Vernalisation-induced changes to the Arabidopsis circadian clock require Polycomb

Repressive Complex 2 and are FLC-independent

Stephanie S. I. Williams[1], Miguel Montez[1], Emily Edwards[1,2], Pirita Paajanen[1], Antony N.

Dodd[1]*

1. John Innes Centre, Norwich Research Park, Norwich NR4 7UH, U.K.

2. Current address: Department of Biosciences, Durham University, Durham DH1 3LE, U.K.

* Corresponding author: antony.dodd@jic.ac.uk

ORCID

SSIW - 0000-0003-0612-1789

AND - 0000-0001-6859-0105

EE - 0009-0003-5573-1337

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Abstract

In many plants, prolonged winter cold and seasonal day-length changes align the transition

to flowering with spring. This occurs through the vernalisation and photoperiod pathways,

respectively. Despite roles for the circadian clock in regulating both pathways, their

mechanisms have mainly been studied in isolation and their interactions are not fully

understood. We found that vernalisation elicits changes in the circadian clock and this is

linked to alterations in several clock-controlled outputs, including photoperiodic flowering.

Importantly, vernalisation-induced changes in specific clock genes are stable and persist

upon return to warmth, providing the circadian clock with a memory of prior long-term cold

exposure. The changes are not systematic and affect specific circadian oscillator genes. This

change requires the epigenetic regulator Polycomb Repressive Complex 2 (PRC2), but not

the major genetic determinants of vernalisation *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C*

(*FLC*). In contrast to their role in *FLC* silencing, core PRC2 components but not accessory

proteins are required for these changes in the clock post-vernalisation. Our work raises the

possibility that long-term cold feeds epigenetic information into the clock as a seasonal

response mechanism, potentially preparing plants for the coming season.

Introduction

Plants monitor seasonal progression in two broad ways: by tracking day-length changes, and through long-term temperature sensing. In flowering plants, these processes align the transition to reproductive development with the seasonal environment. Some Arabidopsis accessions and winter annual crops require prolonged cold to induce flowering (Bastow et al., 2004; Aikawa et al., 2010). This is known as vernalisation and captures long-term information on past environmental conditions. Species including Arabidopsis and rice are also photoperiodic, and initiate flowering in response to seasonal day length changes (Yanovsky & Kay, 2002; Serrano-Bueno et al., 2017). Photoperiodic responses involve the circadian clock, so the interaction between 24 h circadian timing and long-term environmental monitoring forms an important part of plant phenology. As vernalisation is often studied in isolation from photoperiodism and circadian regulation, we wished to investigate how these processes might be linked in controlling long-term developmental processes.

Circadian clocks adapt plants to environmental fluctuations over both 24 h and annual timescales (Eriksson & Millar, 2003; Millar, 2016). They involve self-sustaining cellular oscillators that are entrained to rhythmic environmental cues such as light and temperature. In turn, the oscillator aligns biological processes with the time of day (Pittendrigh, 1960; Millar, 2003; Hsu & Harmer, 2014). In *Arabidopsis thaliana*, the circadian oscillator comprises a series of interlocking transcription-translation feedback loops. These operate alongside post-transcriptional regulation and chromatin modifications (Fujiwara et al., 2008; Jordi Malapeira et al., 2012; Yang et al., 2020), with histone modifications contributing to the rhythms of some oscillator components (Perales & Mas, 2007; J. Malapeira et al., 2012; Song & Noh, 2012; Baerenfaller et al., 2016). The Arabidopsis circadian clock regulates at least 30% of the transcriptome, suggesting that many processes – including development, physiology, and metabolism – are under circadian regulation (Harmer et al., 2000; Covington et al., 2008; Michael et al., 2008).

In addition to photoperiodism, for some plants the floral transition also requires vernalisation.

Vernalisation is thought to prevent premature flowering in autumn when photoperiods may

induce flowering, but environmental conditions do not favour reproduction (Hepworth et al.,

2018). In Arabidopsis, *FLOWERING LOCUS C* (*FLC*) and *FRIDGIDA* (*FRI)* are factors that

specify the vernalisation requirement (Koornneef et al., 1994; Michaels & Amasino, 1999;

Sheldon et al., 1999). *FLC* encodes a MADS-box DNA binding transcription factor which,

prior to vernalisation, is expressed highly in an open chromatin state, allowing its active

transcription. High FLC levels inhibit floral integrator genes including *FT* and *SOC1*,

preventing flowering (Michaels & Amasino, 1999). *FRI* is a transcriptional activator of *FLC*

and its activity is antagonised by vernalisation (Choi et al., 2011). The Col-0 accession of

Arabidopsis has a naturally occurring mutation to the *FRI* allele, rendering it non-functional.

Therefore, Col-0 does not require vernalisation and is described as a rapid-cycling accession

(Gazzani et al., 2003; Michaels et al., 2003).

During the first stages of vernalisation, transient cold temperatures promote transcriptional

FLC shutdown through activity of the long noncoding RNA *COOLAIR* (Csorba et al., 2014).

Longer cold periods upregulate expression of the PHD finger protein VERNALISATION

INSENSITIVE 3 (VIN3) (Sung & Amasino, 2004; Hepworth et al., 2018). VIN3 and its

homolog VERNALISATION 5 (VRN5) act as accessory proteins to the Polycomb Repressive

Complex 2 (PRC2) (Greb et al., 2007), and are both required for the *FLC* silencing during

vernalisation. PRC2 is an evolutionarily conserved chromatin-modifying complex with

methyltransferase activity (De Lucia et al., 2008; Hennig & Derkacheva, 2009), which

mediates a switch from an active to a repressive chromatin state at *FLC* marked by histone

H3 trimethylation at lysine 27 (H3K27me3). Upon the arrival of warmer spring temperatures,

PRC2 spreads this repressive mark across the *FLC* locus, conferring stable epigenetic

silencing and derepressing flowering (Angel et al., 2011; Yang et al., 2017). *FLC* expression

levels are reset for the next generation during embryogenesis (Sheldon et al., 2008; Choi et

al., 2009).

There are interactions between the circadian clock and vernalisation pathway. The clock

regulates *VIN3* expression during mild low temperatures, such that *VIN3* transcript levels

peak at the end of short days (Hepworth et al., 2018). This likely occurs through interaction

of the circadian oscillator components CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and

LATE ELONGATED HYPOCOTYL (LHY) with the vernalisation-responsive *cis*-element

within the *VIN3* promoter (Kyung et al., 2022). Vernalisation is compromised in the *cca1 lhy*

double mutant due to impaired upregulation of *VIN3* at 12˚C (Kyung et al., 2022).

