; Devlin 2002).

LHY and *CCA1* both encode a highly conserved myb-transcription factors which, when over expressed, cause disruption of the clocks normal function (Schaffer *et al.,* 1998; Wang *et al.,* 1998). *TOC1* was first identified by screening for mutants with altered period length and *toc1-1* mutant has altered clock function throughout the life of the mutant plant. The *toc-1* mutant has been identified as acting downstream of the input as the cab2::luc expression produced a shorter period in both red and blue light (Millar *et al.,* 1995).

The promoter of *TOC1* contains an evening element motif (EE) Numerous genes that peak in the evening under the control of the circadian clock also have EE motifs and these have been shown to be sufficient to confer an evening based rhythm (Alabadi *et al.,* 2002; Harmer *et al.,* 2005). *TOC1* positively regulates *CCA1/LHY* in a process yet to be determined. CCA1/LHY proteins then feed back to inhibit *TOC1* expression by binding to an evening element of the *TOC1* promoter, this consequently inhibits further *LHY* and *CCA1* expression (Alabadi *et al.,* 2001).

ZTL is an additional regulator of TOC1, specifically targeting TOC1 for degradation by the 26s proteasome. The rhythm of TOC1 protein was analysed in *ztl* mutants and results showed that TOC1 protein did not cycle in either constant light or dark thus indicating a potential role of ZTL for TOC1 degradation in both day and night (Baxter *et al.,* 2003; Mais *et al.,* 2003).This original model of

the clock has proved to be over simplistic and mathematical modelling has predicted the existence of two additional loops.*TOC1* encodes a member of a family of PSEUDO-RESPONSE REGULATOR (PRR) genes which play a role in clock function. These genes are expressed from dawn, in a specific order, *PRR9, PRR7, PRR5, PRR3* and finally *TOC1*(Matsushika *et al.,* 2000; Eriksson *et al.,* 2003). *PRR7* and *PRR9* which contain a *CCA1*- binding site in their promoters form part of the second loop of the clock and both genes are expressed in the morning. CCA1/LHY positively regulates PRR7/PRR9 (Mas *et al.,* 2000; Matsushika *et al.,* 2000). The method of regulation has yet to be confirmed however it is most probably that this occurs via binding to the *CCA1* promoter biding site. In addition both PRR7 and PRR9 could potentially be involved in light input into the clock by either a direct or indirect manner (Abe *et al.,* 2005; Farre *et al.,* 2005). Lock *et al* used mathematical modelling to identify a key player of the third feedback loop and named it factor Y. In the third clock model factor Y is proposed to activate *TOC1* transcription and then the TOC1 protein represses factor Y, thus proposing that *TOC1* has both a negative and a positive function. In addition *LHY* was also proposed to be a repressor of factor Y thus allowing the network to fit both the WT and the *cca1:lhy* double mutant experiments. Therefore at dawn LHY acts to inhibit expression of both *TOC1* and factor Y (Gocal *et al.,* 2001; Locke *et al.,* 2005).

Interestingly *GIGANTEA* (*GI*) expression levels have been shown to parallel that of the predicted Factor Y expression and as such *GI* has been added to the loop (Gocal *et al.,* 2001; Locke *et al.,* 2005). GI protein contains several putative membrane spanning domains, and *GI* expression has been shown to be repressed via an unknown interaction by TOC1 (Gocal *et al.,* 2001; Locke *et al.,* 2006). At dawn light induces a peak in expression of *GI*, then as the levels of

CCA1/LHY rise they repress this expression by binding to evening elements in the *GI* promoter (Gocal *et al.,* 2001; Locke *et al.,* 2005). Furthermore GI has a role to play in both the input and the output of the clock. As an input to the clock it plays a role in blue light input into the clock (Ewing *et al.,* 1995; Martin-Tryon *et al.,* 2007) and as an output from the clock is involved in the regulation of flowering by inducing *CO* expression in LD (Samach *et al.,* 2000) .

1.1.10.2 Entrainment of the clock

In order to synchronise the circadian clock with the external environment, the clock must be entrained. Many signals contribute to entrainment however light and temperatures have the strongest effects on resetting the clock. In Arabidopsis the circadian clock runs to an intrinsic period of approximately 24 hrs. However this period can vary in different accessions and under different growth conditions (Christie *et al.,* 1999; Michael *et al.,* 2003).

The mechanism of the clock is reset with each diurnal cycle, without which the clock would become desynchronised with the external environment (Devlin 2002). In the absence of the external cues such as in constant light, the rhythm of the clock still occurs, however the clock oscillates with a free running period (Figure:4). It is important that the length of the period is maintained and if the clock deviated from this it would very quickly fall out of phase with time. However entrainment of the clock by the external cues ensures that any deviations do not occur.

Light is the most important cue in entrainment of the clock and the clock can respond differently to light at different times of the day either by advancing or delaying the clock by a mechanism referred to as a gating response (Hicks *et al.,* 1996; Somers *et al.,* 1998) .

The light input pathway is poorly understood but as previously discussed is perceived by the phytochromes and cryptochromes. PhyA has been shown to act as a red light receptor and in addition plays minor roles in absorption of blue light. It is thought that in low light PhyA accumulates at high levels and absorbs blue light (Yanovsky *et al.,* 2002). Interestingly this minor role has a major influence on the clock shortening its period significantly. (Marcelo J. Yanovsky 2000). In addition cry1 has been shown to be required for the transmission of both red and blue light to the clock by PhyA (Devlin *et al.*, 2000).

PhyB, along with PhyD and PhyE are red photoreceptors however PhyD and PhyE only act in the absence of PhyB thus showing possible overlapping functions and partial redundancy of PhyD and PhyE (Devlin 2002) (Somers *et al.,* 1998).

Pr is converted to Pfr by light thus revealing a nuclear localisation signal of PhyB (Ahmad *et al.,* 1995; Chen *et al.,* 2005). Once in the nucleus PhyB is able to interact with a transcription factor called PHYTOCHROME INTERACTING FACTOR 3 (PIF3). PIF3 binds directly to the promoters of both *LHY* and *CCA1* to control entrainment of the clock (Alonso-Blanco *et al.,* 2000; Martínez-García *et al.,* 2000).

The cryptochromes *CRY1* and *CRY2* act as blue light receptors in the entrainment of the clock but with a certain degree of redundancy (Hicks *et al.,* 1996; Somers *et al.,* 1998).

Interestingly in the quadruple mutant *phya:phyb:cry1:cry2* developmental responses are still able to be entrained by light thus suggesting compensatory roles of light input by other photoreceptors (Yanovsky *et al.,* 2000; Alabadi *et al.,* 2001). PHOTs are involved in blue light sensing and as such may also be involved in blue light transmission to the clock. In addition the ZTL family of

proteins could also act as blue light receptors as these contain a LOV domain that undergoes a light induced conformational change. The LOV domain is also similar to the chromophore binding domain of the PHOT light receptors (Gardner *et al.*, 2006).

ELF3 antagonizes light input to the clock during the night and also functions in resetting of the clock (McWatters *et al.*, 2000; Carre 2002). In addition *SUPPRESSOR OF PHYA-105 (SPA1)* and *TIME FOR COFFEE (TIC)* may also antagonize light input to the clock (Hall *et al.*, 2003; Ishikawa *et al.*, 2006).

Temperature plays a role in entrainment of the circadian clock, however the molecular mechanism is not well understood. *PRR7* and *PRR9* are both believed to be involved in the temperature input of the clock as the *prr7/prr9* double mutant do not maintain circadian rhythms after temperature entrainment (Abe *et al.*, 2005; Salome *et al.*, 2005). However it may be possible that this double mutant phenotype is a consequence of the role of these genes in the central oscillator itself (Farre *et al.*, 2005).

1.1.11 Photoperiodic Pathway

Arabidopsis is a facultative long day (LD) plant and as such will flower in both LD and short days (SD), but much earlier in LD conditions. Photoperiodic flowering pathway mutants only demonstrate an effect in one photoperiod, mutants such as *co*, *gi*, *lhy*, *ft*, *fwa*, and *fha* delay flowering under LD but not SD conditions (Redei 1962; Koornneef *et al.,* 1991).

In Arabidopsis the photoperiodic pathway is regulated by photosensory receptors including the five red/far-red light-receptor phytochromes (Phy A-E) the two blue/UV-A light-receptor cryptochromes (cry1 and 2) (Mockler *et al.*, 2003)

Phytochromes and cryptochromes perceive light and are responsible for resetting the circadian clock (Hayama *et al.,* 2004). The underlying mechanism of photoperiodism is not yet fully understood but extensive research supports the hypothesis for the external coincidence mode (Suarez-Lopez *et al.,* 2001) (Figure 4). This model proposes that light must interact at the appropriate time with an endogenous rhythm in order to confer a photoperiodic response (Bünning 1936). This endogenous rhythm is created by the circadian clock, which as well as being intrinsic to the response to photoperiod also regulates hypocotyl elongation, leaf movement and numerous other processes within the plant (Suarez-Lopez *et al.*, 2001). The circadian clock is formed by several genes acting in a negative feedback loop. *LATE ELOGATED HYPOCOTYL (LHY)*, and *CIRCADIAN CLOCK ASSOSIATED* 1 *(CCA1)* negatively regulate the expression of *TIMIMG OF CAB EXPRESSION 1 (TOC1)* and *EARLY FLOWERING 4 (ELF4)* genes. Conversely *TOC1* and *ELF4* positively up regulate *LHY* and *CCA1* expression. These genes all function as part of the central circadian oscillator (Figure 5). *EARLY FLOWERING 3 (ELF3)* has been shown to function on the light input

pathway into the circadian clock, acting between the photoreceptors and the central oscillator (Hicks *et al.,* 1996) (Figure 5). Plants carrying mutations in the *elf3* gene have no detectable circadian rhythms in continuous light and flower early in short days (Hicks *et al.,* 1996).

The circadian clock drives the rhythmic expression of a large number of genes, one of which is *CONSTANS (CO)* a key regulator of the photoperiodic flowering pathway (Hayama *et al.,* 2004). *CO* together with *FT* plays a major role in the photoperiodic pathway, *co* and *ft* mutants are late flowering in LD but are only slightly effected in SD (Koornneef *et al.,* 1991). When over-expressed, both *FT* and *CO* produce early flowering phenotypes in both SD and LD conditions. *CO* is a transcription factor which encodes a B-box zinc finger protein and a CCT domain (Putterill *et al.,* 1995; Robson *et al.,* 2001). It is a transcriptional regulator that activates *FT* expression but is not believed to bind to DNA itself (Hepworth *et al.,* 2002). *FT* encodes a RAF-kinase inhibitor-like protein and is transported to the phloem and meristem to trigger flowering (Kardailsky *et al.,* 1999).

The expression of *CO* follows a rhythmic pattern in both LD and SD with generally low expression levels in the light and a peak of expression in the dark (Suarez-Lopez *et al.,* 2001).However in LD when the light period is longer there is an addition peak in *CO* expression towards the end of the light period. Light stabilises the CO protein in the evening and it's the coincidence of light with this peak in *CO* expression towards the end of the long day that leads to activation of *FT* and flowering. In SD, high levels of *CO* expression only occurs in the dark, there is no induction of *FT* and flowering is delayed (Figure 4.) (Yanovsky *et al.,* 2002)

The regulation of CO stability is also under the control of the photoreceptors which act antagonistically to generate rhythms in *CO* abundance (Valverde *et al.*, 2004) (Figure 5).

The reason why there is no induction of *FT* during the dark even though there are high levels of *CO* expression is due to CO protein being degraded in the dark by a process involving ubiquitination and degradation by the 26S proteosome (discussed later). During the light period this degradation is suppressed by the action of PhyA and cry1 and cry2 (Suarez-Lopez *et al.*, 2001; Yanovsky *et al.*, 2002; Valverde *et al.*, 2004).

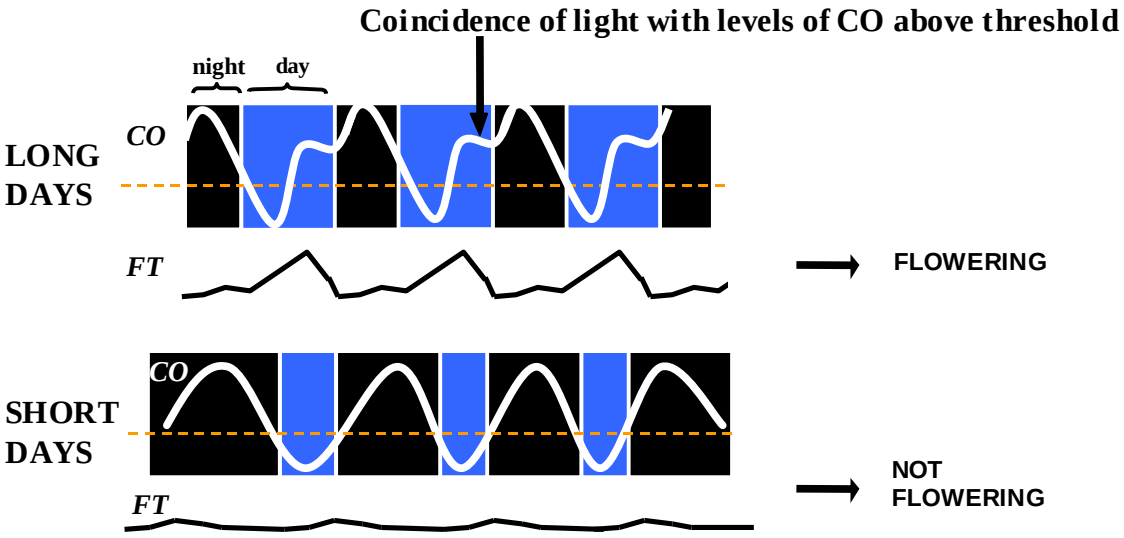


Figure 4. External Coincidence Model (Suarez-Lopez et al. 2001).

ZEITLUPE (ZTL) (Somers *et al.*, 2000), *FLAVIN BINDING KELCH REPEAT F-BOX* (FKF) (Nelson *et al.*, 2000) and *LOV KELCH PROTEIN (LKP)* (Schultz *et al.*, 2001) belong to a family of genes that have all been shown to have an influence on flowering time. Both the *ztl* mutant and *LKP2* over expressers produce a late flowering phenotype in LD and alter, or abolish, a number of circadian rhythms (Somers *et al.*, 2000). A mutation in *fkf1* has less influence on circadian rhythms but leads to a late flowering phenotype in LD because the evening peak of *CO* expression is abolished in the *fkf1* mutant (Nelson *et al.*, 2000). FKF1 targets CYCLING DOF FACTOR 1 (CDF1), a repressor of *CO* expression for degradation in LD therefore enabling the evening peak of *CO* expression to occur which induces early flowering in LD compared to SD.

ZTL, *FKF1* and *LKP2* encode proteins that contain an F-Box (involved in the ubiquitination and protein degradation pathway), a PAS domain, six repeated Kelch motifs and a light sensing and oxygen voltage (LOV) domain (Boss *et al.*,

2004). Recent studies of the LOV domain in *FKF1* has provided evidence that ZTL, FKF1 and LKP2 proteins form a new family of blue light photoreceptors that are possibly implicated in a unique light-signalling pathway (Schultz *et al.*, 2001; Imaizumi *et al.*, 2003). ELF3 and RED and FAR-RED INSENSITIVE 2 (RFI2) have been shown to be negative regulators of *CO* expression however the mode by which they do so has not yet been determined (Hicks *et al.*, 2001; Chen *et al.*, 2006).

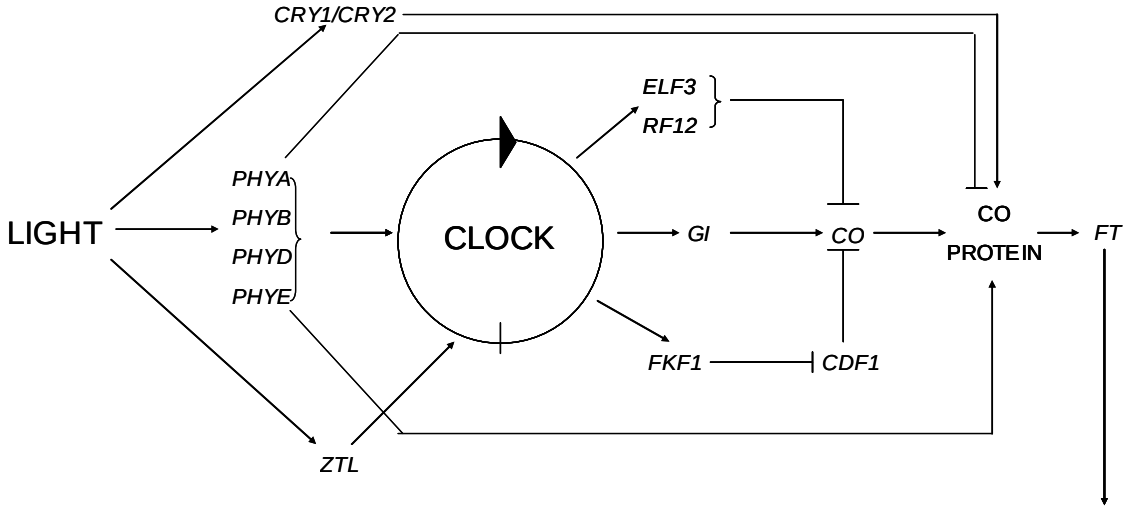


Figure 5. **Illustration of the components of the photoperiodic induction pathway.**

1.1.12 Florigen

Florigen is the floral stimulus that is produced in the leaves in response to an inductive photoperiod. The signal moves through the phloem and sieve elements to the shoot apical meristem (SAM) where floral initiation occurs. Grafting experiments have shown that this floral stimulus is graft transmissible and is conserved between different species and plants of different photoperiodic response types (Zeevaart 1976; Bernier *et al.,* 1993).This graft transmissible signal was first named Florigen however the biochemical nature of this molecule remained elusive for many years. As the perception of light occurs in the leaves and the induction of flowering at the SAM then flowering must occur via a long distance signal.

Recently, the flowering time genes *CO* and *FT* have been implicated in this long distance signalling mechanism reviewed by (Bäurle *et al.,* 2006). Flowering was shown to be promoted through leaf-specific expression of *CO* (Ayre *et al.,* 2004). In addition mis-expression of *CO* and grafting experiments have shown that *CO* acts in the phloem to promote flowering and that this is partly dependent on *FT* (An *et al.,* 2004).

FT protein has been shown to be a component of the florigen signal moving from the leaf to the shoot apex. At the SAM FT protein binds a bZIP transcription factor, FD, to activate floral meristem identity genes (Abe *et al.,* 2005; Wigge *et al.,* 2005). This suggests that *FT* is a component of the florigen signal (Corbesier *et al.,* 2007).

1.1.13 Role of Constans and regulation by GI

As daylength increases, flowering in Arabidopsis is accelerated. *CO* is a gene, which encodes a putative zinc finger transcription factor (Putterill *et al.*, 1995). Consistent with its role in flowering, expression of *CO* was up-regulated by LD, which in turn resulted in up-regulation of floral meristem identity genes, such as *APETALA1 (API)* and *LFY* (Putterill *et al.,* 1995; Simon *et al.,* 1996; Nilsson *et al.,* 1998). The external coincidence model proposes that the timing of *CO* expression needs to coincide with light for accurate measurement of daylength and floral initiation to occur. *CO* transcript is regulated by the circadian clock and peaks between 16-20 hours after dawn. The phase of *CO* expression is dependent on the circadian clock and is crucial for floral initiation (Roden *et al.,* 2002). In non-inductive SD *CO* expression mainly occurs in the dark hence is not able to promote flowering (Suarez-Lopez *et al.,* 2001). However, it is the coincidence of *CO* expression with the light in LD that ultimately leads to the perception of a LD daylength and the promotion of flowering (Yanovsky *et al.,* 2002). The photoreceptors PhyA and cry2 have been shown to stabilise the CO protein in the light whereas CO protein is degraded in the dark via the 26s proteasome thus adding support to the external coincidence model (Valverde *et al.,* 2004). Over-expression of *CO* results in early day length-insensitive flowering whilst loss of *CO* function leads to late flowering in LD (Simon *et al.,* 1996; Samach *et al.,* 2000).

GI has a complex role in both the regulation of the circadian clock and the promotion of flowering in response to photoperiod. Daylength, the circadian clock, light quality and temperature all have an effect on the expression of *GI*. The *gi* mutant has a late flowering phenotype that is more pronounced in LD and was first identified by (Redei 1962). Over-expression of *GI* with the 35S

promoter leads to plants that flower early in LD and SD (Mizoguchi *et al.,* 2005). *GI* functions to promote flowering via *CO* (Suarez-Lopez *et al.,* 2001). However *GI* may act via multiple pathways to promote flowering due to the fact that when 35S:*GI* was introduced into *co* and *ft* mutant plants an intermediate flowering time was observed which indicates that the early flowering phenotype of 35S:*GI* is only partially dependent on *CO* and *FT* function (Mizoguchi *et al.,* 2005). *ELF3* has been shown to regulate flowering through *GI* in a *CO*-independent process which implies a direct regulation of *FT* by *GI* (Kim *et al.,* 2005).

Recent work has shown that MicroRNAs (mi-RNAs) also function as regulators of gene expression. These miRNAs (~2-27bp) that work by targeting complementary mRNA and have been shown to play a role in the regulation of flowering time and flower development (Mallory *et al.,* 2006). Much research is now being focussed on the study of miRNAs which has resulted in the finding that *GI* can induce *FT* independently of *CO* by the regulation of miRNA172 (Jung *et al.,* 2007). In this study miRNA172 did not show any daily oscillations in either LD or SD, its abundance was consistently higher in LD, there was increased levels in blue light and decreased levels in red light and the abundance of miRNA172 was reduced in the *gi-2* mutant (Jung *et al.,* 2007). Theses results indicate that miRNA172 promotes flowering in LD and is regulated by *GI*. Therefore *GI* must have a dual role in the photoperiodic control of flowering by regulating *CO* and miRNA172 abundance which both independently lead to *FT* induction and floral promotion (Jung *et al.,* 2007).

1.1.14 Short Day Plants.

In contrast to Arabidopsis, rice is a SD plant. This SD flowering phenotype allows rice to synchronise its flowering and reproduction with the rainy seasons. Rice along with other monocotyledons such as barley and wheat are plants of major agricultural importance and as such has lead to a large area of scientific study. Sequencing the rice genome in 2005 (International Rice Genome Sequencing Project 2005) has facilitated the identification of rice homologues of Arabidopsis genes that control the floral transition (Izawa *et al.,* 2003).

Flowering time in rice is referred to as heading date (Hd) and a large amount of variation is observed in heading date and photoperiodic response among rice cultivars and strains. Several combinations of crosses of cultivars have lead to the identification of Quantitative trait locus (QTL) in rice and to the identification of at least 15 loci (Hd1 to Hd15). A mapped based cloning approach was use in the identification of *Heading date 1* (*Hd1*), an ortholog of *CO* in *Arabidopsis.* As such *Hd1* has been shown to be involved in the promotion of heading under SD conditions and inhibition under LD conditions (Yano *et al.,* 2001). Hd1 has been shown to encode a GATA1-type protein that has a high degree of similarity to the zinc finger domain and the C-terminal region of *CO* (Yano *et al.,* 2000). Furthermore *Hd1* was shown to be expressed rhythmically in a similar manner to *CO* in Arabidopsis (Kojima *et al.,* 2002).

In addition to *Hd1* other heading date loci have been shown to control photoperiodic responses. These include *Hd2, Hd3, Hd4, Hd5* and *Hd6* (Yamamoto *et al.,* 2000; Abe *et al.,* 2005). *Hd3a* shows a high level of similarity to Arabidopsis *FT* and functions floral promoter (Kojima *et al.,* 2002).In LD *Hd1* has been shown to inhibit the expression of *Hd3a* and promote its expression in SD (Izawa *et al.,* 2002; Kojima *et al.,* 2002). Thus indicating that in rice *Hd1* acts

to repress the expression of *Hd3a* which is the reverse of the role of *CO* on FT in Arabidopsis (Hayama *et al.,* 2003).

Hd5 encodes a putative subunit of a CCAAT-box-binding protein and is involved in inhibition of flowering under LD conditions. *Hd6* encodes an alpha-subunit of protein kinase CK2 and is also involved in inhibition of flowering under LD conditions.

1.2 Ubiquitination and E3 Ligase protein degradation.

The ubiquitin/26S proteasome pathway is the major proteolytic pathway in plants. Recent genetic analysis has implicated this pathway in numerous plant processes including photomorphogenesis and circadian rhythms (Vierstra 2003). Proteins are targeted for degradation by the covalent attachment of ubiquitin molecules. Once ubiqutinated the protein is then able to be recognised by the 26S proteasome complex which then degrades the target but leaves the ubiquitin molecules intact and ready to be used again (Moon *et al.,* 2004).

Protein degradation involves several steps involving E1, E2 and E3 ligases. E1 ligase is a ubiquitin activating enzyme involved in the catalyst of a thiol ester bond between itself and a ubiquitin molecule. E2 ligases are ubiquitin-conjugating enzymes which contain an active cysteine residue and are involved in an ATP-dependent transfer of the ubiquitin molecule from E1 to itself. E3 ubiquitin-protein ligases bind the target protein that is to be degraded and facilitate transfer of the ubiquitin from the E2 enzyme to the target protein. The process is continued until sufficient ubiquitins have been attached to the target protein to enable it to be recognized by the 26S proteosome whereupon the protein is degraded (Vierstra 2003) (Ni *et al.,* 2004)(Figure 6.).

The 26S proteosome is a multi-subunit protein complex consisting of a 20S core proteolytic complex and a 19S regulatory complex. The 20S complex is a cylinder made up of four stacked heptameric rings, with openings at either end of the cylinder. The active site of the complex is internal to the cylinder. The 19S complex regulates access to the opening of the cylinder and is also involved in the unfolding of the target protein allowing interaction with the active sites of the 20S core protein (Ayre *et al.*, 2004; Smalle *et al.*, 2004).

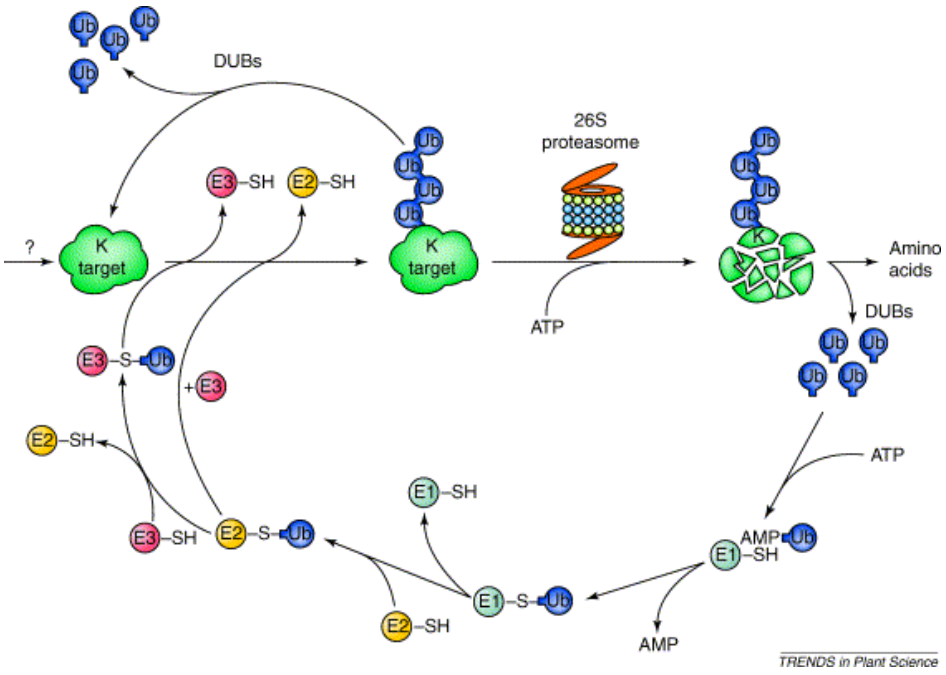


Figure 6. **The Unbiquitination cascade**.Taken from, Vierstra (2003). *Trends in Plant Science*

1.3 Chromatin Modifications and the plant homeodomain (PHD)

In addition to post-translational modifications, gene expression is also controlled at the level of transcription. Chromatin remodelling is one such way in which gene transcription can be governed. Chromatin is made up of tightly bound DNA wrapped around an inner octameric histone core (Figure 7.). This core is comprised of four histones which have a long N-terminal histone tails that extend out of the histone core. It is these tails that are subject to covalent modifications such as phosphorylation, methylation and acetylation (Figure 7.). Not all of these modifications will be on the same histone at the same time however they provide enormous potential for the control of gene expression. Typically methylations of lysine27 histone 3 (H3K17) is associated with chromatin silencing whilst H3K4 is associated with active chromatin (Kouzarides 2007). An example of this is the trimethylation of Histone (H3) at lysine 4 which has been shown to be associated with active FLC expression. Furthermore when the H3 is deacetylated at lysine 9 and 27 this leads to the repression of *FLC* (He *et al.,* 2005).

The addition of these modifications encourages the recruitment of proteins that bind via specific domains, for example the Plant HomeoDomain (PHD). PHD domains were first identified in plants consist off a zinc-binding Cys4-His-Cys3 motif and have been shown to recognise methylation of histones (Kouzarides 2007). These domains are protein-protein interaction domains that are typically involved in chromatin remodelling and have been implicated in the control of flowering time examples of which are the floral repressors *VIN3* and *EBS* (Pineiro *et al.,* 2003; Bastow *et al.,* 2004).

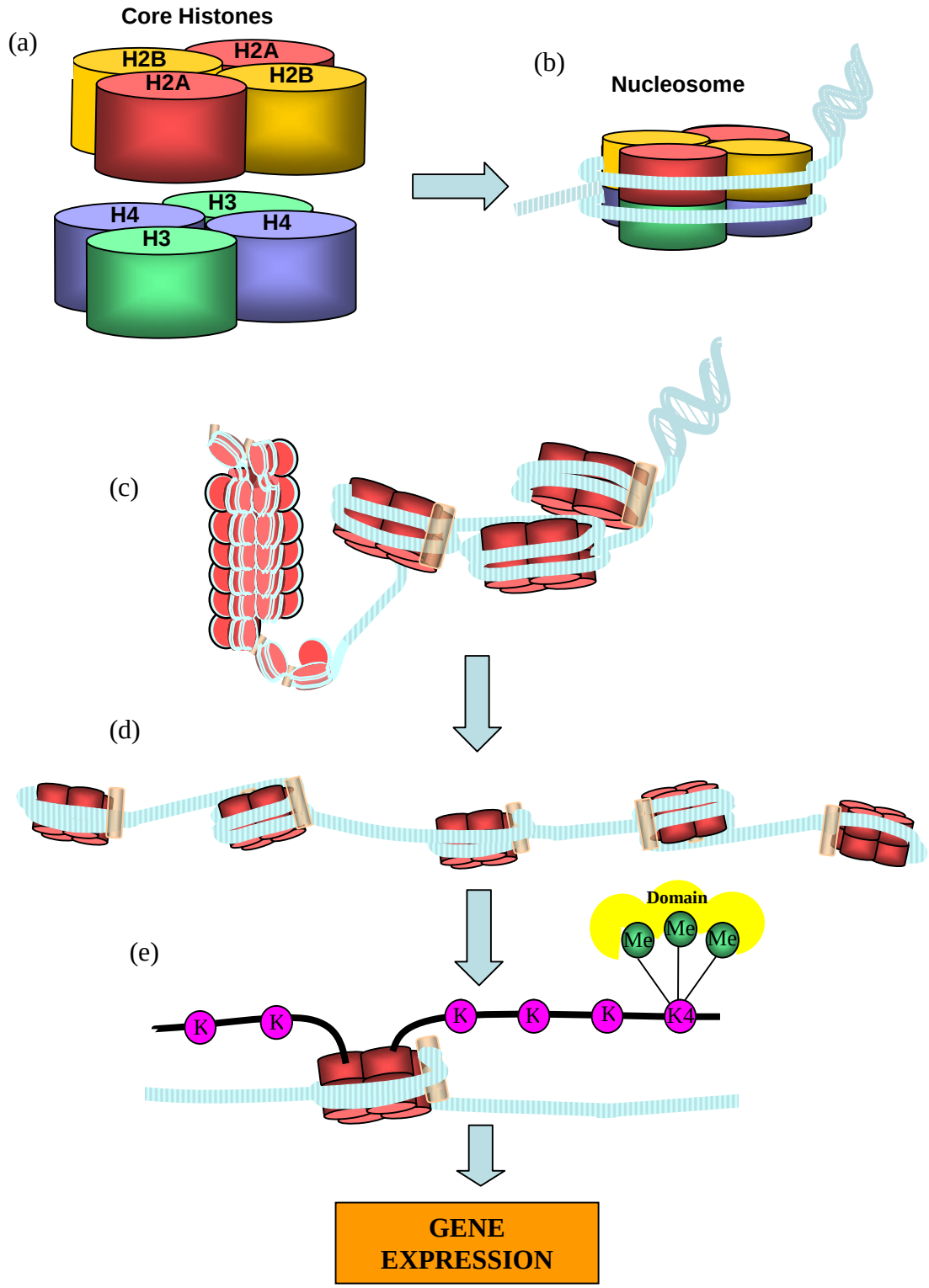


Figure 7. **Illustration of Chromatin remodelling**.
(a) The 8 histone core making up the nucleosome. (b) Chromatin wraps around the nucleosome. (c) Tightly bound chromatin structure. (d) Chromatin loosens during modification. (e) tri-methylation of H3K4 which is recognised by a protein domain leading to active chromatin.

1.4 INTRODUCTION TO DNF

The *dnf* mutant flowers early under SD conditions and at the same time as WT in LD (Figure 8.) however, other growth development aspects such as leaf and stem morphologies and flowering architecture remain the same as WT. It is, therefore, only affected in one photoperiod similar to other photoperiodic flowering pathway mutants such as *ft, co, fwa, gi* and *fha* which also display a phenotype under just one photoperiodic condition. In light of this, *DNF* is postulated to act within the photoperiodic flowering pathway (Koornneef *et al.,* 1998; Mouradov *et al.,* 2002; Simpson *et al.,* 2002).

Sequence analysis of the *DNF* gene (At3g19140) suggests that it may function as an E3 ligase or a PHD domain, therefore may have a role in protein degradation or through chromatin remodelling (Figure 9.) Previous analysis of both *CO* and *FT* expression using quantitative real-time PCR (qRT-PCR) indicated an altered expression pattern of both these genes in the *dnf* mutant. Unlike in WT where *CO* expression levels are low during the light period of a SD, in the mutant elevated levels of *CO* expression are observed at the end of the light period of a SD resulting in the induction of *FT* and early flowering in SD. This suggests that, in the absence of the *DNF* gene, expression of *CO* is de-repressed due to a possible absence of protein degradation.

The *DNF* gene encodes a small protein of 141 aa with a putative membrane spanning domain at the N-terminus and a C-terminal domain (Figure 9). From sequence analysis we know that the C-terminal domain within *DNF* has similarities to both RING-H2 and PHD domains. However the consensus sequence for both these domains is not quite met (Figure 10) In addition the gene sequence of the *dnf* mutant shows that the T-DNA insertion is located within this c-terminal domain (Figure 9).

Ubiquitin E3 ligases contain RING-H2 domains which are involved in targeting proteins for degradation. Many proteins with RING domains such as COP1 (Osterlund *et al.,* 2000) and CULLIN4 (Chen *et al.,* 2006) have been shown to act as or form part of E3 ligases directing the ubiquitination of specific proteins which subsequently targets them for degradation by the 26S proteasome. As *DNF* has sequence similarity to RING-H2 domains it would not be unreasonable to consider *DNF* acting as an E3 ubiquitin ligase in an ubiquitination complex which targets an activator of *CO* transcription for degradation. In the *dnf* mutant this activator is not degraded therefore *CO* expression is elevated in SD. However a study of predicted RING finger domains in the Arabidopsis genome found that DNF is not predicted to be an E3 ligase (Stone *et al.,* 2005).

PHD fingers are protein-protein interaction domains and tend to be found in nuclear proteins which play roles in regulating chromatin. (Aasland *et al.,* 1995; Bienz 2006). VIN3-LIKE 1 (VIL1) and EARLY BOLTING IN SHORT DAYS (EBS) and are examples of PHD domain proteins that have been shown to be involved in the control of flowering time (Pineiro *et al.,* 2003; Sung *et al.,* 2006)

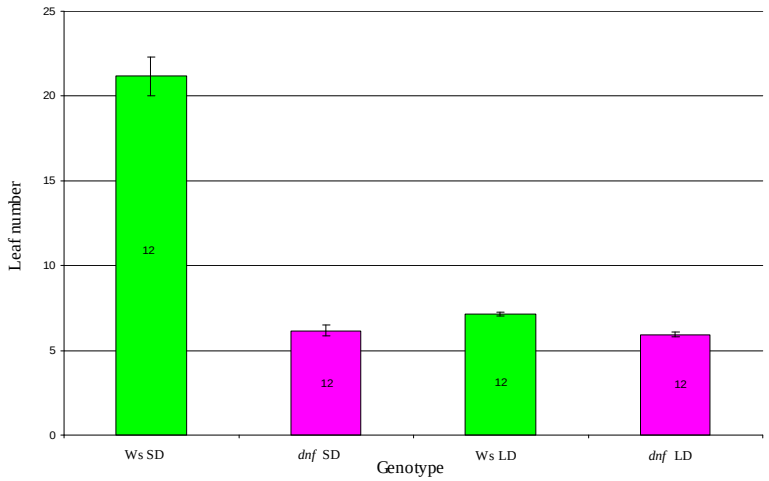


Figure 8. Flowering times of the *dnf* mutant compared to Ws. LD (16h) and SD (8hr) The numbers shown in the middle of each column indicate the number of plants analysed and the error bars indicate standard error.
Flowering time was also scored by days flower and a similar trend was observed.

