DAY NEUTRAL FLOWERING Represses *CONSTANS* to Prevent Arabidopsis Flowering Early in Short Days

Karl Morris[1], Sarah Thornber[1], Lesley Codrai, Christine Richardson, Adam Craig, Ari Sadanandom, Brian Thomas and Stephen Jackson*

Warwick HRI, Warwick University, Wellesbourne, Warwick, CV35 9EF, UK.

[1] These authors contributed equally to this work

* corresponding author; S.Jackson
e-mail: Stephen.jackson@warwick.ac.uk
Tel: 024 76575091
Fax: 02476574500

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: S.Jackson (Stephen.jackson@warwick.ac.uk).

Running Head: *DNF* suppresses *CO* expression in SD.

Key words: Photoperiod, Flowering, Arabidopsis, Critical photoperiod, RING finger,

Manuscript corresponds to approximately 11.6 printed pages

Synopsis

This work describes the flowering time gene *DAY NEUTRAL FLOWERING* (*DNF*),which acts in the same flowering pathway as *CONSTANS (CO)*. DNF is a membrane-bound E3 ligase which represses *CO* expression and plays an important role in maintaining low levels of *CO* expression in SD; it is thus essential for the ability of the Arabidopsis plant to have a different flowering response in LD and SD.

Abstract

The photoperiodic response in Arabidopsis requires the precise regulation of *CONSTANS* (*CO*) expression in relation to the light period during the day. In short days (SD) levels of *CO* expression are normally low during the light period and this results in delayed flowering compared to long days (LD) when *CO* expression rises to high levels before the end of the light period. We have identified a novel flowering time gene called *DAY NEUTRAL FLOWERING* (*DNF*) that acts in the same flowering pathway as *CO*. DNF is a membrane-bound E3 ligase which represses *CO* expression and plays an important role in maintaining low levels of *CO* expression in SD. The effect of DNF on the rhythm of *CO* expression is essential for the photoperiodic response of Arabidopsis, enabling it to have a different flowering response in LD and SD.

Introduction

Many plants regulate the timing of the transition from vegetative to reproductive growth to coincide with favourable seasons of the year. They are able to do this through their perception of, and response to, environmental signals such as temperature and photoperiod (Yanovsky and Kay, 2003; Michaels, 2009). These stimuli are perceived in different organs of the plant, vernalising temperatures are detected in the shoot apical meristem whereas photoperiod is detected in the leaves. Perception of an inducing photoperiod in the leaves results in the production of a systemic flowering signal that moves to the apex where it triggers flower development (Zeevaart, 1976). The identity of this mobile signal in Arabidopsis has been shown to include the FLOWERING LOCUS T (FT) protein (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007), the expression of which is principally regulated by the *CONSTANS* (*CO*) gene (Samach et al., 2000). Key questions still remain, however, regarding the control of both *CO* transcription and the stability/activity of the CO protein (reviewed by Imaizumi and Kay, 2006).

Arabidopsis is a facultative long day plant in which long days (LD) promote more rapid flowering than short days (SD). Different flowering responses to changes in photoperiod are brought about through the interaction of light with the circadian clock-regulated rhythmic expression of *CO*. In SD of 8-10h *CO* expression is low during the light period, whereas in LD of 14-16h the level of *CO* expression rises towards the end of the day, the coincidence of light with high levels of *CO* expression leads to the induction of *FT* and flowering (Suàrez-López et al., 2001). As evidence to support this

model it has been shown that flowering can be induced in SD by constitutive over-expression of *CO*, or by altering the rhythm of *CO* expression such that it is expressed at high levels during the light period of a SD (Onouchi et al., 2000; Roden et al., 2002; Yanovsky and Kay, 2002). In addition to transcriptional regulation, there is also regulation at the level of CO protein stability which is affected by light signals acting through photoreceptors (Valverde et al., 2004). In order to generate the level of sensitivity required to distinguish between photoperiods that may only differ by a couple of hours, both the transcription of *CO* and CO protein stability have to be very tightly regulated.

Transcription of *CO* is known to be controlled by a number of factors one of which is the circadian clock which causes rhythmic oscillations in *CO* expression (Suàrez-López et al., 2001). *GIGANTEA* (*GI*) is also known to affect the expression of *CO* (Suàrez-López et al., 2001; Mizoguchi et al., 2005). GI has been shown to bind a transcriptional repressor of *CO* expression called CYCLING DOF FACTOR 1 (CDF1). The stability of CDF1 is controlled by an F-box protein called FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) (Imaizumi et al., 2003, 2005; Sawa et al., 2007). FKF1 has also been shown to bind to GI in a blue-light-dependent manner. This has led to the proposal of a model in which the CDF1 repressor bound to the *CO* promoter is bound by GI, binding of FKF1 to this complex later on in the day results in the degradation of CDF1 thus allowing *CO* expression to increase at the end of a LD (Sawa et al., 2007). It has recently been shown that other related DOF factors, CDF2, CDF3 and CDF5, act redundantly with CDF1 to repress *CO* expression and delay flowering, and that CDF2 is also targeted for degradation by FKF1 (Fornara et al., 2009). Over-expression of *GI* in the *fkf1* mutant still causes early flowering indicating that GI is able to promote flowering independently of the FKF1-mediated degradation of the CDF proteins (Sawa et al., 2007), however this has been shown to be due to partial redundancy between FKF1 and its close homologs ZEITLUPE (ZTL) and LOV kelch protein2 (LKP2) (Somers et al., 2000; Schultz et al., 2001; Fornara et al., 2009).

Interestingly, Fornara et al. (2009) also demonstrated that this whole layer of regulation of *CO* expression by GI and the CDF proteins can be removed without affecting the rhythm of *CO* expression, or its response to photoperiod. In a quintuple mutant carrying the *gi* mutation combined with mutations in the four *CDF* genes (*CDF 1, 2, 3* and *5*), flowering was responsive to photoperiod and the rhythm of *CO* expression in SD and LD was similar to WT but at slightly elevated levels. This means

that other regulators of *CO* transcription must be generating this photoperiodic-responsive rhythm of *CO* expression and that other factors apart from GI are also able to induce *CO* transcription. The role of GI and CDFs 1, 2, 3 and 5 appears to be to modulate the amplitude of this underlying rhythm of *CO* expression.