Vernalisation also shortens the circadian period independently from *FLC* expression

(Salathia et al., 2006). Furthermore, *FLC* contributes to temperature compensation of the

circadian clock, perhaps through interactions with LUX ARRHYTHMO (LUX) and another

unknown factor (Edwards et al., 2006). *FLC* also binds weakly to the *LHY* promoter, with this

being detectable in *FLC*-overexpressing plants (Spensley et al., 2009). Together, this

suggests crosstalk between the vernalisation pathway, *FLC*, and the circadian clock, which

might provide an additional mechanism to distinguish between similar photoperiods during

autumn and spring (Salathia et al., 2006).

Despite this evidence for interactions between the circadian clock and vernalisation pathway,

it remains unclear how vernalisation modulates the circadian oscillator and whether such

changes impact plant physiology and development. Therefore, we investigated how specific

oscillator components respond to vernalisation, the associated mechanisms within the

vernalisation pathway, and outcomes for both growth and photoperiodic flowering responses.

Results

Vernalisation induces stable alterations to the circadian clock

We wished to determine whether circadian oscillator dynamics are altered by long-term cold

exposure that causes vernalisation. Specifically, we tested whether the oscillator might be

altered after return to control temperatures, following vernalisation. We investigated this with

the Col FRI^{SF2} Arabidopsis genotype (henceforth Col *FRI*), a winter annual derivative of Col-

0 that contains a functional *FRI* allele introgressed from the San feliu-2 accession, and thus

requires vernalisation to flower (Lee et al., 1994). We reasoned that its responses to

prolonged cold may differ from those of summer annual (rapid-cycling) Arabidopsis

accessions such as Col-0. Moreover, Col *FRI* expresses *FLC* highly before vernalisation

(Sung & Amasino, 2004), allowing investigation of the relationship between *FLC* expression

and the circadian clock.

Seedlings were cultivated under control temperature conditions (19 °C) for 11 days, and

subsequently transferred to vernalising conditions (5 °C, 8 h photoperiod) for 8 weeks (Fig.

1A). This is sufficient to fully silence *FLC* expression (Whittaker & Dean, 2017). Next, the

plants were returned to control temperature conditions (19 °C) for 7 days, and subsequently

transferred to constant light (LL) to monitor the circadian clock under free running conditions

(Fig. 1A). This design was to avoid transient effects within the oscillator that might occur

immediately after transfer from the vernalisation treatment to control temperature conditions.

Under these control temperature free running conditions, we harvested aerial tissue for

mRNA at 4 h intervals over 48 h to examine the dynamics of circadian oscillator transcripts.

We compared this with control plants that did not receive a vernalisation treatment. To

control for the effect of aging on the circadian clock (Kim et al., 2016), these non-vernalised

(NV) plants were grown for an equivalent period of developmental time (26 days) under

control temperature conditions (Zhao et al., 2020). Given that the Arabidopsis circadian

oscillator is formed from multiple interlocking transcription-translation feedback loops we

examined the abundance of several transcripts that function within the oscillator (*CCA1*,

PSEUDO-RESPONSE REGULATOR7 (*PRR7*), *TIMING OF CAB EXPRESSION1* (*TOC1*),

EARLY FLOWERING3 (*ELF3*) and *LUX*). We also evaluated whether transcript dynamics

differed between Col-0 and Col *FRI* in NV control plants. In these NV plants, when *FLC* is

expressed highly in Col *FRI,* we observed no obvious differences in dynamics of oscillator

transcripts between genotypes (Supplementary Fig. 1). *FLC* levels were measured after the

8-week cold treatment; *FLC* transcripts were nearly undetectable after return to control

temperatures, demonstrating successful vernalisation (Fig. 1B).

8 weeks of vernalisation (8V) did not substantially alter the dynamics of dawn-expressed

CCA1 upon return to control temperature conditions. In vernalised and NV plants, maximum

CCA1 transcript levels occurred around subjective dawn (timepoints 25 h and 49 h) and

minimum transcript levels around subjective dusk (37 h and 61 h) (Fig. 1C). In contrast,

oscillations of transcripts encoding the circadian clock component *PRR7* were altered by the

prolonged cold treatment (Fig, 1d). In 8V plants, *PRR7* transcript levels increased earlier

during each subjective day compared with the NV plants (29 h and 53 h, *vs.* 33 h and 57 h)

(Fig. 1D). The transcripts reached minimum levels at approximately the same time (Fig.1D).

Oscillations of transcripts encoding the clock component *TOC1* were not altered consistently

between 8V plants and NV plants (Fig. 1E).

ELF3 and *LUX* encode parts of the evening complex (EC) of the circadian oscillator, and

play roles in plant temperature sensing (Nusinow et al., 2011; Silva et al., 2020). In NV

plants, the greatest and lowest *ELF3* transcript levels occurred around subjective dusk and

subjective dawn, respectively. *ELF3* transcript levels appeared to increase earlier in 8V

compared to the NV control, but this was somewhat inconsistent between experimental

repeats (Fig. 1F). Our data suggest that *LUX* transcript oscillations were unaltered by the 8-

week vernalisation treatment (Fig. 1G).

Using rhythmicity detection algorithms, we identified that all transcripts examined were

significantly rhythmic (Supplementary Dataset 1). We detected no systematic change across

all oscillator transcripts in their period length, phase, or amplitude following vernalisation

165 (Fig. 1H; Supplementary Dataset 1). However, this analysis identified that both *PRR7* and

166 *ELF3* transcripts phased earlier in 8V plants compared with NV controls (Fig. 1H;

167 Supplementary Dataset 1). While the period of *ELF3* may be lengthened by vernalisation,

168 the period of *PRR7* was unaltered and remained *circa* 24 hours. Moreover, this analysis

169 suggests that exposure to prolonged cold increases the overall amplitude of the rhythm of

170 *PRR7* (Fig. 1H; Supplementary Dataset 1). Using CircaCompare, a tool for statistical

171 comparison of rhythmic datasets (Parsons et al., 2020), we verified that *PRR7* transcripts

172 phase earlier in plants exposed to prolonged cold compared with NV plants (p = 0.015).

173 Overall, this suggests that following a period of prolonged cold, the timing of maximum

174 *PRR7* transcript levels is altered under constant conditions, and this was consistent across

175 both days of experimentation. This could suggest that the repression of *PRR7* is lifted earlier

176 during the subjective day in 8V compared with NV plants.