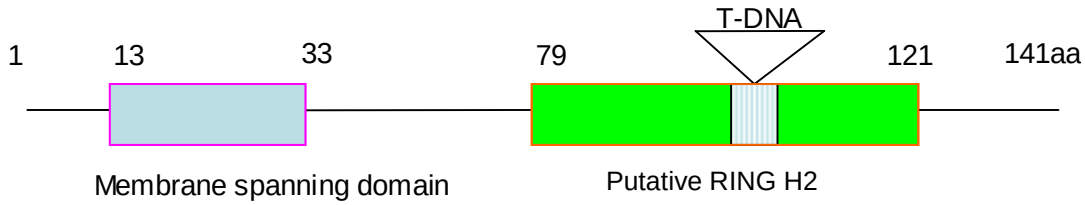


Figure 9. Illustration of DNF. Showing the position of the membrane spanning domain, the putative RING-H2 domain and the T-DNA insertion.

MNEDALEAVRSRTFFAILTVFYSIFRCCLAYCNKGDDDHLIHPSHSLHVIK

ATGINPSVLLSIPVVSFNANAFKDNIECVVCLSKFIDEDKARVLPSCNHCF

HFDFTDTWLHSDYTCPNCRKNVEEIQNHELSLSPNPNSG.

Typical RING H2

C-x(2)-C-x(9 to 39)-C-x(1 to 3)-H-x(2 to 3)-C-x(2)-C-x(4 to 48)-C-x(2)-C.

DNF Putative RING H2

C-x2-C-x(15)-C-x(1)-H-x C-x(1)-H x (8)-H-x(4)-C-x(2)-C.

Figure 10. DNF protein sequence 141 amino acid residues. Membrane spanning domain highlighted blue and putative Ring H2/PHD domain highlighted green

The primary aims of this project are to position *DNF* on the photoperiodic pathway, analyse the spatial expression of DNF, and to identify possible interacting proteins. To address these questions EGFP and epitope tagged DNF fusion protein constructs were made and transformed into WT and *dnf* mutant plants. *dnf:co-2* double mutants were also generated in order to determine whether the *dnf* mutation requires an active *CO* in order to confer the early flowering phenotype. Quatitative RT-PCR was also used to examine the expression of *DNF* in the *dnf* mutant, *DNF* over-expressers and WT plants.

Chapter Two

General Materials and methods

2.1 General materials and methods

2.1.1 Plant material

The wild type (WT) Arabidopsis plants used in this study were Wassilewskija-4 (Ws-4),Landsberg *erecta* (Ler),Columbia-0/2. The mutant Arabidopsis plants used in this study were *dnf*.

The background and stock number of each of the WT and mutant plants used in this study is listed in Table 1.

Germplasm	Background	Reference
dnf	Ws	
Ler	Landsberg *erecta*	
ABO		(Wagner *et al.*, 1991)
Noss	Nossen	(Magliano *et al.*, 2005)
Col-0	Columbia	
Col-2	Columbia	
phyA	Ws-4	(Whitelam *et al.*, 1993)
phyB-1	Columbia	(Reed *et al.*, 1993)
co-2	Ler	(Robson *et al.*, 2001)
cry1 cry2	Col-4	(Mockler *et al.*, 1999)
fha-1	Ler	(Guo *et al.*, 1998)

Table 1. Arabidopsis germplasm information. Mutants are described as specific mutation alleles in corresponding loci. The Background is the ecotype in which the mutation is found. The stock No. is the unique number assigned to the germplasm by the Arabidopsis Biological Resource Centre (ABRC). The *cry1 cry2* double mutant was obtained from Chentao Lin (University of California-Los Angeles). The *phyA* monogenic mutant was obtained from Gary Whitelam (University of Leicester).

2.1.2 Plant growth conditions

Unless otherwise stated plants were grown in Levingtons F2 compost containing 6 parts compost, 1 part sand and 1 part vermiculite. Seeds were stratified in the dark at 4 °C for 4 days, to achieve uniform germination. Plants were then transferred to Sanyo growth chambers and grown at 22 °C in either SDs, or LDs. SDs consisted of 8 hrs WL (100μmols^{-1}m^{-2}) followed by 16 hrs darkness supplied by BriteGro F36WT8 lamps (Sylvania, Germany). LDs consisted of 16 hrs of white light (WL, 100μmols^{-1}m^{-2}) followed by 8 hrs darkness.

2.1.3 Hypocotyl Elongation Assay

Seeds were sterilised and pipetted on to 3 % MS agar, supplemented with 1 % glucose. Seeds were stratified for 3 days in the dark at 4 °C. The seeds were then exposed to white light (100 μmol m^{-2} s^{-1}) for 3 hours and then placed back into the dark for a further 24 hr. Finally seeds were exposed to the appropriate light quality and fluence rate in a Percivil CLF plant Climatics growth cabinet (model 1-3LEDDLL3) for 4 days after which the length of each hypocotyl was measured with a ruler. Far-red LED's 0.1*u*Mol m-² s-¹ , Blue LED 0.4*u*Mol m-² s-¹ , Red LED 2.5*u*Mol m-² s-¹. Light source was measured using Stellar Net (EPP2000C) Spectroradiometer.

2.1.4 Flowering time analysis

Flowering time was measured by either, the number days to flower from when the plants are transferred to 22°C to when the bolt is 1 cm high. Or by number of rosette leaves when the bolt is 1 cm high. For ambient temperature

experiments the flowering time analysis was performed as stated above before being transferred to the different ambient temperatures of 16°C, 18°C, 20°C, 22°C and 27°C. Again flowering time was measured by either number of days to flower or number of rosette leaves when the bolt is 1 cm high.

2.1.5 Arabidopsis crosses

Sepals, petals and immature anthers were removed from the flower buds of the female parent plant using watchmaker's forceps leaving an unfertilized stigma. Stamens bearing dehiscent pollen were taken from the male parent plant and its anther was rubbed directly onto the stigma of the female parent. Stigmas were labelled and wrapped in clingfilm for two days. Mature siliques were collected approximately four weeks after fertilisation.

2.1.6 Arabidopsis Transformation

Arabidopsis plants were transformed using the floral dip method (Clough *et al.*, 1998). 200 ml of LB media containing the appropriate antibiotics was inoculated with *Agrobacterium tumefaciens* Strain GV3101 transformed with the appropriate construct and grown for 2 days at 28 ºC. The culture was centrifuged and the pellet resuspend in 5 % sucrose to an OD_{600} 0.8. Silwet L-77 was added to a concentration of 0.05 % and mixed well. Flowering Arabidopsis plants were dipped into the solution for 5 seconds. Plants were then returned to normal growth conditions and covered by a dome for 1 day. Arabidopsis plants were dipped again as above one week after the initial dipping.

2.1.7 Seed Sterilisation

Seeds (approximately 20mgs) were sterilised in 1 ml 95% ethanol for 5 mins. The ethanol was removed by pipetting and 1 ml of 20% bleach with 0.1% Tween 20 is added. Samples are left in the bleach solution for 5 mins before removing then washing the seeds 5 times with sterile water. The seeds were then suspended in 1ml top media before pipetting onto Murashige and Skoog (MS) agar plates (Murashige *et al.,* 1962) containing sucrose and the appropriate concentration of hygromycin (see below).

2.1.8 Hygromycin selection

Hygromycin is extremely toxic to mammalian cells and great care was undertaken when using. All work with hygromycin was carried out in a laminar flow bench and all waste disposed of as described by COSHH. 2XMS was prepared by the following method: 2XMS4.3g/ MS salts (GIBCO\BRL) with 10g/L sucrose, pH 5.7 (1M KOH), autoclaved before adding 7g/L Agar. Hygromycin was added to the MS media at a working concentration of 20mgs/ ml (1ml of 20mgs/ml hygromycin/L 2XMS). Chalforan cefotaxime (1g powder) + 4mls filter sterilized water was also added to the media to help prevent agrobacterium growth on the plates.

1 L of media was sufficient for 10 large (14cm) plates (approx 80 mls each), two small l (8cm) for positive and negative controls leaving 100mls for top media (without antibiotic selection)

The sterilised seeds were added to 1ml of top media and pipetted onto the plates before placing in a cold room 4ºC in the dark for 2-3 days. The plates were then brought into the light for 4-12hrs to promote germination.

Next the plates were wrapped up in tin foil and placed in the dark for 5 days starting from the beginning of the light period; longer growth in the dark will reduce recovery in the light.

After 5 days the hygromycin sensitive seedlings will be lying on the media with short hypocotyls and hygromycin resistant with long hypocotyls and closed cotyledons. The plates were then placed in a weak light (lab bench) 2-3 days to allow hygromycin resistant seedlings to green up. The transformants were then transferred to soil for growth in appropriate conditions.

2.2 General molecular biology

2.2.1 An over view of the DNF cloning strategy

Both N and C- terminal fusions of *DNF* were generated using NTAP, or EGFP and CTAP tags, respectively. Either the native promoter or an over expressing (P35S) promoter were used to drive expression of downstream targets (Figure 17.).These fusion proteins were initially constructed in pBluescript cloning vectors. The pBluescript (Figure 14.) cloning vectors are designed for DNA cloning and contain the *lac* Z gene encoding the N-terminal fragment of beta-galactosiadase which allows blue/white selection screening of recombinant plasmids. They also contain the selectable marker Ap^R conferring resistance to ampicillin and allows easy replication in *Escherichia coli*.

After selecting positive clones, they were sequenced using M13F and M13 reverse primers, and then further checked for the appropriate protein translation.

The T-DNA insertion in the *dnf* mutant confers resistance to Basta and Kanamycin (Figure 11). It is for this reason that the fusion proteins were sub-

cloned from Bluescript using HindIII and SacI restriction enzymes, into the BIB-HYG vector. This enables the transgenic plants obtained to be selected on hygromycin.

Name	**Sequence 5'→ 3'**
NTAPF	GG**GAATTCTAGA**ATGGTGGTCGACAACAAGTTC
NTAPR	CCGAAT**TCTAGA**CAGTGCGCCGCTGGAGCTGAT
CTAPF	AG**GGATCC**T**CCATGG**AGAGCAGCAGATGGAAG
CTAPR	CG**GAGCTC**TCACTTTGGGGCTTCGGCATC
DNFR (-Stop)	G**GGATCC**ACCGGAATTAGGGTTTGGACT
DNFR (+Stop)	GC**GAGCTC**CTAACCGGAATTAGGGTTTGG
DNFF	CG**GAATTCTAGA**ATGAACGAAGATGCTCTCGAA
EGFPF	CG**GGATCCATGG**TGAGCAAGGGCGAGGAG
EGFPR	CG**GAGCTC**TTACTTGTACAGCTCGTCCAT
P35SF	CG**AAGCTT**GACTAGAGCCAAGCTGATCTC
P35SR	CG**GAATTCTAGA**TCGACTAGAATAGTAAATTGT
PDNFF	TG**AAGCTT**GGTGATTTCGTTGGTTTGTTAT
PDNFR	CG**GAATTCTAGA**CTTAAAAACTTTTGCAATATC

Xba1
BamH1
Nco1
Sac1
EcoR1
HindII

Table 2. Primers for fragments used in the construction of plasmids, incorporating appropriate restriction sites. (F=forward R=reverse).

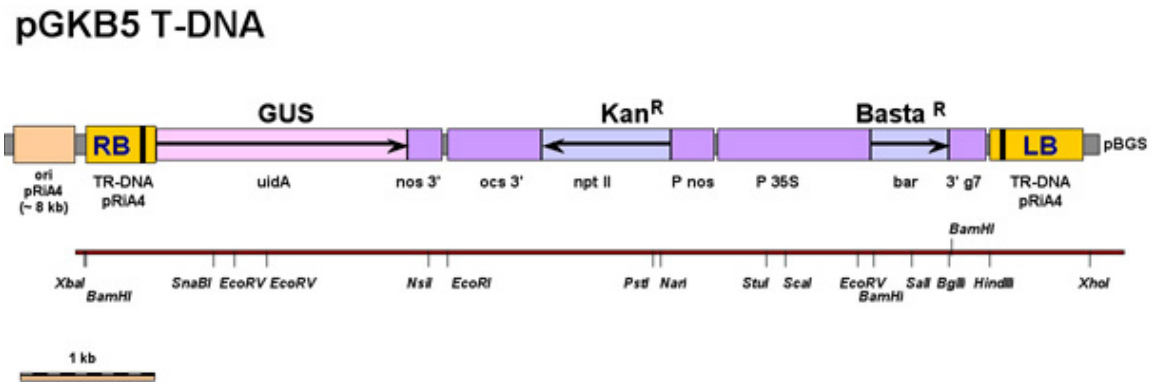


Figure 11. Illustration of T-DNA insertion (Taken from http://weedsworld.arabidopsis.org.uk/Vol2ii/pelletier.html) . LB and RB refer to the left and right borders of the T-DNA.GUS is a reporter gene Kan confering resistance to the antibiotics Kanamycin and Basta confering resistance to the herbicide basta (phosphinotricin)

2.2.2 Polymerase chain reaction (PCR)

2.2.2.1 Standard PCR

Unless otherwise stated Taq DNA polymerase (Invitrogen) was used in standard PCR reactions (20 µl). The components and final concentration of standard PCR reaction components were 1 unit Taq DNA polymerase, 10X PCR buffer (1X), dNTPs (Invitrogen, 2 mM each), $MgCl_2$ (1.5 mM), forward and reverse primers (0.5 µM each), template DNA (0.5 µl) and MiliQ water was used to make up the volume to 20 µl. Amplification cycles (35) were as follows: 95 °C for 30 sec, 56 °C for 30 sec, 68 °C 1 min. Unless otherwise stated amplicons were electrophoresed on agarose gels (2 %, w/v) and visualised on a transilluminator. Amplicons were purified using QIAquick gel extraction kit (Qiagen).

2.2.2.2 Zygosity PCR (Genotyping).

In order to identify T2 transformed lines zygosity PCR's were performed using three primers. (F represents forward primer and R represent reverse primer).

The oligonucleotide primers used to genotype the plants were:-

DNFF (5-TGAATGAAGCCATGTGTCAG-3)

DNFR (5-GGATCAACCCTTCCGTTCTTT-3)

RBR (5- CGAAACGCAGCACGATACG-3).

DNFF and DNFR will amplify a fragment 178bp from Ws plants that do not contain the T-DNA insertion. DNFF and RBR will amplify a fragment 482bp from plants that do contain the T-DNA insertion, the size of the T-DNA insertion prevents amplification with DNFF and DNFR primers. Heterozygous T-DNA lines amplified both a 178bp product from DNFR and DNFR primers and a 482bp product from DNFF and RBR primers. WT lines amplified one 178bp fragment from DNFF and DNFR and homozygous lines amplified one 482bp fragment from the DNFF and the RBR primers.

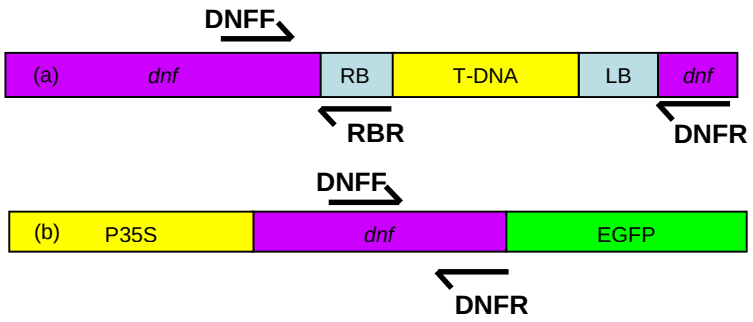


Figure 12. Primer sites for genotyping of *dnf*. (a) *dnf* mutant, (b) Ws

2.2.2.3 High fidelity PCR

Novagen KOD hot start DNA polymerase was used to amplify the fragments to be used in the cloning strategy. This is a high fidelity kit contains a thermostable DNA polymerase which exhibits higher accuracy than other commercially available polymerases thus reducing the risk of error. The components and final concentration of KOD PCR reactions were 1 unit KOD Hot Start DNA Polymerase (1unit/*ul*), 10X PCR buffer for KOD Hot Start DNA Polymerase, (1X), dNTPs (KOD, 2 mM each), $MgSO_4$ (2.5 mM), forward and reverse primers (10 µM each), template DNA (1 µl) and MiliQ water was used to make up the volume to 25 µl. Amplification cycles (35) were as follows: 94 °C for 5 mins,94 °C for 30 sec, 55 °C 1 min and 72°C for 30 secs. Unless otherwise stated amplicons were electrophoresed on agarose gels (1%, w/v) and visualised on a transilluminator. Amplicons were purified using either the QIAquick PCR purification kit or the QIAquick gel extraction kit (Qiagen).

2.2.2.4 Quantitative RT-PCR

Quantitative real-time PCR was used to detect the levels of *CO*, *FT*, *GI*, *CAB* and *DNF* mRNA abundance. RNA was extracted from Arabidopsis plants using Z6 total RNA extraction protocol. 5µg of RNA was DNase treated with 1µl DNase (Roche) and made up to a total of 9 µl with MilliQ water. The RNA samples were then heat at 37 ºC for 1 hour before inactivating the DNase at 75ºC for 10 mins. The RNA samples were then used to synthesise cDNA using the Super Script First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions.

A real-time qRT-PCR assay was performed using a (ABI Prism ® 7900HT) Taqman machine (Applied Biosystems). Each reaction contained

0.4 μM of the forward and reverse primers (Table 3), 6 μl of DEPC treated H2O and 7.5 μl of Applied Biosystems SYBR® GREEN PCR 2xMaster Mix with the exception of *DNF* primers where the concentration was reduced to 0.2 μM..Triplicate reactions were run for each sample.

The cycling parameters consisted of: 95°C for 10 min, followed by 50 cycles consisting of denaturation at 94°C for 15 seconds and annealing/extension at 60°C for 1 min. Melt curve analyses were performed to show that only a single product was being amplified in each reaction.

The raw data was analysed using the default settings of the software for determining both the threshold value and baseline. In each assay, a standard curve for the primer set was generated using 10 fold serial dilutions of and used to determine the relative copy number in unknown samples. Reactions were optimised so that efficiencies were equal to 100 % ± 10%. ABI prism SDS2.1 software version was used to analyse assay results.

Target Gene	**Primer sequence**	**Amplicon bp**
DNF	**F** 5-GACACATGGCTTCAGACT-3	102
	R 5-ACCGGAATTAGGGTTTGGAC-3	
ACTIN (ACT2)	**F** 5-TGTCGCCATCCAAGCTGTTCTCT-3	85
	R 5-GTGAGACACCATCACCAGAAT-3	
FT	**F** 5-GGCCTTCTCAGGTTCAAAACA-3	118
	R 5-TCGGAGGTGAGCGTTGCTA-3	
CO	**F** 5-GAGAAATCGAAGCCCGAGGAGCA-3	79
	R 5-TCAGAATGAAGGAACAATCCCATA-3	
GI	**F** 5-CACAGCTTGCTCTCCACAAC-3	80
	R 5-AAGTGGGTGCTCGTTATTGG-3	
CAB2	**F** 5-CTGAGTTGAAGGTGAAGGAG-3	115
	R ATGGTCAGCAAGGTTCTCTA-3	

Table 3. Primers used for quantitative real-time qPCR analysis. F= forward, R=reverse

2.2.3 Generation of plant transformation constructs

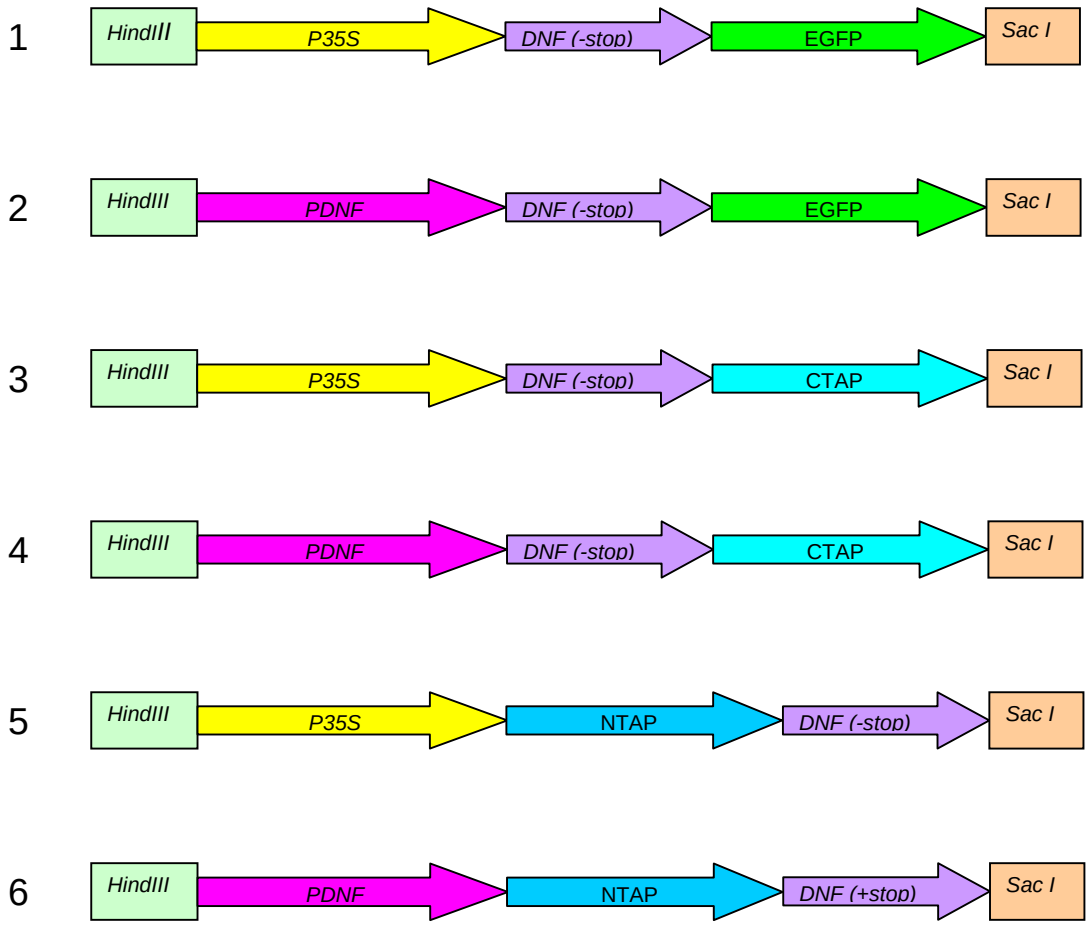


Figure 13.Illustration of *DNF* constructs.(1)P35S:DNF:EGFP,(2) PDNF:DNF:EGFP, (3) P35S:DNF:CTAP,(4) PDNF:DNF:CTAP,(5) P35S:NTAP:DNF,(6)PDNF:NTAP:DNF.35S refers to the constitutive 35S CaMV promoter. HindIII and Sac I refer to restriction. sites to allow digestion from the pBluescript II vector and ligation into BIB-HYG vector. *DNF*(+stop) and *DNF*(-stop) refers to with and without a stop codon respectively

2.2.3.1 Cloning into pBS vector

Gel purified fragments were digested using their specific restriction sites (Table:1) and ligated, firstly into bluescript cloning vector (Figure 14) then subcloned into pBIB-HYG cloning vector. As a general rule a ratio of 1:3 vector to insert is required and this was achieved using the following calculation.

$$\frac{(ng.vector)(kb.insert)}{kb.vector} x(molar.ratio.insert/vector) = ng.insert$$

$$eg\ \frac{(100ng)(0.6)}{3} x\frac{3}{1} = 60ng$$

The reaction components for ligations are 1.5*ul* 5xligation Buffer (1 unit/*ul* Invitrogen T4 DNALigase),1*ul* ATP,1*ul* Ligase (Invitrogen T4 DNA Ligase) 1:3 (vector:insert) made up to final volume 20*ul* with H2O.

.

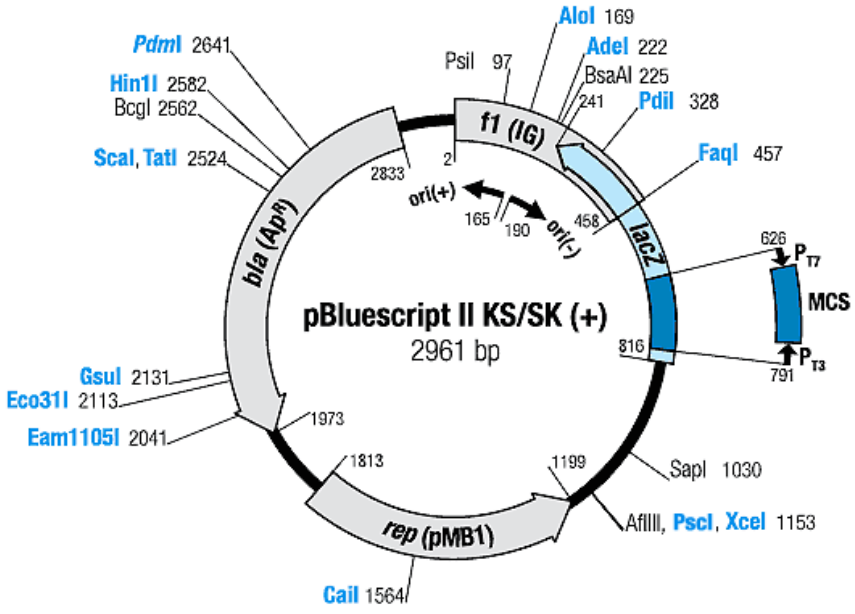


Figure 14. Vector map of pBluescript II
(*taken from,* http://www.fermentas.com/techinfo/nucleicacids/mappbluescriptiiskks.htm)

2.2.3.2 Phenol/Chloroform extraction and Ethanol Precipitation

The samples for precipitation were made up to 100*ul* total volume with sterile H_2O before adding a further 100 *ul* of (Phenol:Chloroform:IsoAmyl Alcohol 25:24:1 pH 6.6-8 (BHD)). The samples were then centrifuged for 5min 13,000g and the supernatant transferred to a new 1.5ml tube. 1/10th 3M Na Acetate and 2x100%EtOH was the added to the samples before placing in -20ºC freezer for 10 mins. The samples were then centrifuged again for 10 mins 13,000g before washing twice 70% EtOH. The pellet was dried before re-suspending in 10*ul* Tris-EDTA with RNASE 10mgs/ml (Invitrogen).

2.2.4 Plasmid transformation into bacterial cells

2.2.4.1. Transformation of Electro-Competent (EC100) cells

Unless otherwise stated all vectors were transformed into EC100 *E. coli* cells (Cambio) before selection on LB agar plates with the addition of the appropriate antibiotics. 20*ul* of 10% autoclaved glycerol was added to 20*ul* of EC100 cells before the addition of 5*ul* of the appropriate plasmid. The solution was pipetted into an electroporation curvette (Bio-Rad) and placed into the electroporator (Bio-Rad MicroPulser™) before pulsing. Immediately 1ml of room temperature SOC media (900mls deionised H20,12g bacto-tryptone, 24g bacto-yeast extract, 4mls glycerol and 20mM glucose, autoclave cool to 60ºC then add 20ml 1M glucose solution) was added to the transformation. The transformations were then left to recover at 37ºC for 1 hour before pipetting onto LB agar plates. Plasmids were prepared from resistant *E. coli* using quiagen plasmid mini-prep kit and the presence of the desired fragment confirmed by

PCR using specific primer (Table:1). Plasmids containing the required cDNA were sequenced using

M13F-(CCCAGTCACGACGTTGTAAAACG)

M13R-AGCGGATAACAATTTCACACAGG).

Clones without sequence errors were transformed into electrocompetant Agrobacterium GV3101 cells by electroparation.

2.2.4.2 Antibiotic selection LB Agar Plates

The plates were prepared using LB-BOUILION MILLER (MERCK) 25g/L, Agar MERCK 15g/L, with the addition of the appropriate antibiotic selection.

2.2.4.3 β galactosidase selection

1ml/L X-Gal (20mg/ml in dimethylformamide), 500*ul*/L isopropylthio-ß-D-galactoside was added to 1 litre of LB (IPTG,2mg/8ml sterile H2O volume adjusted to 10 mls and filter sterilized through a 0.22 micron disposable filter) and Ampicillin (Sigma)100mg/ml.

2.2.4.4 Preparation of electrocompetent *Agrobacterium tumefaciens*

The *Agrobacterium tumefaciens* strain used in this study was C58pGV3101. To propagate GV3101 cells 10 ml LB media containing 25 µg/ml rifampicin and 25 µg/ml gentamycin (to maintain the helper plasmid that carries the gentomycin selectable marker) were cultured over night at 28 ºC. 250 ml LB media was inoculated with the 10 ml over night culture and grown to an OD_{550} 0.5-0.6. The culture was transferred to ice for 30 min and then centrifuged at 3500 rpm for 15 min at 0 ºC. The supernatant was removed and the cells resuspended in 250 ml sterile chilled water. The culture was then centrifuged at 3500 rpm for 15 min at 0 ºC after which the supernatant was removed and the

cells were resuspended in 10 ml chilled 10 % glycerol. The culture was then centrifuged at 5800 rpm for 15 min at 0 ºC, the supernatant was removed and the cells were resuspended in 1 ml 10 % glycerol. Cells were aliquoted into 40 µl aliquots using a dry ice bath and stored at –80 ºC.

2.2.4.5. Transformation of *Agrobacterium tumefaciens*

Transformation of Agrobacterium was essentially the same as that for the transformation of Electro-competent (EC100) (see 2.2.4.1.) except that the incubation was at 28ºC for 1 hour before diluting to 1:1000 and transferring to LB agar plates containing 10 mg/ml rifampicin , 25mg/ml gentamycin and kanomycin 50 mg/ml.

2.2.4.6 Agrobacterium selection

1ml Kanamycin (50mg/ml), 500ul Rifamycin (10mg/ml) and 500ul gentamicin (25mg/ml), (Sigma) was added to 1 litre of LB.

2.2.4.7 Agrobacterium miniprep

A single resistant colony was used to inoculate 5 ml of LB media containing the appropriate antibiotics, which was grown over night at 28 ºC. The culture was centrifuged at 5000 rpm for 15 min. The supernatant was removed and the cells were gently re-suspended in 100 µl of miniprep solution 1 (50 mM glucose, 25 mM Tris.Cl pH 8 and 10 mM EDTA pH 8). 200 µl of miniprep solution 2 (0.2 M NaOH and 1 % SDS) was then added, the solution was mixed gently and place on ice for 4 min. 150 µl of miniprep solution 3 (3 M KoAc pH 4.8) was added, the solution was gently mixed and then centrifuged for 10 min at 13000 rpm. The supernatant was transferred to new tube, 400 µl of phenol/chloroform was added, and the solution was mixed and centrifuged at 13000 rpm for 5 min. The upper aqueous layer was transferred to a new tube, 1

ml 100 % EtOH was added, the solution was mixed and centrifuged at 13000 rpm for 10 min. The supernatant was removed and the pellet re-suspend in 100 µl water. 1 ml 100 % EtOH was added and the solution was placed on ice for 5 min. The solution was then centrifuged for 10 min at 13000 rpm, the supernatant was removed and the DNA pellet was air-dried. Finally the DNA was re-suspended in 25 µl sterile distilled water containing 20 µg/ml RNase A. The presence of desired plasmids was confirmed by PCR using gene specific primers.

2.2.5 Nucelic acid extraction and sequencing

2.2.5.1 CTAB genomic DNA extraction from Arabidopsis

CTAB-buffer B was made up of 100mM Tris/Cl pH 8.01,4M NaCl, 20mM EDTA (autoclaved) and 2% hexadecyltrimethyl ammoniumbromide (Doyle 1987). CTAB-buffer C was made up of 1% CTAB, 10mM EDTA, 50mM Tris 8 and 1M CsCl. Approximately 100 mg of frozen plant material was homogenized using a drill and modified frozen drill bit. 300 µl CTAB-buffer B was added and sample homogenized further. Samples were kept on ice before being transferred to a 65 °C water bath for 10 min. Samples were then centrifuged for 2 min. The supernatant was mixed with 300 µl chloroform/isoamylalcohol (24:1), mixed by pipetting and centrifuged for 2 minutes. The aqueous phase was mixed with 300 µl chloroform/isoamylalcohol and centrifuged for a further 2 minutes. The supernatant was mixed with 300 µl of CTAB-buffer C and incubated at RT for 30 minutes. Samples were then centrifuged for 10 minutes, after which the supernatant was removed and 400 µl 1M CsCl was used to dissolve the pellet. Once the pellet was dissolved 800 µl of 100 % ethanol was added and the samples were centrifuged for 10 minutes. The supernatant was then removed and the pellet washed two times with 500 µl 70 %

ethanol, centrifuging for 10 minutes after each wash. Following the last wash, the supernatant was removed and the samples centrifuged to ensure all traces of ethanol had been removed. The pellets were then allowed to air dry. Once dry each pellet was resuspended in 25 μl TE containing RNase (20 μg/ml). Samples were stored at 4 °C.

2.2.5.2 Total RNA extraction from Arabidopsis

Z6 Buffer was made up of 20mM MES, 20mM EDTA & 8M Guanidine HCL in a small volume of R.O water (50-100mls). Once dissolved made up to a final volume of 400mls with RO water then pH with NaOH to pH7. 200ul of ß-meracptoethanol was added to 40mls of Z6 buffer before grinding (Logemann *et al.,* 1987)**.**

Approximately 100 mg of frozen plant material was homogenized using a drill and modified frozen drill bit. 400ul Z6 Buffer & b-Mercaptoethanol was added whilst the tissue is still frozen and the tissue was further ground. 400ul Phenol:Chloroform:IAA (ratio 25:24:1) was then added to the tubes and vortexed. The samples were then microfuged for approx. 15mins @ 13rpm and the supernatant removed to a clean sterile eppendorf. Approximately $1/20^{th}$ (17.5ul) 1M Acetic acid and 0.7 vol. (245ul) 100% EtOH was added to each sample before placing in to a -20 freezer for at least 20mins. The samples were then mixed well before spinning 10mins @ 13rpm. The supernatant was removed and discarded and the pellet dissolved as much as possible in 3M Sodium Acetate (NaOAc) 500ul to remove carbohydrates. The samples were then spun again 10mins @ 13rpm, supernatant removed and washed with 70% EtOH, spin remove supernatant and wash again with 70% EtOH., before leaving to air dry and re-suspending in 50ul of sterile H_2O. Samples are stored at -70.

2.2.5.3 DNA sequence Analysis

Sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Each reaction contained the appropriate primer (4.2 pico mols), 4 μl of the Terminator Ready Reaction Mix and either 200-500 ng double stranded DNA or 30-90 ng PCR products DNA. Each reaction was made up to 10 μl with sterile distilled H_2O. PCR cycles (25) were as follows: 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. Sequencing electrophoresis was performed by an ABI automated sequencer (Applied Biosystems). Nucleotide sequence data was edited using VectorNTI (Invitrogen) or the EditSeq package within DNASTAR (DNASTAR, Inc).

2.2.6 Protein work

2.2.6.1 Arabidopsis total protein extraction

100 mg of frozen leaf tissue was ground in 500 µl LE buffer (50 mM lithium phosphate pH 7.2, 1 mM monoiodoacetic acid, 120 mM 2-mercaptoethanol, 10 mM Phenylmethanesulfonyl fluoride (PMSF), 10 mM E-64 and 50 mM MG132). Once the sample had thawed 50 µl of 20% lithium dodecyl sulphate (LiDs) was added and the sample was boiled for 45 seconds and then centrifuged for 20 min at 13,000 rpm. Following centrifugation the supernatant was transferred to a new tube and stored at -20°C.

2.2.6.2 SDS-PAGE and Immunoblotting

Proteins were separated on 12% SDS gels and transferred onto nitrocellulose membrane as described in manufactures instructions using Invitrogen **iBlot™ Dry Blotting System**. The membrane was then washed for 5 minutes with PBS. The membrane was blocked using dried milk powder dissolved in PBS for 1 hour. For detection of DNF and EGFP, CTAP and NATP fusion proteins blots were incubated with either anti-GFP (Open-biosystems),anti-TAP (Open-biosystems), or anti-DNF (Eurogentec), monoclonal antibodies at a final dilution of 1:10000 (PBS) for 1 hour. Excess primary antibody was removed by washing the PDF membrane twice with PBS containing 1% Tween 20 and twice with PBS containing 0.5% Tween 20 and 0.5% NaCl (each wash 5 minutes). The PDF membrane was then incubated with Horseradish Peroxidase (HRP)-conjugated to anti-mouse IgG at a final concentration of 1:10000 (PBS) for 1 hr. Excess antibody was removed by washing the PDF membrane three times with PBS containing 1% Tween 20, once with PBS containing 0.5% Tween 20 and 0.5% NaCl and then a finally

once with PBS (each wash 5 min). Antigen-antibody complexes were visualized using ECL detection reagents (Amersham Biosciences).

2.2.6.3 Confocal microscopy

Transgenic *Arabidopsis* leaves were mounted for microscopical observation in water under glass coverslips. The leaves were examined using a Olympus confocal fluoview IX70 laser-microscope. The Argon laser excitation wavelength was 488 nm EGFP emission was detected with the filter set for FITC (505–530 nm). The fluorescence of the images was assessed using the Olympus fluoview software.