Apart from the CDF proteins one other transcriptional repressor of *CO* has been reported called RED AND FAR-RED INSENSITIVE 2 (RFI2) which affects the expression of *CO* and *FT*, and flowering, and this acts primarily in LD (Chen and Ni, 2006). In this paper we describe the identification of a repressor that regulates the rhythm of *CO* expression in SD. This factor, called DAY NEUTRAL FLOWERING (DNF), is crucial in enabling Arabidopsis to distinguish between LD and SD as loss of this repressor alters the rhythm of *CO* expression and the critical photoperiod for flowering with the result that Arabidopsis flowers at the same time in 8h SD as in 16h LD.

Results

Isolation of the early flowering *dnf* mutant

A mutant that flowered early in 8h SD was isolated from a screen of the INRA-Versailles T-DNA knockout mutant population. The mutant is in the Wassilewskija (Ws) background and has been called *day neutral flowering* (*dnf*). The mutant is only affected in flowering time in one photoperiod, flowering early in SD but at the same time as WT in LD (Fig.1), indicating that the mutation affects the photoperiodic flowering pathway. As rosette leaf number is taken as a measure of flowering time we checked that the *dnf* mutation did not affect leaf production. The rate of leaf development in the *dnf* mutant grown in SD was shown to be the same as that in WT plants (Supp. Fig.1). The phenotype of the mutant resembles WT in all other aspects suggesting that the mutation does not have any pleiotropic effects and specifically affects the flowering pathway.

As the T-DNA carried a gene for phosphinothricin resistance (PPT^R), following a back-cross to Ws, the F2 population (approx. 500 lines) was analysed for segregation of the early flowering phenotype with the PPT^R gene. All early flowering lines were PPT resistant suggesting linkage between the *dnf* mutation and the PPT^R gene. A ratio of 1 early flowering (PPT^R): 2.7 late/intermediate flowering (PPT^R): 0.96 late flowering (PPT^S) was obtained. The reason for the slightly skewed ratio is unknown but a 4:1 ratio rather than a 3:1 ratio was observed for both the flowering phenotype and PPT

resistance (of 492 plants in total, 92 were early flowering whilst 400 were late/intermediate flowering, and 102 were PPT^S whilst 390 were PPT^R). It is therefore possible that the *dnf* mutant may contain more than one T-DNA insertion affecting flowering time.

Isolation of the *DNF* gene

A fragment of the T-DNA sequence was used to probe a genomic library made from the *dnf* mutant to isolate clones containing a T-DNA insertion and flanking DNA sequences. Analysis of the flanking sequences of the clones obtained showed that the T-DNA insertion was located within the coding sequence of a putative RING finger domain gene, At3g19140. This gene encodes a small protein of 141 aa that according to the TAIR annotation is predicted to be localised in the endomembrane system. Using the bioinformatic protocols outlined by Emanuelsson et al. (2007), it was shown to have a predicted cleavable signal sequence at the N-terminus followed by a transmembrane domain which is the typical structure of a class I membrane protein (Fig. 2, von Heijen 1988). Type I membrane proteins are orientated such that the C-terminal part of the protein is in the cytoplasm. The C-terminal domain of DNF contains a consensus sequence of a RING-S/T domain, which is a modified RING finger domain (Stone et al., 2005). RING domains are present in E3 ubiquitin ligases that are involved in targeted protein degradation by the proteosome. Functional analysis of all predicted RING domain proteins in Arabidopsis found that of the predicted RING-S/T proteins tested, which included At3g19140 (DNF), none had detectable E3 ligase activity when assayed with Arabidopsis UBC8, UBC10, UBC11, UBC35 or UBC36 as the E2 conjugating enzyme (Stone et al., 2005). It is possible, however, that some or of all of them may function as E3 ligases specifically with one of the other E2s that were not tested, as Arabidopsis has 37 E2 conjugating enzymes.

PHD domains are closely related to RING finger domains and have a similar consensus sequence to the RING domain. PHD domains are protein-protein interaction domains typically involved in chromatin remodelling (Bienz, 2006), both EARLY BOLTING IN SHORT DAYS (EBS) and VERNALISATION INSENSITIVE 3 (VIN3) are examples of PHD domain proteins involved in the control of flowering time (Pineiro et al., 2003; Sung and Amasino 2004). In the case of DNF, however, the similarity to the PHD consensus breaks down after the cysteine in position 3 (Fig. 2), and so it is unlikely to act as a PHD domain protein. Apart from some sequence similarity to other

proteins in the RING/PHD domain region, DNF does not show any homology to other plant proteins in the databases.

To confirm that the T-DNA insertion in At3g19140 is responsible for the early flowering phenotype of the *dnf* mutant in SD, the WT *DNF* allele with 1.1kb of upstream sequence was cloned from Ws genomic DNA and transformed into the *dnf* mutant to test for complementation. Fig. 3A shows that the *DNF* transgene restores WT flowering to the *dnf* mutant; this complementation confirms that At3g19140 encodes the *DNF* gene. As only one line showing full complementation was obtained (the other lines were later flowering than *dnf* but were not completely restored to WT flowering), and as the complementation effect was unstable and frequently lost in subsequent generations, we also recreated the early flowering phenotype of the *dnf* mutant by down-regulating the At3g19140 gene in WT plants through RNAi. The whole *DNF* coding sequence was used for the RNAi construct, as BLAST searches showed that no other Arabidopsis gene has any significant sequence similarity to *DNF* and thus the RNAi construct would target *DNF* specifically. Several RNAi lines were obtained that all exhibited early flowering to a similar extent as the original *dnf* mutant (Fig. 3B) and this was stable in successive generations. Expression levels of *DNF* in the two RNAi lines (4 and 10) used in subsequent experiments was shown to be greatly reduced compared to Ws in SD at ZT5 (Suppl. Fig.2), a timepoint at which *DNF* expression levels are known to be high in Ws (see below). The complementation and RNAi results confirm that mutation of the At3g19140 gene results in early flowering.