177 *Vernalisation alters rhythms of clock output transcripts*

178 Next, we investigated whether vernalisation-induced changes in oscillator dynamics extend

179 to circadian regulated outputs. We quantified the abundance of several transcripts involved

180 in flowering and growth, two major seasonal processes under circadian regulation, and

181 several PRR7-regulated transcripts (Liu et al., 2013). Differences between the accumulation

182 of these transcripts in vernalised and non-vernalised plants were assessed over 24 h of

183 constant light (Fig. 2).

184 The circadian clock underpins the regulation of photoperiodic flowering by modulating the

185 timing of peak *CONSTANS* (*CO*) expression. *CO* is a B-box-type zinc-finger transcription

186 factor that directly promotes the floral transition through transcriptional activation of

187 *FLOWERING LOCUS T* (*FT*) (Song et al., 2015). *CO* transcripts were upregulated at the end

188 of the day and during the subjective night in vernalised plants (Fig. 2A). Rhythmic *CO*

189 expression is partly supported by PRR oscillator components (Nakamichi et al., 2007;

190 Hayama et al., 2017) and the CYCLING DOF FACTOR (CDF1-5) protein family (Fornara et

191 al., 2009). Transcripts encoding the blue-light photoreceptor FLAVIN-BINDING KELCH

REPEAT F-BOX 1 (FKF1), which mediates degradation of CDF proteins (Sawa et al., 2007),

was unaltered following vernalisation (Fig. 2B); however, *CDF2* transcripts were upregulated

at some timepoints (Fig. 2C). The circadian clock also exerts control over vegetative growth.

PHYTOCHROME INTERACTING FACTORs (PIFs) act downstream of the clock to regulate

growth and shade-avoidance responses. *PIF4* and *PIF5* transcripts are regulated by *PRR7*

and the EC (Nusinow et al., 2011; Zhang et al., 2020), and transcript levels were unaltered

following vernalisation (Fig. 2D, E). Similarly, there were no vernalisation induced changes in

RVE2 transcript levels; RVE2 is also involved in hypocotyl elongation and flowering

(Fig. 2F).

SALT TOLERANCE (*STO,* known also as *BBX24*) and *ELONGATED HYPOCOTYL 5* (*HY5)*

participate in light signalling and photomorphogenesis (Indorf et al., 2007) (Catalá et al.,

2011). These are repressed by PRR7 (Liu et al., 2013) but were unaltered following

vernalisation (Fig. 2G, H). Expression of iron (Fe) homeostasis genes are also regulated by

the circadian clock (Hong et al., 2013). Specifically, the *FERRITINS FER1*, *FER3,* and *FER4*

are PRR7-repressed (Liu et al., 2013); we identified an increase in *FER3* transcript levels

following vernalisation at 33 h of constant light (Fig. 2I).

The circadian clock influences endogenous ABA levels and the expression of genes involved

in its biosynthesis (Covington et al., 2008; Liu et al., 2013). Moreover, over a third of PRR7

targets contain ABA-responsive elements. Following vernalisation, the abundance of *ABA*

DEFICIENT1 (*ABA1*) transcripts increased around the time of its peak expression (25 h and

29 h, Fig 2. j), suggesting a possible increase in oscillatory amplitude.

C-REPEAT/DRE BINDING FACTORs (*CBFs*) are crucial for cold acclimation and are

induced rapidly by low temperatures (Fowler et al., 2005). *PRR7* can regulate *CBF*s by

binding to their promoters to repress expression around dusk (Liu et al., 2013; Kim et al.,

2024). Following vernalisation, *CBF2* transcript abundance was greater in vernalised plants,

specifically at 29 hours of constant light (Fig. 2K).

Vernalisation attenuates photoperiod-regulated differences in flowering time

In Arabidopsis, both vernalisation and photoperiod length regulate the transition to flowering.

Arabidopsis is a long-day plant, so flowering is accelerated by longer photoperiods (e.g.,

16 h). The circadian clock regulates photoperiodic flowering through stabilisation of CO

protein at specific times during the day (Nakamichi et al., 2007; Andrés & Coupland, 2012).

Given that the expression of specific circadian clock transcripts was altered following

vernalisation (Fig. 1) and *CO* mRNA was upregulated in vernalised plants (Fig. 2A), we

hypothesised that this might change the critical daylength threshold for photoperiodic floral

induction. To investigate this, following initial growth under short days, we exposed

Arabidopsis plants to vernalising long-term cold treatments of two different durations (4

weeks, 8 weeks) alongside a non-vernalised control, and then transferred the plants from all

three conditions into either short (8 h light / 16 h dark), equinox (12L:12D), or long (16L:8D)

photoperiods under control temperature conditions (19 °C) (Fig. 3A). To understand how the

duration of vernalisation treatment and subsequent photoperiod impacted the developmental

stage of the transition to flowering, we compared the total leaf number at time of

inflorescence emergence.

Longer vernalisation treatments advanced the developmental stage at which flowering

occurred (Fig. 3B). This acceleration of flowering is consistent with the well-established

vernalisation response of Col *FRI*. Exposure to prolonged cold also caused Col-0 to flower at

an earlier developmental stage (Fig. 3C). This was conserved across all photoperiods

tested; even under short day conditions, flowering in Col-0 was accelerated by both 4-and 8-

week vernalisation treatments relative to non-vernalised plants (Fig. 3C, D). In Col-0 under

16 h photoperiods, there was no further acceleration of flowering when vernalisation was

extended from 4 to 8 weeks. This is because plants flowered with the same number of

leaves and after the same number of days after both durations of vernalisation (Fig. 3C, E).

Interestingly, exposure to an 8-week vernalisation treatment altered the importance of

photoperiod for flowering in both Col-0 and Col *FRI* (Fig. 3B, C). When plants received either

no vernalisation treatment or 4 weeks of cold, there was a photoperiod-dependent effect on

flowering. Under longer photoperiods, plants flowered at an earlier developmental stage,

with 8 h and 12 h photoperiods delaying flowering significantly (Fig. 3B, C). This response

was lost after 8 weeks of cold exposure and there was no difference between the total leaf

number at bolting between 12 h and 16 h photoperiods in both genotypes (Fig. 3B, C). In Col

FRI, only 8 weeks of cold was sufficient for flowering under short days within the duration of

the experiment (Fig. 3B).

Together, this suggests that exposure of Arabidopsis to prolonged cold for 8 weeks may

override the photoperiodic requirement for flowering, allowing plants flower at an earlier

developmental stage. Furthermore, this response may be conserved regardless of the

genotype's vernalisation requirement.