Chapter 3

Physiological characterisation of the *dnf* mutant

3.1 Introduction

When seedlings emerge from below the ground following germination they perceive the light and undergo a process called de-etiolation. The buried seedling emerges from the ground and reaches towards the light and switches from etiolated to de-etiolated development. The apical hook straightens out, the cotyledons open up and hypocotyl extension is inhibited. This de-etiolated development involves complex interactions between phytochromes and cryptochromes . Phytocromes are responsible for perceiving red and far red light and cryptochromes for receiving blue light. Mutations in phytochrome and cryptochromes genes, *phy-B, phy-A cry-1/2,* affect the perception of red (R), far red (FR) and blue (B) light respectively.

Generally under low light intensities, development is initially under the control of PHYA. Then as the seedling reaches higher light intensities phyA is degraded as the control of development is overtaken by phyB and the cryptochromes (Fankhauser *et al.,* 1997; Sullivan *et al.,* 2003). As R, FR and BL all play a role in the de-etiolation process, defects in light perception at these wavelengths will affect the hypocotyl length of the developing seedling (Lin *et al.,* 1998; Reed *et al.,* 2000). *Cry* mutants lead to a defect in blue light perception, and such mutants will have a longer hypocotyl when grown in blue light compared to WT as blue light is no longer effective in inhibiting hypocotyl elongation. The same holds true for *phyA* mutants when grown in FR light and *phyB* mutants when grown in red light (Johnson *et al.,* 1994; Bagnall *et al.,* 1995).

The regulation of hypocotyl elongation can be measured by continuous illumination at different specific light wavelengths and is an easy way of assessing any defects in light mediated development reviewed by (Christian

Fankhauser 2004). Light quality also affects flowering with different wavelengths having different effects. For example, mutations such as *phyB* and *cry2* that cause early and late flowering respectively and also impair de-etiolation in RL and BL respectively (Reed *et al.,* 1993; Lin *et al.,* 1998). Furthermore PHYA plays a promotive role in flowering in Arabidopsis and the *phyA* mutant flowers later than wild-type plants when grown in LD (Johnson *et al.,* 1994).

Many genes involved in controlling the floral transition have been identified through mutants that either accelerate or delay flowering and have been commonly known as flowering time mutants (Redei 1962; Koornneef *et al.,* 1991) (Chapter 1).

Photoperiodic flowering pathway mutants only exhibit their effect in one photoperiod leading to plants that flower either early in SD or late in LD. For example, a mutation in *GI* gene leads to late flowering plants in LD but little or no effect is observed in short days (Fowler *et al.,* 1999).

By studying the epistatic interactions between genes important information such as the position and pathway which they function can be obtained. For example *co* and *gi* have been shown to be fully epistatic to *CRY2* (El-Din El-Assal *et al.,* 2003). This work demonstrated that *CRY2* needs *CO* and *GI* function in order to promote flowering.

The duration of day/night cycles in any 24 hr period can be measured in plants and requires a clock to measure time and photoreceptors to determine if it's day or night. As plants develop they often become synchronised with changes in the seasons and initiate developmental processes at times that anticipate the season in which they will be required. Wightman Garner and Henry Allard were plant physiologists who first reported the response of plants to

daylength (photoperiodism), work which has had a major influence on plant science and horticulture (Garner *et al.,* 1920). They went on further to classify plants as SD, LD or day neutral plants according to their specific response to critical day length (CDL). During their development plants go through a transition from a vegetative state to a reproductive flowering state and this transition is marked by the CDL. This CDL can alter with both the age of the plant and the environment in which it is grown. Critical day length for facultative LD plants such as Arabidopsis can be described as that photoperiod above or below which time to flower is minimal (Vince-Prue 1975). In short day plants if the photoperiod is longer than the CDL then flowering would be delayed. In long days plants if the photoperiod is shorter than the CDL then flowering would also be delayed (Vince-Prue 1975).

In addition to the four main flowering pathways and CDL, changes in ambient temperature and light quality also effect flowering time. When the temperature is reduced from 22°C to 16C° a delay in flowering is observed (Blázquez *et al.,* 2003). Furthermore exposure to low R:FR light ratios associated with the shade avoidance response leads to a promotion of flowering (Cerdán *et al.,* 2003).

The *dnf* mutant flowers early in SD but shows no effect in LD (Figure 20.). This phenotype is typical of photoperiodic mutants and as such lead to the belief that *dnf* lies on the photoperiodic flowering pathway.

As sessile organisms plants require the ability to respond rapidly to changes in the ambient temperature. These fluctuations in temperature can be during day night cycles or as the seasons change form colder winter conditions through to a warmer summer climate. There have been widespread studies on

how plants respond to extensive periods of cold (vernalisation) and many species require vernalisation before floral initiation. This strategy ensures that flowering will only occur in more favourable conditions such as spring or summer (Chapter 1). However little is understood about the processes involved in responding to the ambient temperature changes.

In Arabidopsis the onset of flowering is under the control of endogenous and environmental cues. Ambient temperature is one environmental cue that can have a profound effect on growth and flowering time. Increasing ambient temperature produces enhanced growth of Arabidopsis hypocotyls and advanced seed development (Halliday *et al.,* 2003).

The effect of different ambient temperatures on the growth of Arabidopsis can produce great variation in flowering time and leaf morphology. For example when Arabidopsis is grown at 23ºC it will flower earlier than when grown at 18ºC (Samach *et al.,* 2005)

The pathways controlling temperature inputs form a complex and integrated network many of which are also influenced by other external cues such as light. A photoreceptor mutant *phyB,* has an early flowering phenotype in both LD and SD. PhyB plays a significant role in shade avoidance, regulating flowering to altered ratios of red to far red light. At 23º in SD, PhyB represses flowering, a process that requires a functional PHYTOCHROME AND FLOWERING TIME 1 (PFT1) (Cerdán *et al.,* 2003). In *phyB* mutant plants this repression no longer occurs and therefore leads to an early flowering response. However when the *phyB* mutant is grown in SD at 16°C the plants flower as late as WT and therefore the early flowering phenotype is temperature dependent.

In this chapter we aim to characterise the *dnf* mutant with respect to light signalling, CDL, analysis of the flowering time of *dnf:co-2* double mutants and the effect of ambient temperature on its flowering.

3.2 Aims and objectives

To determine if light perception is altered in the *dnf* mutant.

To analyse the epistatic relationship between *CO* and *DNF*.

To determine the critical day length of the *dnf* mutant.

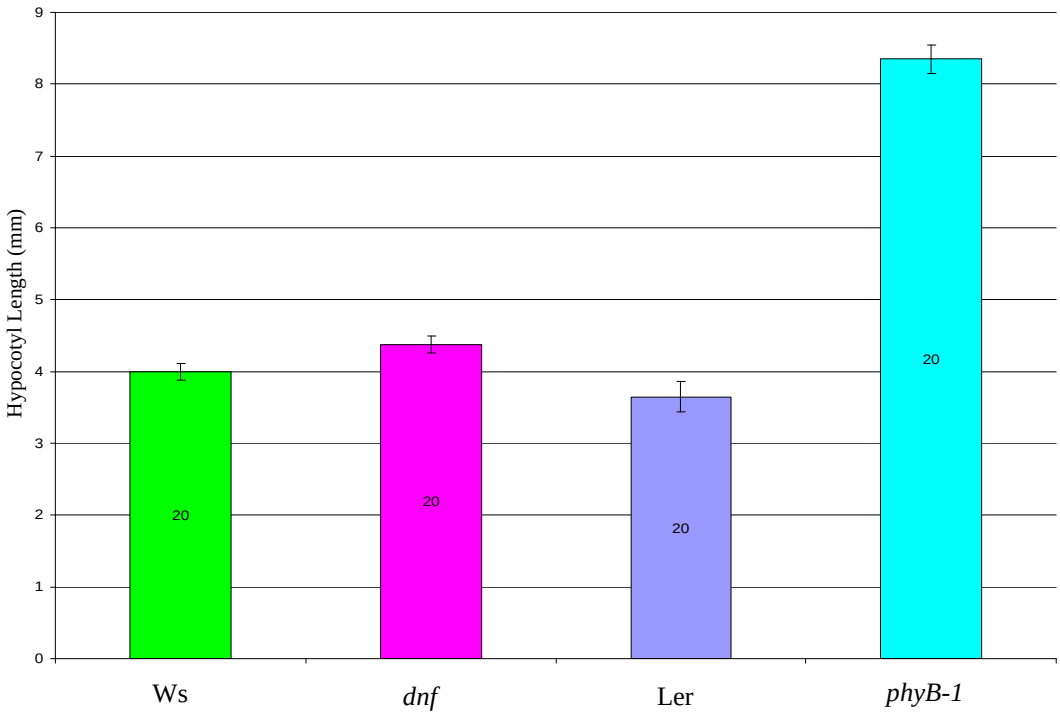
To determine if the early flowering phenotype or morphology of the *dnf* mutant is affected by different ambient temperatures.

3.3 Results

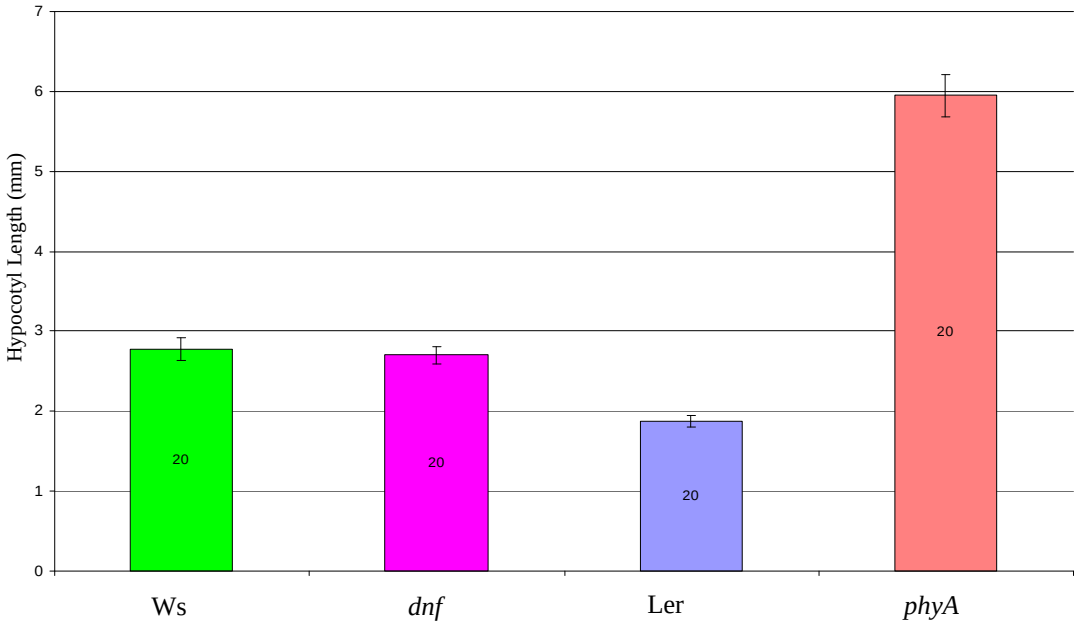
3.3.1 There is no defect in light perception in the *dnf* mutant.

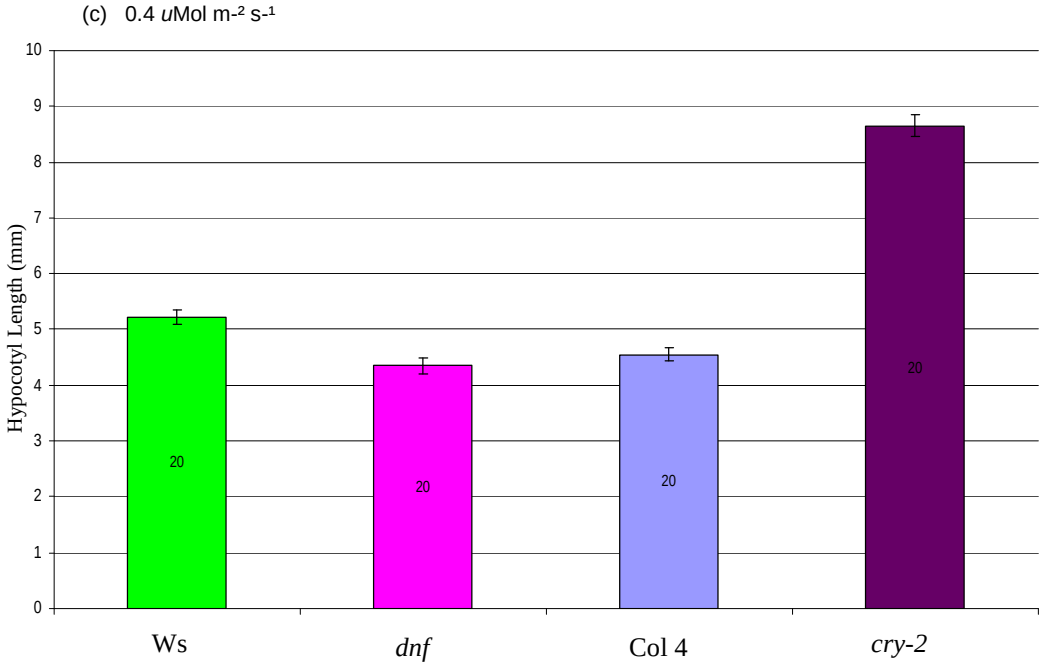
The de-etiolation of the *dnf* mutant in different light qualities was analysed to determine if the *dnf* mutant has a light perception defect. The hypocotyl elongation of the mutant was tested by growing in constant red, far-red and blue light, seedlings were also grown in the dark as a control. When grown under the different light fluencies no difference was observed between the hypocotyl lengths of the *dnf* mutant compared to Ws. Under R light the *phyB-1* mutant had an extended hypocotyl when compared to wild-type Ler (Figure 15.(a)) and likewise for the *phy-A* mutant grown in FR light (Figure 15.(b)) and the *cry-2* mutant compared to wild-type COL-4 grown in BL(Figure 15.(c)). When grown in constant dark all the seedlings showed elongated hypocotyls. From these results we can conclude that there is no defect at the fluence rates examined in the *dnf* mutant.

(a) 2.5 *u*Mol m-² s-¹



(b) 0.1 *u*Mol m-² s-¹





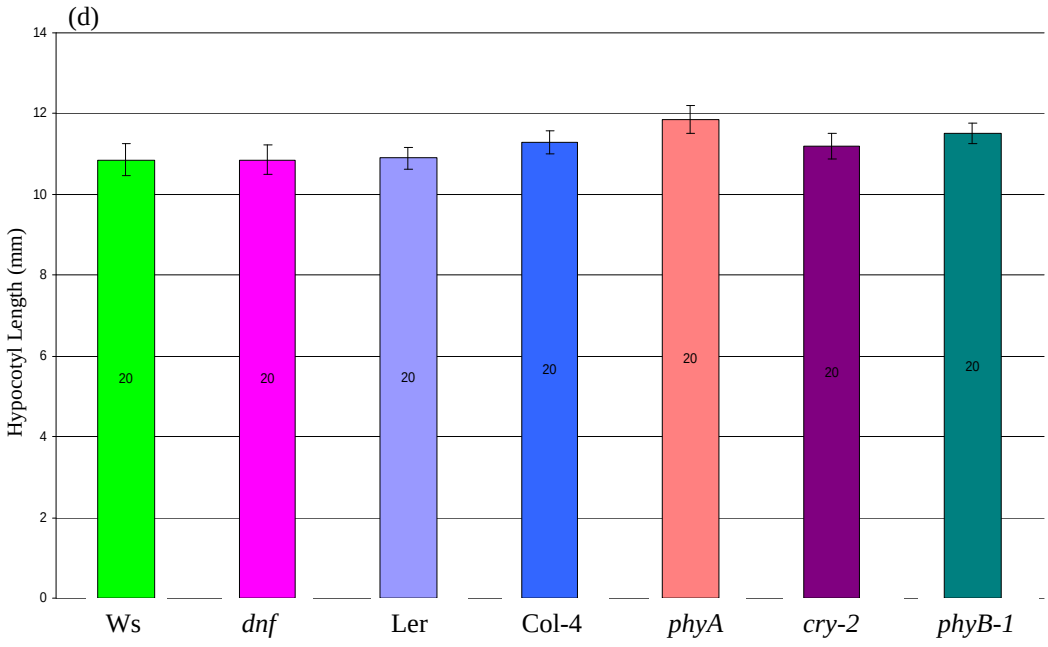


Figure 15, Hypocotyl length of young seedlings, (a) hypocotyl elongation in constant R *phyB-1* in *Ler* background (b) hypocotyl elongation in constant FR *phyA* in *Ler* background. (c) hypocotyl elongation in constant blue light *:cry-2* in COL-4 background. (d) hypocotyl elongation in the dark. *dnf* is in Ws background. The numbers shown in the middle of each column indicate the number of plants analysed and the error bars indicate standard error. The *dnf* mutant lacked the elongated hypocotyl phenotype under R,FR and B light conditions.

3.3.2 The *dnf* mutant requires an active *CO* to remain early flowering and lies on the same flowering pathway.

Photoperiodic flowering pathway mutants only exhibit their effect in one photoperiod leading to plants that flower either early in SD or late in LD. For example, a mutation in *CO* gene leads to late flowering plants in LD but little or no effect is observed in short days (Putterill *et al.,* 1995) .

As the early flowering phenotype of the *dnf* mutant is only observed in SD and not LD it is believed that *DNF* lies on the photoperiodic pathway. *CO* lies on the photoperiodic pathway therefore by crossing the *dnf* mutant with the *co-2* mutant we can determine if *dnf* requires an active *CO* for the early flowering phenotype and thus if *DNF* is acting on the photoperiodic pathway.

If the *dnf:co-2* double mutants exhibit an early flowering response then this would indicate that the *dnf* mutation is causing the early flowering through a *CO*-independent pathway. However if the crosses have a late flowering phenotype then this would indicate that the early flowering of *dnf* does require an active *CO* and therefore positioning *DNF* on the same pathway as *CO*.

To test whether the *DNF* is acting in the same pathway as *CO* the *dnf* mutant (Wassilewskija (Ws) accession of *Arabidopsis*) was crossed with the *co-2* mutant (Landsberg *erecta* (Ler) accession of *Arabidopsis)* (see methods) (crosses performed by Dr Karl Morris and Mrs Lesley Codrai). The F_2 population was screened however no double mutants were identified in this generation. F_3 seed was collected from a double heterozygous F_2 line (DNF*dnf:*CO*co*) which has been selfed and its progeny screened for flowering time. Again no double mutants were identified but 3 lines that were heterozygous for the *dnf* mutation and homozygous for the *co-2* (DNF*dnf:coco*) were identified. F_4 progeny from

these three F_3 lines were screened for double mutants and flowering time was scored by days to flower.

A PCR-based assay was used to identify the presence of the *dnf* mutation using primers DNF (F) (5'- TGAATGAAGCCATGTGTCAG -3') DNF(R) (5-GATCAACCCTTCCGTTCTTT-3) and RB1 (5- AAACGCAGCACGATACG-3). All 3 primers were used to amplify a 178 bp from DNF(F) and DNF(R) (Ws) and 482 bp product from DNF(F) and RB1 (*dnf* mutant) (see methods) (Figure 16.).Plants that amplify both bands will therefore be heterozygous lines.Then genotyping was undertaken by sequencing the region of *CO* that contains the *co-2* point mutation.

Primers CO-Span 2 F (5'-AGCTCCCACACCATCAAACTTCA-3') and CO-Span 2 R (5'-CTTGGCATCCTTTATCACCTTCTT-3') were used to amplify a 987 bp region of *CO* including the position of the co-*2* mutation. PCR fragments were purified using the Qiaquick PCR purification kit (Qiagen), proceeded by sequence analysis using CO-Span 2 F primer to search for the presence of the *co-2* point mutation (see methods).

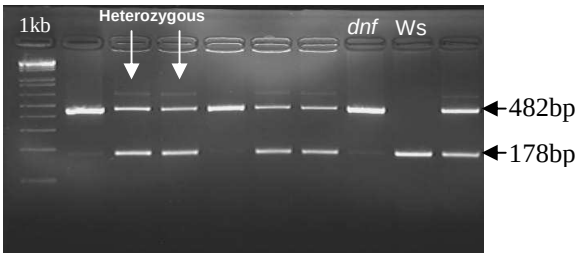


Figure 16. Example of zygosity PCR products indicating the expected band size for WT (Ws) , dnf mutant (*dnf*) and heterozygous for dnf (D*d*)

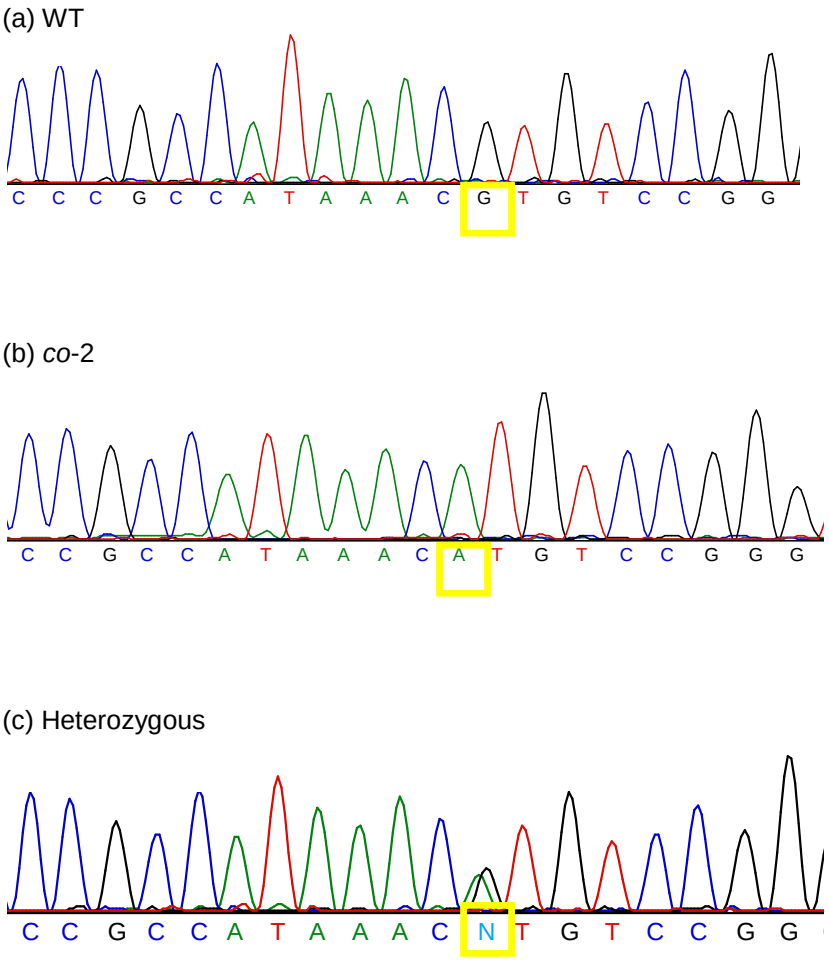
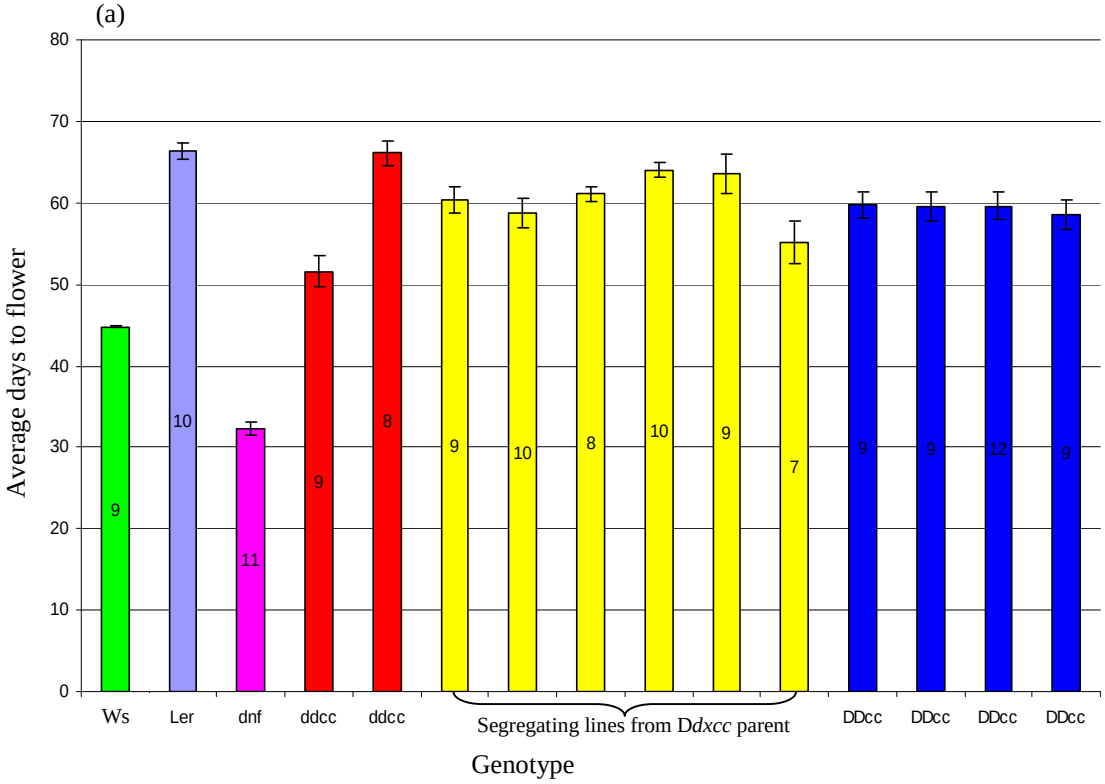


Figure 17. **Example of sequencing for the *co-2* mutation** (a)*WT* without CO mutation (b) sequence showing the single point mutation of *co-2* (A replacing G) (c)heterozygous for the *co-2* mutation.

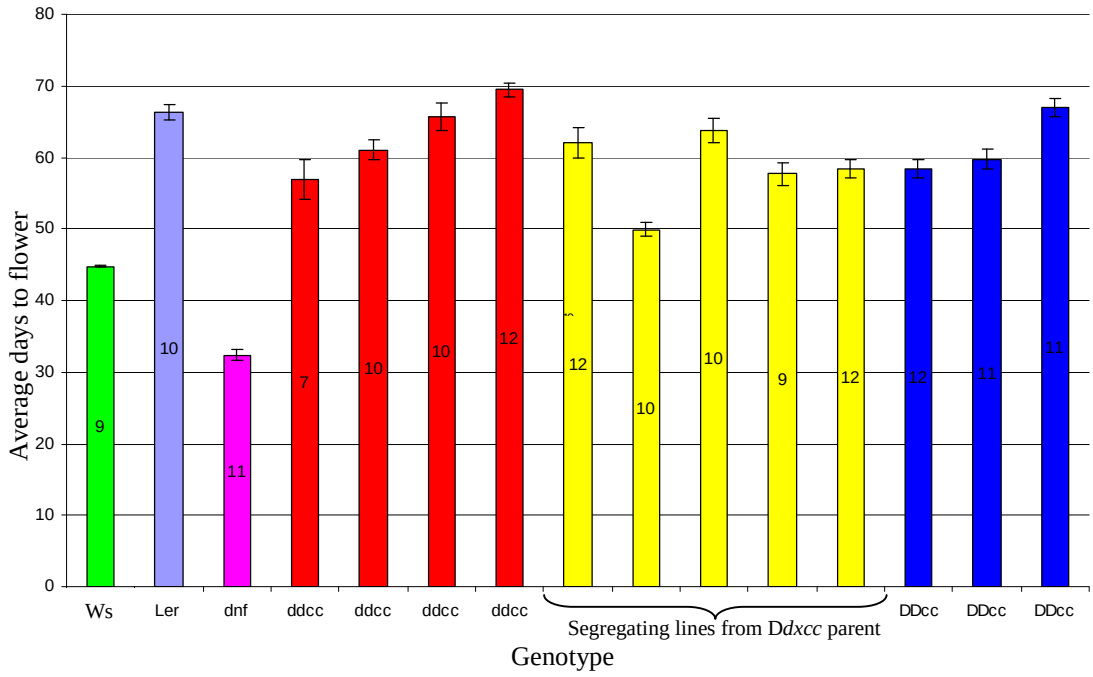
Of the genotypes outlined below *dnfdnfcoco*, *DNFDNFcoco* and the segregating lines from DNF*dnfcoco* parent all flowered late with respect to *dnf* mutant (between 50-70 days) (Figure 18.). Under SD conditions wild type Ws flowers on average at 45 days compared to the *dnf* mutant which flowered at approximately 20 days after germination. In addition the wild type Ler takes an average about 68 days to flower however the *co-2* mutant which is in a Ler background failed to germinate so this data could not be shown. Previous

analysis of the *co-2* mutant grown under SD has demonstrated little difference between the flowering time of the mutant and the Ler control as *CO* is only effective in LD (Putterill *et al.*, 1995). The DNFDNF:*coco* (dark blue bars) representing the *co-2* mutant in the Ler xWs crossed background produce an intermediate flowering time between Ler and Ws which is representative of the cross between the two ecotypes. Comparing *dnfdnf:coco* to DNFDNF:*coco* there is no difference in flowering time therefore the *dnf* mutation has no effect in the presence of the *co-2* mutation. The yellow columns are plants from DNF*dnf:coco* parent and as such are segregating for *dnf* mutation but all flowered at similar times to DNFDNF:*coco*. From this data we can conclude that *dnf* requires an active CO for the early flowering phenotype as the double mutants lead to a late flowering phenotype which was observed in all three double mutant lines.

(a)


Average days to flower
80
70
60
50
40
30
20
10
0
9
10
11
9
8
9
10
8
10
9
7
9
9
12
9
Ws
Ler
dnf
ddcc
ddcc
Segregating lines from D*dxcc* parent
DDcc
DDcc
DDcc
DDcc
Genotype

(b)


Average days to flower
80
70
60
50
40
30
20
10
0
9
10
11
7
10
10
12
12
10
10
9
12
12
11
11
Ws
Ler
dnf
ddcc
ddcc
ddcc
ddcc
Segregating lines from D*dxcc* parent
DDcc
DDcc
DDcc
Genotype

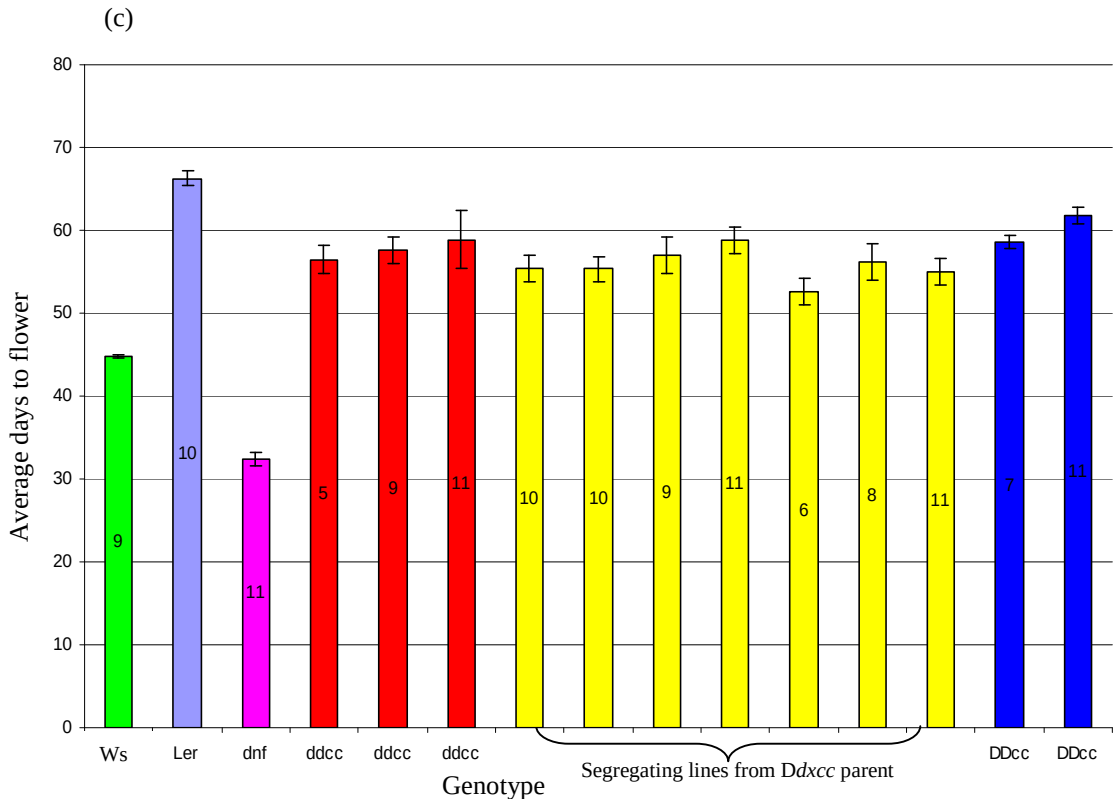
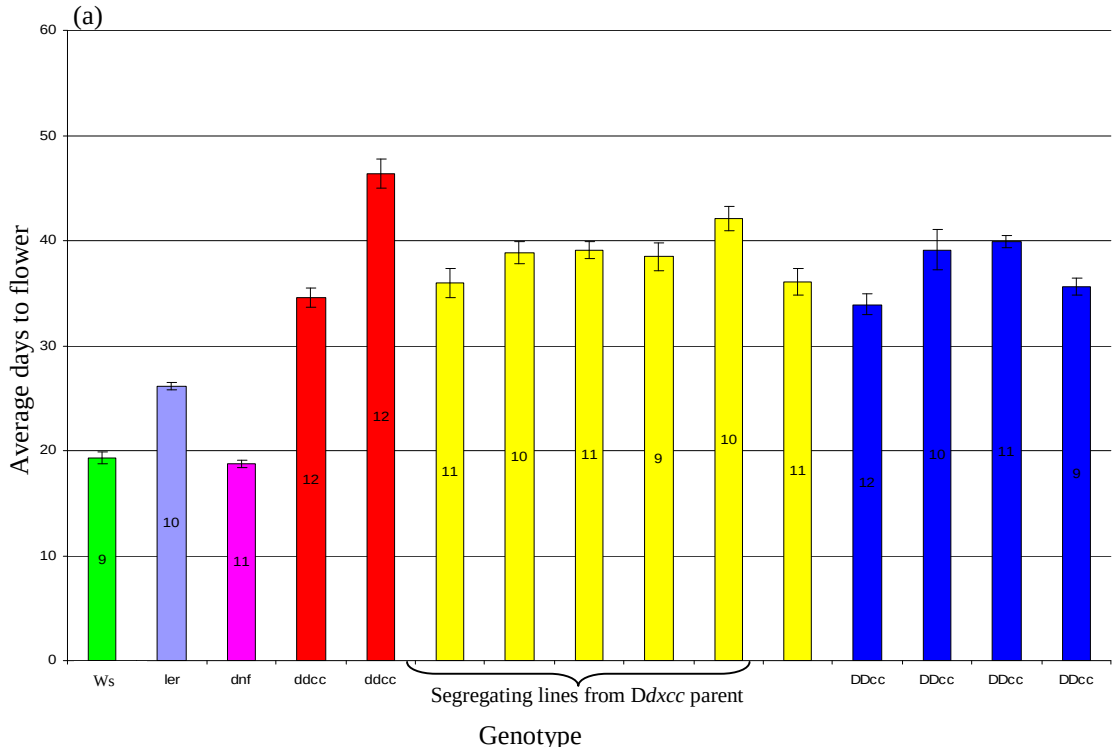


Figure 18. Flowering times of co-2dnf crosses grown under short days. (a) line 1 (b) line 2 (c) line 3.The numbers shown in the middle of each column indicate the number of plants analysed and the error bars indicate standard error.
ddcc= double mutants, *Ddcc*= heterozygous for the *dnf* mutation homozygous for *co-2* mutations, *DDcc*= WT for dnf homozygous for *co-2* mutations (Ler:Ws cross).
Flowering time was also scored by leaf number at flowering and a similar trend was observed.

3.3.3 The *dnf:co-2* double mutant flowered late when grown under LD.

Under LD the *co-2* mutant flowers late due to the lack of an active CO and under SD the *CO* mutation has no effect on flowering time as its role in floral promotion is only observed under LD conditions (Figure 19.). As *DNF* is also believed to lie on the photoperiodic pathway we would not expect to see an effect of the *dnf* mutation in LD. Of the genotypes outlined below *dnfdnf:coco,* DNFDNF*:coco* and the segregating lines from DNF*dnf:coco* parent all flowered late with respect to Ws, Ler and the *dnf* mutant (between 35-45 days). When comparing *dnfdnf:coco* to DNFDNF*:coco* it can be seen that on average the double mutants flowered at around the same time. If the double mutants did not show this effect it might indicate that *DNF* lies on a different flowering pathway. The fact that the *dnfdnf:coco* double mutants flowered late in LD adds further support to the findings of the crosses grown under short day conditions.


