

DAY NEUTRAL FLOWERING does not act through GIGANTEA and FKF1 to regulate *CONSTANS* expression and flowering time

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The regulation of *CONSTANS* (*CO*) gene expression and protein levels is the critical factor in determining a plant's response to photoperiod, flowering is induced when high levels of *CO* protein are present in the light. The regulation of *CO* transcription is mediated in part by GIGANTEA (*GI*), FKF1, and the CYCLING DOF FACTORS (*CDFs*), and factors affecting the levels of these proteins will also affect *CO* expression. The DAY NEUTRAL FLOWERING (*DNF*) protein is an E3 ligase involved in repressing *CO* expression in the early part of the day. In this article we present evidence to support the argument that *DNF* is not acting through the *GI*/*FKF1*/*CDF* regulatory mechanism to repress *CO* expression, but that it acts on another transcriptional activator of *CO*.

Photoperiod is one of the main factors regulating flowering time in plants and some plants are able to respond to very small changes in photoperiod. In the facultative long day (*LD*) plant *Arabidopsis* a lot is now known about the molecular mechanisms underlying this response. These mechanisms centre on the transcriptional and post-transcriptional regulation of the *CONSTANS* (*CO*) protein which directly induces the production of the FLOWERING LOCUS T (*FT*) protein that moves to the apex to induce flowering. The photoperiodic response of a plant is determined by the time at which the *CO* protein is produced during the light period of the daily light/dark cycle. In *Arabidopsis* the *CO* protein is only produced at significant levels from about 10 h after dawn, which explains why

Arabidopsis starts to flower earlier only when the photoperiod is greater than 10 h. A strict control of flowering in response to changing daylength therefore requires a very tight control of *CO* transcription and protein levels.

DAY NEUTRAL FLOWERING (*DNF*) is a negative regulator of *CO* expression which acts at a particular time of the day, inhibiting *CO* expression specifically between 4–7 h after dawn.¹ This repression prevents *CO* expression, and consequently *FT* expression and flowering, in 8 h short days (*SD*). In the *dnf* mutant this repression is absent and *CO* transcript is allowed to accumulate as early as 4 h after dawn, leading to the induction of *FT* by 6 h after dawn. As a result the *dnf* mutant is induced to flower early in photoperiods as short as 6 h, and exhibits maximal induction in photoperiods of around 8 h which are non-inductive photoperiods for *WT* plants.

Double mutant analysis has shown that the *dnf,co-2* double mutant is late flowering as expected because the effect of the *dnf* mutation in de-repressing *CO* expression to induce flowering is not observed as the *co-2* mutation means that flowering is not able to be induced. *DNF* acts upstream of *CO* as demonstrated by the effect of the *dnf* mutation on *CO* expression.¹ The *dnf* mutation does not affect the expression of GIGANTEA (*GI*), however the *dnf,gi-11* double mutant is also late flowering indicating that both active *GI* and *CO* proteins are required to observe the early flowering caused by the *dnf* mutation.¹ In *gi* mutants *CO* expression is either not activated or is continually repressed^{2,6} thus masking the effect of the *dnf* mutation in the *dnf,gi-11* double mutant.

Key words: flowering, photoperiod, *Arabidopsis*, *constans*, *gigantea*, *FKF1*, short days, long days, daylength