Long-term cold alters growth rates upon return to warmth

The circadian oscillator is associated with vegetative growth regulation of Arabidopsis, and

changes in oscillator function can alter growth rates (Dodd et al., 2005; Graf et al., 2010;

Chew et al., 2022). Because we observed vernalisation-induced changes in circadian

oscillator components and some output transcripts, we hypothesized that there might be

changes in growth after long-term cold. To investigate this, we used an experimental design

whereby seedlings were vernalised, returned to control temperature conditions, and then

transplanted to compost to monitor the visible leaf area of the whole rosette as a proxy for

growth. We also compared the growth rate of Col *FRI* with Col-0, to determine whether

winter annual accessions might have different growth rates compared with rapidly cycling

summer annuals after a period of cold.

To examine differences in plant size we fitted a linear mixed model (LMM) using genotype,

treatment, and time as fixed effects, and this also accounted for repeated measures of

individual plants. We observed an increase in leaf area with time ($p < 0.001$), reflecting

growth of all plants under control temperatures. This also identified a three-way interaction

between treatment, genotype, and time ($p < 0.01$), suggesting that the effect of winter cold

on growth is genotype dependent. Post-hoc testing confirmed that under control temperature

conditions, there was no differences in overall rosette area between Col *FRI* or Col-0 plants

($p = 0.50$). However, whilst vernalisation did not alter the rosette size of Col *FRI* (Fig. 4A, $p =$

0.86), it did reduce the visible leaf area of Col-0 plants (Fig. 4B, $p < 0.01$) compared to non-

vernalised controls (Fig. 4A-C).

To compare the relative growth rates independently of initial (starting) plant size, the leaf

area was normalised to the first measurement at the beginning of the experiment. Using this

approach, we could control for differences in growth between the genotypes that occurred

previously, during the cold temperature treatment. Overall, vernalised plants grew relatively

faster than non-vernalised plants for both genotypes (Fig. 4D-F). This was the case both

when comparing treatments using a LMM across the entire time course (Fig. 4D, E; $p <$

0.001) and when comparing the relative change in rosette size between the start and end

the measurements (Fig. 4F; $p < 0.001$). Under control temperature conditions, there was no

difference between the growth rate of Col-0 and Col *FRI* (Fig. 4F; $p > 0.5$), supporting the

analysis of absolute leaf area (Fig. 4C). Comparisons between the growth rates of vernalised

plants suggested that effect of vernalisation on growth rate varied between genotypes, such

that Col-0 grew faster than Col *FRI* (Fig. 4F).

Together, this suggests that Col-0 plants had smaller rosettes than Col *FRI* after

vernalisation, but they may compensate for this though accelerated growth upon return to

control temperature conditions. In comparison, Col *FRI* plants achieved a larger rosette the

during the vernalisation treatment but have slower growth upon transfer to control

temperature conditions.

Vernalisation-induced alterations in the clock are not determined by FRI or FLC

FRI and FLC are the major determinants of vernalisation requirement in Arabidopsis, and

variation in *FLC* silencing is a strong contributor to flowering time post-vernalisation (Napp-

Zinn, 1957; Shindo et al., 2005). It has been suggested previously that the response of the

circadian clock to vernalisation occurs independently from *FLC* expression (Salathia et al.

2006). To assess the roles of FRI and FLC in this process, we examined the response of the

circadian clock to 8 weeks of cold in Col-0 – which lacks a functional *FRI* allele – and

FLClean, where the entire *FLC* gene was removed by gene editing (Nielsen et al., 2024). To

allow comparison of multiple genotypes, we screened two timepoints under free running

conditions that corresponded to 1 h after subjective dawn (25 h in constant light) and 1 h

before subjective dusk (35 h in constant light). These timepoints were chosen to capture the

vernalisation-induced changes in circadian oscillator transcripts that occurred within 48 h

time courses (Fig. 1).

Non-vernalised Col *FRI* plants had higher *PRR7* transcript levels at 35 h compared with 25 h

(Fig. 5A). In vernalised Col *FRI* plants, *PRR7* transcripts levels did not differ between these

timepoints, indicating altered rhythms of *PRR7* transcripts following exposure to prolonged

cold (Fig. 5A). This is consistent with earlier experiments (Fig. 1D, H). Similarly, in Col-0,

PRR7 transcripts levels did not differ between the timepoints after vernalisation (Fig. 5B).

Loss of *FLC* in *FLClean* also did not impact the effect of vernalisation on *PRR7* dynamics

(Fig. 5C). *CCA1* and *TOC1* transcripts responded similarly across the genotypes,

demonstrating specificity of the response to certain transcripts within the circadian oscillator

(Supplementary Fig. 2A, B). These results suggest that the effects of vernalisation on

specific clock transcripts are uncoupled from the roles of *FRI* and *FLC* in the vernalisation.

Changes to circadian clock transcripts after vernalisation require the activity of core PRC2

components

Vernalisation depends on the epigenetic silencing of the *FLC* gene. This relies on PRC2

components, including the core proteins VRN2 and CLF, and PRC2 accessory proteins VIN3

and VRN5. Our data suggest that despite entrainment to control temperature conditions,

prior exposure to long-term cold is stably registered within the clock. Thus, we set out to

determine whether epigenetic regulation contributes to this. We used a variety of PRC2

mutants known to affect *FLC* silencing, and compared the effect of vernalisation on the

accumulation of circadian oscillator transcripts.

VIN3 is an early cold-induced component of the vernalisation pathway that is essential for

FLC silencing (Sung & Amasino, 2004). Therefore, we investigated whether VIN3 contributes

to the regulation circadian clock dynamics following prolonged cold. The response of *PRR7*

to winter cold was unaltered in *vin3*-4 (Fig. 5D), suggesting that *VIN3* is not required for

these responses. VRN5 is a homolog of VIN3 and also required for *FLC* silencing (Greb et

al., 2007). We observed that the response of *PRR7* to vernalisation was also unaltered in

vrn5-8 (Fig. 5E). Temperature-dependent growth, conveyed through NTM1-LIKE 8 (NTL8)

protein concentration, also participates in long-term cold sensing through the regulation of

VIN3 (Zhao et al., 2020). However, it remains unclear whether this indirect cold sensing

mechanism may regulate a wider set of cold-responsive genes. We observed no changes in

PRR7 transcript levels between vernalised and non-vernalised plants in the gain-of-function

ntl8-D mutant compared with Col *FRI* (Fig. 5F). In this mutant, *NTL8* is constitutively active

in, leading to misregulation of *VIN3* even in the absence of cold (Zhao et al., 2020).