(a)
Average days to flower
60
50
40
30
20
10
0
9
10
11
12
12
11
10
11
9
10
11
12
10
11
9
Ws
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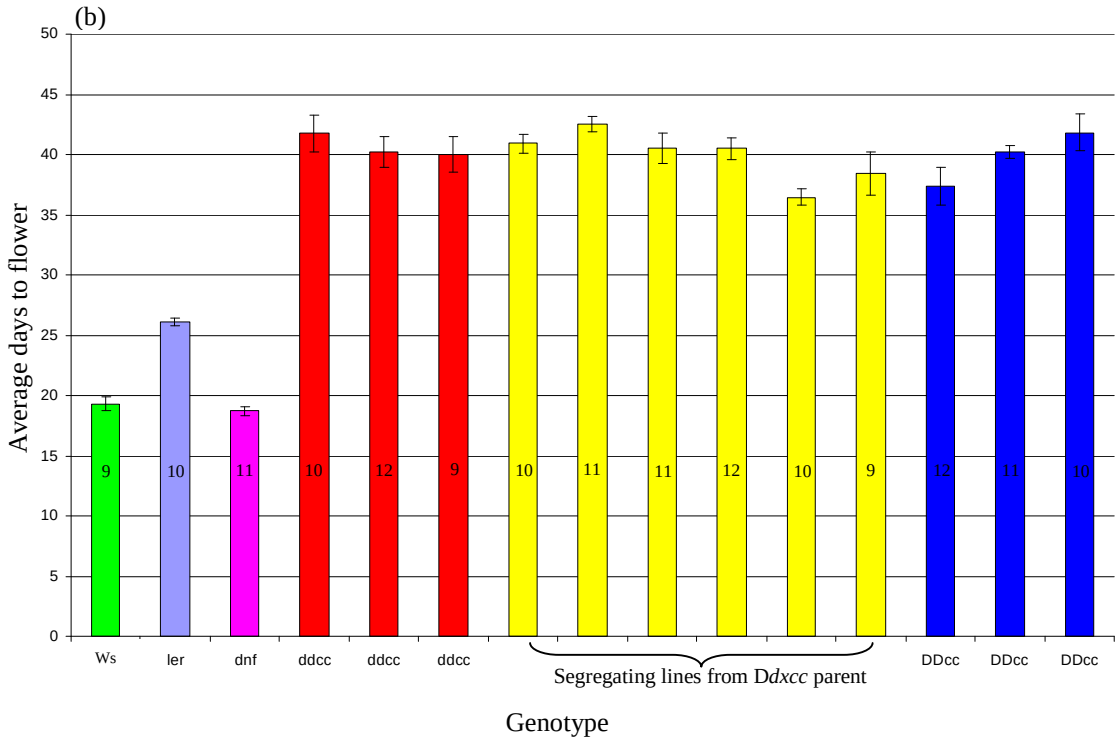
dnf
ddcc
ddcc
DDcc
DDcc
DDcc
DDcc
Segregating lines from D*dxcc* parent
Genotype


(b)
Average days to flower
50
45
40
35
30
25
20
15
10
5
0
9
10
11
10
12
9
10
11
11
12
10
9
12
11
10
Ws
ler
dnf
ddcc
ddcc
ddcc
DDcc
DDcc
DDcc
Segregating lines from D*dxcc* parent
Genotype

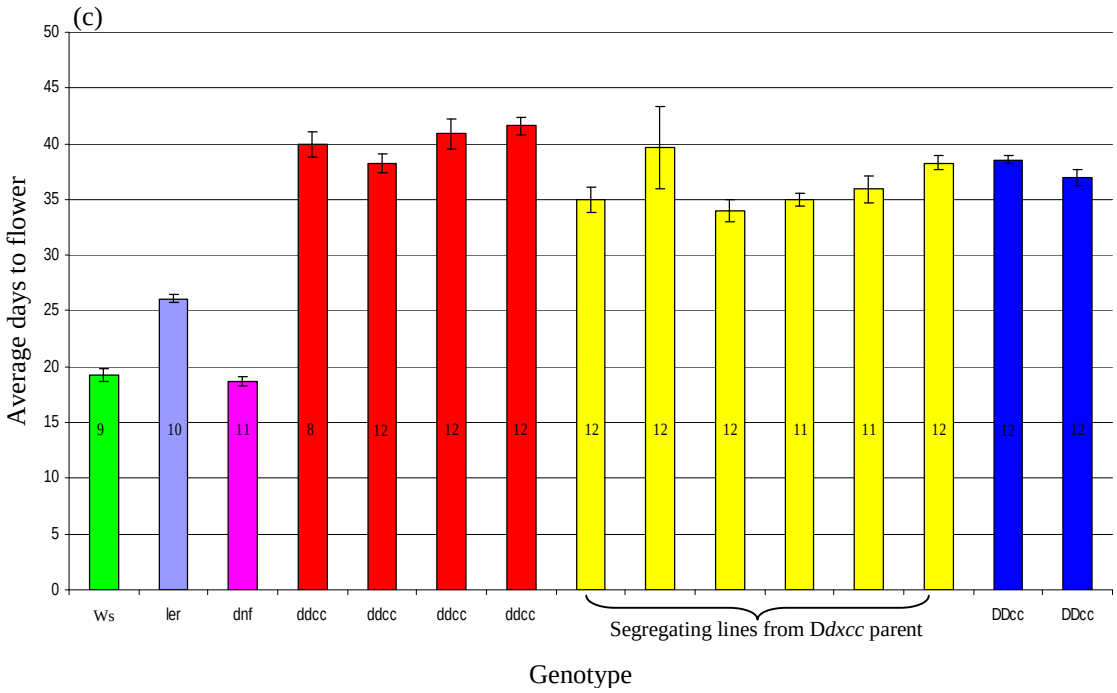


Figure 19. Flowering times of *co-2* x *dnf* crosses grown under long days. (a) line 1 (b) line 2 (c) line 3 The numbers shown in the middle of each column indicate the number of plants analysed and the error bars indicate standard error.
ddcc = double mutants, *Ddcc*= heterozygous for the dnf mutation homozygous for *co-2* mutations, *DDcc*= WT for dnf homozygous for *co-2* mutations (LerxWs cross).
Flowering time was also scored by leaf number at flowering and a similar trend was observed.

3.3.4 The critical daylength of the *dnf* mutant is between 4 and 6 hours

The *dnf* mutant flowers early when grown under short days at 22°C i.e. 8 hours light and 16 hours dark but at the same time as Ws plants when grown under long days 22°C 16 hours light 8 hours dark (Figure 20.).

In order to determine the CDL for the *dnf* mutant the mutant along with the Ws control was grown at 4, 6, 8, 12 and 16hrs day length and flowering time scored by leaf number. At 4 hours light there was very little difference between the flowering time of the *dnf* mutant and Ws plants. The *dnf* mutant plants grown in a 6 hour daylength flowered much earlier than the corresponding wild type (Figure 21.), flowering with an average of 14.28 leaves compared to Ws which was flowering at 34.55 leaves. When the light was increased to 8 hours the *dnf* mutant was flowering with an average of 7.41 leaves compared to the 25.18 leaves of Ws. In 16 hour light periods very little difference between the flowering times of the *dnf* mutant compared to WT was observed. This data indicates that the critical daylength of the *dnf* mutant is between 4 and 10 hours which is different to Ws which lies between 8 and 16 hrs.

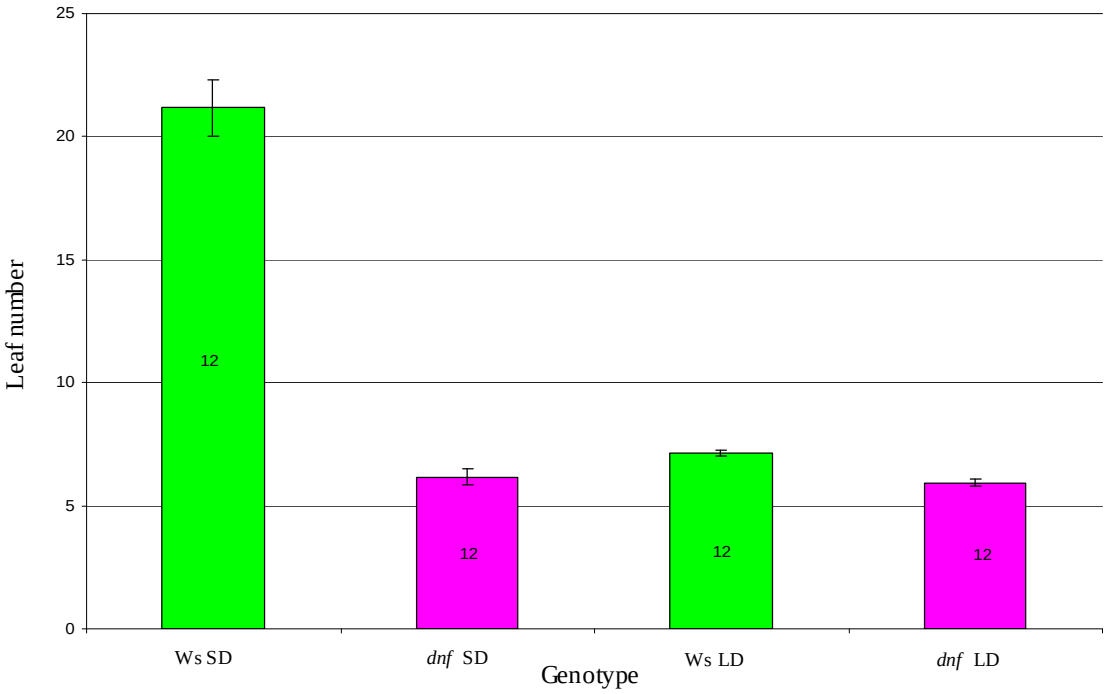


Figure 20. Flowering times of the *dnf* mutant compared to Ws. LD (16h) and SD (8hr) at 22ºC. The numbers shown in the middle of each column indicate the number of plants analysed and the error bars indicate standard error.
Flowering time was also scored by days to flower and a similar trend was observed.

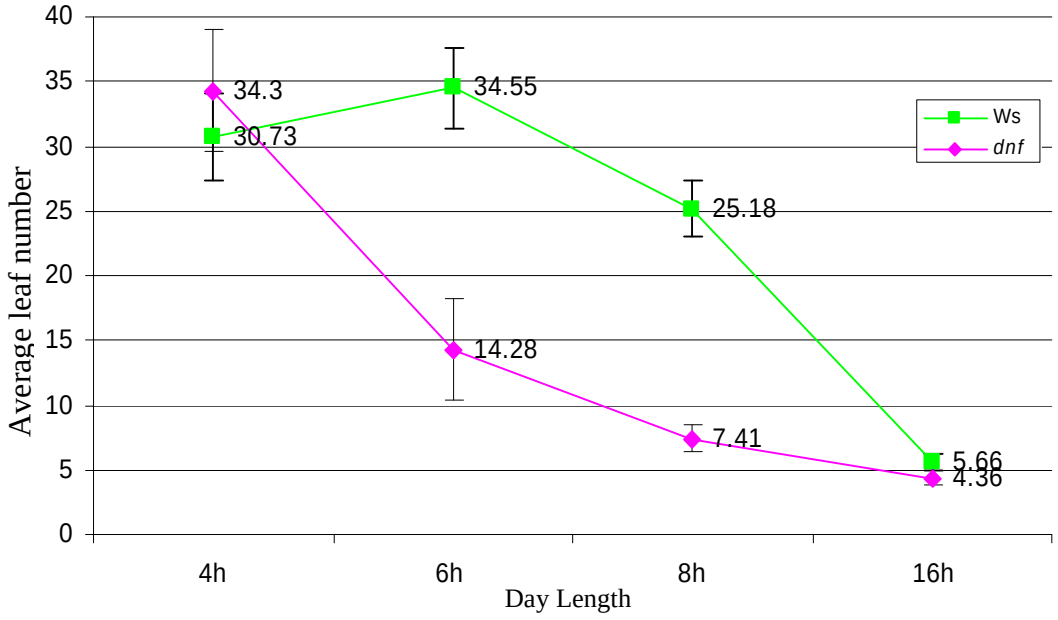


Figure 21. The critical day-length of the *dnf* mutant and WT Ws plants. The numbers represent the average leaf number at flowering and the error bars indicate standard error where n=12.

3.3.5 The early flowering phenotype of the *dnf* mutant is restricted to SD and is temperature dependent.

We have already demonstrated through hypocotyl elongation experiments that there is no defect in light perception in the *dnf* mutant, however (Halliday *et al.*, 2003) have shown that hypocotyl elongation and flowering time can be separated at lower temperatures. We wanted to establish if the effect of reduced temperatures had an effect on plant growth and development and on the early flowering phenotype of the *dnf* mutant that had be previously observed at 22ºC SD. We grew the *dnf* mutant along with the wild type Ws control in SD photoperiods at a range of temperatures 16ºC, 18ºC, 20ºC, 23ºC and 27ºC and examined the flowering time responses. As previously described the *dnf* mutant flowered significantly earlier than WS in SD at 22ºC a phenotype that is also observed at 23ºC and 27ºC, scored by both days to flower and leaf number (Figure 22 and Table 4.). However when grown at the lower temperatures of 16ºC, 18ºC and 20ºC this early flowering phenotype is no longer apparent and the *dnf* mutant flowers significantly later than Ws (Table 4). Figure 22 illustrated that on average the *dnf* mutant flowers 15 days earlier and with 8 fewer leaves than Ws at 22ºC. This early flowering phenotype is also observed at the higher temperatures of 23ºC and 27ºC where the mutant flowers 8 and 3 days earlier and with 3 and 2 fewer leaves than Ws respectively. However when the mutant is grown alongside Ws at 16ºC then the early flowering phenotype is abolished and *dnf* flowers on average 9 days later and with 8 more leaves than Ws. This later flowering phenotype is also observed at 18ºC and 20ºC when *dnf* flowers 8 and 5 days later and with 5 and 3 more leaves respectively. These results indicate that the early flowering phenotype of the *dnf* mutant is restricted to SD and is temperature dependent.

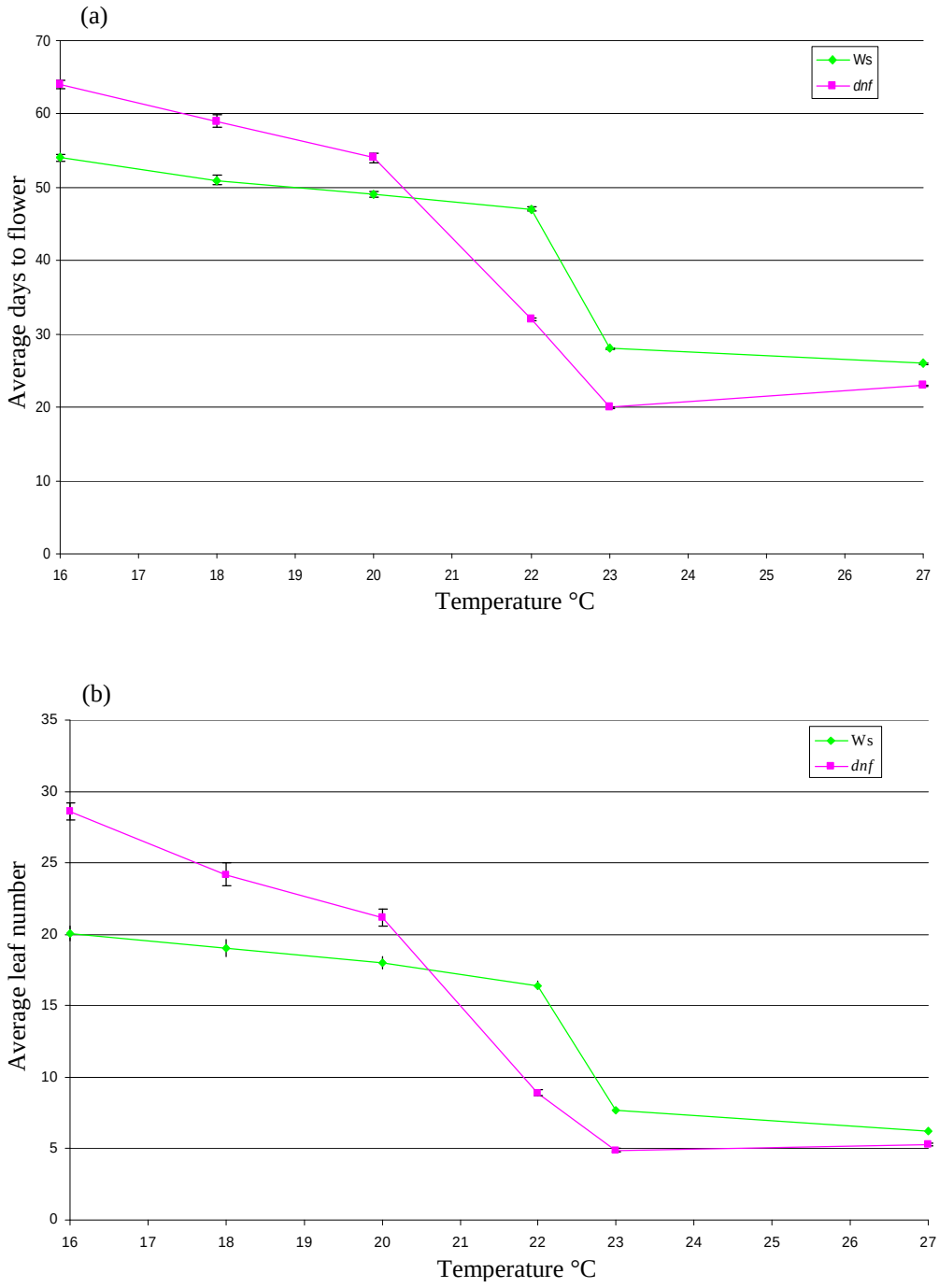


Figure 22. Flowering times of the *dnf* mutant compared to WS in SD at 16º,C 22ºC and 27ºC.
Flowering time shown as (a) average days to flower, (b) average leaf number. The numbers shown in the middle of each column indicate the number of plants analysed and the error bars indicate standard error.
When grown at 23ºC *dnf* flowers significantly earlier than Ws (p=2.23E-25, see appendix Table 12.)

Temperature	Flowering phenotype of *dnf.*	***P*-value for *dnf* and Ws** Average days to Flower	***P*-value for *dnf* and Ws** Average leaf Number at Flowering
16ºC	Late	3.35E-21	9.17E-26
18ºC	Late	1.4E-16	1.48E-13
20ºC	Late	2.24E-3	1.51E-3
22ºC	Early	6.67E-39	4.24E-35
23ºC	Early	2.23E-25	2.2E-20
27ºC	Early	1.29E-12	9.7E-08

Table 4. Statistical data showing differences in flowering times of the *dnf* mutant and Ws grown at different ambient temperatures. 16ºC,18ºCand 20ºC show the *p*-vaules when *dnf* flowers later than Ws and 22ºC, 23ºC and 27ºC show the *P*-values when *dnf* flowers later than Ws. *P*-values obtained from two-tailed t-test assuming equal variances.(see appendix Table 12.)

3.3.6 The adult leaf morphology appears altered in the *dnf* mutant when compared to WT grown in SD at 16°C.

Photographic images were taken of the plants at young and adult stages grown at 16ºC and 22ºC. Compared to the Ws wild-type plants, *dnf* mutant adult plant developed shorter more rounded rosette leaves when grown at 16ºC SD (Figure 23). In addition the petioles appear longer in Ws and the rosette leaves narrower and longer. This difference is not observed in the young plants or in the adult plants grown at 22ºC SD.

In an attempt to determine if there were any quantifiable differences in leaf morphology we next measured the leaf and petiole length at flowering for both the *dnf* mutant and Ws grown at 16, 18, 20 and 22ºC SD.

When the plants are grown at lower temperatures the developmental processes begin to slow down. However this is more exaggerated in the *dnf* mutant compared to Ws. Figure 25 shows that the average leaf length of Ws is

longer than that of *dnf* but there is little difference in the average petiole length. In addition both the leaf and petiole length in Ws begin to rise throughout development before gradually declining before floral initiation at around the 18th leaf. In the *dnf* mutant a similar pattern is observed but the peak in length of the leaves and petioles is extended before a gradual decline in length until flowering at approximately 30 leaves. At 18ºC a similar pattern is observed, with Ws having a longer average leaf length than the *dnf* mutant but the length of the petioles appears to be indistinguishable. When grown at 20ºC the Ws leaf length is again on average larger that that of the *dnf* mutant, but again the petiole lengths are indistinguishable. At 22ºC the there is a dramatic difference between the *dnf* mutant and Ws. The leaf and petiole development in Ws is very similar to that at lower temperatures. However as the *dnf* mutant flowers early then there are much fewer leaves at floral initiation.These results indicate that at lower temperatures developmental processes slow down and that is more exaggerated in the *dnf* mutant. The reason why the wild-type Ws had longer leaves however has yet to be elucidated.

The blade area for leaf 7 was measured in the *dnf* mutant and Ws grown in the differing ambient temperatures. At all temperatures Ws had a larger blade area than that of the *dnf* mutant (Table 5.). At 16ºC the average blade area of the *dnf* mutant was 19.7% smaller than Ws at 18°C 12.76%, at 20°C 26.4% and at 22°C 25%. These results are consistent with the increased leaf length observed in the Ws plants (Figures 21-24).

Figure 23. Leaf morphology at flowering (a) Ws-wild type young plant (b) *dnf* mutant young plant (c) Ws-wild type adult (d) *dnf* mutant adult. Grown under SD at 16°C. The Ws and *dnf mutant* plants appear indistinguishable when young plants.

Figure 24 Leaf morphology at flowering (a) *dnf* mutant young plant (b) Ws-wild type young plant (taken when *dnf* was flowering) (c) *dnf* mutant adult showing early flowering phenotype (d) Ws-wild type young plant. Grown in SD at 22°C

Parameter	**wild-type**	**(n)**	***dnf* mutant**	**(n)**
Area of leaf 7 (mm²) 16ºC SD	407± 29	(10)	303±22	(10)
Area of leaf 7 (mm²) 18ºC SD	437±43	(10)	280±53	(10)
Area of leaf 7 (mm²) 20ºC SD	457±39	(10)	351±25	(10)
Area of leaf 7 (mm²) 22ºC SD	356±7	(10)	204±5.5	(10)

Table 5. Measurements of blade area for wild-type Ws and *dnf* mutant plants when grown under different temperatures in SD. Blade area was measured on leaf 7 after bolting. (mean ± SE;n=10)

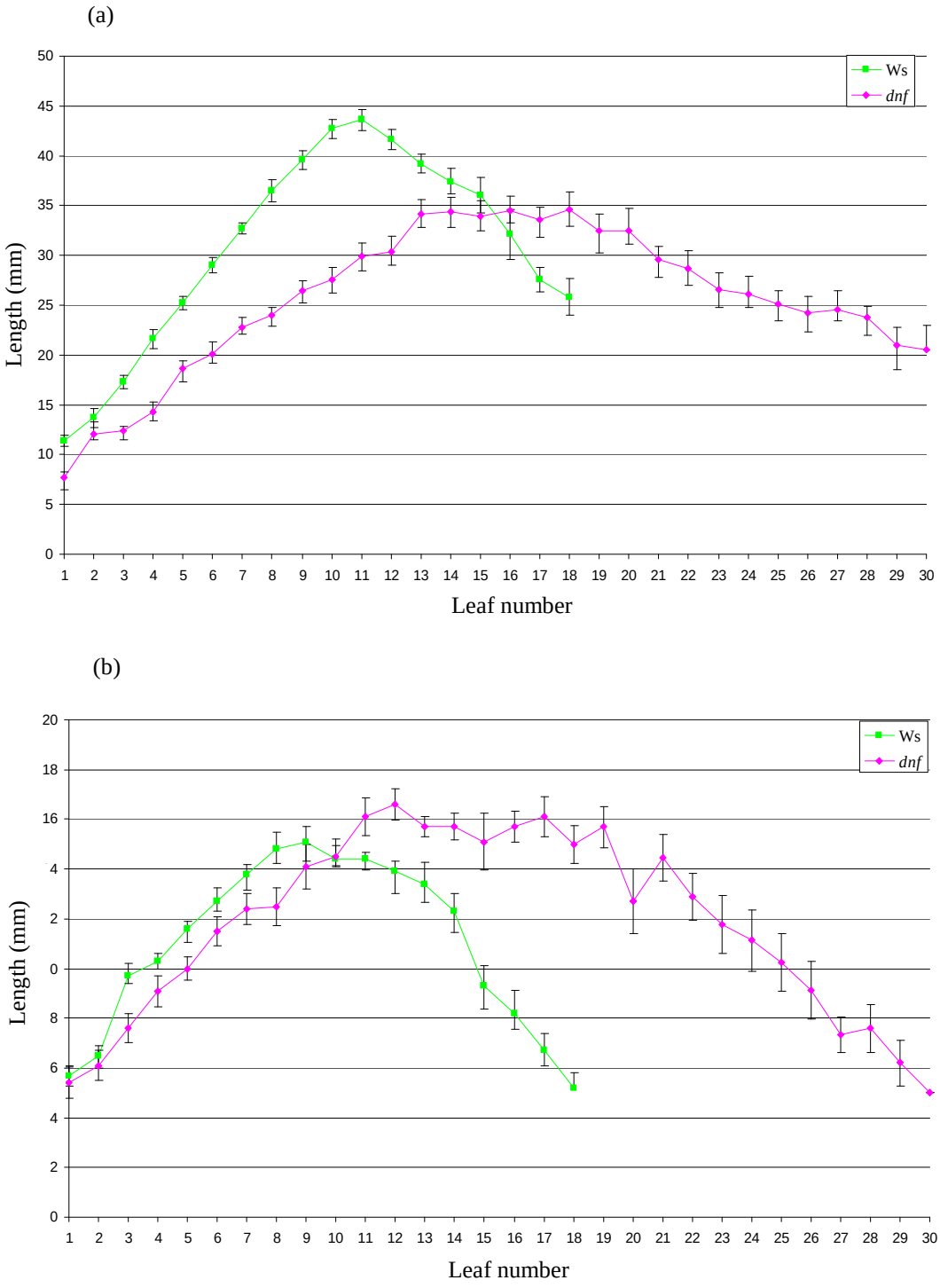
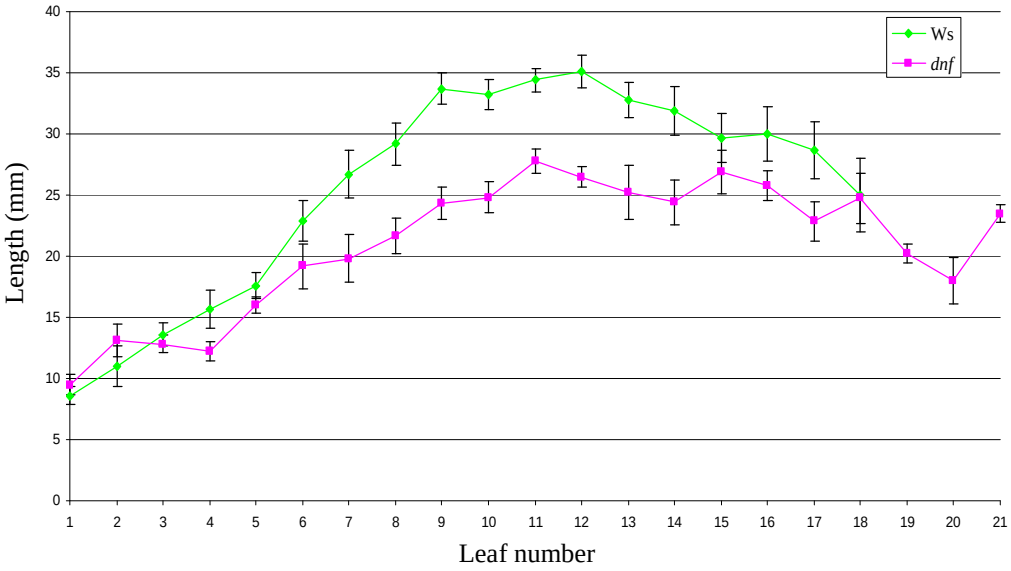


Figure 25 Effect of ambient temperature on leaf development at 16ºC in Ws and the *dnf* mutant.
(a) Average leaf length (b) Average petiole length. Samples analysed at flowering. Error bars indicate standard error where n=10.

(a)



(b)

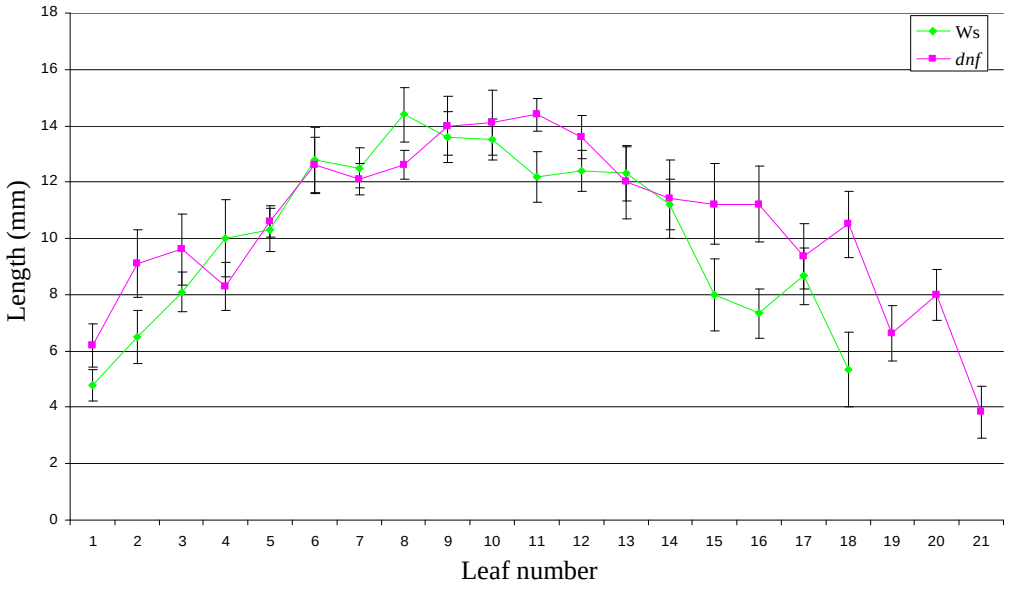
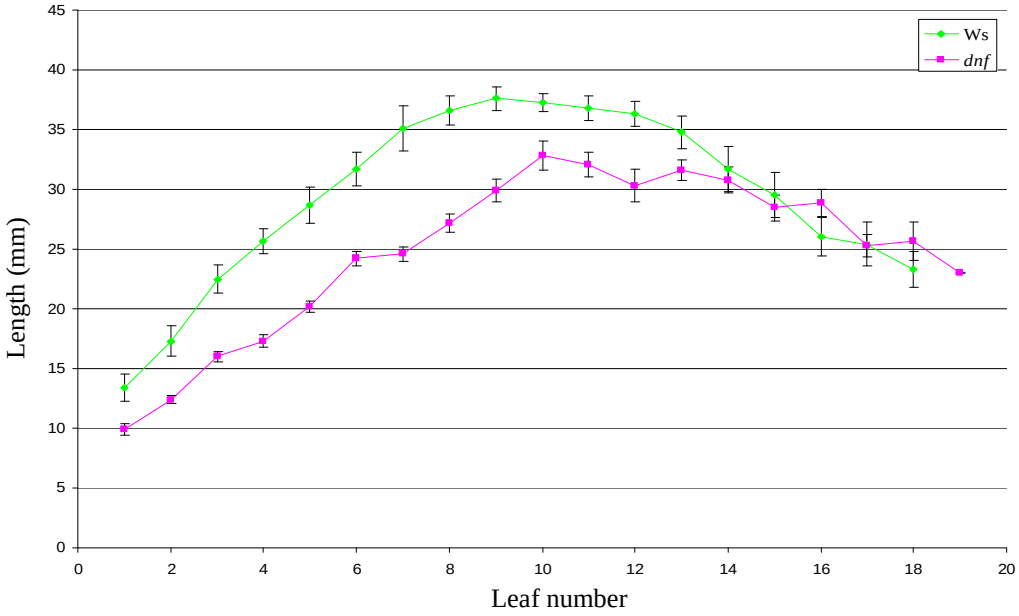


Figure 26 Effect of ambient temperature on leaf development at 18ºC in Ws and the *dnf* mutant. (a) Average leaf length (b) Average petiole length. Samples analysed at flowering. Error bars indicate standard error where n=10.

(a)



(b)

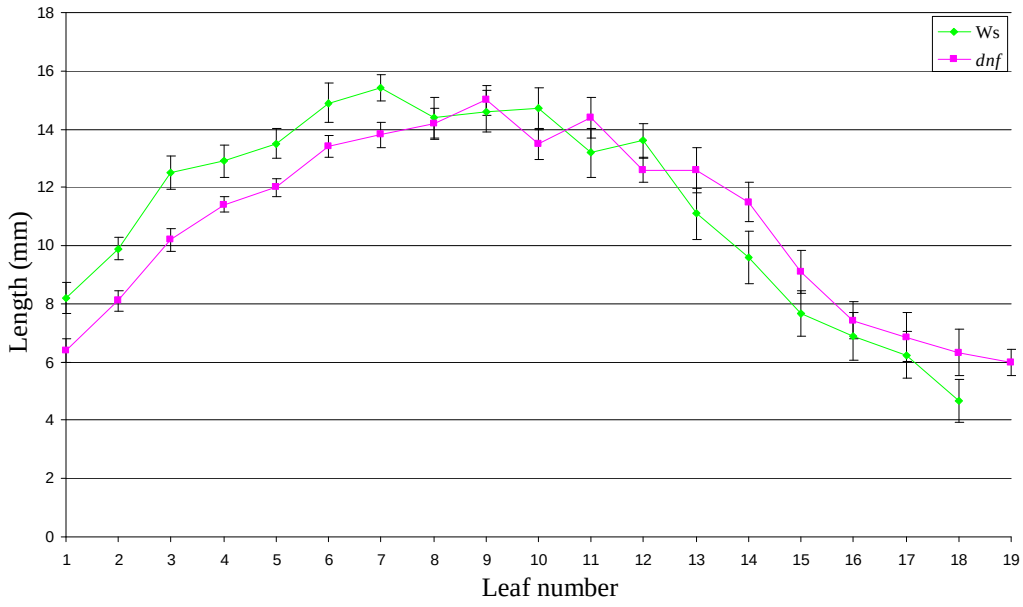
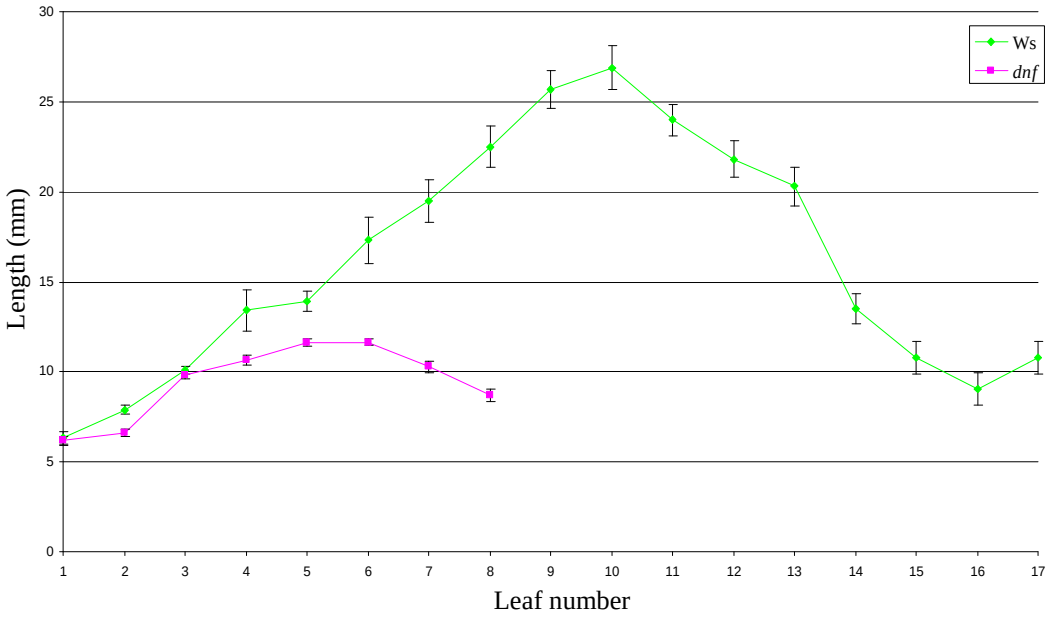


Figure 27. Effect of ambient temperature on leaf development at 20ºC in Ws and the *dnf* mutant. (a) Average leaf length (b) Average petiole length. Samples analysed at flowering. Error bars indicate standard error where n=10.

(a)



(b)

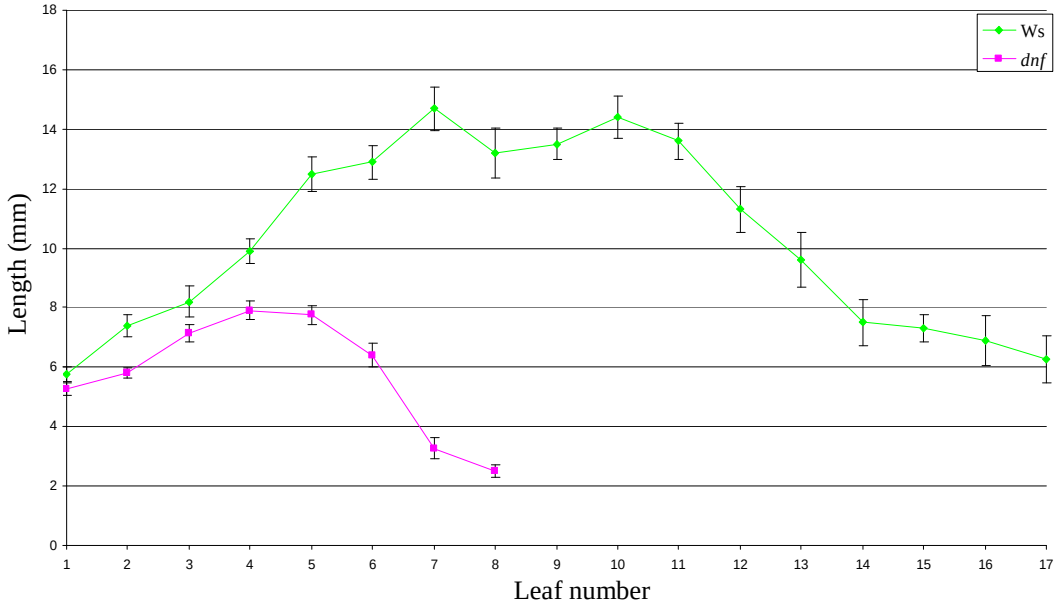


Figure 28. Effect of ambient temperature on leaf development at 22ºC in Ws and the *dnf* mutant. (a) Average leaf length (b) Average petiole length. Sample analysed at flowering. Error bars indicate standard error where n=10.