Abbreviations: *SD*, short days; *LD*, long days; *h*, hours; *WT*, wild type

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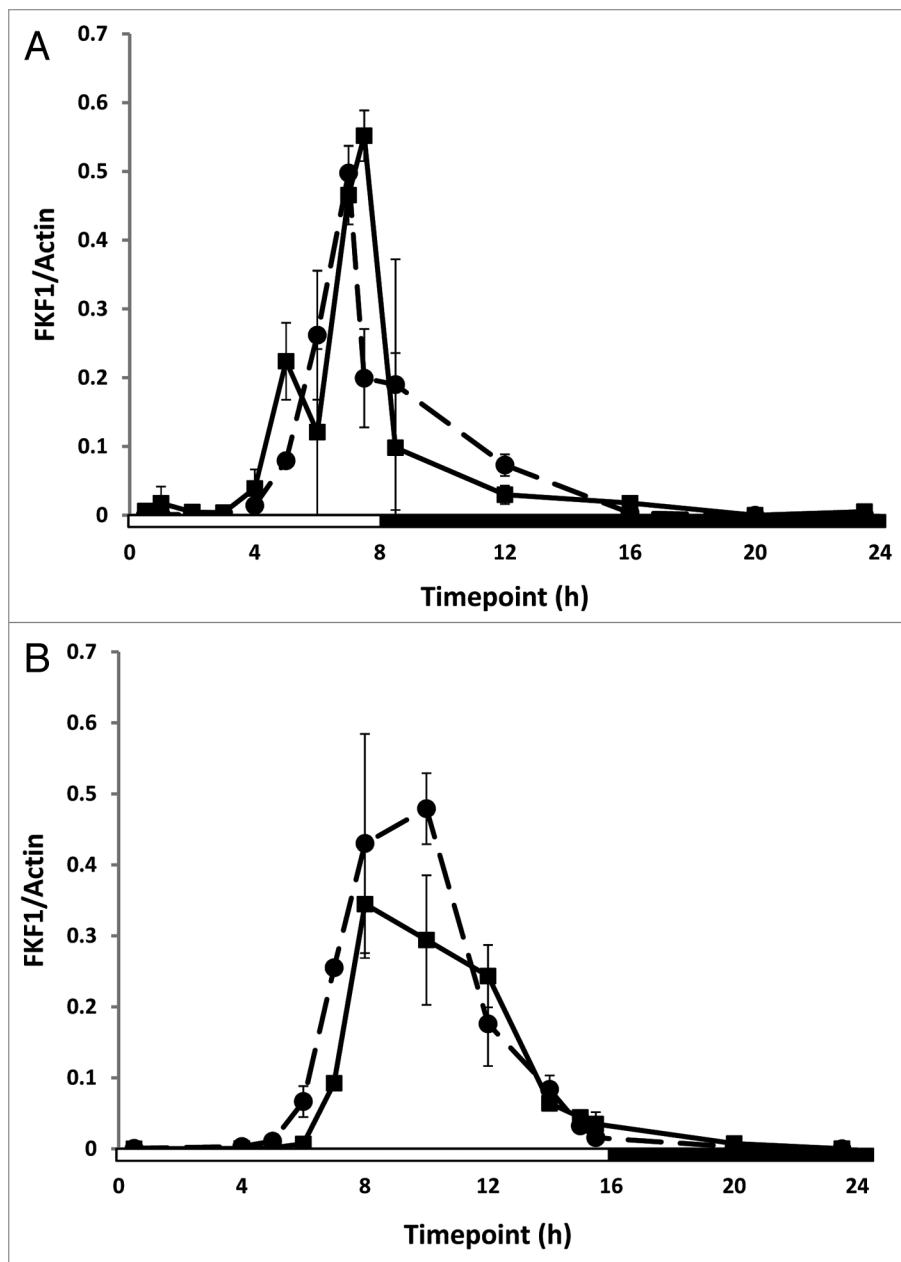


Figure 1. Relative levels of *FKF1* gene expression in the *dnf* mutant (circles, dotted line) and WT plants (squares, solid line) in 8 h SD (A) and 16 h LD (B). Expression levels are normalised to the β -Actin gene. Transcript levels were measured by quantitative RT-PCR of two replicate rosette leaf samples, with three technical replicates performed for each sample. White and black bars depict light and dark periods respectively. Error bars show standard deviation.

The mechanism of action of the *DNF* protein is as yet unknown, however it does have a RING-ST domain characteristic of E3 ubiquitin ligases, and it has been shown to possess E3 ligase activity in vitro.¹ It is thus likely that *DNF* is an E3 ligase which targets an activator of *CO* transcription for degradation in the early part of the day (between 4–7 h after dawn). *DNF* is only expressed between 4–6 h

after dawn, shortly after this time *DNF* protein is presumably no longer present to degrade the activator of *CO* transcription which is then allowed to accumulate and induce expression of *CO* later in the day (8 h or more after dawn). *GI* is known to activate *CO* expression,^{2,3} however as *GI* expression and protein levels are high by 4 h after dawn in SD,⁴ at the same time that *DNF* expression is high,

DNF cannot be targeting *GI* for degradation or repressing its expression. Another possibility is that *DNF* may be regulating the level of *FKF1* which interacts with *GI* in a light dependent manner to regulate *CO* expression through the degradation of the CYCLING DOF FACTOR (CDF) repressors.^{5,6} As *FKF1* protein levels closely match the levels of its RNA in WT plants,⁷ it would suggest that *DNF* is not acting to delay the accumulation of *FKF1* protein. However, the fact that *FKF1* expression only increases around 7–10 h after dawn,⁷ which is after the expression of *DNF* has diminished, raises the possibility that *DNF* may repress the expression of *FKF1* in the early part of the day (through targeted degradation of a transcriptional activator of *FKF1*) thus restricting *FKF1* expression to the latter part of the day.

To test this possibility we examined *FKF1* expression in the *dnf* mutant compared to WT plants in both SD and LD (Fig. 1). No difference in the expression of *FKF1* was observed in the *dnf* mutant compared to WT in either photoperiod indicating that the expression profile of *FKF1* is not regulated by *DNF*. Thus *DNF* is not affecting *CO* expression through altered regulation of either *FKF1* or *GI*, and so it must be repressing the expression of *CO* through the targeted degradation of another, as yet unidentified, transcriptional activator of *CO*. Fornara et al.⁶ showed that the whole layer of regulation of *CO* transcription mediated by *GI*, *FKF1* and the CDF repressors can be removed without abolishing the rhythmic cycling of *CO* expression. One or more transcriptional activators must therefore exist that cause the rhythmic cycling of *CO* expression, and we propose that *DNF* regulates the level of this activator(s), targeting it for degradation between 4–7 h after dawn therefore preventing the induction of *CO* expression during this period of the day.

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