Therefore, this further supports our finding that *VIN3* activity is not required for altered

circadian clock dynamics following prolonged cold and suggests that this independent cold

sensing pathway does not have a broader effect on the circadian clock. There were also no

effects of these mutants on post-vernalisation dynamics of *CCA1* and *TOC1* transcripts

(Supplementary Fig. 2A, B).

VERNALISATION2 (VRN2) and CURLY LEAF (CLF) are core subunits of the PRC2 and

participate in epigenetic silencing of *FLC* and other targets during cold (Wood et al., 2006;

Greb et al., 2007). In both *vrn2-1* and *clf-81* mutants, the effect of vernalisation on *PRR7*

dynamics is lost (Fig. 5G, H). This indicates that the PRC2, and VRN2 in particular, is

required for the response of the circadian clock transcript *PRR7* to winter cold.

To examine further the effect of the *vrn2-1* mutant upon *PRR7* transcript dynamics, we

conducted an additional experiment using identical conditions, but sampling at several

daytime timepoints when the post-cold phase shift of *PRR7* is greatest under free running

conditions (Fig. 5I). In vernalised Col *FRI*, *PRR7* transcript levels were greatest after 29 h of

constant light, and lower after 33 h (Fig. 5I). In contrast, in the vernalised *vrn2-1* mutant,

PRR7 transcript abundance was unaltered between 29 h and 33 h of constant light (Fig. 5I).

At 29 h, *PRR7* transcript abundance was greater in vernalised Col *FRI* compared with

vernalised *vrn2*-1 (Fig. 5I). In combination with the data in Fig. 5G, this indicates that core

PRC2 components, particularly VRN2, are necessary for vernalisation-induced alterations in

PRR7 dynamics under free running conditions.

Discussion

Here, we studied the effect of vernalising cold treatments upon the Arabidopsis circadian

clock. We focused on the winter-annual accession Col *FRI*, as we reasoned that its clock

might respond differently to these treatments compared with the more commonly used Col-0

rapid cycling accession. The are existing demonstrated links between the circadian clock,

the vernalisation pathway, and the floral repressor *FLC* (Edwards et al., 2006; Salathia et al.,

2006; Spensley et al., 2009; Kyung et al., 2022). This motivated us to study the effect of

vernalisation on the temporal dynamics of specific oscillator components and circadian clock

outputs.

We identified that after vernalisation, *PRR7* transcripts in Col *FRI* phased earlier under free

running conditions compared with plants grown in control temperatures, despite the same

entrainment regime (Fig. 1D). This did not occur for other key clock components such as

CCA1 and *TOC1* (Fig. 1C, E), suggesting differential sensitivity of distinct clock components

to environmental factors (Webb et al., 2019; Paajanen et al., 2026). *PRR7* is positioned

within the morning loop of the circadian oscillator (Nakamichi et al., 2010; Farré & Liu, 2013).

It acts downstream of the phytochrome photoreceptors and participates in temperature

compensation mechanisms alongside other *PRR*-family genes (Kaczorowski & Quail, 2003;

Salome et al., 2010; Shor et al., 2017; Martín et al., 2018). Thus, it can integrate both light

and temperature signals into the clock to regulate oscillator dynamics. Moreover, *PRR7*

transcription is influenced by internal metabolic signals communicated *via* the sugar-

responsive transcription factor bZIP63 and the energy-sensing kinase SnRK1 (Haydon et al.,

2013; Frank et al., 2018). Collectively, this supports the idea that *PRR7* may act as a

signalling hub through which both external and internal cues are integrated across the plant

(Webb et al., 2019). Our data imply that in addition to this, *PRR7* registers past

environmental history through alteration of its phase in response to prolonged cold

temperatures (Fig. 1, 5).

In nature, vernalisation ensures that flowering is delayed until plants have been exposed to a

sufficient period of cold, preventing premature flowering during inductive photoperiods of

autumn. Previous research using high temporal resolution leaf movement assays identified a

modest vernalisation-dependent shortening of the Arabidopsis circadian period (up to

approximately 1 h shorter) that occurs independently from *FRI* and *FLC* (Salathia et al.,

2006). Given that shorter period circadian clock mutants often have early flowering

phenotypes (summarised in Wang et al. (2025)), these faster paced clocks have been

proposed to support photoperiodic flowering in spring (Salathia et al., 2006). The sampling

resolution of our experiments was insufficient to determine whether the same period

changes occurred for oscillator transcripts. However, we did observe that vernalisation

eliminated the delay in flowering that occurs between long-day and equinox photoperiods, in

both Col *FRI* and in Col-0 (Fig. 3B-E). This suggests that prolonged exposure to cold

temperature modulates the sensitivity of photoperiodic responses, despite Col-0 not having a

vernalisation requirement for flowering. We also identified upregulation of *CO* transcripts at

the end of a 12-hour day in vernalised plants (Fig. 2A), which could arise from altered

circadian regulation. Since increased amplitude of *CO* expression alters overall *FT* levels

and accelerates flowering (Ito et al., 2012), this could contribute to the flowering phenotypes

we observed (Fig. 3B).

It was interesting that growth rates were affected by vernalisation (Fig. 4), because growth is

also regulated by photoperiod length – albeit through distinct mechanisms (Liu et al., 2021;

Wang et al., 2024). The metabolic daylength measurement system that underpins seasonal

growth regulation is based on combined circadian clock- and photoperiod-controlled

regulation of starch degradation (Liu et al., 2021; Li & Gendron, 2025). *MYO-INOSITOL-1-*

PHOSPHATE SYNTHASE 1 (*MIPS1*) is required to support leaf growth under long days,

with a critical photoperiod for the light-sensitive phase for *MIPS1* expression to occurring

after ZT12 in long-days (Wang et al., 2024). We speculate that the same winter cold-induced

change in circadian dynamics could also extend to other circadian outputs, including *MIPS1*

expression. If so, this could change the critical photoperiod for growth, thereby explaining

why vernalised plants grow relatively more than non-vernalised plants.