3.4 Discussion

Firstly we determined if light perception was altered in the mutant thus leading to early flowering phenotype. When *dnf* was grown alongside light perception mutants at different light fluencies no de-etiolation phenotype was observed and the seedlings grew at the same rate as wild type Ws (Figure 19.). This data suggests that there is no defect in light perception in the *dnf* mutant.

A major aim of this chapter was to position *DNF* within the LD photoperiodic promotion pathway. To this end the epistatic relationship between *DNF* and *co*, was examined. Being able to cross *co-2* and *dnf* should have been straight forward as the *co* mutation lies on chromosome V and *dnf* on chromosome III. However no homozygous double mutants were identified until the F4 generation. Initial genotyping for the *co* mutation in the F2 population was only undertaken on plants that were found to be homozygous for the *dnf* mutation, however if we had looked for the *co* mutant in heterozygous *dnf* plants then we would have more than likely identified double mutants in the F3 populations. As yet the reason that no homozygous double mutants were identified in the F2 population remains unclear. However analysis of the *dnfco-2* double mutants showed that they flowered late when grown under SD conditions.

The fact that the *co-2 dnf* double mutants flowered late indicates that *dnf* requires an active CO to exhibit the early flowering phenotype in SD. The cross between *dnf* and *co -2* is complicated by the fact that *dnf* is in a Ws background and *co-2* is in Ler. Ws plants flower earlier than Ler in SD therefore a straightforward cross between the two ecotypes may result in a range of flowering times and this could well be why we see a range in the crosses. Nevertheless the *dnf:co-2* double mutants all flowered later than Ws control and only slightly earlier than the Ler control. In addition the double mutant also

displays a similar flowering time to that of the DNFDNF:*coco* lines, this adds further proof that the *dnf* mutant requires an active CO (Figure 18.). This data indicates that *CO* is epistatic to *DNF* and confirms that *DNF* functions in the LD photoperiodic promotion pathway.

The CDL of the *dnf* mutant was analysed to determine if the early flowering phenotype is restricted to a specific day length. Interestingly the *dnf* mutant remains early flowering when the daylength is reduced from 8 to 6 hrs, this is more than likely to be as a result of altered *CO* expression in the *dnf* mutant in these photoperiods (Chapter 4). This altered *CO* expression is not observed at 4 hours which accounts for the fact that when the daylength is reduced to 4 hours the early flowering phenotype is abolished and the mutant flowers around the same time as Ws. The results demonstrate that the *dnf* mutant has an altered critical photoperiod of between 4-10 hours compared with Ws which has a critical photoperiod of between 8-16 hours (Figure 21.).

A further aim of this chapter was to determine if *dnf* remains early flowering when grown at lower temperatures in SD. Consistent with previous results we found that the *dnf* mutant flowered early in SD at 22°C compared to WT flowering approximately 6 days earlier and with 4 fewer leaves than Ws. The early flowering phenotype of *dnf* remained true at 27°C but the difference was to a lesser extent with *dnf* flowering approximately 2 days sooner and with 2 fewer leaves than Ws. In addition at 27°C the WT flowered earlier than when grown at 22°C with respect to both leaf number and days to flower, however the *dnf* mutant flowered slightly later at 27ºC but with approximately the same number of leaves at both temperatures.

When the growing temperature was reduced to 16°C the effect on the flowering time of the *dnf* mutant was remarkable. At this lower temperature the

early flowering phenotype of the *dnf* mutant was completely reversed and the *dnf* mutant flowered later than Ws. On average *dnf* flowered approximately 9 days later than Ws and with 8 more leaves. In addition the flowering time of Ws plants was increased when grown at 16°C. At this lower temperature Ws plants flowered on average 7 days later and with 12 more leaves than when grown at 22°C. These results suggest that the early flowering response of the *dnf* mutant is temperature conditional.

These findings show similarity to that of the *phyB* mutant, which as previously discussed flowers early then WT at 22°C, but then as late as WT when grown at 16°C. However the early flowering phenotype of the *phyB* mutant shows an effect in SD and LD at 22°C, compared with *dnf* where the effect is restricted to SD photoperiod. In addition as PhyB perceives red light and when grown under red light the *phyB* mutant develops extended hypocotyls a phenotype that was not however observed when *dnf* was grown under the same conditions (see hypocotyl elongation above). Therefore there is no defect in the PhyB response pathway in the *dnf* mutant. Furthermore in the *phyB* mutant there is no effect on the level of *CO* mRNA and PhyB has been shown to be a repressor of CO protein (Cerdán *et al.,* 2003). Red light has also been shown to inhibit flowering whilst FR and blue light lead to the promotion of flowering at the level of the CO protein (Valverde *et al.,* 2004). However we have demonstrated that *dnf* requires an active CO to remain early flowering (see above) and in addition the *dnf* mutant does have an effect on the level of CO mRNA (Chapter 4).

In all ambient growth temperatures tested there was little difference in petiole length between the mutant and Ws. However at 22ºC, due to the early flowering phenotype of *dnf* the vegetative development stage was greatly

reduced. The results for the petiole length are in contrast to those found in the *phyB* mutant which has elongated petioles at both 22ºC and 16ºC (Halliday *et al.*, 2003).

Consistent with previous findings by Halliday *et al*. 2003 wild type plants grew slower at 16ºC than at 22ºC with only a slight extended developmental phase and with on average 5 more leaves. This is in great contrast to the *dnf* mutant which shows a vastly extended developmental phase at 16ºC compared to 22ºC and flowered on average with approximately 20 more leaves.

Under all temperatures the leaf length appears longer in Ws compared to *dnf* with more pronounced difference being observed at 22ºC. This was also true for blade area where the Ws leaf was consistently larger than *dnf* at all temperatures. Interestingly these differences were not as clear cut in the younger leaves (up to leaves 6) and it was only when the later leaves appeared that any differences could be clearly seen. Similar results have been observed in the *phyD* mutant which when grown in SD at 16ºC produced leaves at a similar rate to that of WT for the first seven leaves but there after the rate of leaf production slowed considerably (Halliday *et al.,* 2003). In addition to the rate of leaf formation the rosette leaves of the *phyD* mutant are also smaller than that of WT but only in LD at 16ºC (Halliday *et al.,* 2003). It appears that leaf production continued for longer in the *dnf* mutant due to its later flowering at lower temperatures than Ws.

These results may indicate a possible role for *dnf* in vegetative leaf development at lower temperatures. However it is not yet known if this is restricted to SD. The early flowering phenotype of *dnf* is known to exist only in SD at temperatures between 22ºC-27ºC, but we do not know if a change in temperature in LD has any effect on the flowering time of the mutant.

By growing the mutant along with the appropriate controls under different wavelengths, photoperiods and temperatures we have been able to establish that there is not a light perception defect in the mutant, the CDL is between 4-10 hours and that the early flowering phenotype is temperature dependent with prolonged leaf development at lower temperatures. Furthermore by generating *dnf:co-2* double mutants we have also established that *CO* is epistatic to *DNF*.

Chapter 4

Molecular characterisation

of the *dnf* mutant.

4.1 Introduction

Genetic approaches have been used in the identification of many genes that are involved in the photoperiodic flowering pathway. Mutations in these genes show either a delay or acceleration in flowering. For example GI is known to regulate flowering by the regulation of *CO* mRNA abundance. The *gi* mutant contains reduced levels of *CO* mRNA and flowers late, furthermore when *GI* is overexpressed the levels of *CO* mRNA are increased and flowering is accelerated (Suarez-Lopez *et al.,* 2001).

CO is a central component of the LD photoperiodic pathway. The *CO* gene encodes a protein with a conserved carboxyl-terminal domain known as the CCT domain and a nuclear protein with two zinc-fingers at the amino terminus (Putterill *et al.*, 1995). The regulation of *CO* is both at the transcriptional and post transcriptional level. The transcription of *CO* mRNA is controlled by the circadian clock, it is repressed by CDF1 which is degraded by FKF1/GI complex, and shows a rhythmic expression pattern (Sawa *et al.*, 2007). The expression of *CO* is such that it is high in the morning and shows a gradual decline throughout the day before rising towards the late afternoon (Suarez-Lopez *et al.*, 2001). Phytochromes and cryptochromes have been shown to regulate *CO* at a post-translational level. Red light acting through PhyB delays flowering by promoting degradation of CO protein by the proteasome. In contrast PhyA, Cry1 and Cry2 photoreceptors stabilize CO protein in FR and BL thus leading to the promotion of flowering. (Johnson *et al.,* 1994; Guo *et al.,* 1998; Valverde *et al.,* 2004). Furthermore a small four member SUPPRESSOR OF PHYA 1-4 (SPA1-4) protein family has been shown to physically interact with CO to inhibit flowering in SD by controlling CO stability (Laubinger *et al.*, 2006).

In LD the level of *CO* transcription increases in the late afternoon resulting in a rise in *CO* mRNA. The increased levels of *CO* mRNA subsequently lead to increased levels of CO protein (Figure 29(a)). The action of PhyA and cryptochromes has the effect of stabilising the CO protein at the end of the day (Valverde *et al.,* 2004). Recently *cop1* mutants have been shown to have a dramatic increase of CO protein (but not CO mRNA) in the night, thus indicating that COP1 works to degrade CO during the night (Jang S 2008). The increased levels of CO protein induce gene transcription of the floral integrators *FT* and *SOC1*. *FT* and *SOC1* are the immediate downstream targets of *CO* and induce the expression of the floral meristem identity genes and ultimately floral promotion.

In SD conditions darkness occurs before the rise in *CO* mRNA, the CO protein is degraded by COP1 and the 26s proteasome and floral initiation is delayed (Figure 29(b)).

In summary, under LD conditions *CO* mRNA falls to a low level rapidly after dawn, rises about 12hours after dawn staying high throughout the night before going down again the following dawn, therefore *CO* mRNA peaks with the exposure of light which stabilises the CO protein so it accumulates towards the end of a LD (Suarez-Lopez *et al.,* 2001) . In contrast, in short days *CO* mRNA remains consistently low throughout the day peaking only during the night when CO protein is degraded so does not accumulate and does not cause the promotion of flowering (Figure 29 (a) and (b)).

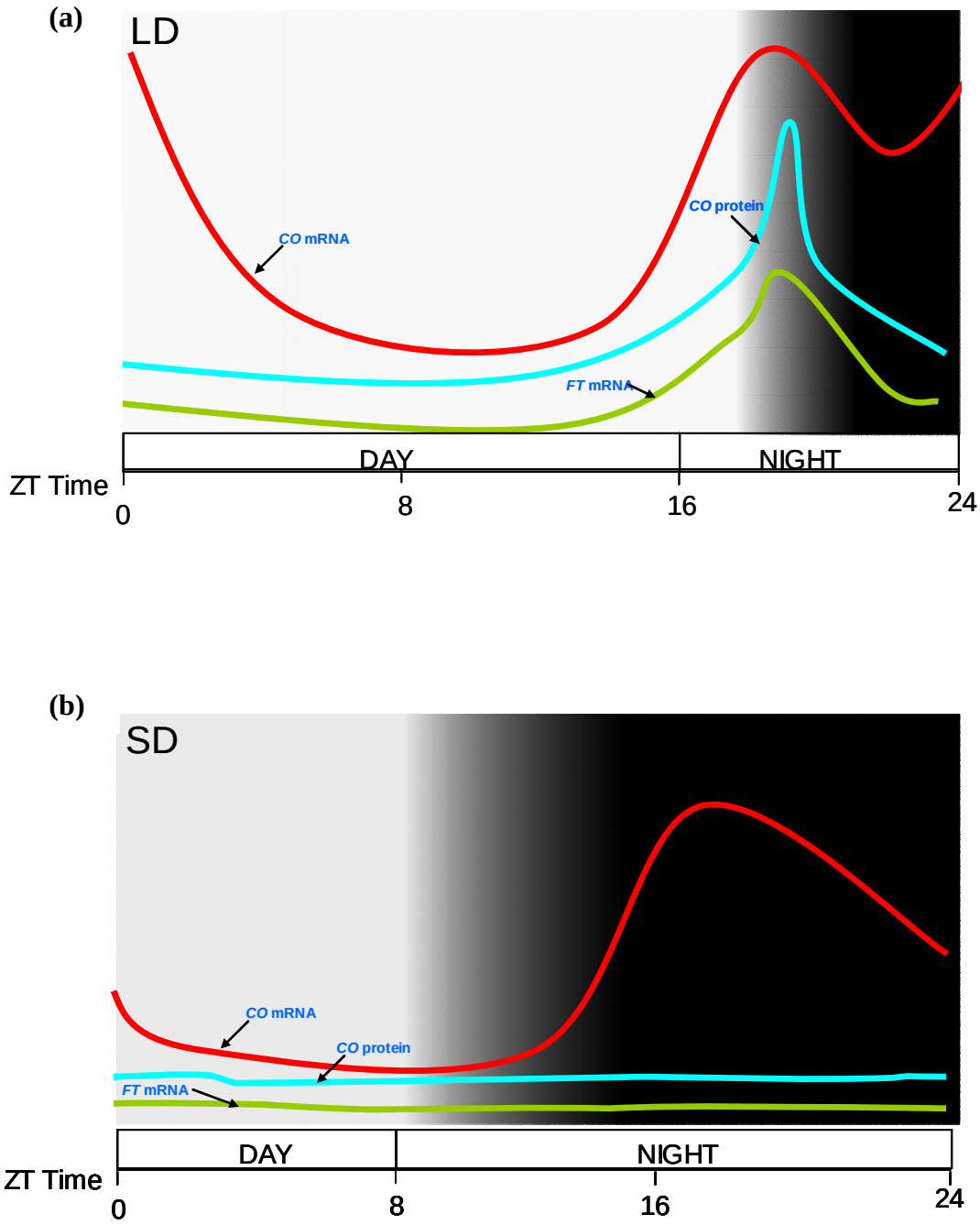


Figure 29. Model illustrating *CO* mRNA, CO protein and *FT* mRNA expression in Arabidopsis grown under (a) LD and (b) SD conditions.

FT was first identified in the classic flowering-time screens described by Koornneef *et al*., (1991). It is a strong promoter of flowering and produces an extremely early flowering phenotype when over expressed in transgenic plants (Boss *et al.,* 2004). The *FT* gene encodes a small 23kD protein with sequence similarity to RAF kinase inhibitor proteins (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999).

Recent work has shown that the FT protein induced locally in the leaf, moves to the shoot apex where its protein interacts with FD to induce transcription of floral meristem identity genes (Abe *et al.,* 2005; Wigge *et al.,* 2005). These results led to the hypothesis that *FT* is a component of the mobile flowering signal Florigen (Corbesier *et al.,* 2007).

In addition to induction by *CO, FT* and *SOC1* also respond to other cues, such as developmental age and vernalisation and as such integrate several flowering-time pathways (Jo Putterill 2004). However *FT* expression is predominantly regulated by photoperiod and is one of the early targets of *CO* as inactivation of *FT* delayed flowering in constitutive *CO* over-expressers (Onouchi *et al.,* 2000), whereas the vernalisation/autonomous pathways have a greater effect on the expression of *SOC1* than the photoperiod pathway (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999; Wigge *et al.,* 2005) Recent expression analysis has demonstrated that *SOC1* expression is suppressed in the *ft* mutant, even in the 35S:CO background which implies that *CO* regulates *SOC1* via *FT* (Schmid *et al.,* 2003; Yoo *et al.,* 2005).

GI is a large nuclear localised protein which is involved in the photoperiodic flowering, red light signalling and circadian clock function (Koornneef *et al.,* 1991; Fowler *et al.,* 1999; Huq *et al.,* 2000). Mutations that cause loss of function of GI lead to a late flowering phenotype which can be corrected by over-expression of *CO* in the mutant background (Suarez-Lopez *et al.,* 2001). GI is involved in the transcriptional regulation of *CO* and the *gi* mutation causes down regulation of *CO* expression in LDs. In addition *GI* is thought to interact with the clock genes *CCA1* and *LHY*, as when these genes are over expressed the circadian expression of *GI* is disrupted. However, the *gi-3* mutation affects the expression of *CCA1* and *LHY* reducing their transcript abundance (Fowler *et al.,* 1999). Therefore is seems that all three transcription factors have an effect on each other. In addition Locke et al. (2005) characterised double loss-of-function *lhy* and *cca1* alleles and developed a mathematical model showing a possible interlocking feedback loop in which *GI* forms part of a second feedback loop which positively regulates *TOC1* expression which in turn negatively regulates *GI* (Figure 30) This link between *GI* and *TOC1* is required to maintain period length, circadian amplitude and mediating part of the light input into the clock (Locke *et al.,* 2005).

Support for Locke's model has come from the work of Gould et al. (2006). Gould demonstrated *GI* expression was increased at higher temperatures and that this expression increased the levels of *TOC1*. Furthermore *TOC1* positively influenced the expression of *LHY/CCA1*, but decreased the expression of *GI*. However at lower temperatures the expression of *GI*, *TOC1* and *LHY* was decreased with *LHY* to a lesser extent. Therefore it is postulated that *GI* regulates *TOC1* and that this regulation is temperature dependent, thus maintaining the

amplitude of *CCA1* and *LHY* in order to preserve accurate clock function (Gould *et al.*, 2006) (Figure 30).

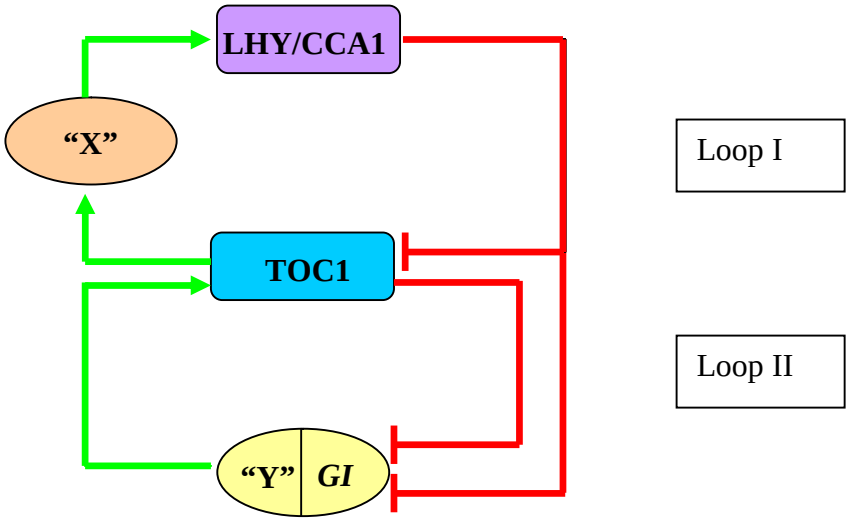


Figure 30. Schematic representation of the proposed model for the circadian clock. X and Y are hypothetical proteins and GI is proposed to form part of the 'Y' protein (Locke *et al.*, 2005). Redrawn from (Ueda 2006).

GI mRNA is regulated by daylength and cycles under the control of the circadian clock peaking at 8-10 hours after dawn. This rhythmic expression is regulated by day length and *GI* transcripts peak earlier in SD than in LD (Fowler *et al.*, 1999). Furthermore David et al (2006) demonstrated that GI protein levels oscillate in both SD and LD peaking earlier in SD and that this expression closely follows the expression of *GI* mRNA. The fact that *GI* is expressed in SD to similar levels as LD without leading to floral promotion suggests that *GI* modifications or interactions only occur in LD and that these modifications are prevented in SD (David *et al.*, 2006).

Based on genetic analysis GI, CO and FT have all been placed on the photoperiodic pathway and have been given a functional hierarchy of GI-CO-FT (Redei 1962; Koornneef *et al.,* 1991; Koornneef *et al.,* 1998; Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999; Samach *et al.,* 2000; Suarez-Lopez *et al.,* 2001) (Figure 33).

Light is a key environmental signal for plants and plays a major role in gene expression and plant development. Light dark cycles entrain the central oscillator (or circadian clock) to an approximate 24 hour period. When plants are deprived of a light/dark transition (e.g. transferring to constant light) the expression of clock controlled genes still follows a rhythmic pattern which can be observed often for several days (Millar 1999; McClung 2000).

Chlorophyll a/b binding protein *CAB* is an example of such a clock control gene and is expressed rhythmically. As such this gene has often been used as a marker for circadian regulation in plants. The rhythmic expression of *CAB* has been well characterised and seedlings transferred to continuous light (LL) exhibit a robust circadian rhythm of *CAB* expression with the period of expression being approximately 24.5h in LL (Millar *et al.,* 1995). Therefore in order to maintain a 24-h period in natural conditions the transition from a light/dark cycle must shift the phase of the oscillator by approximately 0.5hrs each day, this process is known as entrainment (Figure 31). Light signals play a major role in entrainment of the clock and plants must be able to adapt to different growth conditions as in seasonal variation. This ensures that gene expression and plant development occur under all photoperiods and at the most appropriate time.

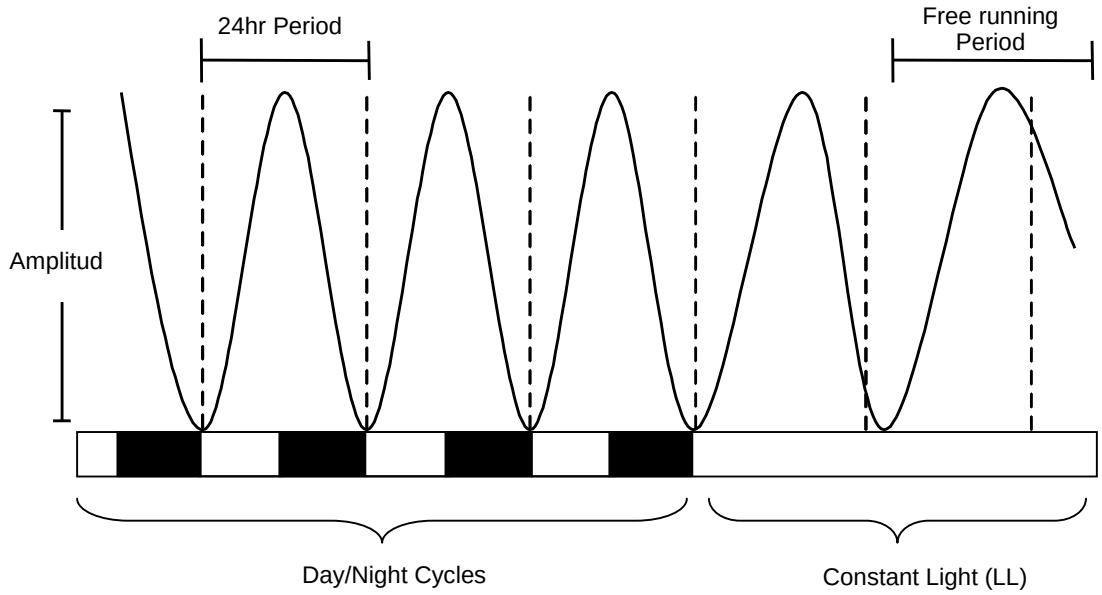
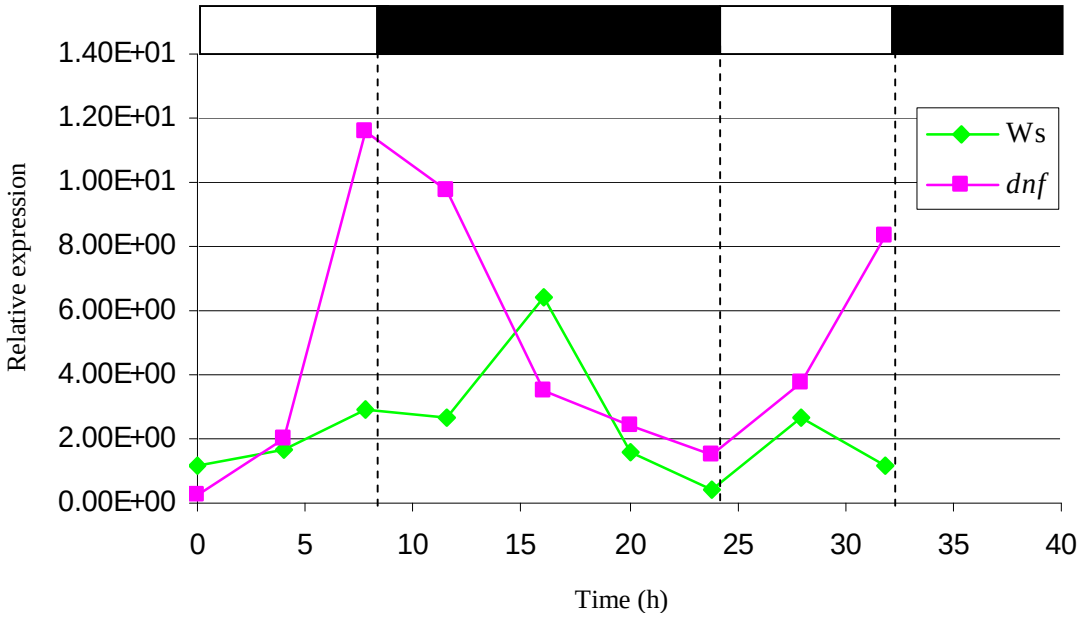


Figure 31.Schematic representation of the rhythmic expression a clock controlled gene. The transition form the day/night cycle resets the clock to an approximate 24hr period a process called entrainment. Without entrainment the rhythm reverts to a free running period.

The *dnf* mutant has been positioned on the photoperiodic pathway through crosses with the *co-2* mutant. However the main aim of this chapter is to determine the location of *DNF* within this pathway. To this end qRT-PCR analysis was used to examine the expression of *CO, FT GI*, and *CAB* in both the *dnf* mutant and WT (Ws). Using real-time analysis of both *CO* and *FT* had previously revealed altered expression patterns in the *dnf* mutant compared to Ws (Dr Stephen Jackson Warwick HRI personal communication) (Figure 32).

(a)



(b)

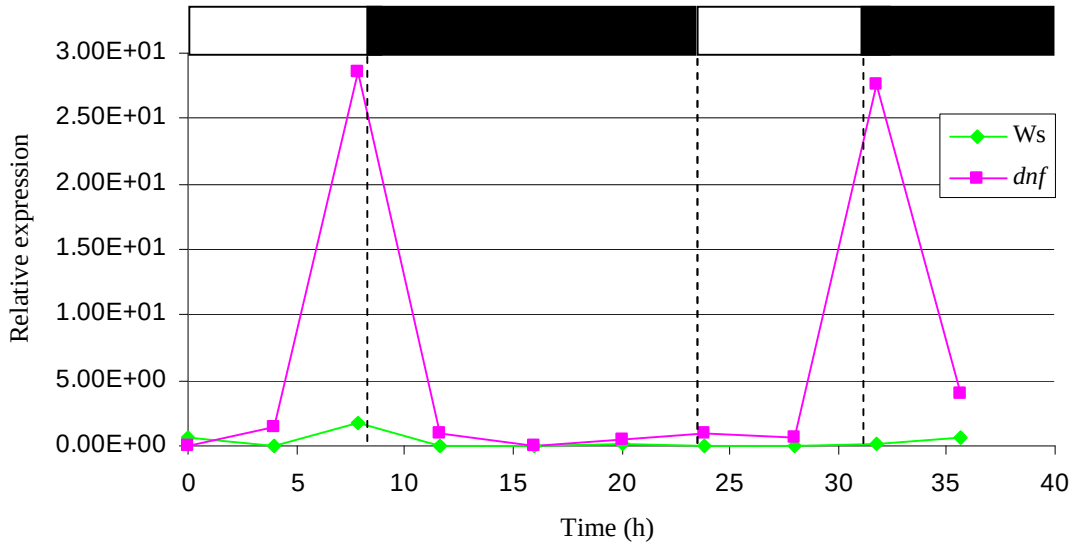


Figure 32. Quatitative real time PCR analysis of key photoperiodic pathway genes (a) qRT-PCR analysis of *CO* expression in the *dnf* mutant. (b) qRT-PCR analysis of *FT* expression in the *dnf* mutant (Dr Stephen Jackson Warwick HRI personal communication).

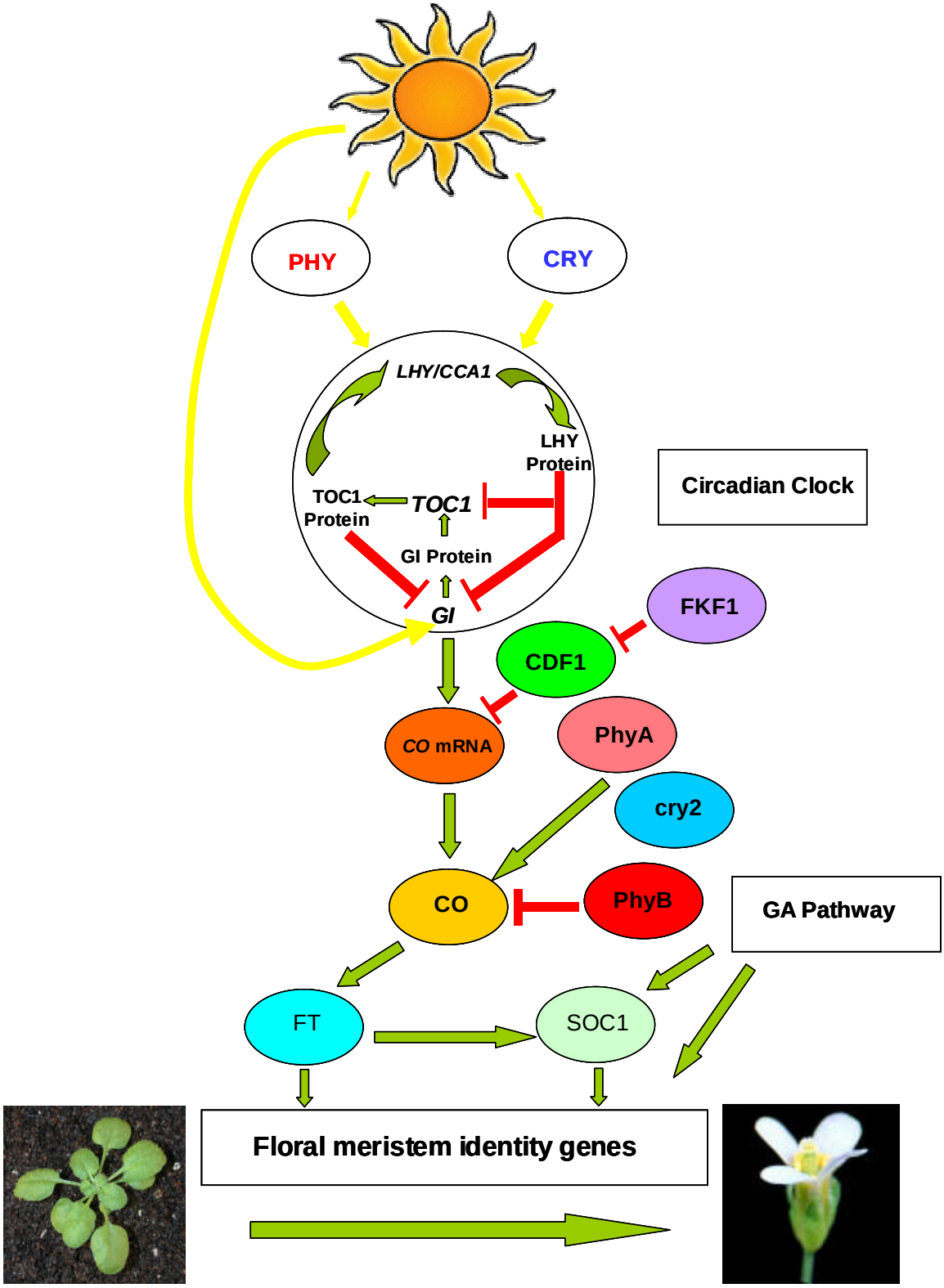


Figure 33. Schematic representation of the Photoperiodic Flowering Pathway. Light is perceived by the phytochromes and cryptochromes that entrain the circadian clock. The clock controls the expression of *CO* and *GI* regulates flowering by regulating the levels of *CO* mRNA. Activated CO protein then induces the expression of the floral integrators FT and SOC1 which in turn control the expression of the floral meristem identity genes to induce flowering.

4.2 Aims and Objectives

To determine the position of *DNF* within the photoperiodic pathway.

To investigate the role that *DNF* plays in the control of flowering.

4.3 Results.

4.3.1 DNF is expressed between 4-6hrs in SD.

The expression profile of the *DNF* gene was determined using plants grown in SD and LD. The transcripts were analysed in the *dnf* mutant and Ws plants in SD. As expected, no transcript was detectable in the *dnf* mutant plants. Figure 34 shows that in SD the expression profile of *DNF* is very low but peaks at around 4-6 hrs after dawn. Before 4 hrs and after 6 hrs there is no expression of *DNF* which suggests that this gene is tightly regulated.

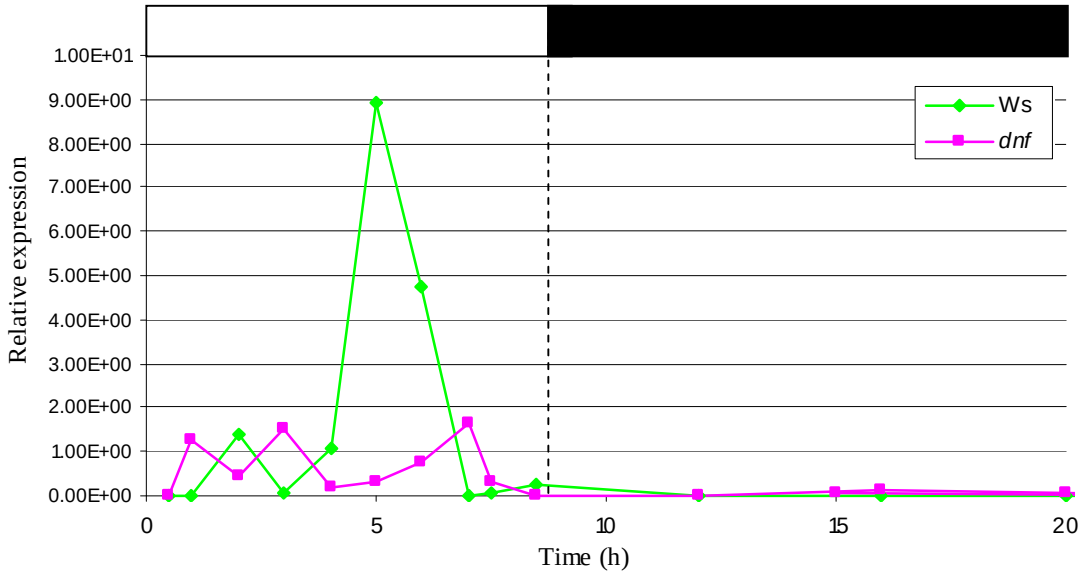


Figure 34. Expression of *DNF* in WT and *dnf* plants. Plants grown in SD and data normalised to ß-actin. Black and white rectangles indicate lights on light off respectively

4.3.2 The expression of CO is altered in the *dnf* mutant in SD.

In order to confirm previous findings of altered CO expression in *dnf*, we monitored the expression of *CO* in the *dnf* mutant and Ws. Total RNA was isolated from plants grown under non-inductive SD photoperiods at 22ºC. *CO* transcript levels were then compared between the *dnf* mutant and Ws.

In the SD grown *dnf* mutant the level of *CO* transcript is significantly increased when compared to wild type (Ws-4). In figure 35 two clear peaks can be identified the first at approximately 8 hrs and the second at 16 hrs after dawn. This expression profile is consistent with both the previous findings of Dr Stephen Jackson (see above) and the early flowering *dnf* phenotype. Interestingly the two peaks occur 8 hours apart, an expression pattern that is not dissimilar to that previously observed in LD for the *CO* transcript, however in that case the two peaks were shown to be at 16 and 24 hrs (Hayama *et al.,* 2003).The increased levels of *CO* expression proved to be significant by using a two-tailed T-test assuming equal variances P=0.0296 (Appendix Table 9.).