To show that the early flowering in SD caused by the *dnf* mutation was not dependent upon the Ws genetic background (as Ws itself is early flowering compared to Columbia (Col) or Landsberg (Ler) ecotypes), the *dnf* mutation was introgressed into the Col background through 4 back-crosses. After each back-cross lines containing the T-DNA insertion were selected based upon their resistance to PPT. Following 4 rounds of back-crossing, PPT^R lines were selfed to produce a segregating population containing homozygous mutant lines. The progeny of these selfed lines were screened for flowering time and PPT^R. All of the lines that showed 100% PPT^R were also early flowering compared to WT Col (Fig. 3C), these lines were genotyped to confirm that they were homozygous for the *dnf* mutation. Therefore the *dnf* mutation can also cause early flowering in the Col ecotype and is not dependent upon the Ws genetic background.

A search for other mutant alleles of the *DNF* gene yielded only one line where a T-DNA insertion disrupts the *DNF* open reading frame (GABI-Kat line 857H08). Plants homozygous for this insertion line did not flower early in SD as expected. However this is probably because the position of the insertion is right at the 3' end of the *DNF* gene, only 5bp upstream from the TAG stop codon, and thus it is possible that functional DNF protein could still be produced in these plants. Analysis of *DNF* transcript levels in this GABI-Kat insertion line showed that *DNF* transcript levels were unaffected by the insertion and accumulated to the same level in SD as in WT Columbia plants (Suppl. Fig.2). *DNF* expression in the *dnf* mutant is not above background levels.

DNF is an E3 ligase

As the DNF protein contains a RING-S/T domain we tested whether DNF had E3 ligase activity. We expressed and affinity purified DNF without the N-terminal putative signal peptide sequence as a GST fusion from *E. coli* (the complete DNF protein containing this sequence could not be resolubilised from the pellet following extraction). Ubiquitination activity was observed for the purified GST-DNF fusion protein in the presence of yeast E1 and the human E2 Hubc5b, and to a lesser extent with human E2 Hubc5a, (Fig. 4, lanes 5 and 6). This ubiquitination was dependent upon the presence of the E1, E2, and GST-DNF, and the level of activity varied depending upon which E2 was present. DNF primarily directed ubiquitination of one major protein in the *E.coli* extract and this was the DNF protein itself. This was shown by probing the immunoblot with a GST antibody which bound to the GST tag of the expressed DNF protein (Fig. 4, lower panel), thus DNF has autoubiquitination activity. DNF may also ubiquitinate other plant proteins which are not present in the *E.coli* extract, the fact that it does not ubiquitinate many *E.coli* proteins suggests that it may only target specific proteins for ubiquitination. Our findings contrast those of Stone et al. (2005) who did not detect any ubiquitination activity when assaying the recombinant full length protein together with a selection of Arabidopsis E2s; UBC8, UBC10, UBC11, UBC35 or UBC36.

Over-expression of *DNF*

Down-regulation or mutation of *DNF* causes early flowering in SD; *DNF* must therefore be involved in the repression of flowering in SD. We produced Ws plants over-expressing *DNF* to see whether this would cause the plants to be delayed in

flowering. Interestingly the over-expressing lines were all early flowering compared to WT, but not as early as the *dnf* mutant (Fig.5). This is unlikely to be due to co-suppression as RNA expression levels in the over-expressing lines was shown to be much higher than Ws (Suppl. Fig.2). A similar observation was reported for over-expression of the floral repressor *EARLY BOLTING IN SHORT DAYS* (*EBS*), where the over-expressers had a similar early flowering phenotype as the *ebs* mutant (Pineiro et al. 2003). This was thought to be due to the disruption of the formation of complexes necessary for floral induction by either the mutation, or by over-expression, which could cause sequestering of other proteins in the complex and prevent formation of fully active complexes.

The *dnf* mutant has an altered critical photoperiod

As the *dnf* mutant has an altered response to photoperiod we tested whether this is reflected in an altered critical photoperiod for flowering. This was done using small purpose built light boxes in which the fluorescent lights were timed to come on for 4, 6, 8, 10, 12, 14 or 16 h per day, so that we could define the critical photoperiod for flowering. Whilst WT plants showed a delay in flowering time once the daylength was reduced to 10h or less, flowering of the *dnf* mutant was only delayed once the daylength was reduced to 6h or less (Fig.6). In very short photoperiods (4h) the *dnf* mutant exhibited a WT late-flowering response. Thus flowering time in the *dnf* mutant is only accelerated compared to WT in short photoperiods of between 4 and 10h. The accelerated flowering in the mutant compared to WT means that DNF must act to repress flowering. The fact that the difference in flowering time between the mutant and WT plants is only observed when the daylength is somewhere between 4 and 10h suggests that DNF only represses flowering between 4 and 10h after dawn. At or before 4h, or after 10h, in the light DNF does not affect flowering because the mutant behaves as WT in photoperiods of these lengths.

DNF acts in the same pathway as *CO* and *GI* and downstream of the circadian clock

Defects in photoperception, or circadian timing, are known to affect flowering time (Yanovsky and Kay 2003) and so the *dnf* mutant was analysed for defects in light perception and/or in the function of the circadian clock. Hypocotyl elongation in red, far-red and blue light was found to be normal (Supp. Fig.3) indicating that perception of these wavelengths of light is unaffected in the *dnf* mutant. Mutants that are defective in

the perception of these wavelengths of light were included as controls to show that the light treatments used were appropriate to detect such defects in light perception. The circadian clock was analysed by looking at *CAB* gene expression in continuous light. The phase of *CAB* gene expression in the *dnf* mutant upon transfer from light/dark cycles into continuous light was indistinguishable from WT (Supp. Fig.4). This suggests that the *dnf* mutation affects neither photoperception pathways nor the clock, and that it acts downstream of these processes in the photoperiodic pathway to influence flowering time in SD.