By disrupting components of the vernalisation pathway, we identified that the PRC2 subunit

VRN2 is required for the cold-induced phase shift of *PRR7* transcripts (Fig. 5A, E). *VRN2*

mRNA is expressed constitutively and its protein is stabilised under prolonged cold (Gibbs et

al., 2018). This suggests a potential link between proteins that form part of the vernalisation

pathway and circadian clock function (Salathia et al., 2006). In Arabidopsis, *VRN2* is also

associated with seed dormancy (Auge et al., 2017) and development (Roszak & Köhler,

2011), root architecture (de Lucas et al., 2016), hypoxia stress responses (Labandera et al.,

2021), leaf growth (Osborne et al., 2025), and flowering under short days (Labandera et al.,

2021). Evidence for a direct interaction between the circadian clock and VRN2 remains

limited (Xi et al., 2020; Osborne et al., 2025). One explanation is that the interaction may be

context-dependent and detectable only following cold exposure. Alternatively, the

relationship between VRN2 and the clock may be mediated indirectly by unknown

intermediate factors. Through exploration of public datasets, we generated a list of 771

potential candidate genes that could influence *PRR7* dynamics following vernalisation

(Supplementary Dataset 2). These genes are stably down-regulated during vernalisation, as

is expected if they are repressed through the action of VRN2-PRC2 during cold (Xi et al.,

2020), but are not targets of *VIN3* or *VRN5* (Franco-Echevarría et al., 2023). Interestingly,

this change in *PRR7* dynamics appears to be FLC-independent. This is consistent with post-

cold *PRR7* dynamics being unaltered by the absence of *FLC* expression in Col-0 and

FLClean. Moreover, loss of VIN3 or VRN5 – which have critical roles in *FLC* silencing – had

no effect on the vernalisation-induced response of *PRR7*.

Our data suggest that following vernalisation, there are adjustments to the circadian clock.

This might help to align whole-plant physiology with the springtime environment. It could

potentially extend to other circadian regulated processes, ensuring they are regulated

appropriately for the season. For example, the phase of the 'autumn clock' may favour

processes associated with resource allocation and cold tolerance. On the other hand, the

'spring clock' may favour accelerated growth following winter dormancy and reinforce the

reproductive transition. Although we focused on Arabidopsis, vernalisation is part of the

seasonal life cycle in many other plant species (Li et al., 2013; Wu et al., 2015; Bouché et

al., 2017; O'Neill et al., 2019). Therefore, our work could provide insights into the regulation

of seasonal responses in plants including those that hold agronomic importance, such as

other Brassicas and cereal crops.

Materials and methods

Plant material and growth conditions

Background accessions of *Arabidopsis thaliana* (Arabidopsis) that were used were

Columbia-0 (Col-0) and Col *FRI^{sf2}* (gifted from Caroline Dean at the John Innes Centre (Lee

et al., 1994)). All mutant germplasm was in the Col *FRI* background and donated by Caroline

Dean (John Innes Centre). These were *vin3-4,* which contains a T-DNA in the second intron

of *VIN3* (Bond et al., 2009); *flc-2,* a fast-neutron allele containing a 30 kb deletion spanning

the *FLC* locus (Michaels & Amasino, 1999); *FLClean,* a CRISPR-Cas9 deletion of the entire

FLC genomic sequence (Nielsen et al., 2024); *vrn2-1* (Yang et al., 2017); *vrn5-8,* a T-DNA

insertion line (SALK_136506) (Greb et al., 2007) crossed into the *FRI* background; *clf-81*, an

EMS line with a point mutation (Kim et al., 1998) crossed into the *FRI* background, and *ntl8-*

D3, a truncated NTL8 protein caused by a deletion in the C-terminal transmembrane domain

resulting in enhanced nuclear localisation (Zhao et al., 2021).

For cultivation, Arabidopsis seeds were surface sterilised using 70% (v/v) ethanol for 10

minutes with agitation, washed 3 times with sterile water, and suspended in 0.1% (w/v) agar.

Sterilised seeds were pipetted onto half strength Murashige and Skoog (MS) media with

0.8% (w/v) agar (pH 6.8), without added sucrose, and stratified in darkness at 4°C for 3 days

before being moved into growth chambers. Cultivation occurred in PHCBi MLR-352 growth

chambers.

Circadian time-course sampling for RT-qPCR

Following stratification, seeds were grown under controlled temperature conditions (19 °C,

12L:12D, ~ 50 µmol m^{-2} s^{-1}) for 11 days. On day 12 after transfer to growth chambers, plants

were moved to vernalisation conditions (5°C, 8L:16D, ~ 20 µmol m^{-2} s^{-1}) for 8 weeks.

Following this, to investigate persistent changes within the circadian oscillator, plants were

initially returned to control temperature growth conditions (19 °C, 12L:12D, ~ 50 µmol m^{-2} s^{-}

^{1}) for 7 days. On day 8 of this condition, to monitor the circadian oscillator under free running

conditions, plants were transferred to constant light and temperature. Sampling commenced

after 24 h of constant light to avoid sampling during transitory effects of the final dawn. To

control for potential effects of developmental age on circadian clock gene expression (Kim et

al., 2016), non-vernalised controls were grown under control temperature conditions for an

amount of developmental time that was equivalent that elapsed under the vernalising

conditions, using an approach described previously (Zhao et al., 2020). Where possible, the

growth of non-vernalised seedlings was staggered such that sampling occurred on the same

day as vernalised seedlings.

For 48-hour time course sampling, after 24 hours of constant light, aerial plant tissue was

collected every 4 hours for 48 h (from 25 h to 73 h of constant light). For experiments

investigating vernalisation pathway mutants, after 24 hours of constant light, aerial plant

tissue was collected after 25 h and 35 h of constant light, as these timepoints allowed

efficient discrimination of effects of the vernalisation mutants upon circadian clock gene

expression across multiple mutant backgrounds. To gain a more detailed understanding of

circadian clock transcript dynamics in the *vrn2-1* mutant following 8 weeks of vernalisation,

plants were grown as described previously and samples were collected under constant light

conditions after 25 h, 29 h, 33 h, 35 h and 37 h of constant light. For each time point, aerial

tissue from five individual plants was pooled to generate one biological replicate. A total of

three biological replicates were obtained per time point. Harvesting was destructive,

precluding repeated measurements on the same plant. Tissue was immediately frozen in

liquid N_2 and stored at -70 $^\circ$C until processing.

RNA isolation and RT-qPCR

Total RNA from Arabidopsis seedlings was prepared using RNeasy Plant MINI kits (Qiagen)

with on-column DNase treatment using the RNAse-Free DNase Set (Qiagen), as per

manufacturer's instructions. RNA was eluted using nuclease-free water and sample quality

was assessed (Nanodrop, Thermo-Scientific). RNA was stored at -70°C until required.