The SD expression profile of *CO* in wild-type Ws is masked by the higher expression profile in the *dnf* mutant. However when the *dnf* mutant data is removed the expression in Ws can clearly be seen, beginning to raise around 12 to 20 hrs after dawn with a peak at around 16 hrs. (Figure 36) This profile matches previous findings of *CO* expressions in SD shown by (Hayama *et al.,* 2003)

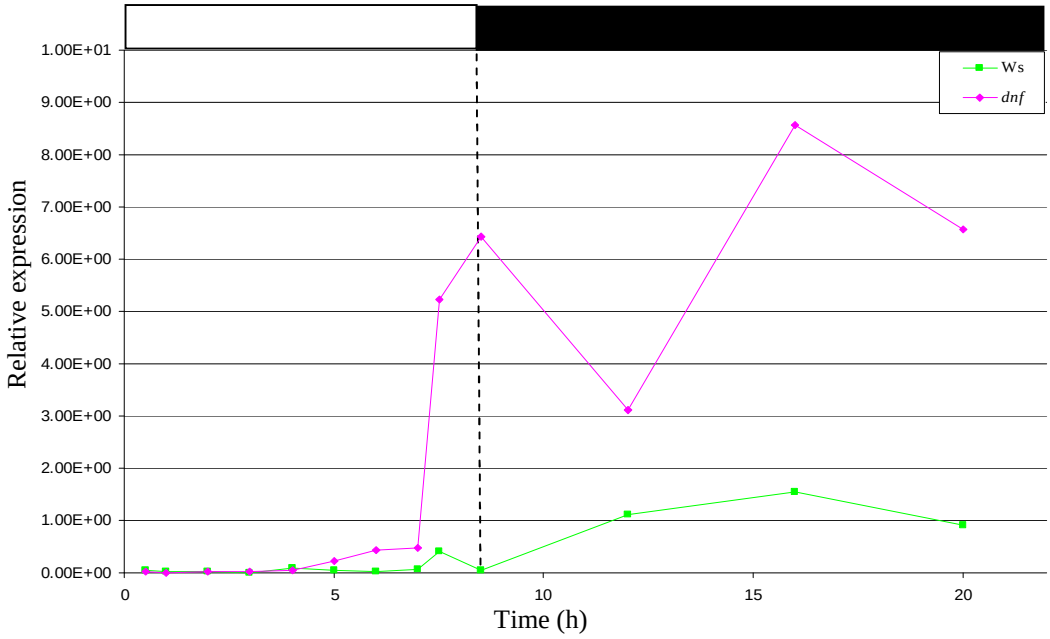


Figure 35. Expression of *CO.* in WT and *dnf* plants. Plants grown in SD and data normalised to ß-actin. Black and white rectangles indicate lights on light off respectively. For each time point rosette leaf tissue was combined from approximately 10 plants from leaf 5-6.

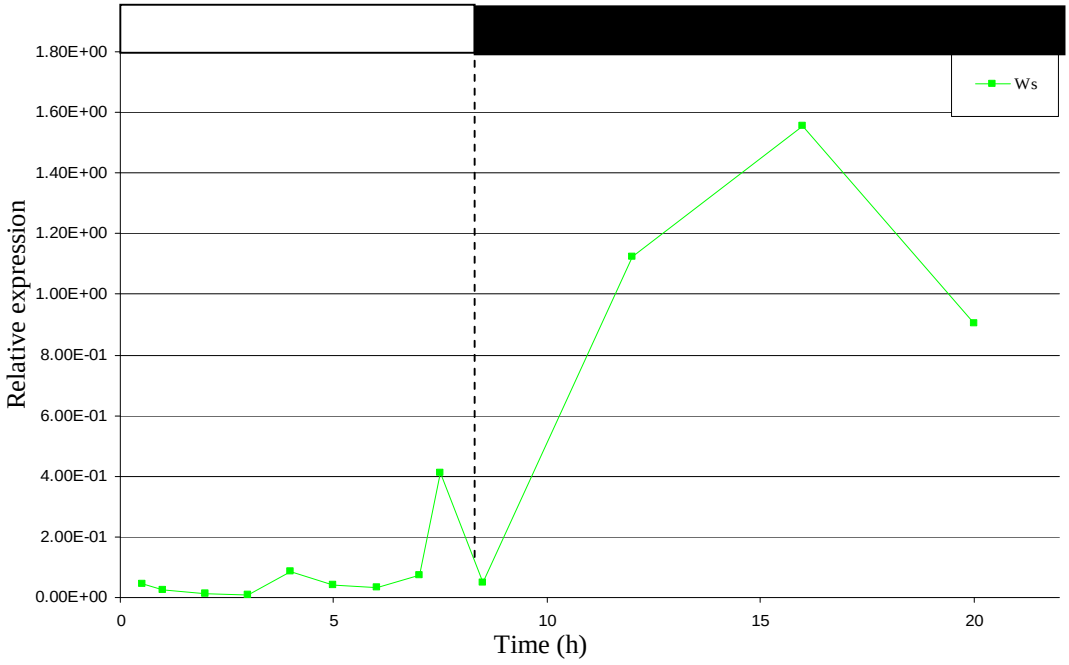


Figure 36. Expression of *CO.* in WT plants. Plants grown in SD and data normalised to ß-actin. Black and white rectangles indicate lights on light off respectively. For each time point rosette leaf tissue was combined from approximately 10 plants from leaf 5-6.

4.3.3 The expression of *CO* in SD at 16ºC is unaltered in the *dnf* mutant.

We have already established that the *dnf* mutant is no longer flowers early when grown under SD at lower temperatures such as 16ºC and in fact the lower temperature grown *dnf* flowers later than WT (Chapter 3). When grown at 22ºC SD the *dnf* mutant demonstrates an increased level of *CO* expression which is consistent with the early floral promotion. To test whether the late flowering of the *dnf* mutant at 16ºC is due to reduced levels of *CO* transcript we looked at the expression of *CO* in samples collected from both Ws and *dnf* mutant lines grown at this reduced temperature. Samples were collected at ZT 2, 4, 6, 7, 7.5, 8 and 10 hours. As can be seen in Figure 33 there is little difference between the levels of *CO* transcript between Ws and the *dnf* mutant which would explain why *dnf* is no longer early flowering at 16ºC. Any difference in expression did not prove using two-tailed T-test assuming equal variances, P=0.325 (Appendix Table 10.).

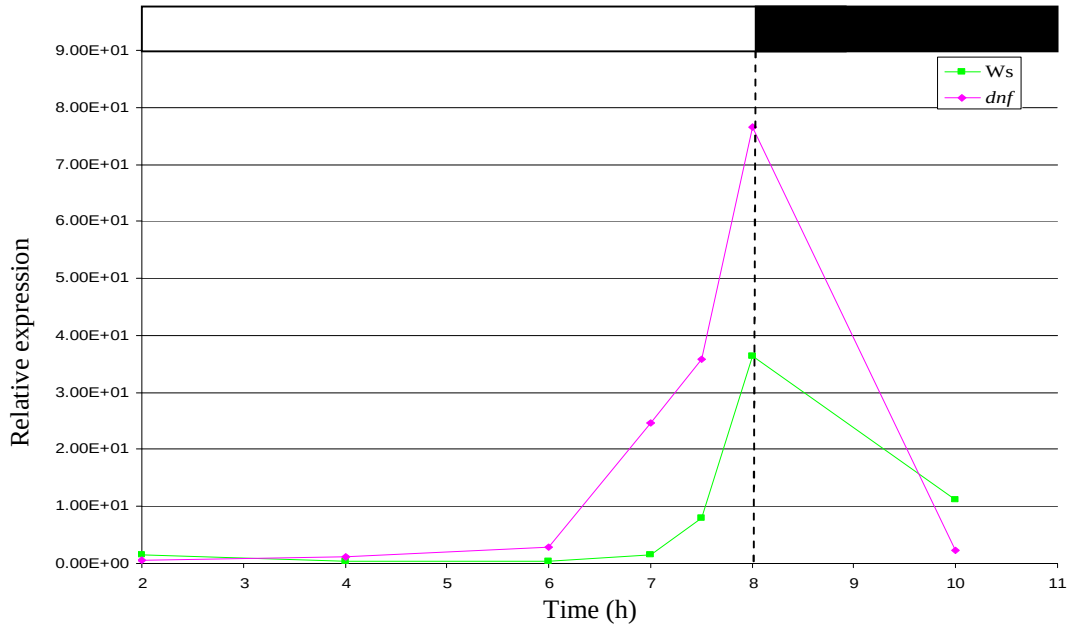


Figure 37. Expression of *CO*. in WT and *dnf* plants at 16°C. Plants grown in SD and data normalised to ß-actin. Black and white rectangles indicate lights on light off respectively. For each time point rosette leaf tissue was combined from approximately 10 plants from leaf 5-6.

4.3.4 The *dnf* mutation leads to an increase in *FT* transcript levels in SD.

The levels of *FT* were strongly elevated in the *dnf* mutant in SD with levels starting to rise toward the end of the day and remaining high for the first part of the night before falling back down before dawn (Figure 34).The levels of *FT* rose to between 4-5 fold higher than that of wild-type Ws where levels are consistently low. The increase in levels of *FT* in *dnf* follows the increase in expression of *CO* which might be expected as *CO* is known to induce *FT* expression.

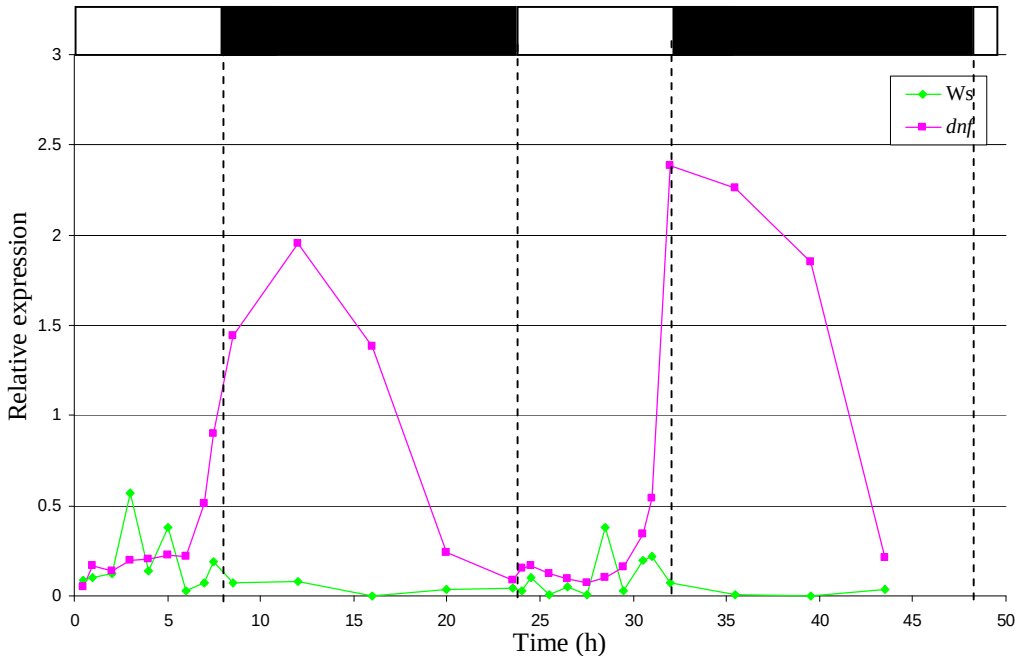


Figure 38. Expression of *FT* in WT and *dnf* plants. Plants grown in SD and data normalised to ß-actin. Black and white rectangles indicate lights on light off respectively. For each time point rosette leaf tissue was combined from approximately 10 plants from leaf 5-6.

4.3.5 The *dnf* mutant does not affect *GI* expression under short days.

To test whether *DNF* lies upstream of *GI* on the photoperiodic pathway we examined the expression levels of *GI* in the *dnf* mutant and found that the *dnf* mutation did not cause any dramatic changes in the level of the *GI* transcript (Figure 35). In both cases the expression profiles rise around 4hrs after dawn peaking toward the end of the SD before falling completely during the night. Although the levels of *GI* expression appear slightly lower in the *dnf* mutant the difference is not significant with a P value of 0.356 (Appendix Table 11.).

The fact that the *dnf* mutant causes an increase in both *CO* and *FT* expression levels without changing the *GI* mRNA expression pattern indicates that *DNF* lies upstream of *CO* but downstream of *GI* on the photoperiodic pathway.

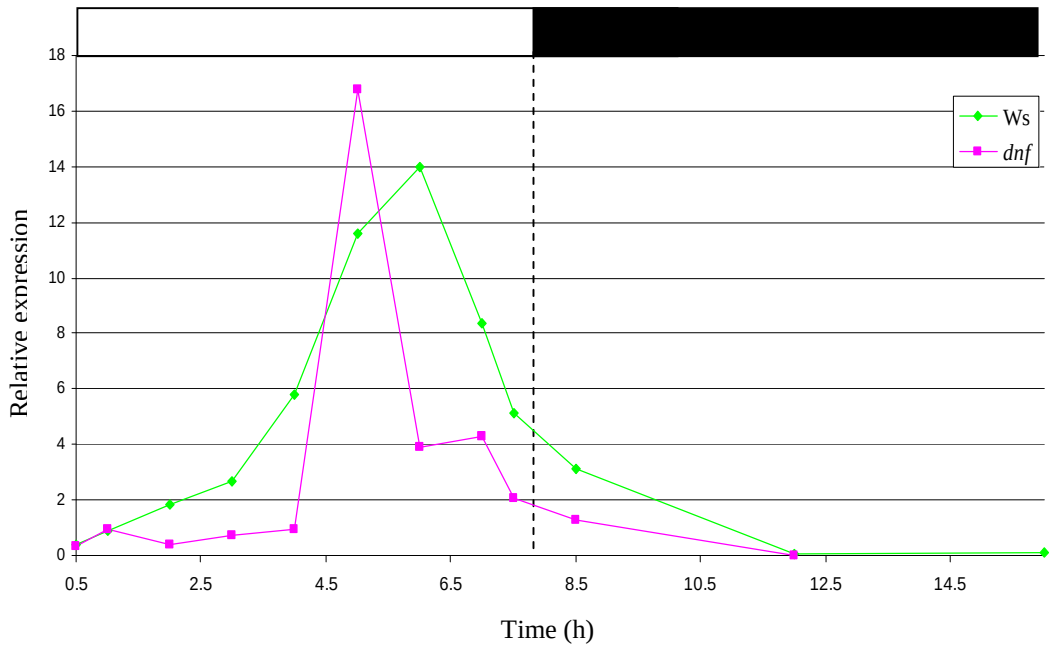


Figure 39. Expression of *GI* in WT and *dnf* plants. Plants grown in SD and data normalised to ß-actin. Black and white rectangles indicate lights on light off respectively. For each time point rosette leaf tissue was combined from approximately 10 plants from leaf 5-6.

4.3.6 The robust rhythm of *CAB* expression does not appear to be altered in the *dnf* mutant.

We have already demonstrated that the perception of light is unaffected in *dnf* (Chapter 3).However the early flowering phenotype may be caused by a defect in circadian regulation. In order to determine if the *dnf* mutation caused the free running period of the circadian rhythm to either lengthen or shorten we examined the expression of *CAB* in the *dnf* mutant and compared this to *CAB* expression in wild-type Ws. Plants were grown under 8hr SD conditions until they were young seedlings before transferring to LL conditions. The plants were then sampled at specific time points for 2 days.

After transferring to LL conditions the expression of the *CAB* gene in *dnf* compared to Ws is indistinguishable with both following a similar circadian expression pattern (Figure 36).The fact that there is no difference in the phase of *CAB* gene expression indicates that there is no effect on circadian regulation in the *dnf* mutant.

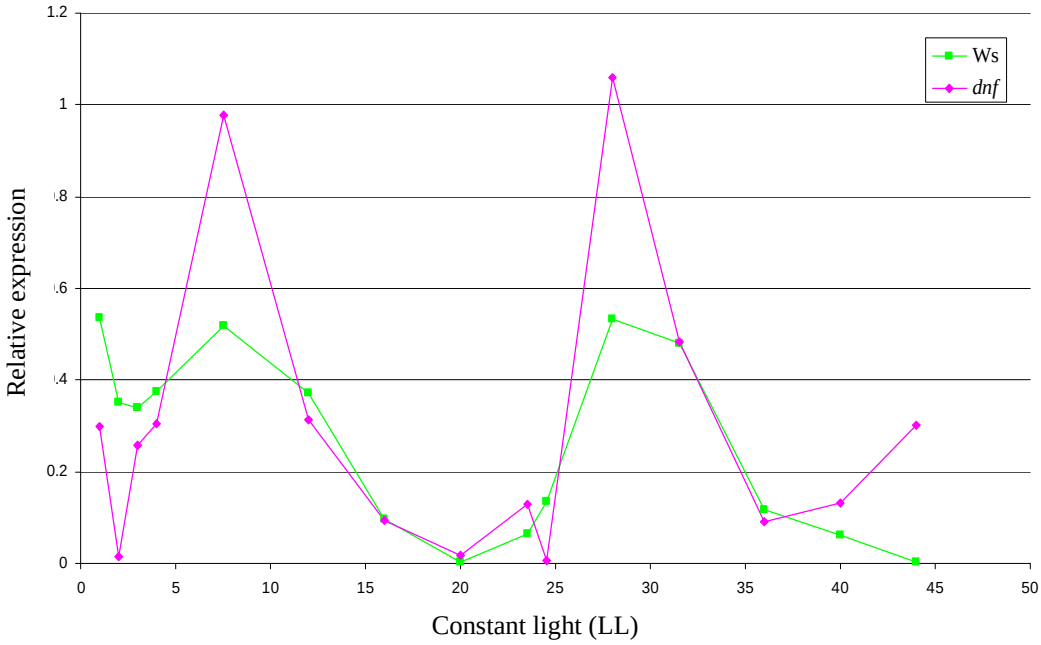


Figure 40. Expression of *CAB*.in WT and *dnf* plants.
Plants grown in SD until 4 true leaves before transferring to LL. For each time point rosette leaf tissue was combined from approximately 10 plants from leaf 5-6.
Data normalised to ß-actin.

4.4 Discussion

Under SD there is a precise peak in the expression of *DNF* between 4-6hrs after dawn indicating a tight regulation of the gene. This tight peak in expression fits neatly with the critical photoperiod data (Chapter 3) in which we observed no difference in flowering response between the *dnf* mutant and Ws in photoperiods of only 4 hrs. It was only when the photoperiod was increased beyond 4 hrs that a difference was observed.

Interestingly the peak in *DNF* expression in SD occurs just before the peak in *CO* expression (Figure 34 and 35). If *DNF* is involved in the transcriptional regulation of *CO* then you would expect the expression of *DNF* to be earlier than the expression of *CO*. When analysing *CO* expression in the *dnf* mutant it was found to be significantly higher by using a two-tailed T-test assuming equal variances P=0.0296 (Appendix Table 9.). This result is consistent with the early flowering phenotype of the mutant and previous findings of Dr Stephen Jackson.

The wild-type plants grown in SD demonstrate that *CO* expression begins during the night between 12 to 20 hrs after dawn with a peak at around 16 hrs. In the *dnf* mutant the expression of *CO* appears to begin to rise at about 7hrs before falling around 20hrs after dawn. This expression pattern is very similar to that described for LD wild-type plants (Suarez-Lopez *et al.,* 2001). This suggests that the early flowering phenotype of the *dnf* mutant in SD is due to an inappropriate induction or loss of repression of *CO*, thus it is postulated that *DNF* could act a repressor of *CO* transcription.

The *DNF* gene contains a predicted membrane spanning domain and a putative RING finger domain. Although the mechanism by which *DNF* could

repress *CO* is unknown there are two possible ways in which this may occur. Firstly DNF contains sequence similarity to a putative RING-H2 domain. Many RING-H2 domains are known to be E3 ligases and are involved in targeting specific proteins for degradation by the 26S proteasome (Stone *et al.,* 2005). These RING-H2 domains contain a characteristic set of histidine and cysteine residues that bind two zinc ions and interact with an E2 binding domain. Therefore it is possible that DNF could function as an E3 ligase specifically targeting an activator of CO for degradation by the proteasome. However a study of all RING finger proteins in the Arabidopsis genome found that the DNF protein was unlikely to form an active RING domain (Stone *et al.,* 2005).

An alternative explanation is that DNF is acting as a transcriptional repressor of *CO*. The putative RING finger domain also shows similarity to Plant Homeodomain (PHD) sequences. Many of these domains are thought to mediate protein-protein interaction and are present in transcriptional regulators involved in chromatin remodelling (Pineiro *et al.,* 2003). Other PHD proteins have previously been identified as being involved in flowering, these include VIN3-LIKE 1 (VIL1) and EARLY BOLTING IN SHORT DAYS (EBS) (Pineiro *et al.,* 2003; Sung *et al.,* 2006). The mechanism of action of *DNF* is still under study.

Growing the *dnf* mutant at 16ºC SD completely abolished the early flowering phenotype that is observed at 22ºC SD. It is hypothesised that the early flowering phenotype is a consequence of the increased levels of *CO* expression in the *dnf* mutant at 22ºC. Therefore we would expect that as *dnf* no longer flowers early at reduced temperatures then the levels of *CO* expression would revert back to WT levels.

As expected there is little difference in the levels of *CO* transcript

between Ws and the *dnf* mutant which would account for the later flowering phenotype of our mutant at 16ºC.

This reversal of the early flowering phenotype at lower temperatures is not unique and has been observed in other early flowering mutants such as *phyB* (Halliday *et al.*, 2003). The Halliday lab also found elevated levels of *CO* expression in the *phyB* mutant at both 16ºC and 22ºC, however as the increase in *CO* expression was relatively small it was not deemed to be significant. This is in contrast to the *dnf* mutant when at 22ºC the level of *CO* expression is significantly higher than in Ws suggesting that *DNF* functions through the *CO*-dependent photoperiodic pathway.

In addition to photoperiod, temperature is recognised as a major environmental factor affecting of flowering time. The precise reason for the reversal of the early flowering phenotype of the *dnf* mutant has yet to be clarified. It may be possible that the role of *DNF* is restricted to higher temperatures and at lower temperatures other predominant regulators of flowering come into effect. This is true of phyB which acts as a repressor of *CO* at 23ºC but at 16ºC phyB no longer represses *CO*, instead phyE replaces phyB as a repressor of *CO* (Cerdán *et al.*, 2003; Halliday *et al.*, 2003). Due to time constraints no further analysis was performed on the *dnf* mutant at 16ºC, however further research in this area would provide a greater insight into the possible mechanism involved in the control of flowering at lower temperatures.

The overexpression of *FT* causes early flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) and since *FT* is regulated by *CO*, we tested whether the upregulation of *CO* in the *dnf* mutant results in the upregulation of *FT*. We found that *FT* was strongly elevated in the *dnf* mutant with levels starting to rise toward

the end of the day and being high during the first part of the night before falling back down to a low level before dawn. Thus the high levels of *FT* in *dnf* follow the increased expression of *CO* as expected consistent with the ides that the de-repression of *CO* in the *dnf* mutant results in the induction of *FT* and flowering in SD.

It is interesting to note that the elevated levels of *FT* in *dnf* are between 4-5 fold higher than that of wild-type Ws, and that *FT* expression oscillated with a diurnal rhythm. This diurnal expression of *FT* in the *dnf* mutant closely parallels that of *FT* expression in WT in LD described by (Suarez-Lopez *et al.,* 2001) in which *FT* mRNA was shown to cycle with a diurnal pattern that follows that of *CO* expression.

An altered pattern of *FT* expression has been observed in the early flowering mutant *ebs* however no elevated levels of *CO* transcript was observed indicating that this gene acts independently of *CO* and is a repressors of *FT* under SD (Pineiro *et al.,* 2003). Interestingly the *spa1* mutant also flowers early specifically under SD, it too shows an increased level of *FT* mRNA during the night phase (Laubinger *et al.,* 2006). SPA1 is believed to control the levels of CO protein as SPA1 was shown to physically interact with CO both in vivo and in vitro (Laubinger *et al.,* 2006).

Mutants in *gi* display reduced amplitude of *CO* expression and late flowering (Suarez-Lopez *et al.,* 2001). Some early flowering mutants have been shown to lead to a change in expression of *GI*. For example the *elf-3* mutant flowers early in both short and long days and leads to an increase in *GI* expression in both photoperiods (Fowler *et al.,* 1999).

In order to determine if there was any difference in expression levels of *GI* in the *dnf* mutant we examined the *GI* transcript in the *dnf* mutant and Ws. The results showed that the *dnf* mutation did not cause any dramatic changes in the level of the *GI* transcript ((Figure 35). Therefore we can conclude that as the signalling downstream of *GI* is altered but not the levels of *GI* transcript itself, then *DNF* must lie downstream of *GI* and upstream of *CO* on the photoperiodic pathway.

The major effect of *GI* on flowering in LD is through the up-regulation of *CO* mRNA levels (Suarez-Lopez *et al.,* 2001). However in SD, although *GI* expression is detected in the light period *CO* is not up-regulated. This suggests that that *GI* might be modified in SD so that interactions only take place in LD (David *et al.*, 2006). The *dnf* mutant results in an increase in both *CO* and *FT* expression levels without changing the *GI* mRNA expression pattern. Therefore it is possible that DNF is involved in the regulation of GI interactions SD thus preventing an increase in *CO* mRNA and that in the *dnf* mutant this level of control is lost allowing *CO* transcript to increase leading to the promotion of flowering.

The plant circadian clock regulates leaf movement and the rhythmic expression of many different genes including *CAB* whose expression is low during the night and peaks in the mid morning (Millar *et al.*, 1996). The expression of *CAB* can be monitored as an indication of circadian clock function. The *elf4* mutant has been shown to affect the rhythm of *CAB* such that the mutant lost its rhythmcity of *CAB* expression in LL after one 24hr cycle (Doyle *et al.*, 2002). When we compared the expression of *CAB* in the *dnf* mutant to that of Ws in LL, no difference between the two could be found. This finding shows that the

clock circadian regulation is not altered in the *dnf* mutant

The aims of this chapter were to investigate the role that *DNF* plays in the control of flowering and to determine the position of *DNF* within the photoperiodic pathway. To this end we used qRT-PCR to examine differences in expression patterns of genes involved in the photoperiodic flowering pathway. We discovered that the *DNF* transcript is expressed rhythmically with a precise peak between 4-6 hrs. This expression profile occurs just before the WT expression of *CO* which has been shown to peak between 8-16hrs in LD and 12-20 hrs in SD (Hayama *et al.*, 2003). Furthermore the expression of *CO* in the *dnf* mutant is altered and shows a similar transcriptional profile to that of *CO* observed in WT in LD. This increase in the expression of *CO* in the *dnf* mutant in SD leads to an increase in expression of *FT* which is consistent with the early flowering phenotype of the mutant. In addition the expression of *GI* and *CAB* is unaltered in the *DNF* mutant, thus indicating that *DNF* must lie downstream of *GI* and upstream of *CO* on the photoperiodic pathway and that the clock function is not altered.

This combined with the change in the expression profile of *CO* indicates that the role of *DNF* must be to prevent the expression of *CO* during the day in SD possibly through GI modifications and therefore preventing flowering in SD.

Chapter 5

Expression of TAP and EGFP tagged DNF fusion proteins.

5.1 Introduction

As described in Chapter 1 complementation of the *dnf* mutant was carried out by re-introducing the wild-type *DNF* gene under the control of its endogenous promoter. Successful complementation of the mutant confirmed that the mutated *dnf* gene is responsible for the observed early flowering phenotype in SD. Other mutants also show similar flowering phenotypes to that of *dnf*. *EARLY BOLTING IN SHORT DAYS (EBS)* has been shown to be a repressor of flowering in SD and may act as a repressor of *FT* (Gomez-Mena *et al.*, 2001). *Early flowering 4 (elf4)* is a mutant that also leads to early flowering in SD and as is the case in *dnf*, shows an increased level of *CO* transcription. However this mutant has been shown to disrupt the rhythm of the circadian clock associated gene *CCA1* which induces flowering by controlling the phase of a clock output gene such as *CO* that is specific to floral to regulation of flowering (Doyle *et al.*, 2002).

The primary aims of this chapter were to look at the sub-cellular localisation of DNF and identify any protein partners of DNF. To this end EGFP linked to DNF was expressed in Arabidopsis and the fusion protein used to examine the cellular and sub-cellular localisation of DNF via confocal microscopy. This method has been used in the analysis of CO and demonstrated that CO was present in the nucleus of plants grown in BL, FR,WL but not RL (Valverde *et al.*, 2004).

With the aim to identify interacting proteins of DNF an epitope tag was fused to *dnf to* allow purification via TAP tag columns. Once purified, the associated proteins can be separated by 2D electrophoresis and proteins can be excised. After which time they can be analysed by matrix-assisted laser

desorption/ionisation time-of-flight (MALDI-TOF) MS in order to generate a peptide map of each protein. Identification of individual proteins can then be performed by comparison with protein databases. MALDI-TOF MS has been routinely used in the identification of constituents of protein complexes, recent applications include the identification of the components of the Arabidopsis 26S proteasome (Yang *et al.,* 2004) and analysis of molecular changes at the protein level in *Arabidopsis* after vernalisation (Mi *et al.,* 2007).

Another powerful tool for proteomic- based investigations is the yeast two-hybrid system. A protein of interest X is expressed as a fusion protein to a DNA binding domain (DBD-X or ‘bait`). A cDNA library is constructed thus creating the second protein of interest Y which is fused to the activation domain (AD-Y or ‘prey`).The AD-Y fusion vector is introduced into a yeast strain containing the DBD-X fusion partner by transformation. It is only if proteins X and Y physically interact with one another that the DBD and AD brought together and activate the expression of the downstream reporter genes e.g. *gal1-lacZ* the *beta* galactosidase gene.

This technology has already been employed to investigate the protein-protein interactions in *Arabidopsis* for example in the identification of protein interactors of a plant defence gene *NPR1* and in the search for protein components of the VIN3 complex, a protein essential for the vernalisation response.(Despres *et al.,* 2000; Sung *et al.,* 2006)

The advantage of using the TAP-tag pull down procedure over the yeast two-hybrid system is that a protein can serve as bait in its native environment and complexes of multiple proteins can be isolated whereas the yeast two hybrid

system only identifies interactions between two proteins reviewed by (Aebersold *et al.,* 2003).

Dr Stephen Jackson has previously demonstrated functional complementation of the *dnf* mutant with using wild-type DNF gene (Figure 41.). Functional complementation of the *dnf* mutant with the epitope–tagged fusion proteins would show that the tagged DNF protein was functional and therefore interacting with its protein partners.

5.2 Functional complementation

After complementing the *dnf* mutant with the wild-type *DNF* gene a range of flowering times was observed in the T_2 generation with some lines showing full complementation with the restoration of WT flowering time. Testing of these lines in the T_3 generation showed that they were homozygous for the complementing transgene. Lines with an intermediate flowering phenotype are likely to be heterozygous for the transgene.

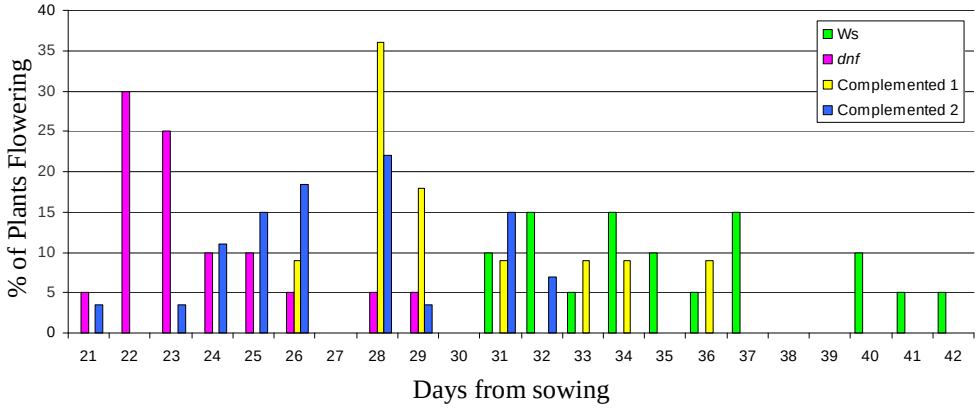


Figure 41. Complementation of the *dnf* mutant. Flowering Times of two independent lines showing complementation (Dr Stephen Jackson Warwick HRI personal communication).

5.3 Aims and Objectives

To examine cellular and sub-cellular localisation of EGFP-DNF fusion proteins under the control of both the native (PDNF) and over expressing P35S promoter.

Creation of both N and C terminal TAP tag expressing plant lines that can be used in pull down experiments thus allowing purification and identification of possible interacting proteins of DNF.

5.4 Production of transformed plants

Conventional cloning was used in the construction of the six plasmids outlined in figure 42 and prior to transformation PCR-amplification performed across each junction using gene specific primers and the sequenced was analysed in order to confirm that the reading frame was maintained in the constructs to enable correct read-through of the genes (Chapter 2).

The *dnf* mutant was transformed by the floral dip method with each of the six plasmid constructs illustrated in figure 42 (Chapter 2). T_0 seed from the transformed mutant was then selected on hygromycin plates in order to identify resistant plants. The resistant plants were transferred as young seedlings (approximately 10 days old) to soil and T_2 seed collected. In addition leaf tissue was collected from and PCR-amplification was performed. These PCR's used hygromycin oligonucleotide primers HYG F and HYG R which amplified a 468bp fragment. An additional PCR was performed using internal fusion protein oligonucleotide primers (DNFF and EGFPR, DNFF and CTAPR, DNFR and NTAPF, which amplified a 1046, 212 and 384 bp products respectively (Table 6). All amplicons were visualised on a 2% agarose gel, an example of which can be seen in figure 43.

The hygromycin resistant T_2 plants that were also positive in the PCR assays were then grown in SD 22°C and flowering time scored by both days to flower (data not shown) and leaf number.

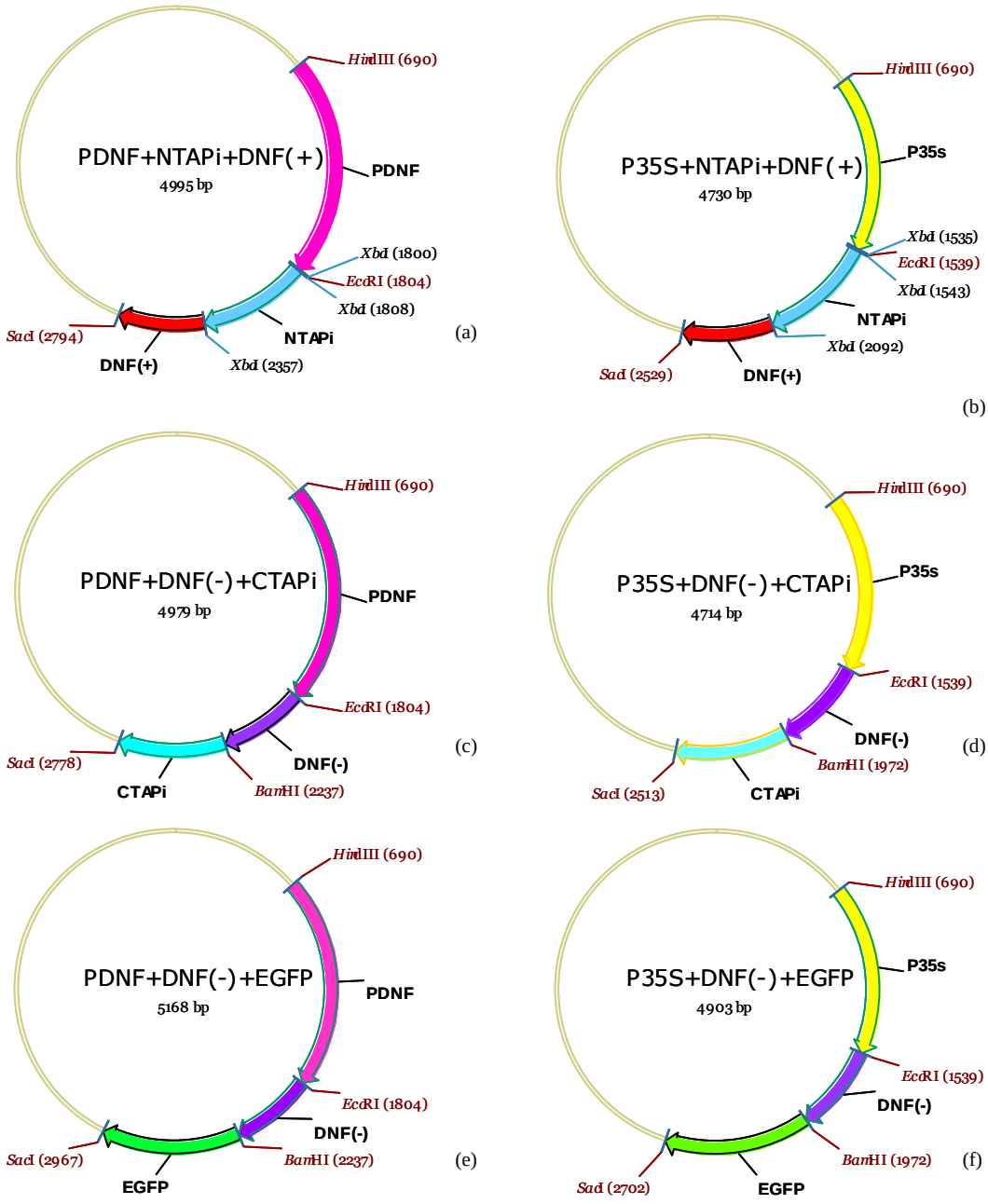


Figure 42. Illustration of completed construction in pBIB-hyg vectors. DNF(+)= DNF with the addition of a stop codon, DNF(-)= DNF without the addition of a stop codon. (a) PDNF:NTAP:DNF pBIB-hyg, (b) P35S:DNF:NTAP pBIB-hyg, (c) PDNF:DNF:CTAP pBIB-hyg,(d) P35S:DNF:CTAP pBIB-hyg, (e) PDNF:DNF:EGFP pBIB-hyg,(f) P35's:DNF:EGFP pBIB-hyg.