To investigate whether *DNF* is acting in the same pathway as *CO* to affect the photoperiodic flowering response, the *dnf* mutant (Ws) was crossed into the *co-2* (Ler) mutant background. Due to technical difficulties, homozygous double mutant lines were not identified until the F4 generation. To make allowance for possible variation in flowering time caused by background flowering QTLs that would segregate after crossing Ler and Ws, three different homozygous *dnf,co-2* double mutant lines were analysed together with their sibling lines that were only homozygous for the *co-2* mutation but which carried the WT *DNF* allele. These plants were grown in both LD and SD and scored for flowering time. It should be noted that the late flowering phenotype caused by the *co* mutation is normally only observed in LD (Putterill et al., 1995) and the effect of the *dnf* mutation is only observed in SD. The *dnf* mutation caused early flowering in SD in Ler plants that had the WT *CO* allele (*ddCC*). In SD (as well as in LD) the double mutant lines (*ddcc*), however, flowered as late as their siblings carrying just the *co-2* mutation (*DDcc*) showing that the *co-2* mutation is epistatic to the *dnf* mutation (Fig.7A & B). The late flowering of the double mutants in SD means that functional CO protein is required for the early flowering phenotype of the *dnf* mutant in SD, and thus that *DNF* and *CO* are acting in the same flowering control pathway.

The *gi-11* mutant, which is in the Ws background, was used to cross with the *dnf* mutant. The *dnf,gi-11* homozygous double mutant flowered as late as the *gi-11* mutant in SD (Fig.7C). This shows that GI function is required for the *dnf* mutation to cause early flowering, and that therefore *DNF* is also acting in the same flowering pathway as *GI*.

<u>Localisation and expression of DNF</u>

As DNF is predicted to be a type 1 membrane protein we investigated the intra-cellular localisation of a DNF-GFP fusion protein. GFP fluorescence was observed in the plasma membrane of leaf epidermal cells of plants transformed with a 35S:DNF-GFP construct, and there also appears to be evidence of the DNF:GFP protein in endomembrane structures within the cell (Fig.8A). DAPI staining indicates that the bright globular structures showing fluorescence are not nuclei but some other cellular compartment. Following plasmolysis of the leaf tissue the GFP fluorescence is still observed in the plasma membrane which has become detached from the cell wall (Fig. 8B). In this case the DNF:GFP protein does not seem to be as evenly distributed throughout the membrane as in non-plasmolysed tissue.

The expression of *DNF* was examined in WT and *dnf* plants in both SD and LD. Expression levels were very low (undetectable by RNA gel blots), therefore real-time PCR was necessary for quantification. No expression was detectable in *dnf* mutant plants indicating that it is probably a null mutation. Expression of *DNF* in WT plants was observed at very precise times of the day. In SD expression of *DNF* was observed in the period 4-6 h after dawn (ZT4-ZT6, Fig.9A). Up until ZT3 there is very little expression of *DNF* and expression levels had fallen to zero again by ZT7, suggesting that there is very tight regulation of its expression. Interestingly, the expression profile of *DNF* fits nicely with the critical photoperiod data which shows that in the first 4h of the day there is no difference in flowering response between *dnf* and WT plants, only in SD photoperiods greater than 4h is a difference in flowering time observed (ie. just after the point when *DNF* expression is observed in WT plants). In LD the expression pattern is very different with a major peak in *DNF* expression occurring between ZT12-ZT15 (Fig.9B), there is a minor peak in expression between ZT4-ZT6 at the same time as in SD and the expression levels at this time of day are similar in LD and SD (Supp. Fig.5A), but the induction at this time is small in comparison to the later peak. The reason why *DNF* is expressed so highly in LD when its absence in the *dnf* mutant has no effect on flowering in LD is unclear. The large second peak in expression at ZT12-ZT15 is not observed in SD when the plants are in the dark, which indicates that light is required for *DNF* expression, or that *DNF* expression may be repressed in the dark.

DNF expression was analysed in different organs of the plant to examine where it is expressed. It was found to be expressed in leaves, stem, roots and flowers with highest expression in rosette leaves (Supp. Fig.5B). No obvious circadian regulation of *DNF* expression was observed when Ws plants were sampled for 3 days in continuous

light following transfer from SD conditions (Supp. Fig.6). Diurnal peaks in expression are observed at ZT4 in both the first short day and following the dark period in the first subjective day as expected, however in continuous light *DNF* appears to be de-regulated and expressed at continuously high levels.

To address the question of how the *dnf* mutation affects flowering time only in SD, the expression of *CO* and *FT* was analysed in the *dnf* mutant compared to WT. In the *dnf* mutant *CO* expression is the same as WT in LD (Fig. 10A) consistent with the lack of effect of the *dnf* mutation on flowering in LD. In SD, however, the expression of *CO* is altered such that it starts to rise by 4 h after dawn and is expressed at high levels before the end of an 8h SD (Fig. 10B). The usual night-time peak of *CO* expression is also observed. The elevated levels of *CO* transcript in the light before the end of the SD must result in elevated CO protein levels, because induction of *FT* is also observed before the end of the SD in the *dnf* mutant. The induction of *FT* expression follows that of *CO* and occurs between 4 and 6 h after dawn (Fig. 10C). *CO* and *FT* expression was also induced in SD in the *DNF* RNAi lines, and this occurred at the same time as in the *dnf* mutant, demonstrating that the altered expression pattern of *CO* and *FT* is due to the *dnf* mutation and not due to some second site mutation in the *dnf* mutant. The induction of *CO* and therefore *FT* in SD explains the early flowering phenotype of the *dnf* mutant and the RNAi lines in SD. The role of *DNF* must therefore be to prevent the expression of *CO* during the light period of a SD, thus enabling the plant to prevent flowering and continue vegetative growth in SD.

As *GI* is known to affect the expression of *CO*, the expression of *GI* in the *dnf* mutant was also investigated. The expression of *GI* in the *dnf* mutant over a SD (8h) time-course was found to be very similar to its expression in WT plants, with expression increasing around ZT4 to peak before the end of the SD before falling to low levels in the dark (Supp. Fig.7, Fowler et al., 1999). The fact that the *dnf* mutation causes alterations in both *CO* and *FT* expression without significantly affecting the expression of *GI* indicates that *DNF* acts upstream of *CO* but not of *GI* in the photoperiodic pathway.