For RT-qPCR analysis, 1 µg of total RNA was used to synthesise cDNA using a High-

Capacity cDNA Reverse Transcription kit following manufacturer's instructions, using

random primers (Applied Biosystems, ThermoFisher). Relative transcript abundance was

measured using a LightCycler 480 II (Roche), using four technical replicates per sample and

cDNA that was diluted 1:100 using nuclease-free water. *PROTEIN PHOSPHATASE 2A*

SUBUNIT A3 (*PP2AA3*) was used as a refence transcript as its expression is arhythmic

(Supplementary Fig. 3) and appears unaltered by cold treatments (as determined by EFP

browser, Waese et al., 2017). Total reaction volume was 10 µl, containing: 5 µl 2x RT-qPCR

BIO SyGreen LO-ROX (PCR Biosystems); 2.5 µl template cDNA; 0.4 µl 10 mM forward

primer and 0.4 µl 10 mM reverse primer. Primers used are detailed in Supplementary Table

1. Absolute quantification of Ct values was determined using the Second Derivative

Maximum method (Guescini et al., 2008). Relative transcript abundance was calculated

using the 2^ΔΔCt method and normalised to Col FRI NV at 25 h in constant light.

Circadian time-course data analysis

For RT-qPCR time-series spanning 48 h, data were analysed with several methods that are

used to examine properties of oscillatory data, including JTK_CYCLE (Hughes et al., 2010)

and meta2d within the R package MetaCycle, using default parameters (Wu et al., 2016).

Fast Fourier Transform-Non Linear Least Squares Method (FFT-NLLS) used BioDare2, with

no detrending (biodare2.ed.ac.uk, Zielinski et al., 2014), and CircaCompare was used when

the period length was approximately 24 h (Parsons et al., 2020). Phase was reported in

circadian units, i.e. using time-series aligned to a 24 h period (sometimes known as "modulo

tau"). Changes in phase, period and amplitude following vernalisation were calculated by

taking the mean of these parameters for 8-week vernalisation treated samples and

subtracting this from these parameters from the non-vernalised dataset.

To examine the transcript abundance of circadian clock outputs, RT-qPCR was performed on

samples collected between 25 h and 49 hours under constant light normalised to the refence

transcripts *PP2AA3* and *UBQ10*. Data were analysed at each time point between

vernalisation treatments using Welch's two-sample t-tests. To account for multiple testing, p-

values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure.

Growth assays

To investigate plant growth dynamics under control temperatures, following vernalisation,

Col-0 and Col *FRI* seeds were sown on 0.5 MS agar and stratified. They were initially grown

under control temperature conditions (19 ˚C, 12L:12D, ~ 50 µmol m^{-2} s^{-1}) for a minimum of

11 days, then transferred to vernalising conditions for 8 weeks (5˚C 8L:16D, ~ 20 µmol m^{-2}

s^{-1}). Following this, 12 plants were transplanted to compost (Levington F2 starter mix) in 24-

compartment horticultural trays and returned warm control conditions for 10 days.

Germination and growth of non-vernalised seedlings was deliberately delayed so that these

plants were transferred to compost at the equivalent developmental stage as vernalised

seedlings, to allow side by side comparison (Zhao et al., 2020).

During the period following prolonged cold, plants were imaged daily at approximately the

same time of day using a camera positioned directly above the compost trays. A calibration

scale was always included in images. Visible leaf area was measured using FIJI software

(Schneider et al., 2012). Using this, images were manually thresholded so the entire rosette

was isolated from the compost background. Thresholded images were converted to binary

format, allowing measurement of the area of each thresholded region.

Flowering time assays

Seedlings were grown initially on 0.5 MS agar under short day conditions (8L:16D) and

19˚C, to prevent photoperiodic transition to flowering. After this growth period (minimum of

11 days), plants were transferred to vernalising conditions (5˚C 8L:16D, ~ 20 µmol m^{-2} s^{-1})

for either 0, 4, or 8 weeks. Plants receiving the NV or 4-week vernalisation treatments (*vs.*

full 8 week vernalisation) were given additional growing days under warm short days to

account for developmental differences incurred during the cold period (Zhao et al., 2020). In

this experiment, the growth of non-vernalised seedlings was not staggered relative to the

vernalised seedlings, and plants were transferred to compost at equivalent developmental

stages but on different days. Following vernalisation, plants were transplanted onto compost

(Levington F2 starter mix) and transferred to control temperatures (19˚C) under 8L:16D,

12L:12D or 16L:8D photoperiods. These were chosen as standard short day, equinox or long

day protocols with Arabidopsis. Plants were monitored regularly until bolting, and flowering

time was scored as below.

Flowering was classified as the day at which the terminal inflorescence bud exceeded

10 mm. Total leaf number (TLN) was also measured, to estimate the developmental stage at

flowering. The TLN included all primary leaves in the rosette, including cotyledons, but

excluded cauline and auxiliary leaves. The total number of days under control conditions

following vernalisation was also recorded, to calculate days to bolting (DTB). For

comparisons of TLN and DTB with genotypes, two-way ANOVA was used with vernalisation

treatment length and photoperiod as interacting factors P-values were adjusted using the

Tukey's HSD test for post-hoc contrasts.

Analysis of growth dynamics

To compare differences in visible leaf area (VLA) between genotypes and growth conditions

at specific time points, two-way ANOVA was performed with genotype and growth condition

as fixed factors. Post-hoc pairwise contrasts were conducted using Tukey's HSD test to

correct for multiple testing. Relative leaf area was calculated as follows (day 3 was the first

day of imaging):

$$relative\ VLA = \frac{VLA\ on\ day\ x - VLA\ on\ day\ 3}{VLA\ on\ day\ 3}$$

To compare growth over time, repeated measurements of both absolute and relative VLA

were analysed using linear mixed models (LMM) using the lmerTest package (Kuznetsova et

al., 2017). Because each plant was measured at multiple time points, plant identity was

modelled as a random factor to account for the non-independence of repeated observations.

Area was modelled as a function of time, genotype, treatment, and their interactions. To

account for the uncertainty in estimating random effects, P-values for fixed effects were

obtained using Satterthwaites approximation using the lmerTest package (Kuznetsova et al.,

2017). Pairwise contrasts were conducted using the emtrends function in the emmeans

package (Lenth et al., 2025), with Tukey's adjusted p-values for multiple testing:

$$lmer(area \sim genotype * treatment * time + (1 \mid Plant\_ID))$$

Statistical analysis

All statistical analyses and graphing was performed using R v4.4.1.