Primer Name	**Sequence**
M13F	CCCAGTCACGACGTTGTAAAACG
M13R	AGCGGATAACAATTTCACACAGG
HYG F	CAGCGTCTCCGACCTGAT
HYG R	GACCGATTCCTTGCGGTC
DNF F	CACATTCTTCGCGATTCTGA
DNF R	TCAGAATCGCGAAGAATGTG
EGFP R	ACTGGGTGCTCAGGTAGTGG
NTAP F	AGAGCAGCAGATGGAAGAGC
CTAP R	GACAAACGACGCATTCGATA

Table 6. Primer sequences used in the identification of plasmid constructs and transformed lines.

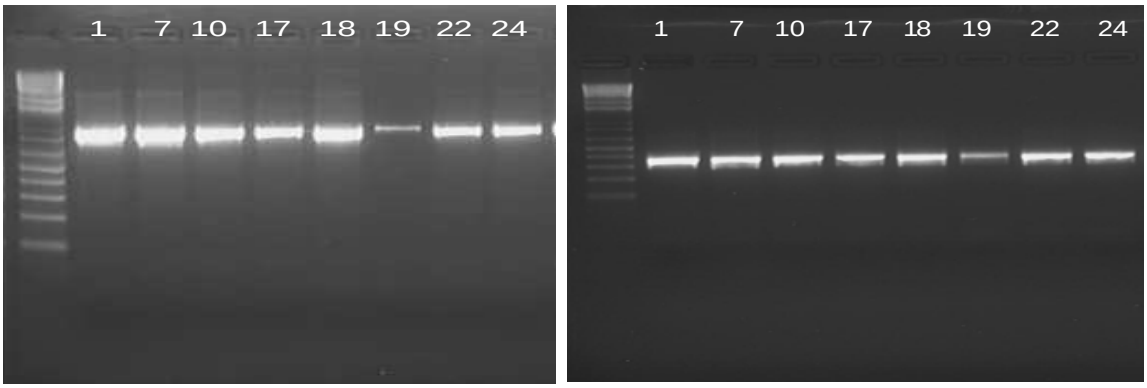


Figure 43. Example of PCR amplifications performed on transformed lines. (a):DNF Forward+EGFP Reverse internal PCR (b):Hygromycin Primers.

5.5 Results

5.5.1 Complementation of the *dnf* mutant using CTAP-Tag DNF constructs.

Each of the 4 TAP tags constructs (Figure 42 a-d) were used to transform the *dnf* mutant. Only three independent lines of PDNF:DNF:CTAP grew successfully to flowering and seed production. The flowering time of these three lines is shown in the chart below (Figure 44). Line 1 and 2 did not show evidence of complementation and flowered around the same time as the *dnf* mutant. However line 3 produced some plants with intermediate flowering times similar to results observed previously by Dr Stephen Jackson.

Eight independent lines that had been transformed with the P35S:DNF:CTAP were obtained. Analysis of T_2 plants from these lines showed that they all obtained some degree of complementation although not full complementation back to WT flowering. The best of these lines was line eight. Seed from 12 plants from line eight were selected again on hygromycin plates to determine the zygosity of the hygromycin resistant gene (Table 7). Five out of the 12 batches of seed grew on hygromycin and the rest died soon after germination. If the progeny of the T_2 plants are heterozygous for the hygromycin gene then we would expect to obtain a 3:1 ratio (resistant to non resistant) when their seeds are germinated on hygromycin, however this was not observed in any of the lines. The reason for this might be explained by poor germination. From the lines that did germinate only one (8.9) proved to be fully resistant and therefore homozygous for the hygromycin resistant gene. Tissue from this line was collected and used for Western blots to determine if the protein is being expressed (Figure 50.).

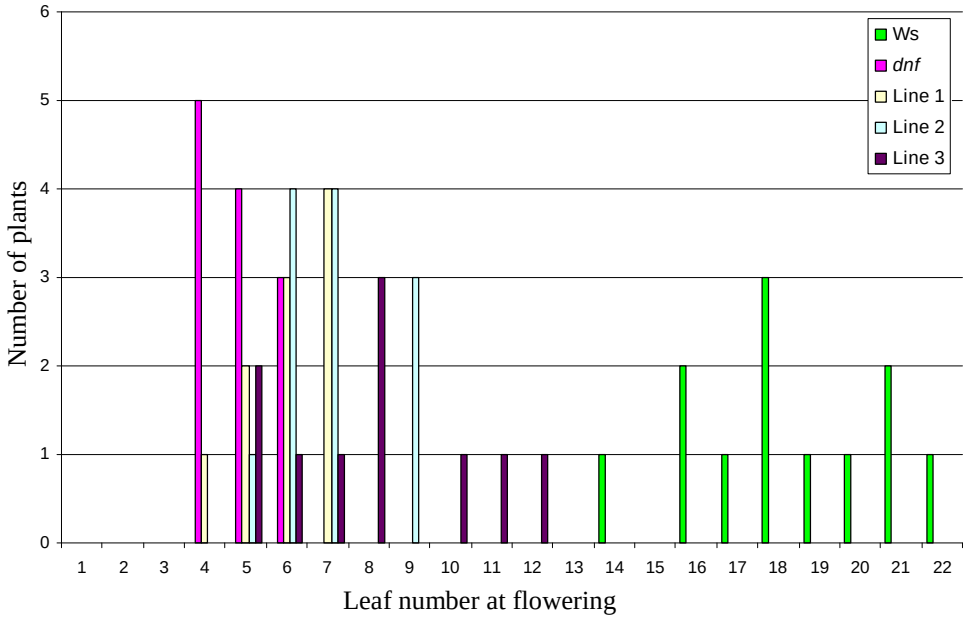
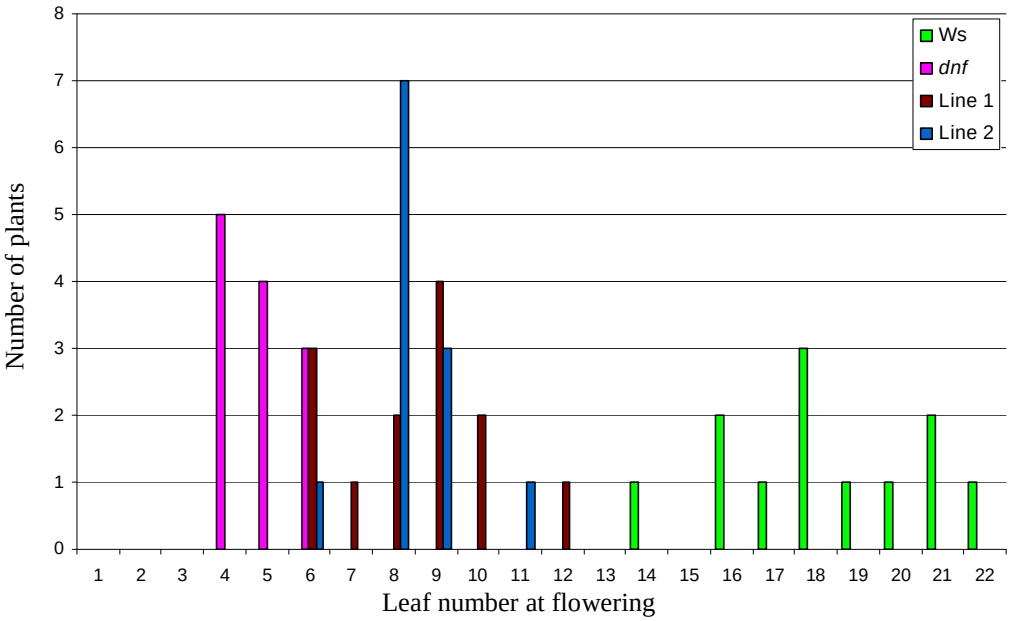
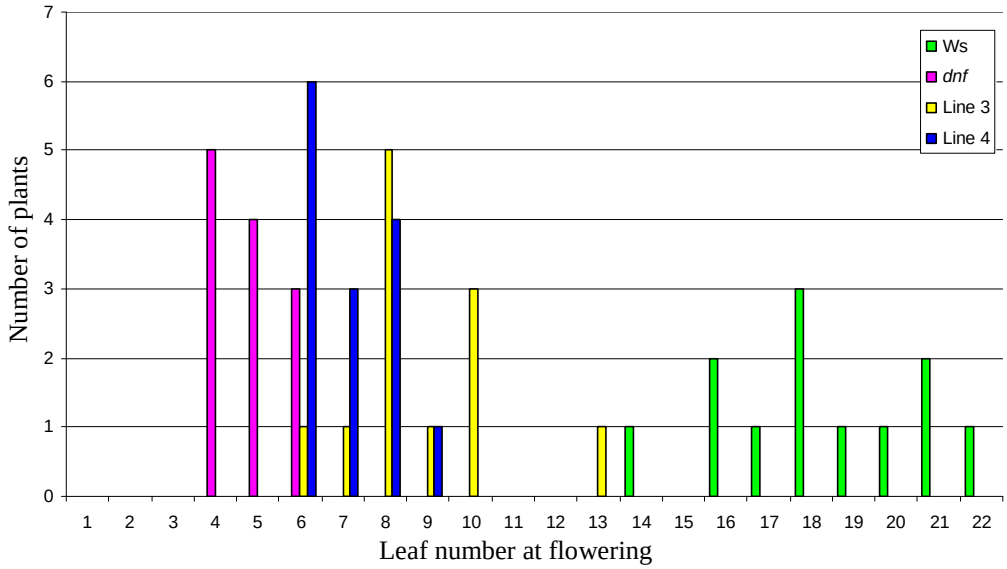


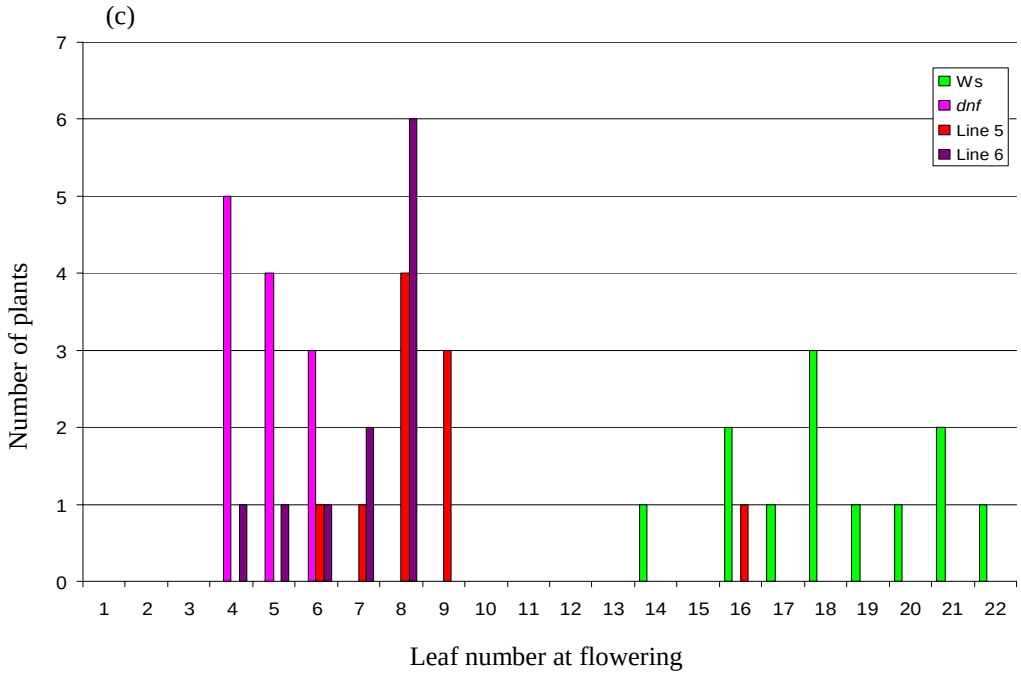
Figure 44. Flowering times of T_2 lines of the *dnf* mutant transformed with PDNF:DNF:CTAP. Flowering times scored by leaf number at flowering.
Flowering time was also scored by leaf number at flowering and a similar trend was observed.

(a)


Ws
dnf
Line 1
Line 2
Number of plants
Leaf number at flowering

(b)


Ws
dnf
Line 3
Line 4
Number of plants
Leaf number at flowering



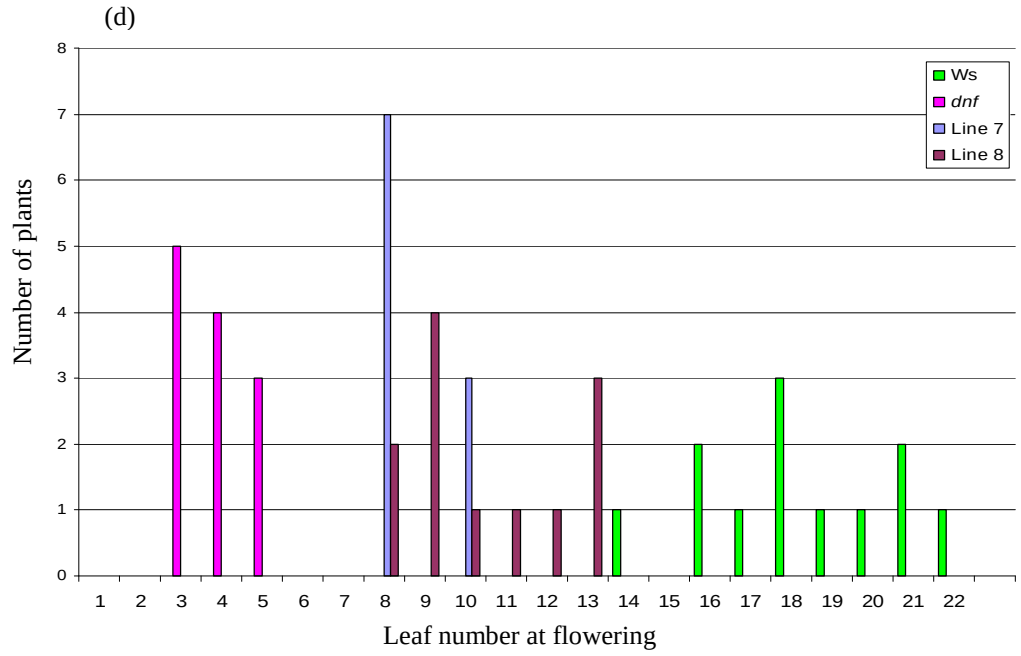


Figure 45. Flowering times of T_2 lines of the *dnf* mutant transformed with P35S:DNF:CTAP.
Flowering times scored by leaf number at flowering.(a) Line1 and 2 (b) Line 3 and 4(c) Line 5 and 6 (d) Line 7 and 8.
Flowering time was also scored by leaf number at flowering and a similar trend was observed.

P35S:CTAP:DNF			
T3 Lines			
Plant number	**Hygromycin Resistant**	**Non Resistant**	**Approximate Ratio**
8.1	65	36	2:1
8.2	36	72	1:2
8.3	44	96	1:2
8.4	48	40	1:1
8.9	124	0	1:0

Table 7. Zygocity scoring for the hygromycin resistance gene in the *dnf* mutant transformed with P35S:DNF:CATP.

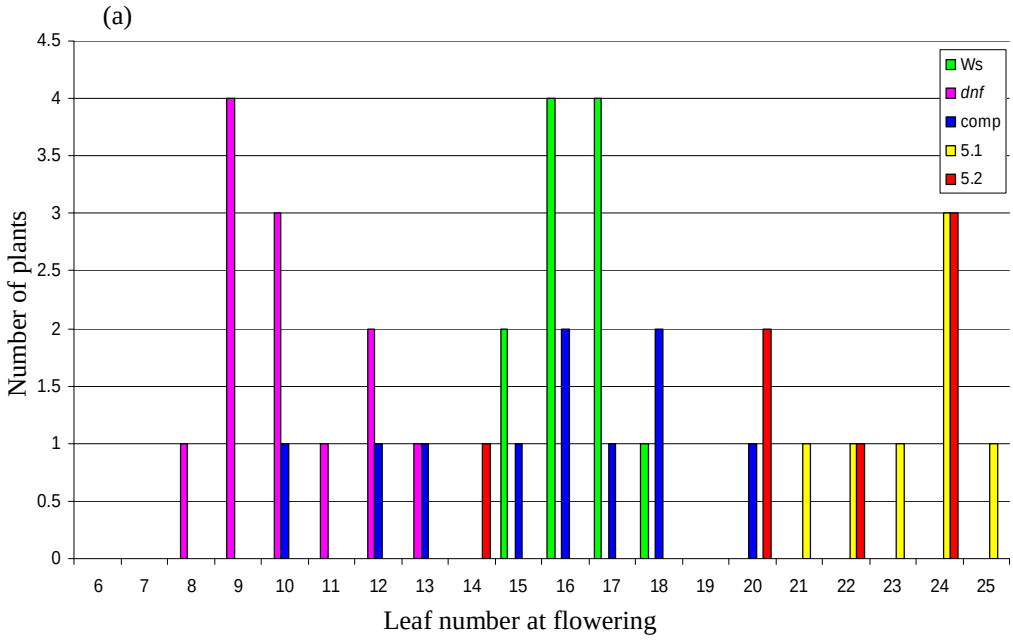
5.5.2 Complementation of the *dnf* mutant using NTAP-Tag DNF constructs.

Limited success was achieved complementing with the CTAP-tag, however the NTAP-tags linked to P35S proved to be more successful. None of the NTAP-tag linked to the native (PDNF) promoter complemented the mutant (data not shown), however 3 independent lines (Lines 5, 8 and 21) for P35S:NTAP:DNF appeared to have at least in part, restored the flowering time to that of WT. Again seed from these T_2 plants was collected and grown on hygromycin plates in order to determine the zygosity for the hygromycin resistant gene (Table 8). The T_2 parents of these lines would be segregating for the hygromycin resistance gene. Therefore some of the progeny will be resistant whilst other's sensitive.100% resistance will be homozygous for the hygromycin resistant gene whilst a 3:1 ratio would be expected from heterozygous lines. Results showed that none of these lines are homozygous for the hygromycin resistance gene and that line 8 produced the expected ratios for segregating heterozygous plants. The ratios for line 5 and 21 were not as expected for either homozygous or heterozygous plants again this could be explained by poor germination of the seed.

Not all seedlings grown on hygromycin plates survived transformation to soil, however seed from two of each line that did survive was collected. This T_3 seed was grown alongside the *dnf* mutant, Ws and a previously complemented line by Dr Stephen Jackson. Flowering time was scored by leaf number at flowering. These results indicate that line 8 and 21 both produced plants that appear to partially complement the *dnf* mutant (Figure 46 b and c). However line 5 produced progeny that fully restored the WT flowering (Figure 46 (a)). Furthermore line 5 flowered later that WT which may be due to it being driven

by a strong promoter. It was also noted that the *dnf* mutant flowered slightly later than in previous experiments which can be explained by the fact that this set of experiments were performed in different growth cabinets.

The fact that these plants complemented the early flowering phenotype indicates that the P35S:NTAP:DNF fusion protein can lead to functional complementation of the mutant. Line 5 was chosen to take forward for protein expression work using Western Blots and in addition the expression profile of the *DNF* gene was also examined in this line.



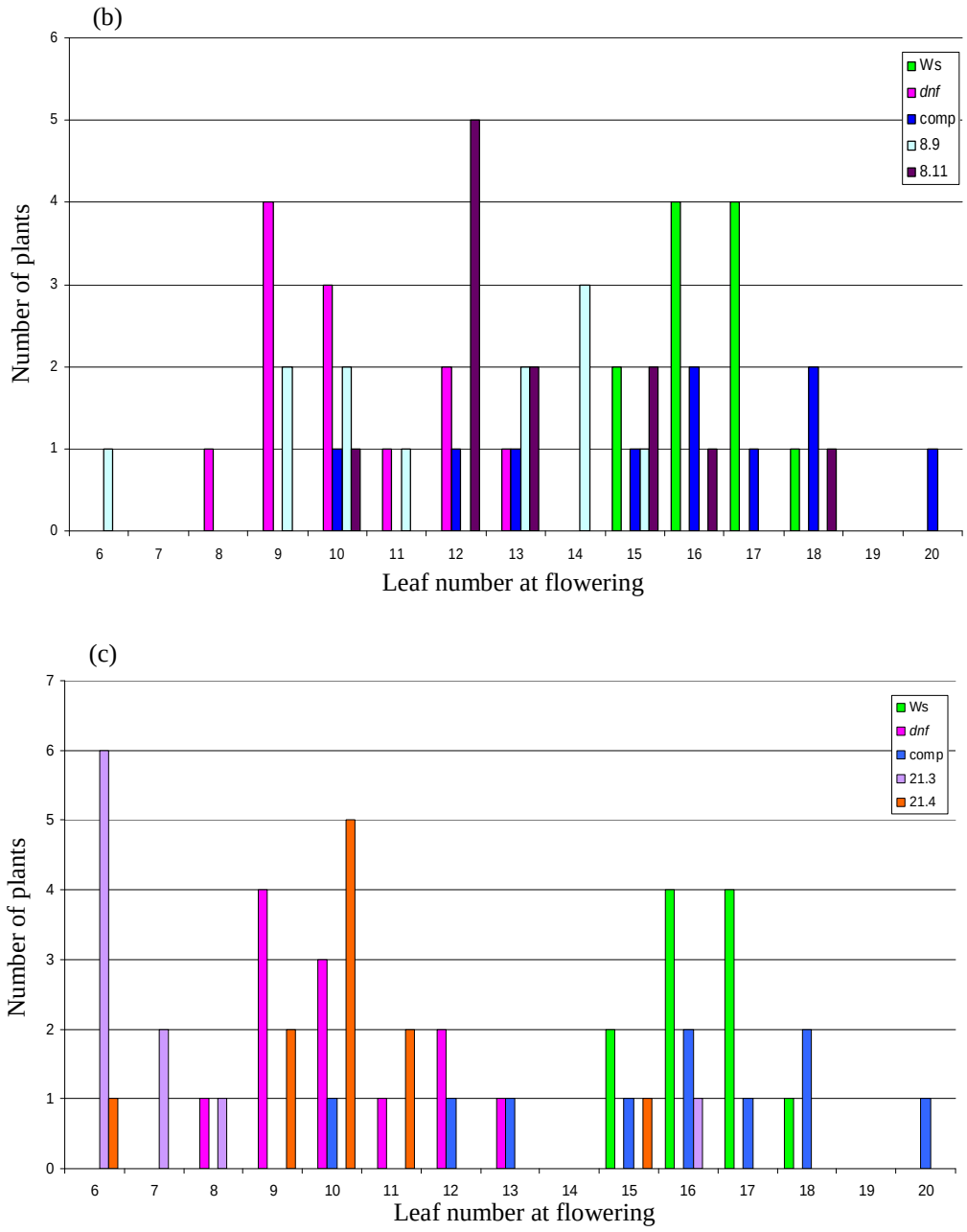


Figure 46. **Flowering times of T_2 lines of the *dnf* mutant transformed with P35S:NTAP:DNF.**
Flowering times scored by leaf number at flowering T_3 plants (a) Line 5 (b) Line 8 (c) Line 21.
Comp = previously complemented lines (Dr Stephen Jackson).
Flowering time was also scored by leaf number at flowering and a similar trend was observed.

P35S:NTAP:DNF			
T3 Lines			
Plant number	**Hygromycin Resistant**	**Non Resistant**	**Approximate Ratio**
5.1	4	24	1:6
5.2	2	8	1:4
8.9	88	12	7:1
8.11	42	14	3:1
21.3	100	16	6:1
21.4	9	60	1:7

Table 8. Zygocity scoring for the hygromycin resistance gene in the *dnf* mutant transformed with P35S:NTAP:DNF.

5.5.3 The *DNF* message seems to be unstable in the dark.

Previous analysis using real-time qRT-PCR has indicated that the *DNF* gene is expressed between 4-6hrs in SD and not in the dark (Chapter 4). Real-time analysis was performed on P35S:NTAP:DNF (Line5) in order to determine the expression profile of *DNF* in this complemented line. Total RNA was isolated from the leaves of the line 5, the *dnf* mutant and Ws over a time course grown in SD (8hr light 16hr dark), using the Z6 extraction method, then DNase treated before being subsequently reverse transcribed to cDNA (Chapter 2). This cDNA was then used for the qRT-PCR amplification of the *DNF* gene using *DNF* specific primers and normalised to ß-Actin (chapter 2).

You would anticipate that any gene expressed from the 35S promoter would be constitutively high and this expression to be independent of temperature, day length or light regime. However when *DNF* was driven by the 35S promoter a clear peak in expression can be seen which begins to rise at 4 hours light before declining at approximately 8 hours just before the end of the short day. This suggests that the *DNF* message is post transcriptionally regulated and unstable in the dark even when overexpressed. This expression profile is not dissimilar to that of the *DNF* gene observed in WS plants (Chapter 4).

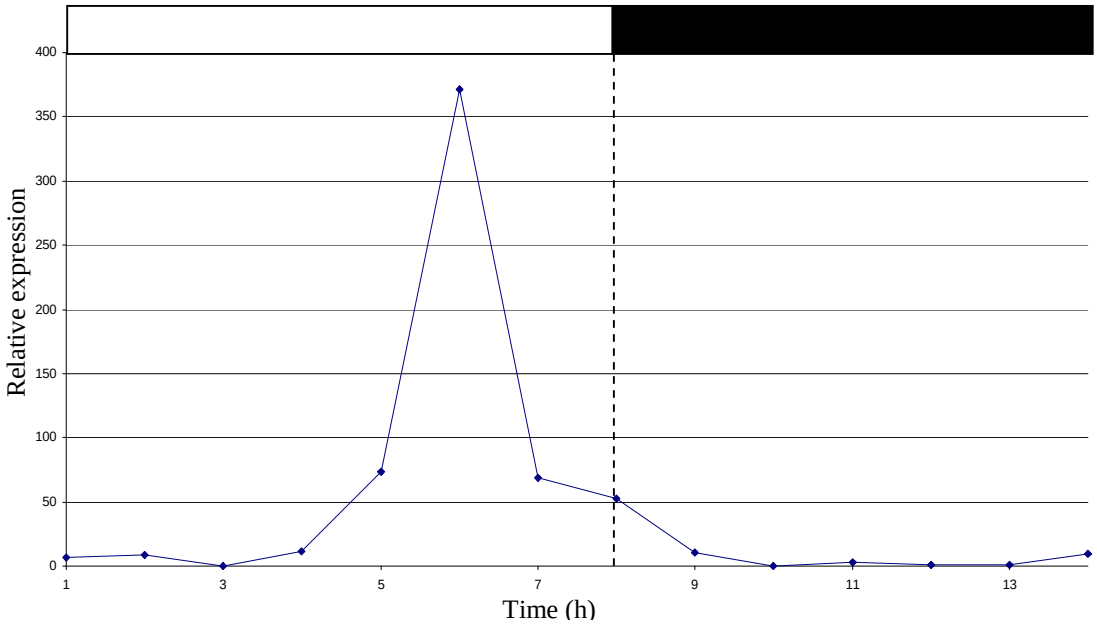


Figure 47. The expression of *DNF* normalised to ß-Actin in P35S:NTAP:DNF. Line 5 grown under short day conditions. White and black rectangles indicate lights on and lights off, respectively.

5.5.4 The EGFP fusion proteins did not complement the early flowering phenotype of the *dnf* mutant.

The *dnf* mutant was transformed with PDNF:DNF EGFP and P35S:DNF:EGFP epitope-tagged constructs. Analysis of the flowering time of T_2 plants revealed that transgenic plants flowered at the same time as the *dnf* mutant and therefore did not lead to functional complementation. The exact reason for this is uncertain, however it is possible that the addition of EGFP to the C-terminal disrupts the function of the DNF protein. This is similar to the results of the CTAP fusion proteins which also showed limited complementation whilst the NTAP fusion proteins were more successful with one line fully complementing the mutant. It was initially believed that the EGFP fusion constructs were not being properly expressed however confocal microscopy analysis revealed that the fusion protein was being expressed (see below).

5.5.5 Expression of the P35SDNF:EGFP fusion protein is localised to the cytoplasm and nucleus of Arabidopsis cells.

EGFP allows the direct visualisation of gene expression and the subcellular localization of fusion proteins in living cells.

Plants transformed with either P35S:DNF:EGFP or PDNF:DNF:EGFP were visualised for EGFP expression using confocal microscopy with a Leica SP2 linked to a Leica DM RE7 upright microscope (University of Warwick biological sciences department) and an Olympus FV300 linked to an Olympus IX70 inverted microscope (University of Warwick HRI).

The *dnf* mutant was transformed with the PDNF:DNF:EGFP and P35S:DNF:EGFP constructs in an attempt to generate stably transformed plants

in which to analyse the expression of DNF linked to EGFP. As previously described the early flowering phenotype of the *dnf* mutant was not complemented by expression of either of the two constructs. However confocal microscopy analysis results indicate that expression of the P35S:DNF:EGFP fusion protein is localised to the cytoplasm and the nucleus (Figure 48 (a)). Furthermore, analysis of the PDNF:DNF:EGFP highlighted a significant difference in protein localisation, since EGFP expression was restricted to the cytoplasm and nuclear staining was not observed (Figure 48 (b)). Validity of these results were confirmed via a negative (*dnf* mutant) and positive (ER and plasma membrane localised GFP) controls. No fluorescence was observed with the negative control and ER and membrane localised fluorescence was clearly visualised in the positive controls (Figure 49 (c,d)) Western blot analysis was undertaken on the EGFP expressing lines (see below).

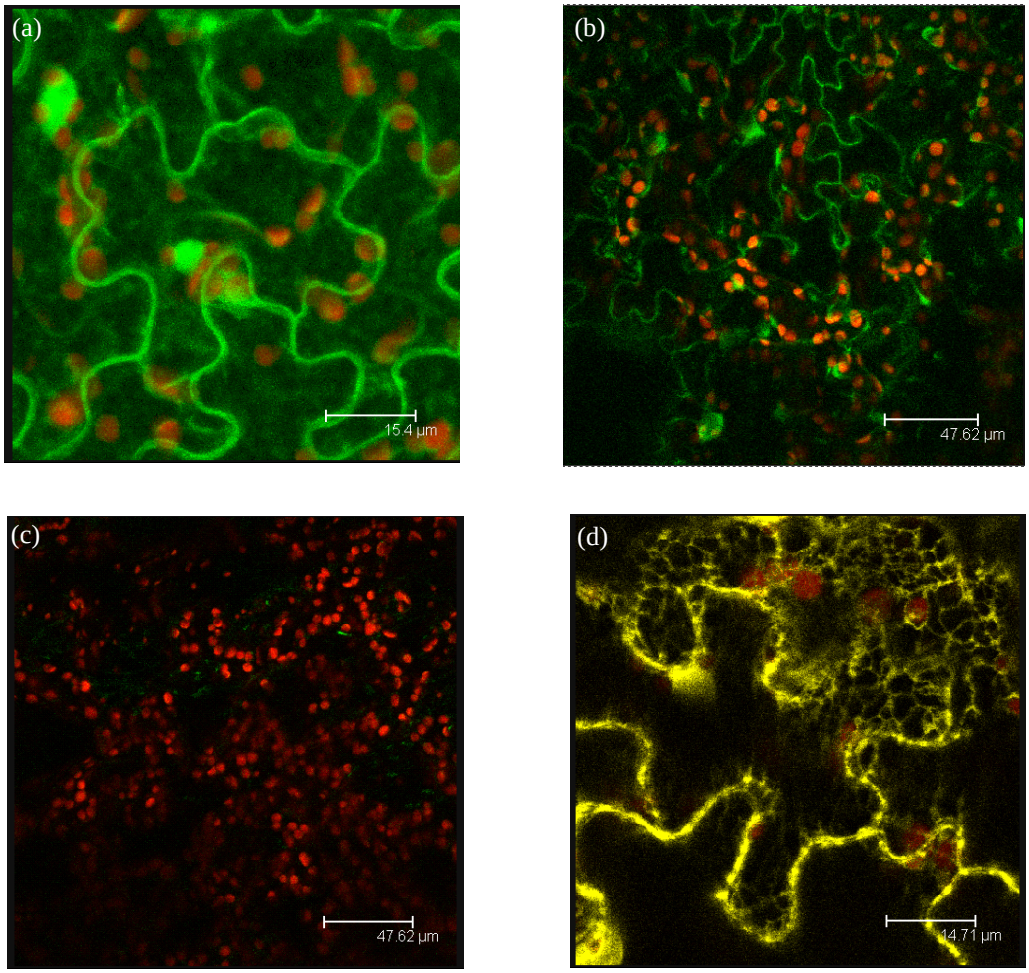


Figure 48. GFP fluorescence in Arabidopsis plant stably transformed with the PDNF:DNF:EGFP and P35S:DNF:EGFP construct. Images taken using a Leica SP2 linked to a Leica DM RE7 confocal microscope (a)P35S:DNF:EGFP (b) PDNF:DNF:EGFP (c) Negative Ws control (d) ER localised positive control (Obatined from Dr Isabelle Carré University of Warwick)

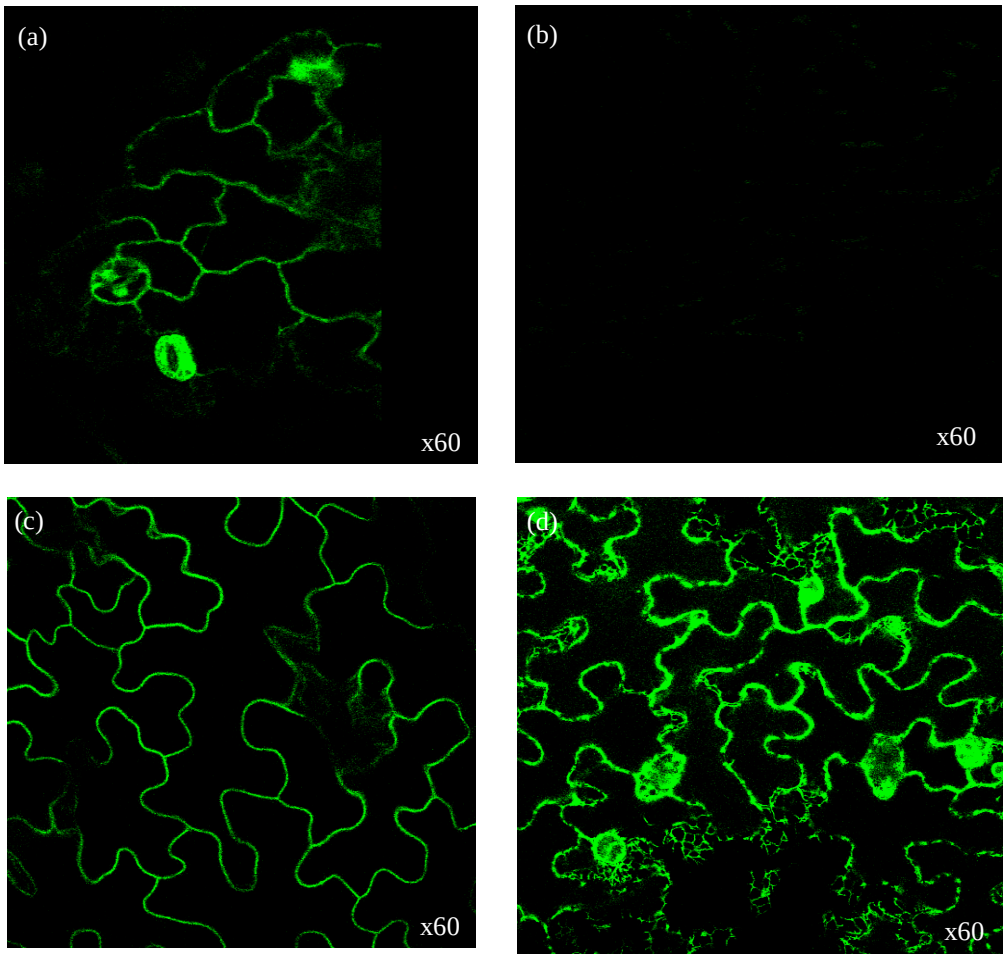


Figure 49. GFP fluorescence in Arabidopsis plant stablely transformed with the PDNF:DNF:EGFP and P35S:DNF:EGFP construct. Images taken using Olympus FV300 linked to an Olympus IX70 inverted confocal microscope. (a)P35S:DNF:EGFP (b) Negative Ws control (c) Plasma membrane localised positive control (donated by Dr Benjamin Kemp University of Warwick HRI) (d) ER localised positive control (Obatined from Dr Isabelle Carré University of Warwick)

5.5.6 Western transfer and immunodetection

Leaf tissue was extracted as described (Rohila *et al.*, 2004). The proteins to be analysed were resolved by SDS-PAGE and transferred to a nitrocellulose transfer membrane using iBlot™ Gel transfer system (Invitrogen). Protein blots were then incubated with either anti-GFP (Open-biosystems), anti-TAP (Open-biosystems), or custom made anti-DNF (Eurogentec), monoclonal antibodies at a final dilution of 1:10000 (PBS) for 1 hour. Excess primary antibody was removed by washing the PDF membrane before incubating with Horseradish Peroxidase (HRP)-conjugated to anti-mouse IgG at a final concentration of 1:10000 (PBS) for 1 hr (Chapter 2). Excess antibody was removed by washing then Antigen-antibody complexes were visualized using ECL detection reagents (Amersham Biosciences).