Discussion

DNF is a novel flowering time gene that encodes a repressor of flowering; this is demonstrated by the fact that the *dnf* mutation causes early flowering in SD when flowering of WT plants is normally delayed. In 8h SD conditions, the *dnf* mutant

flowers as early as WT and *dnf* plants flower in 16h LD conditions. The fact that it is induced to flower as much in 8h photoperiods as it is in 16h photoperiods indicates that it has lost the repression of flowering normally present in 8h SD. The *dnf* mutant exhibits an altered critical photoperiod, being induced to flower early in photoperiods as short as 6h compared to WT which requires longer 10h photoperiods to attain the same level of induction (Fig.6). In 4h photoperiods the flowering of *dnf* is as late as WT, and this correlates to the fact that *DNF* is not expressed before ZT4 (Fig. 9A) and therefore there will be no difference between *dnf* and WT up until this time of day. The absence of *DNF* expression in the *dnf* mutant from ZT4 onwards results in a lack of inhibition of *CO* expression, and therefore *CO* expression starts to increase in the mutant around ZT4 with significant levels of expression by ZT6 (Fig.10B). The high levels of *CO* expression at ZT6 in the mutant results in the induction of *FT* at this time, and thus early flowering of the *dnf* mutant is able to occur in SD photoperiods as short as 6h.

DNF is expressed between ZT4 and ZT6 and the difference in *CO* expression between the *dnf* mutant and WT plants is observed between ZT4 and ZT7; DNF must therefore prevent the induction of *CO* specifically between ZT4 and ZT7. After ZT7, when *DNF* expression in WT plants has fallen to low levels, *CO* expression is no longer repressed and transcripts start to accumulate as the photoperiod becomes increasingly longer and more inductive. In 16h LD *CO* expression starts to increase earlier in the *dnf* mutant than in WT but overall the expression profiles of *CO* in the *dnf* mutant and WT later on in the day are very similar (Fig. 10A). The reason for this may be that other mechanisms (such as the degradation of CDF proteins by FKF1) are also acting to increase *CO* expression towards the end of a LD and this may mask the effect of the *dnf* mutation. This probably explains why there is no significant effect of the loss of the DNF repressor in the *dnf* mutant on flowering time in LD. The high level of expression of *DNF* at the end of a LD is curious given that it does not act to repress *CO* expression at this time, it may be that an interacting co-factor which is also required for the DNF-mediated repression of *CO* expression is missing at this time of day.

DNF is thus an important regulator of the rhythm of *CO* expression, but it is not acting through the GI/FKF1/CDF regulatory mechanism to modulate the amplitude of the rhythm because the effect of the *dnf* mutation on *CO* expression in SD and LD is different to the constitutively high levels of *CO* expression observed in the *cdf1-R cdf2-1 cdf3-1 cdf5-1* quadruple mutant (cf. Fig.10 and Fornara et al.2009). Without the DNF-mediated repression of *CO* transcription between ZT4 and ZT7 the rhythm of *CO*

expression would start to increase after ZT4 and Arabidopsis would not able to distinguish between LD and SD (except if the SD was 4h or less), the specific timing of *DNF* expression is thus crucial in establishing a photoperiodic flowering response.

The mechanism by which DNF represses *CO* transcription is unknown but it could be through the ubiquitin/proteosome degradation pathway. DNF contains a RING-S/T domain and we have shown that it has E3 ligase activity. DNF may specifically target an activator of *CO* transcription for degradation at specific times of the day (between ZT4 and ZT7). As the levels of the GI protein, which is known to promote *CO* expression, have been shown to be high at that time of day (David et al., 2006) DNF cannot be degrading GI, although it could be targeting another transcriptional activator protein that may interact with GI to induce *CO* expression. DNF is a membrane-bound E3 ligase. The auto-ubiquitination of DNF could be a mechanism by which it re-cycles and regulates the amount of DNF protein present in the membrane and cytosol, such a mechanism is known to occur in yeast and humans (Platta et al., 2007, 2009).

In summary, we have shown that DNF affects the rhythm of *CO* expression, particularly between ZT4-ZT7, and that this regulation is involved in determining the critical photoperiod of the flowering response in Arabidopsis, as without it Arabidopsis plants flower early even in days with photoperiods as short as 6h.

Materials and Methods

Plant growth conditions

All Arabidopsis seed, including the T-DNA mutant population and the mutants *co-2* (Koornneef et al., 1991) *phyA-1* (Whitelam et al., 1993), *phyB-1* and *cry2* (originally called *hy3*and *hy4* respectively, Koornneef et al., 1980), was obtained from the Nottingham Arabidopsis Stock Centre (NASC). This is apart from the GABI-Kat line 857H08 which was obtained from Bernd Weisshaar at Bielefeld University, Germany, and the *gi-11* mutant (Richardson et al., 1998) which was obtained from Jo Putterill, University of Aukland, New Zealand.

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 2 days to achieve uniform germination before being transferred to Sanyo MLR-350 growth cabinets and grown at 22 °C in either SD, or LD. SD consisted of 8h white light

(100μmolm^{-2}s^{-1}) followed by 16h darkness, LD consisted of 16h of white light followed by 8h darkness. Lighting was supplied by BriteGro F36WT8 fluorescent lamps (Sylvania, Germany). Critical photoperiod experiments were performed in small purpose built light boxes when the fluorescent lights (50μmolm^{-2}s^{-1}) were timed to come on for 4, 6, 8, 10, 12, 14 or 16 h per day. Flowering time was scored as the number of rosette leaves when the plant had developed a bolt of 1cm. The variation in flowering times observed between different experiments is probably due to the growth cabinets not maintaining exactly the same temperatures; the variation observed within an experiment is much less.

Hypocotyl elongation assay

Seeds were sterilised in 20% bleach, washed 5 times in sterile water then pipetted onto 0.7% agarose plates. The plates were then transferred to a Percival growth cabinet (CLF plant Climatics model 1-3LEDDLL3). The seedlings were grown for 4 days at 22ºC under continuous single fluence light provided by LEDs, red (2.5μmolm^{-2}s^{-1}), FR (0.1μmolm^{-2}s^{-1}), blue (0.4μmolm^{-2}s^{-1}), irradiances were measured using an EPP2000 fibre optic spectrometer (StellarNet UK Ltd.). Seedlings were also grown in the dark as a control. The length of the hypocotyl was then measured.

Complementation of the *dnf* mutant

Primers O3p5 and O3p9 (see Supp. Table 1) were used to PCR a 1.6kb fragment consisting of the full length WT allele of the *DNF* gene plus 1.1kb of upstream sequence from Ws genomic DNA using KOD Hot Start proof-reading DNA Polymerase (Novagen). This was cloned into the Sma I site of pUC 18, sequence verified and then subcloned into pGVPT hygromycin transformation vector (Becker et al., 1992) using the Hind III and Sst I sites. This construct was electroporated into Agrobacterium GV3101, and *dnf* mutant plants were transformed by the floral dip method (Clough and Bent, 1998). Transformed seed were selected on hygromycin plates (20μg/ml).

Overexpression and RNAi of *DNF*

The coding sequence of the DNF gene was amplified by PCR using O3attB1 and O3attB2 primers (Supp. Table 1) and KOD DNA Polymerase. The fragment obtained was cloned into the Gateway pDONR^{TM}207 vector using BP clonase, and sequence verified. The insert was then transferred into the Gateway CaMV 35S overexpression vector pB2GW7 and the RNAi vector pB7GWIWG2 (http://www.psb.ugent.be/gateway/index.php) by the LR reaction. These constructs were transformed into GV3101 and then by floral dip into WT Ws plants. Transformed

plants were selected by spraying young plants with BASTA (0.02% Challenge, BAYER).

GFP constructs

The CaMV *35S* and *DNF* promoters (P35S and PDNF respectively), the *DNF* coding sequence (without the stop codon) and the *GFP* coding sequence were PCR amplified using the following primers (for = forward, rev = reverse); P35Sfor, P35Srev, PDNFfor, PDNFrev, DNFfor1, DNFrev1, EGFPfor, EGFPrev (Supp. Table 1).

The PCR fragments were subcloned into pBluescript vector using the restriction sites present in the primer sequences. The fragments were sequence verified. The *GFP* coding sequence fragment was subcloned behind the *DNF* coding sequence, and the CaMV *35S* or *DNF* promoter fragments were cloned in front of the *DNF-GFP* fusion protein sequence. The whole promoter fusion protein sequence was then subcloned into the BIB-HYG transformation vector using the HindIII and SacI restriction sites. The constructs were transformed into WT Ws plants by floral dip and transformants selected on hygromycin plates (20μg/ml).

Expression analysis

Quantitative real-time PCR was used to detect the levels of *CO, FT, GI, CAB* and *DNF* mRNA abundance. Plants were grown in either SD or LD and samples from 4 plants were harvested at the 4/5 leaf stage and pooled for each RNA extraction. 5μg of total 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 incubated 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. Real-time PCR assays were performed using a Taqman machine (ABI Prism ® 7900HT, Applied Biosystems). Each reaction contained 0.4 μM of the forward and reverse primers (see below), 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 s and annealing/extension at 60 °C for 1 min. 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 a cDNA sample where expression was known

or expected to be high. Reactions were optimised so that efficiencies were equal to 100 % ± 10%. Melt curve analyses were performed to show that only a single product was being amplified in each reaction. ABI prism software version SDS2.1 was used to analyse the assay results. Duplicate RNA samples were assayed for each timepoint (ie. 8 leaf samples per timepoint), each real-time PCR assay for each RNA sample had three technical replicates. The expression levels of β-Actin were used to normalise the expression of the target genes between samples.

Primer sequences; DNFfor2, DNFrev2, Actinfor, Actinrev, FTfor, FTrev, COfor, COrev, GIfor, GIrev, CABfor, CABrev (Supp. Table 1).

Ubiquitination assay

A DNF clone lacking the first 39 n-terminal amino acids containing the putative signal peptide sequence was cloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) to produce an in-frame fusion with the GST tag. All recombinant fusion proteins were retained mostly in the insoluble fraction of *E. coli* strain BL21 (DE3)*pLysS*, the insoluble fraction was solubilized and dialysed according to the protein refolding kit (Novagen), the soluble protein was used for *in vitro* ubiquitination assays. *In vitro* ubiquitination assays were carried out as described previously (Hardtke et al., 2002). Each Reaction (50 μL final volume) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM ATP, 0.2 mM dithiothreitol, 10 mM phosphocreatine, 0.1 unit of creatine kinase (Sigma), 2 μg purified His-Ubiquitin, 50 ng of yeast E1 (Biomol), 150 ng E2 UbcH5b or UbcH5a (Biomol) and 1 μg of refolded GST-DNF. The reactions were incubated at 37 °C for 3 h and stopped by adding 4×SDS-PAGE sample buffer (125mM Tris-HCl, pH 6.8, 20% [v/v] glycerin, 4% [w/v] SDS, and 10% [v/v] β-mercaptoethanol) at 100°C for 5 min and analyzed by SDS-PAGE electrophoresis followed by immunoblotting.

ImmunoBlotting

Immunoblots were performed with mouse monoclonal anti-Ub antibodies (Roche) and rabbit anti-GST antibodies (Novagen). The primary antibodies were used at 1:5000 dilution and the secondary horseradish peroxidase (HRP) conjugated secondary antibodies were used at a 1:20000 dilution. Amersham ECL-plus western blotting chemiluminescence detection kits were used to detect levels of HRP and develop the blots on light sensitive autoradiograph films.

Confocal microscopy

Sections of *Arabidopsis* leaves were mounted for microscopical observation in water under glass coverslips. Plasmolysed leaf samples were prepared by immersing them in 0.8M Mannitol for 20 mins. The leaves were examined using an 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.

Mutant crosses and introgression

The *dnf* mutant was always used as the male parent in the crosses so that F1 progeny from successful crosses could be selected for on their resistance to PPT. For introgression of the *dnf* mutation into Columbia progeny from the cross, and from each of the subsequent rounds of back-crossing, were selected for PPT resistance. After 4 rounds of back crossing, PPT^R plants were selfed and lines homozygous for the *dnf* mutation were selected.