Western blots of total protein extracted from the *dnf* mutant young plants (approximately 4-6 leaves) transformed with the P35S:DNF:CTAP revealed the presence a very faint band around the expected size of 36.1 kda (Figure 50). In contrast to this the P35S:NTAP:DNF fusion protein produce a strong positive band around 36kda which is similar to the predicted size of the NTAP:DNF fusion protein being 36.7kda**.** However the intensity of this band is very strong and therefore the exact size is difficult to predict with a great deal of accuracy. No similar band is observed in either the Ws of the *dnf* control. These result are interesting as these 2 lines also showed evidence of functional complementation with the P35S:NTAP:DNF line 5 fully complementing the mutant phenotype and the CTAP line 8 only partial complementing.

Looking at figure 51 a clear band can be identified at around 44kda for the P35S:DNF:EGFP fusion protein which is approximately the predicted size of the DNF:EGFP fusion at 44.2kda. This band does not appear in the *dnf* mutant or the Ws control however a weak band of similar size can be observed in number 4 of PDNF:DNF:EGFP. None of the EGFP fusion proteins showed any evidence of functional complementation.

Any addition of fusion proteins to a gene of interest is capable of effecting its function therefore it is possible that by when adding EGFP to the C-terminal of DNF we still get protein produced, however the fusion protein could affect the binding of interacting proteins to DNF and thus affect its function.

We have shown that the *DNF* RNA is only weakly expressed with a tight peak of expression and as such you would expect the protein accumulation to follow a similar pattern. Therefore the timing of sample collection would be particularly important analysing expression of the fusion proteins.The anti-DNF antibody failed to recognise to any of the fusion proteins or DNF in the WS control (data not shown).

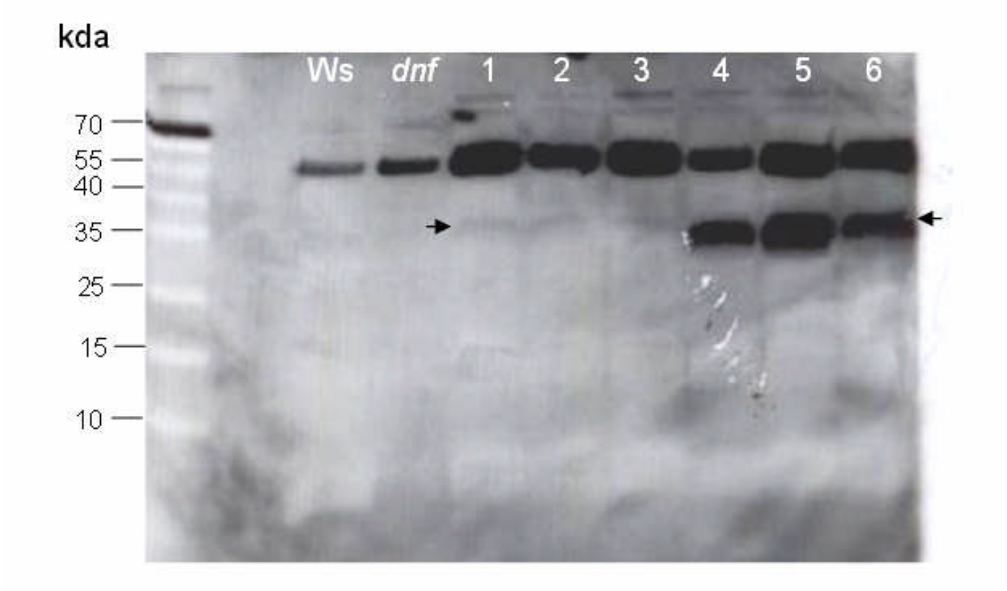


Figure 50.Western blot analysis of CTAP and NTAP expression in Arabidopsis. Total protein extracted from Ws, *dnf*, P35S:DNF:CTAP and P35S:NTAP:DNF . Western blot probed with an anti-TAP monoclonal antibody. 1,2,and 3 tissue extracted from line 8 P35S:DNF:CTAP, 4 5 and 6 tissue extracted from line 5 P35S:NTAP:DNF

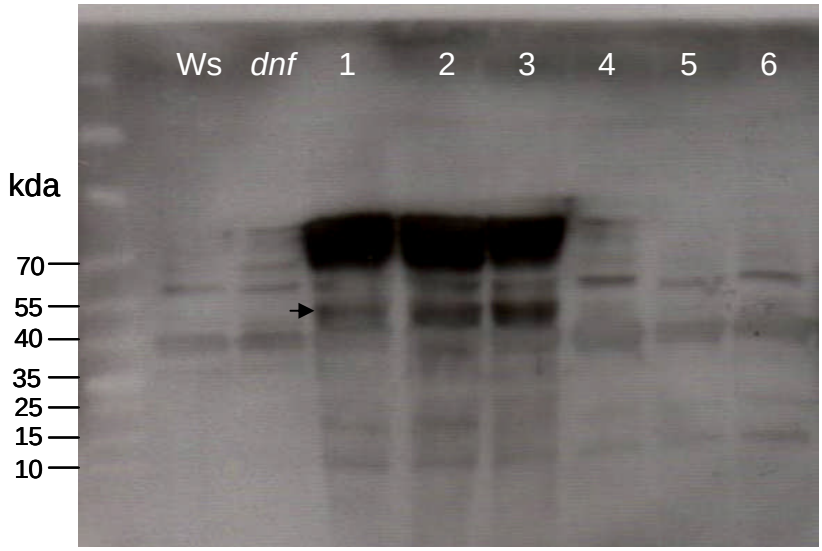


Figure 51.Western blot analysis of EGFP expression in Arabidopsis. Total protein extracted from Ws, *dnf*, PDNF:DNF:EGFP and P35S:DNF:EGFP . Western blot probed with an anti-EGFP monoclonal antibody. 1,2 and 3 tissue extracted from P35S:DNF:EGFP 4,5 and tissue extracted from PDNF:DNF:EGFP.

5.6 Discussion

The *dnf* mutant was transformed with 6 different fusion protein constructs. From the transformed lines only a small number showed any evidence of complementation, possibly down to poor germination.

The CTAP fusion produced varying degrees of complementation similar to previous findings observed by Dr Stephen Jackson and can be attributed to the semi-dominance of the *dnf* mutation. In addition western blot and immunodection on plant tissue from P35S:DNF:CTAP also produced a small visible band around the expected size of the fusion protein. More successful complementation was obtained from the NTAP fusion with one of the independent lines fully complementing the mutant. Western blot analysis of this line showed that a lot of fusion protein was being produced. However attempts to complement the mutant with the EGFP fusion protein were unsuccessful. Interestingly, both EGFP and CTAP tags are attached to the c-terminal of DNF. Therefore it is possible that the C-terminal TAP tags may have adverse effects on the DNF protein by disrupting its function or by rendering the protein unstable.

This could also explain why only one fully functional complemented line was obtained using a N-terminal TAP tag fusion.

Although the EGFP fusion did not complement the mutant we did observe fluorescence.

This fluorescence was observed when driven by the native promoter and the P35S promoter however no fluorescence could be seen in the nucleus with the native promoter. The reasons for the observed differences are unclear. To date, there is no evidence to support the presence of a Nuclear Localisation

Signal (NLS) in DNF which would actively facilitate movement of the protein into the nucleus as observed for P35S:DNF:EGFP protein localisation.

One possible explanation for the presence of DNF in the nucleus is passive diffusion. It has been shown that due to its small size (26kDa), GFP is able to passively diffuse through nuclear pores (Gaudin *et al.*, 2001).As DNF is also a small protein (15.9 kDa) it is possible that the combined EFGP:DNF fusion protein (combined size 44.2 kDa) is also able to enter the nucleus by passive diffusion. Proteins of <40 kD have been shown to pass through nuclear pores by simple diffusion (Kaffman *et al.*, 1999). Alternatively if the fusion protein is to large it is possible that it is free GFP that was visualised in the nucleus as this is known to be able to diffuse through the nuclear envelope (Baulcombe *et al.*, 1995). However when using the EGFP antibody no visible band was observed corresponding to the size of free EGFP.

Protein expression in the P35S:DNF:EGFP transformed plants was observed in the cytoplasm and the nucleus but only in the cytoplasm in the PDNF:DNF:EGFP transformed plants. The lack of nuclear localisation in the PDNF:DNF:EGFP plants may be due to lower expression levels and/or the timing of tissue visualisation.

The disruption of function by the addition of tags is not unique for example when *ft-9* mutant was transformed with FT:GFP flowering occurred but it was less active than the wild-type FT protein (Corbesier *et al.*, 2007).

In addition Rubio *et al* 2005 found when they added a CTAP to the CSN3, and NTAP to COP1 they not longer had expression of the fusion protein,however expression was observed when the tags were placed at the opposite terminals of the genes (Vicente Rubio 2005). In another study Abe *et al*., 2008 attached C and

NTAP tags to their gene of interest and found that two of the lines attached to the C-terminal delayed flowering but when attached to the N-terminal flowering was similar to that of the wild type (Abe *et al.*, 2008).

The fact that the P35S:NTAP:DNF functionally complemented the mutant and produced a positive immunodection confirms that the *dnf* mutation is responsible for the early flowering phenotype and that this TAP tag fusion is functional. It is interesting that the qRT-PCR gave a similar expression profile to that of *DNF* in WT Ws plants even though it was being driven by the P35S. This suggests that the message maybe very unstable and rapidly degraded in the dark. Given this we would expect the DNF protein to be tightly regulated and possibly also very unstable. Therefore the timing of sample collection would be of up most importance for samples that will be used in western blots and immunodection.

The successfully complemented TAP tags lines that have been checked for protein expression can be used in pull-down experiments followed by Mass Spectroscopy with an aim to identify possible interacting proteins of DNF. Furthermore Chromatin immunoprecipitation (Chip assay) could also be performed to determine a possible role for DNF in chromatin remodelling. However due to time constraints this procedure was unable to be performed.

The P35S:NTAP:DNF fusion protein transformation produced 3 independent lines all showing different degrees of complementation, with line 5 fully complementing. Therefore if work on this mutant is continued it would be advisable to re-introduce this particular construct into the mutant in order to obtain more fully complemented independent lines.

Chapter 6

General discussion

6.1 General Discussion

In order to maximise survival and reproductive success it is essential that plants flower at the right time. This is achieved by a complex interaction of environmental cues with a network of genetic pathways (Chapter 1). Together these co-ordinate either the induction or repression of flowering such that it occurs at an optimal time. Temperature and daylength are both environmental cues that play a major role in the transition to flower. Through molecular and genetic studies many genes such as *GI* and *CO* have been identified that induce flowering in response to daylength and have subsequently been placed in the photoperiodic pathway (Alonso-Blanco *et al.*, 2000; Suarez-Lopez *et al.*, 2001). The *DNF* gene is also proposed to act in the photoperiodic pathway due to the fact that its effect is only observed in one photoperiod. Dr Stephen Jackson had previously complemented the *dnf* mutant by introducing a WT copy of the gene into the mutant. These transformed lines produce progeny that restored WT flowering time thus indicating that the mutation in the *DNF* gene is responsible for the early flowering phenotype that is observed.

The *DNF* gene encodes a small protein of 141 aa with a putative membrane spanning domain at the N-terminus and a C-terminal domain that has some homology to both RING-H2 and PHD domains but does not quite meet the consensus sequence for either of these domains. RING-H2 domains are present in E3 ubiquitin ligases, many of which are involved in protein degradation. PHD domains are protein-protein interaction domains typically involved in chromatin remodelling examples of which have been shown to be involved in the control of flowering. It appears that due to the early flowering phenotype in SD that the repression of flowering in SD has been lost in the *dnf* mutant and therefore *DNF* must play a role in the repression of flowering in SD.

The primary aim of this thesis was to characterise the *DNF* gene with respect to the role it plays as a floral repressor. With this aim in mind a number of strategies were employed including, production of transgenic plants, intra-cellular localisation, crossing the mutant with *co-2* mutant and molecular characterisation techniques. This concluding chapter expands on the summaries presented in the previous chapters.

6.2 Conclusions

DNF is a novel gene of unknown biological function. Apart from the early flowering phenotype no phenotypical differences were observed between the mutant and the WT Ws in SD at 22ºC. However a clear difference in leaf morphology was observed when grown in SD at 16ºC. Mutants of the photoperiodic pathway only exhibit their affect in one photoperiod, so it was for this reason that we believed *DNF* forms part of this pathway as *dnf* only affects flowering in SD but not in LD.

A primary aim of this thesis was to confirm that *DNF* does indeed lie on the photoperiodic pathway. With this objective in mind the *dnf* mutant was crossed to the *co-2* mutant and resulting progeny scored for flowering time in SD. This crossing was complicated by the fact that the *dnf* is in the Ws ecotype and the *co-2* mutant in Ler ecotype. However progeny from all isolated double mutants flowered around the same time or later than either Ws or Ler controls in both LD and SD. The fact that the double mutant flowered late indicates that *CO* is required for the early flowering phenotype of the *dnf* mutant and that *CO* is epistatic to *DNF*. Had the double mutants flowered with intermediate flowering times then this would have positioned the mutant on a different flowering pathway. These results also suggests that *DNF* acts upstream of *CO* in the photoperiodic flowering pathway.

Having confirmed that *DNF* was acting in the photoperiodic pathway we then went on to establish the position of *DNF* within this pathway. Quantitative RT-PCR was used to analyse the expression of *CO* and *FT* in the *dnf* mutant grown in SD. This revealed that the expression of *CO* was shifted into the light period in the mutant and that this in-turn increased the expression of *FT*. In wild type plants grown in SD *CO* expression is limited to the dark therefore does not coincide with the light period and flowering is delayed. However the peak in *CO* expression in the SD grown *dnf* mutant does occur in the light therefore enabling CO protein to be produced in the light leading to the expression of *FT* and early flowering in SD.

GI triggers flowering in LD by acting upstream of *CO* therefore the next logical progression in positioning *DNF* was to analyse the expression of *GI*. With this aim in mind we once again used qRT-PCR to look at the expression of the *GI* gene in the *dnf* mutant and Ws. Results indicated that the expression of *GI* is unaltered in the mutant. However we know that the expression *CO* and *FT* is altered in the mutant in SD. The fact that *GI* expression is unaltered whilst *CO* is altered indicates that *DNF* must lie downstream of *GI* and upstream of *CO* on the photoperiodic pathway. Furthermore as the *dnf* mutant flowers early due to an inappropriate induction of *FT* by CO in SD then the role of *DNF* must therefore be to prevent the expression of *CO* during the light period of a SD. In LD the *dnf* mutation had no effect on the level of CO expression thus explaining why there is no difference in flowering time between the mutant and Ws plants in LD (Performed by Dr Karl Morris data not shown).

In addition to the expression of *GI*, *CO* and *FT* qRT-PCR was also used to determine when the *DNF* gene is expressed. No expression was detectable in *dnf* mutant plants and expression levels were very low in Ws, however a very precise peak in expression

was observed between 4-6 hrs after dawn (ZT4-ZT6) this suggests that *DNF* expression and probably protein activity are tightly regulated and fits in neatly with the critical photoperiod data which shows no difference between the mutant and Ws in the photoperiods of 4 hours of the day (see below). This is also interesting as this peak appears just before the peak in *CO* expression which would be anticipated if *DNF* is involved in the regulation of *CO* in SD.

The circadian clock is a key component of the Arabidopsis flowering pathway and as such plays a pivotal role in timing of expression of genes which are involved in the promotion of flowering (Chapter 3). Many of the genes such as *CAB* which are regulated by the clock show a rhythmic expression pattern and as such analysis of any changes in the expression of these genes can be used to determine if there is any alteration in the period or phase of the clock. In order to determine if this was the case in our mutant we looked at the expression of *CAB* gene and compared this to that of Ws. No difference was observed between them and therefore we can conclude that neither the phase nor the period of the circadian clock was altered in our mutant.

Other early flowering mutants such as *phyB* have been shown to have a default in light perception (Halliday *et al.,* 1994) therefore it may be possible that the *dnf* mutant also has altered light perception . To test this, the mutant was grown alongside the appropriate controls in different light fluencies and the hypocotyl length measured. The results indicated no effects of hypocotyl elongation in red, far-red or blue light indicating that the mutant perceives light normally as Ws. From this we can conclude that there is no defect in light perception in *dnf.*

Results thus far had indicated that the circadian clock functions correctly and light is perceived normally. In addition there is no altered expression of *GI,* yet *CO* and *FT* expression is altered. Furthermore the *dnf:co-2* double mutants were late

flowering. These results confirmed that *DNF* does lie on the photoperiodic pathway and that it is positioned between *GI* and *CO* and involved in the repression of *CO* transcription in SD.

Short day photoperiods are typically of 8hrs light and 16 hours dark and under these conditions *dnf* flowers early. We were interested in establishing if the early flowering of *dnf* in SD was due to an altered critical photoperiod in the mutant. To this end the mutant was grown alongside Ws in 4, 6, 8 and 16hr photoperiods and flowering time scored. The results showed a clear difference in the critical photoperiod between the mutant and Ws. When the daylength was reduced to 4hrs the flowering time of Ws and *dnf* was approximately the same however in the photoperiods of 6 hours we began to observe the early flowering phenotype of the *dnf* mutant. This remained true until the daylength was extended to 16 hours, at which point the mutant once again flowered at the same time as Ws. These results suggest that the CDL of DNF falls between 4 and 10hrs compared to Ws where the CDL was between 8 and 16 hrs. Interestingly the expression of the *DNF* gene was shown to increase after 4hrs light which is the time that the difference in the flowering response to photoperiod between Ws and the *dnf* mutant first becomes apparent.

In an attempt to determine the localisation of the DNF protein, *EGFP* was fused to the C-terminal of *DNF* gene with expression driven by both the P35s and the native promoter. These constructs were used to transform the *dnf* mutant and resulting plants were analysed for complementation of the early flowering phenotype. It was initially believed that these transformations had been unsuccessful as no evidence of complementation was observed. However on analysing plant tissue under a confocal microscope fluorescence was observed. This fluorescence was apparent in the cytoplasm and the nucleus when driven by the P35s but only in the cytoplasm when

under the control of the native promoter. Western blot analysis of total protein extracted from the EGFP expressing lines were performed which detected the presence of the P35s:DNF:EGFP, however protein was not detected for the PDNF:DNF:EGFP.

Key to the success of this strategy was the functional complementation of the *dnf* mutant. To this end DNF was epitope tagged at the N- and C-terminals with TAP tag fusion proteins in order to purify protein complexes formed *in planta* and identify any possible interacting proteins of DNF. Complementation proved difficult with the CTAP tags, however NTAP fusion proteins were more successful producing three independent lines that showed evidence of complementation with one line fully complementing the mutant. This data confirms that as previously demonstrated, the mutation caused by the insertion of the TDNA insertion in the *DNF* gene is responsible for the *dnf* mutant phenotype. After successful complementation it was our intention to use these lines in pull down experiments then Mass spectroscopy with a view to identify possible interactors of DNF. However due to time constraints this procedure has not yet been undertaken.

Thus far all the experiments had been performed at an ambient temperature of 22°C, however we were also interested in determining if the mutant phenotype remains true when grown at different ambient temperatures. To this end we grew the mutant alongside the Ws at different temperatures and scored the flowering time. Our results indicated that when grown at lower temperatures the mutant flowers later than the Ws indicating that the early flowering phenotype is temperature dependent and that the action of DNF as a floral repressor is restricted to temperatures above 20°C. This phenomenon is not unique and is also observed in the *phyB* mutant whose phenotype is also restricted to higher temperatures (Halliday *et al.,* 2003).

6.3 A model for *DNF* in the control of flowering.

The proteins GI, CO and FT form the basis of the photoperiodic pathway that results in the promotion of flowering in LD. GI promotes the accumulation of *CO* in LD such that it peaks between 10-12 hrs after dawn, just before the end of the light period. This increase in *CO* mRNA leads to an accumulation of CO protein which is stabilised in the light enabling it to activate *FT* and promote flowering. Under SD *CO* mRNA accumulates during the night and the CO protein that is produced is rapidly degraded therefore *FT* is not induced and there is no promotion of flowering.

CDF1 is clock controlled protein and negatively regulates *CO* transcription by binding directly to AAAG-elements within the *CO* promoter. FKF1 acts together with GI to degrade CDF1 towards the middle of a LD which then allows *CO* mRNA levels to rise (see Chapters 1 and 4). Degradation of CO is suppressed by the action of PhyA, Cry1 and Cry2 (Suarez-Lopez *et al.,* 2001; Yanovsky *et al.,* 2002; Valverde *et al.,* 2004). In addition PhyB has been shown to be required for the degradation of the CO protein in the early part of the day (Valverde *et al.,* 2004).

During the night CO protein is ubiquitinated and then degraded by the 26S proteasome (Valverde *et al.,* 2004). This degradation of CO by the 26S proteasome is dependent on the presence of SPA1, SPA3 and SPA4 (Laubinger *et al.,* 2006) (see Chapters 1 and 4). Simon et al. (1996) demonstrated that expression of a steroid-inducible CO fusion protein from the CaMV35S promoter was sufficient to promote early flowering in both LD and SD. (Simon *et al.,* 1996). Furthermore altering the expression of *CO* such that it is expressed in SD at high levels during the day also results in an early flowering phenotype (Roden *et al.,* 2002; Yanovsky *et al.,* 2002). This demonstrates that the level of CO protein is crucial in determining the flowering response to photoperiod.

Whilst the *dnf* mutant flowers early in SD there is no difference in the expression of *GI* and it has been shown that light perception is not affected in the mutant (see Chapters 3 and 4). However the expression of *CO* is altered in SD such that it is shifted into the light period, peaking at ZT 7-8 (Figure 36). Furthermore the expression of *FT* is also increased, presumably caused by the increase in *CO* expression and subsequent CO protein levels, ultimately leading to the early flowering SD phenotype of the *dnf* mutant. Increased *FT* expression has also been observed in other early flowering mutants such as *ebs,* however *CO* expression was shown to be unaltered (Gomez-Mena *et al.,* 2001). The fact that *CO* is epistatic to *DNF* indicates that *DNF* is specifically involved in the photoperiodic flowering pathway.

6.3.1 The question now arises as to how *DNF* fits into the flowering model?

As described above GI induces *CO* expression, however GI protein levels have been shown to be high in SD when *CO* is not expressed (David *et al.,* 2006). This suggests that there must be a preventative mechanism in place in SD that stops *CO* from being induced by GI and *DNF* could fit neatly into this role.

Components of the photoperiodic pathway either promote or repress flowering. Mutations in genes that promote flowering such as *CO, GI* or *CRY* (Putterill *et al.,* 1995; Fowler *et al.,* 1999; Hongwei Guo 1999) results in late flowering in LD phenotypes and mutations in genes that have been shown to repress flowering such as *EBS* and *SPA* flower early in SD (Gomez-Mena *et al.,* 2001; Laubinger *et al.,* 2006). From the *dnf:co-2* crosses we know that *DNF* acts as part of the photoperiodic pathway and as *dnf* flowers early therefore *DNF* must be acting as a floral repressor either directly or indirectly (Figure 52) . Our experiments have shown that DNF acts to repress *CO* transcription in SD.

The expression profile of *DNF* in WT in SD has been shown to peak at ZT 4-6, we propose that this represses *CO* expression during the day until it is observed to increase in WT plants after ZT 10. In the *dnf* mutant this repression of *CO* expression does not occur and the *CO* mRNA levels start to rise after ZT 5 (Chapter 4). This earlier induction of *CO* expression enables the mutant to flower early in 8hr SD, no effect of the mutation is observed on flowering in LD because *CO* expression is normally induced in LD conditions in WT plants.

As previously discussed in Chapter 4 the mechanism by which DNF could repress *CO* could be by either of two processes. Firstly by acting as an E3 ligase, specifically targeting an activator of *CO* transcription for degradation by the proteasome. Or DNF could act as a transcriptional repressor of *CO* by mediating protein-protein interactions involved in chromatin remodelling.

In a study of all RING finger proteins in the Arabidopsis genome it was found that the DNF protein was unlikely to form an active RING domain (Stone *et al.*, 2005). Therefore it is more feasible that the putative RING finger domain is a PHD protein that is involved in chromatin remodelling. VIL1 and EBS are examples of other PHD proteins that have previously been identified as also being involved in flowering regulation (Pineiro *et al.*, 2003; Sung *et al.*, 2006).

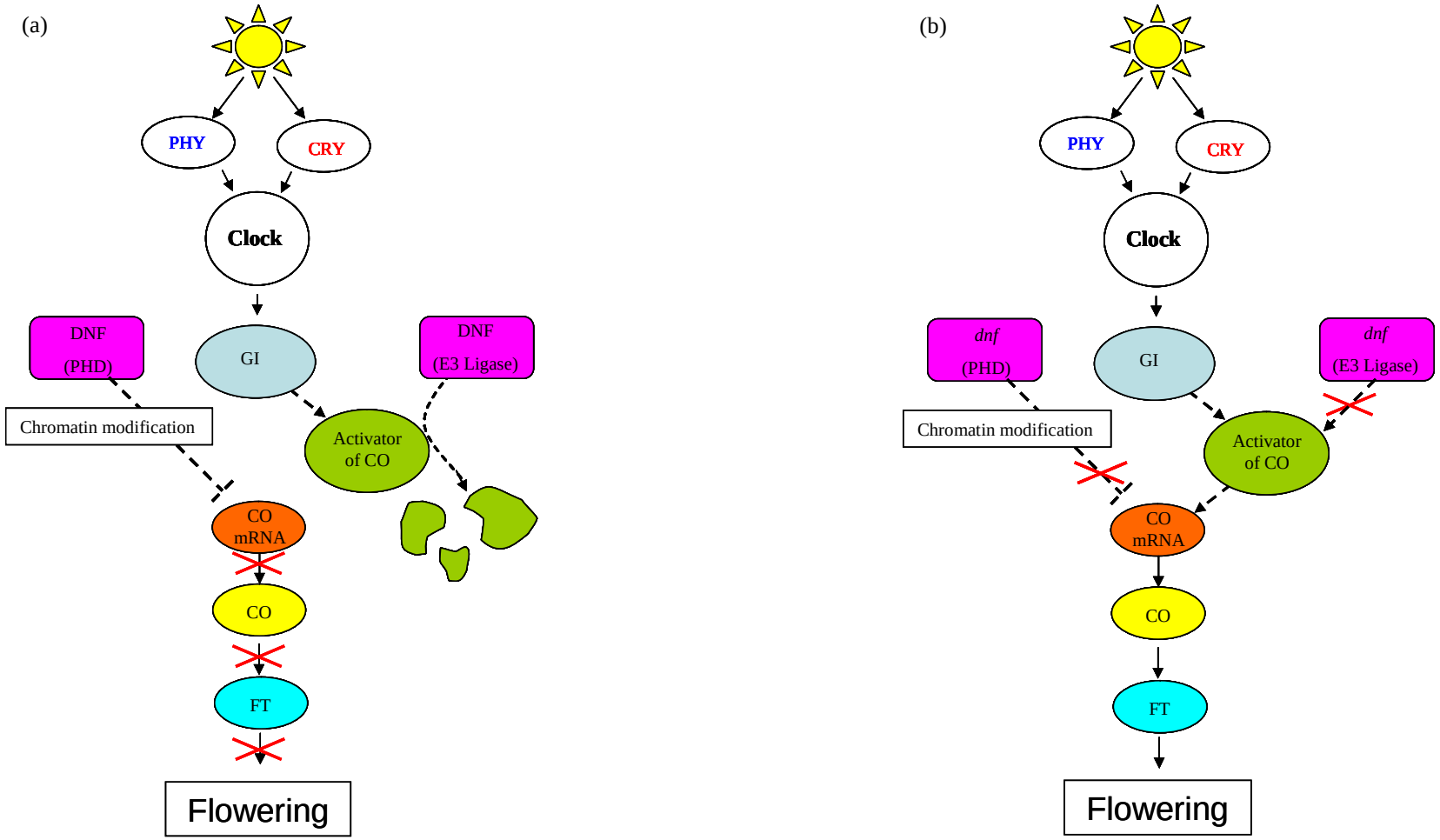


Figure 52. Repression of flowering in SD through the action of DNF on *CO* transcription. A possible mechanism of action of DNF to repress flowering in SD either through chromatin remodelling or targeted proteolysis. (a) WT DNF showing a classical SD flowering response (b) Mutant *dnf* leading to early flowering in SD. Dashed lines are hypothetical interactions.

6.4 Future work

(i) To determine the precise mechanism by which DNF represses *CO* expression in SD.

As we have fully complemented the mutant with P35S:NTAP:DNF then this would be an ideal line to be used in-vitro pull downs and mass spectroscopy analysis. Identification of interacting proteins would help further elucidate the precise role that *DNF* plays in the repression of flowering in SD photoperiods. This construct could also be used to re-transform the *dnf* mutant with a view to obtain more independent lines that also fully complement the mutant.

In addition, yeast-2-hybrid could be undertaken with a view to identify possible protein interactors of DNF. Chromatin immunoprecipitation (ChIP-on-ChIP) could also be undertaken to examine the hypothesis that *DNF* is involved in the repression of flowering by chromatin remodelling.

(ii) To further investigate the role that temperature plays on the early flowering time of the *dnf* mutant.

Experiments such as determining if temperature affects flowering of the mutant in LD, combining different light fluencies with different temperatures and looking at the CDL under different temperatures are possible suggestions for future investigations on the effect of temperature.

Due to time constraints the analysis of *CO* expression at 16°C was only undertaken on a limited number of samples and only up to ZT10 (Chapter 4). In SD *CO* peaks during the night in WT plants therefore little can be elucidated from the results obtained. A full time course experiment using mutant and Ws plants grown in SD at 16°C followed by qRT-PCR analysis would confirm if the repression of *CO* transcription by *DNF* is restricted to higher temperatures. It would also be interesting to look at the expression of *DNF* and *FT* under these growth conditions.

Appendix

	dnf	Ws
Mean	2.397667123	0.335544373
Variance	10.07428038	0.268525579
Observations	13	13
Pooled Variance	5.171402979	
Hypothesized Mean Difference	0	
df	24	
t Stat	2.311889121	
P(T<=t) one-tail	0.014840093	
t Critical one-tail	1.710882316	
P(T<=t) two-tail	0.029680185	
t Critical two-tail	2.063898137	

Table 9. The expression profile of the *CO* is significantly different in the *dnf* mutant to that of Ws in SDs at 22 °C T-test: two-sample assuming equal variances. DF stands for degrees of freedom. T-stat is the t-value calculated from the data. P(T<=t) two tail shows the probability of getting the calculated t value by chance alone. T critical two tail shows the t value that must be exceed in order for the difference between the means to be significant.

	dnf	Ws
Mean	20.56071953	8.478469298
Variance	800.8671415	169.1947332
Observations	7	7
Pooled Variance	485.0309373	
Hypothesized Mean Difference	0	
df	12	
t Stat	1.026353879	
P(T<=t) one-tail	0.162484886	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.324969772	
t Critical two-tail	2.178812792	

Table 10. The expression profile of the *CO* is not significantly different in the *dnf* mutant and Ws in SDs at 16 °C. T-test: two-sample assuming equal variances (see Table 9. for details).

t-Test: Two-Sample Assuming Equal Variances		
	dnf	Ws
Mean	2.25546301	3.851457871
Variance	19.33585061	21.01074763
Observations	14	14
Pooled Variance	20.17329912	
Hypothesized Mean Difference	0	
df	26	
t Stat	-0.940138947	
P(T<=t) one-tail	0.177899696	
t Critical one-tail	1.705616341	
P(T<=t) two-tail	0.355799392	
t Critical two-tail	2.055530786	

Table 11. The expression profile of the *GI* is not significantly different in the *dnf* mutant and Ws in SDs at 22 °C. T-test: two-sample assuming equal variances (see Table 9. for details).

Leaf number at flowering

t-Test: Two-Sample Assuming Equal Variances

Leaf Number 16ºC	*dnf*	*Ws*
Mean	28.30769	17.64103
Variance	12.37652	5.34143
Observations	39	39
Pooled Variance	8.858974	
Hypothesized Mean Difference	0	
df	76	
t Stat	15.82539	
P(T<=t) one-tail	4.58E-26	
t Critical one-tail	1.665151	
P(T<=t) two-tail	9.17E-26	
t Critical two-tail	1.991675	

t-Test: Two-Sample Assuming Equal Variances

Leaf Number 18ºC	*dnf*	*Ws*
Mean	24.2	16.45946
Variance	17.58333	4.810811
Observations	25	37
Pooled Variance	9.91982	
Hypothesized Mean Difference	0	
df	60	
t Stat	9.492802	
P(T<=t) one-tail	7.38E-14	
t Critical one-tail	1.670649	
P(T<=t) two-tail	1.48E-13	
t Critical two-tail	2.000297	

t-Test: Two-Sample Assuming Equal Variances

Leaf Number 20ºC	*dnf*	*Ws*
Mean	21.475	18.725
Variance	13.3839744	15.07628
Observations	40	40
Pooled Variance	14.2301282	
Hypothesized Mean Difference	0	
df	78	
t Stat	3.26019281	
P(T<=t) one-tail	0.00082576	
t Critical one-tail	1.66462542	
P(T<=t) two-tail	0.00165152	
t Critical two-tail	1.99084752	

Days to Flower

t-Test: Two-Sample Assuming Equal Variances

Days to Flower 16ºC	*dnf*	*Ws*
Mean	59.10256	46.94872
Variance	18.9892	14.52362
Observations	39	39
Pooled Variance	16.75641	
Hypothesized Mean Difference	0	
df	76	
t Stat	13.11114	
P(T<=t) one-tail	1.68E-21	
t Critical one-tail	1.665151	
P(T<=t) two-tail	3.35E-21	
t Critical two-tail	1.991675	

t-Test: Two-Sample Assuming Equal Variances

Days to Flower 18ºC	*dnf*	*Ws*
Mean	64.08	53.51351
Variance	15.82667	10.97898
Observations	25	37
Pooled Variance	12.91805	
Hypothesized Mean Difference	0	
df	60	
t Stat	11.35552	
P(T<=t) one-tail	6.98E-17	
t Critical one-tail	1.670649	
P(T<=t) two-tail	1.4E-16	
t Critical two-tail	2.000297	

t-Test: Two-Sample Assuming Equal Variances

Days to Flower 20ºC	*dnf*	*Ws*
Mean	54.95652	50.95
Variance	24.48696	44.61282
Observations	46	40
Pooled Variance	33.83111	
Hypothesized Mean Difference	0	
df	84	
t Stat	3.186171	
P(T<=t) one-tail	0.001012	
t Critical one-tail	1.663198	
P(T<=t) two-tail	0.002024	
t Critical two-tail	1.98861	

t-Test: Two-Sample Assuming Equal Variances

Leaf Number22ºC	*dnf*	*Ws*
Mean	8.923077	20.05128
Variance	1.388664	8.31309
Observations	39	39
Pooled Variance	4.850877	
Hypothesized Mean Difference	0	
df	76	
t Stat	-22.3117	
P(T<=t) one-tail	2.12E-35	
t Critical one-tail	1.665151	
P(T<=t) two-tail	4.24E-35	
t Critical two-tail	1.991675	

t-Test: Two-Sample Assuming Equal Variances

Days to flower 22ºC	*dnf*	*Ws*
Mean	32.12820513	48.20513
Variance	8.16734143	7.430499
Observations	39	39
Pooled Variance	7.798920378	
Hypothesized Mean Difference	0	
df	76	
t Stat	-25.42160675	
P(T<=t) one-tail	3.33557E-39	
t Critical one-tail	1.665150648	
P(T<=t) two-tail	6.67114E-39	
t Critical two-tail	1.991675163	

t-Test: Two-Sample Assuming Equal Variances

Leaf Number 23ºC	*dnf*	*Ws*
Mean	4.869565	7.659091
Variance	0.482213	0.741543
Observations	23	44
Pooled Variance	0.65377	
Hypothesized Mean Difference	0	
df	65	
t Stat	-13.4082	
P(T<=t) one-tail	1.1E-20	
t Critical one-tail	1.668636	
P(T<=t) two-tail	2.2E-20	
t Critical two-tail	1.997137	

t-Test: Two-Sample Assuming Equal Variances

Days to Flower 23ºC	*dnf*	*Ws*
Mean	20.82609	27.61364
Variance	2.422925	2.475159
Observations	23	44
Pooled Variance	2.457479	
Hypothesized Mean Difference	0	
df	65	
t Stat	-16.8276	
P(T<=t) one-tail	1.12E-25	
t Critical one-tail	1.668636	
P(T<=t) two-tail	2.23E-25	
t Critical two-tail	1.997137	

t-Test: Two-Sample Assuming Equal Variances

Leaf Number 27ºC	*dnf*	*Ws*
Mean	5.291667	6.218182
Variance	0.764184	0.581145
Observations	48	55
Pooled Variance	0.666322	
Hypothesized Mean Difference	0	
df	101	
t Stat	-5.74637	
P(T<=t) one-tail	4.85E-08	
t Critical one-tail	1.66008	
P(T<=t) two-tail	9.7E-08	
t Critical two-tail	1.983731	

t-Test: Two-Sample Assuming Equal Variances

Days to Flower 27ºC	*dnf*	*Ws*
Mean	23.375	26.07273
Variance	3.260638	2.476094
Observations	48	55
Pooled Variance	2.841179	
Hypothesized Mean Difference	0	
df	101	
t Stat	-8.10274	
P(T<=t) one-tail	6.46E-13	
t Critical one-tail	1.66008	
P(T<=t) two-tail	1.29E-12	
t Critical two-tail	1.983731	

Table 12. Statistical results of varying ambient temperatures on flowering time. Flowering scored by leaf number and days to flower. T-test: two-sample assuming equal variances (see Table 9. for details).

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