Genotyping the *dnf* mutation was done in a single PCR reaction using three PCR primers; DNFF and DNFR designed to the *DNF* gene each side of the T-DNA insertion site in the *dnf* mutant, and the RBR primer designed to the right border of the T-DNA (Supp. Table 1).

DNFF and DNFR amplify a fragment 178bp from the *DNF* gene that does not contain the T-DNA insertion, whereas DNFR and RBR amplify a fragment 482bp from the mutated *dnf* gene containing the T-DNA insertion, the size of the T-DNA insertion prevents amplification from DNFF and DNFR primers in the mutant. Homozygous *dnf* mutant lines only produce the 482bp fragment, homozygous *DNF* lines only the 178bp fragment, whilst heterozygous T-DNA lines will amplify both fragments.

Genotyping the *co-2* mutation was done by PCR amplifying the region containing the position of the single base change (Putterill et al., 1995) (using primers CO-Span 2F and CO-Span 2R) and sequencing the fragments obtained. Plants homozygous for the *co-2* mutation posses an A at that position whereas WT plants posses a G, heterozygous plants have a mix of G and A at that position.

Genotyping the *gi-11* mutation was also done by PCR using primers designed to the 5' deleted region of the *GI* gene in the *gi-11* mutant (Fowler et al., 1999); GI-For6 and GI-Rev5.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At3G19140 (DNF), At3G18780 (β-Actin), At5G15840 (CO), At1G22770 (GI), At1G29930 (CAB).

Supplemental Data

The following materials are available in the online version of this article.

Supp. Fig.1. Rate of leaf production in *dnf* and Ws plants.

Supp. Fig.2. *DNF* expression in RNAi and over-expressing lines, and the GABI-Kat insertion line in 8h SD.

Supp. Fig.3. Hypocotyl elongation of Ws and *dnf* mutant plants in different light qualities.

Supp. Fig.4. Analysis of circadian CAB expression.

Supp. Fig.5. A. *DNF* expression at ZT5 in LD and SD. B. *DNF* expression at ZT5 in SD in different tissues.

Supp. Fig.6 *DNF* expression following transfer from one SD to continuous light.

Supp. Fig.7. *GI* expression in *dnf* and Ws plants.

Supplemental Table 1. Primers used in this study.

Acknowledgements

The authors would like to acknowledge the Station de Génétique et d'Amélioration des Plantes INRA, Versailles, France and to NASC (Nottingham, UK) for the production and distribution of the T-DNA mutant population. Thanks to Linda Brown for technical assistance. Thanks also to Jo Putterill for the *gi-11* seed.

This work was funded by the BBSRC.

Figure Legends

Figure 1. Flowering times in LD and SD.

Average leaf number at flowering of Ws and *dnf* mutant plants in LD (16h light, 8h dark) and SD (8h light, 16h dark). Error bars show standard deviation, n= 20 plants

Figure 2. Predicted domains of the DNF protein.

Schematic of the DNF protein showing predicted domains and the site of the T-DNA insertion in the *dnf* mutant. The amino acid sequence of the DNF RING-S/T domain is illustrated together with the consensus sequences for RING-S/T and PHD domains.

Figure 3. Flowering time of a complemented *dnf* mutant line, *DNF* RNAi plants, and Columbia introgression lines in 8h SD.

A. Flowering times of Ws, the *dnf* mutant, and a homozygous complemented mutant line (*dnf* mutant expressing the *DNF* transgene driven by *DNF* promoter sequences). B. Flowering times of several independent *DNF* RNAi lines (RNAi of the *DNF* gene in Ws) compared to the original *dnf* mutant and Ws plants. C. Leaf number at flowering of homozygous progeny from selfed plants derived from four rounds of back-crossing of the *dnf* mutant into Columbia (Col). Flowering times of Ws, *dnf* and Col are also shown. Error bars show standard deviation, n=12-15 plants.

Figure 4. E3 ubiquitin ligase activity of DNF.

GST-DNF was expressed, and purified from *E. coli* and tested for ubiquitination activity in the presence of yeast E1, human E2 (Hubc5b or Hubc5a) and ubiquitin. The

immunoblots were probed with anti-Ub antibodies (top panel) to detect ubiquitinated *E. coli* proteins. Anti-GST antibodies (bottom panel) were used to detect GST-DNF.

Figure 5. Flowering times of *DNF* over-expressers in SD.

Flowering time of *35S:DNF* over-expressing lines compared to Ws and the *dnf* mutant in 8h SD. Error bars show standard deviation, n=12 plants

Figure 6. Critical photoperiod of *dnf* and Ws.

Average leaf number at flowering of Ws and *dnf* mutant plants grown in photoperiods of different lengths ranging from 4 to 16 h light. Error bars show standard deviation, n=12 plants

Figure 7. Flowering times of double mutants.

Average leaf number at flowering in SD (A) and LD (B) of three different homozygous *dnf,co-2* double mutant lines (*ddcc*) compared to their siblings that carry the WT *DNF* allele but are still homozygous for the *co-2* mutation (*DDcc*), and those carrying the WT *CO* allele but homozygous for the *dnf* mutation (*ddCC*). Flowering times of Ws, the *dnf* mutant and Ler are also shown for comparison. Error bars show standard deviation, n=15-20 plants. C. Average leaf number at flowering of Ws, *dnf, gi-11,* and *dnf,gi-11* double mutant plants in SD. Error bars show standard deviation, n=10 plants

Figure 8. Intracellular localisation of DNF protein.

Localisation of DNF-GFP fusion protein in leaf epidermal cells of 35S-DNF-GFP plants before (A), and after plasmolysis (B). Panel i). GFP fluorescence, panel ii). GFP with transmitted light. Arrows in panel Ai). indicate possible internal

endomembrane structures within the cell containing the DNF:GFP protein. Arrowheads in panel Bi). show where the plasma membrane has separated from the cell wall.

Figure 9. Expression pattern of *DNF*.

A. Expression of *DNF* in WT plants in 8h SD. B. Expression of *DNF* in WT plants in 16h LD. Expression levels were determined by qRT-PCR and are normalised to β-*Actin*. Data points represent an average of 2 experimental replicates each with 3 technical replicates. Error bars represent standard deviation.

Figure 10. Expression of *CO* and *FT* in the *dnf* mutant.

A. Expression of *CO* in Ws and the *dnf* mutant in 16h LD. B. Expression of *CO* in Ws, *dnf*, and *DNF* RNAi lines 4 and 10 in 8h SD. C. Expression of *FT* in Ws, *dnf*, and *DNF* RNAi lines 4 and 10 in 8h SD. Expression levels were determined by qRT-PCR and are normalised to β-Actin, but as different standard curves were used for the LD and SD analysis, the levels between experiments cannot be compared. White and black bars represent light and dark periods respectively. Data points represent an average of 2 experimental replicates each with 3 technical replicates. Error bars represent standard deviation.

References

Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992). New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol Biol **20,** 1195-1197.

Bienz, M. (2006). The PHD finger, a nuclear protein-interaction domain. Trends Biochem Sci **31,** 35-40.

Chen, M., and Ni, M. (2006). RFI2, a RING-domain zinc finger protein, negatively regulates *CONSTANS* expression and photoperiodic flowering. Plant J **46,** 823-833.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16,** 735-743.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316,** 1030-1033.

David, K.M., Armbruster, U., Tama, N., and Putterill, J. (2006). Arabidopsis GIGANTEA protein is post-transcriptionally regulated by light and dark. FEBS Letters **580,** 1193-1197.

Emanuelsson, O., Brunak, S., von Heijne, G. and Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. Nature Protocols **2**, 953-971.

Fornara, F., Panigrahi, K.C.S., Gissot, L., Sauerbrunn, N., Ruhl, M., Jarillo, J.A. and Coupland, G. (2009). Arabidopsis DOF transcription factors act redundantly to reduce *CONSTANS* expression and are essential for a photoperiodic flowering response. Developmental Cell **17**, 75-86.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G. and Putterill, J. (1999). GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J **18**, 4679-88.

Hardtke, C.S., Okamoto, H., Stoop-Myer, C. and Deng, X.W. (2002. Biochemical evidence for ubiquitin ligase activity of the Arabidopsis COP1 interacting protein 8 (CIP8). Plant J **30**, 385-94.

Imaizumi, T., and Kay, S.A. (2006). Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci **11,** 550-558.

Imaizumi T., Tran, H.G., Swartz, T.E., Briggs, W.R. and Kay, S.A. (2003). FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. Nature **426**, 302-306.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A., and Kay, S.A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of *CONSTANS* in Arabidopsis. Science **309,** 293-297.

Jaeger, K.E., and Wigge, P.A. (2007). FT protein acts as a long-range signal in Arabidopsis. Curr Biol **17,** 1050-1054.

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

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

Mathieu, J., Warthmann, N., Kuttner, F., and Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Curr Biol **17,** 1055-1060.

Michaels, S.D. (2009). Flowering time regulation produces much fruit. Curr Opin Plant Biol **12**, 75-80.

Mizoguchi, T., Wright, L., Fujiwara, S., Cremer, F., Lee, K., Onouchi, H., Mouradov, A., Fowler, S., Kamada, H., Putterill, J., and Coupland, G. (2005). Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. Plant Cell **17,** 2255-2270.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K., and Coupland, G. (2000). Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among Arabidopsis flowering-time genes. Plant Cell **12,** 885-900.

Pineiro, M., Gomez-Mena, C., Schaffer, R., Martinez-Zapater, J.M., and Coupland, G. (2003). EARLY BOLTING IN SHORT DAYS is related to chromatin remodeling factors and regulates flowering in Arabidopsis by repressing *FT*. Plant Cell **15,** 1552-1562.

Platta, H. W., El Magraoui, F., Schlee, D., Grunau, S., Girzalsky, W., and

Erdmann R. (2007). Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling. J Cell Biol. **177:**197–204.

Platta, H. W., El Magraoui, F., Baumer, B.E., Schlee, D., Girzalsky, W., and Erdmann R. (2009). Pex2 and Pex12 function as protein-ubiquitin ligases in peroxisomal protein inport. Mol Cell. Biol **29**, 5505-16.

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

Richardson, K., Fowler, S., Pullen, C., Skelton, C., Morris, B. and Putterill, J. (1998). T-DNA tagging of a flowering time gene and improved gene transfer by *in planta* transformation of Arabidopsis. Aust J Plant Phys **25**, 125-130.

Roden, L.C., Song, H.R., Jackson, S., Morris, K., and Carre, I.A. (2002). Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **99,** 13313-13318.

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

Sawa, M., Nusinow, D.A., Kay, S.A., and Imaizumi, T. (2007). FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science **318,** 261-265.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M. and Kay, S.A. (2001). A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell **13**, 2659-2670.

Somers, D.E., Schultz, T.F., Milnamow, M. and Kay, S.A. (2000). *ZEITLUPE* encodes a novel clock-associated PAS protein from Arabidopsis. Cell **101**, 319-329.

Stone, S.L., Hauksdottir, H., Troy, A., Herschleb, J., Kraft, E., and Callis, J. (2005). Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. Plant Physiol **137,** 13-30.

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

Sung, S. and Amasino, R.M. (2004). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature **427**, 159-164.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor Regulation of CONSTANS Protein in Photoperiodic Flowering. Science **303,** 1003-1006.

von Heijen, G. (1988). Transcending the impenetrable: how proteins come to terms with membranes. Biochimica et Biophysica Acta **947**, 307-333.

Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.C., Cowl, J.S. and Harberd, N.P. (1993). Phytochrome A null mutants of Arabidopsis display a wild-type phenotype in white light. Plant Cell **5**, 757–768.

Yanovsky, M.J., and Kay, S.A. (2002). Molecular basis of seasonal time measurement in Arabidopsis. Nature **419,** 308-312.

Yanovsky, M.J., and Kay, S.A. (2003). Living by the calendar: how plants know when to flower. Nat Rev Mol Cell Biol **4,** 265-275.

Zeevaart, J.A.D. (1976). Physiology of flower formation. Annual Review of Plant Physiology **27,** 321-348.