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Steve Penfield, and Martin Howard for scientific advice.

Data availability

Source data for this study are in Supplementary Dataset 3.

Conflicts of interest

None declared.

Figure legends

Figure 1: Exposure of Col *FRI* to prolonged cold affects the transcriptional dynamics of

circadian oscillator components under subsequent control conditions. **(A)** Arabidopsis Col

FRI seedlings were initially grown under control temperature conditions (12L:12D 19 $^\circ$C) for

11 days before transfer to vernalising cold temperatures (8L:16D, 5 $^\circ$C) for 8 weeks (8V).

Following this, plants were returned to 12L:12D 19 $^\circ$C for a further 7 days. On day 8, plants

were transferred to constant light (LL) conditions. Sampling of aerial tissue for RNA started

after 25 h of constant light with tissue collected every 4 hours for 48 hours. Non-vernalised

(NV) plants were grown for an equivalent developmental time under control temperature

conditions (19 $^\circ$C). For each timepoint, aerial tissue from up to five individual plants was

pooled to generate each biological replicate. **(B)** *FLC* transcript levels in Col *FRI* following

vernalisation, compared with non-vernalised control plants. Different letters indicate

statistically significant differences ($p < 0.05$), from one-way ANOVA followed by with post hoc

Tukey's HSD test. **(C-G)** Relative abundance of circadian oscillator transcripts *CCA1*, *PRR7*,

TOC1, *ELF3* and *LUX.* Solid lines indicate mean, with shaded ribbon ± s.e.m ($n = 3$). Grey

shading indicates subjective night. **(H)** The magnitude and change in direction of free

running period, phase and amplitude of circadian oscillator transcripts in Col *FRI*, when

comparing vernalised and non-vernalised plants. Direction of post-vernalisation change

calculated by subtracting the parameter from vernalised samples from the parameter from

non-vernalised samples. Point indicates mean difference ± s.e.m (n = 2). Each row

represents a different analysis method. In **(B-G)**, NV = non-vernalised, 8V = 8 weeks

vernalised. Transcript levels expressed relative to *PP2AA3*, as determined by RT-qPCR.

Figure 2. Vernalisation alters the dynamics of transcripts that are outputs from the circadian

clock. The abundance of transcripts downstream of the circadian clock that are associated

with **(A-C)** flowering time, **(D-F)** growth, and are **(G-K)** known downstream targets of *PRR7*

were compared between non-vernalised plants (NV) and those given an 8-week

vernalisation treatment (8V) then returned to control conditions and measured under

constant light (LL). Transcript levels expressed relative to *PP2AA3* and *UBQ10*, as

measured by RT-qPCR. Grey shading represents subjective night. Data are mean ± s.e.m (n

= 2-3). Asterisks represent significant differences between vernalised and non-vernalised

plants determined by Welch's two-sample t-tests, p-values adjusted for multiple testing using

the Benjamini–Hochberg false discovery rate (FDR) procedure, * $p < 0.05$, ** $p < 0.01$.

Figure 3: Vernalisation eliminates flowering time differences between equinox and long day

conditions**. (A)** Arabidopsis seedlings of Col *FRI* and Col-0 were initially grown under control

temperature conditions (8L:16D 19 °C) for at least 11 days before transfer to vernalising cold

temperatures (8L:16D, 5 °C) for up to 8 weeks. Differences in cultivation time under control

conditions ensured plants were at the same developmental stage after cold treatment. Plants

were subsequently grown on compost under control temperatures (19°C) under either

8L:16D, 12L:12D or 16L:8D photoperiods. **(B, C)** Total leaf number at flowering and **(D, E)**

days until bolting under short day (8L:16D), equinox (12L:12D), or long day (16L:8D)

conditions following either 0, 4, or 8 weeks of vernalising cold in Col *FRI* and Col-0. Groups

in which no plant flowered during the timeframe of the experiment are indicated as DNF (did

not flower). Different letters indicate statistically significant differences (p < 0.05), from two-

way ANOVA followed by with post hoc Tukey's HSD test, p < 0.05. NV = not vernalised;

4V = 4 weeks vernalisation; 8V = 8 weeks vernalisation.

Figure 4: Vernalisation-requiring Col *FRI* and rapid cycling Col-0 have differing growth

responses after long-term cold. Arabidopsis seedlings of Col *FRI* and Col-0 were initially

grown under control temperature conditions (12L:12D 19 °C) for 11 days before transfer to

vernalising cold temperatures (8L:16D, 5 °C) for 8 weeks (8V; NV = not vernalised).

Following this, plants were returned to 12L:12D at 19 °C and visible leaf area was monitored.

Data shown as absolute rosette area across time of individuals plants for **(A)** Col *FRI* and

(B) Col-0. Bold line represents mean of all replicates with error bars representing (± s.e.m.).

(C) Difference between the absolute rosette area of the first and last timepoints. Relative

change in rosette size normalised to initial rosette area (on day 3) for **(D)** Col *FRI* and **(E)**

Col-0. **(F)** Fold increase of normalized visible rosette size between the first and last

timepoints. **(A, B, D, E)** Comparison of growth trajectories was analysed using a linear mixed

effects model accounting for repeated measures of individual plants followed by with post

hoc Tukey's HSD test. ns, not significant; * p < 0.05, ** p < 0.01, *** p < 0.001. **(C, F)**

Different letters indicate statistically significant differences (p < 0.05) from two-way ANOVA

followed by with post hoc Tukey's HSD test (n = 10-12).

Figure 5: The Polycomb Repressive Complex 2 (PRC2) is necessary for altered dynamics

of the circadian oscillator transcript *PRR7* after prolonged cold. Arabidopsis seedlings were

grown initially under control temperature conditions (12L:12D 19 °C) for 11 days before

transfer to vernalising cold temperatures (8L:16D, 5 °C) for 8 weeks (8V). Non-vernalised

(NV) plants were grown for an equivalent amount of developmental time under control

conditions. Following this, plants were returned to 12L:12D 19 °C for a further 7 days. On

day 8, plants were transferred to constant light conditions (LL). **(A-H)** Relative abundance of

key circadian oscillator transcript *PRR7* in following a period of prolonged cold, compared

with non-vernalised control plants collected following 25 or 35 hours under constant light.

Different letters indicate represent statistically significant differences between transcript

levels within each graph (p < 0.05; ANOVA with post-hoc Tukey's HSD tests for pairwise

comparisons). **(I)** Time-series of relative *PRR7* transcript abundance in vernalised Col *FRI*

plants (8V, blue), compared with *vrn2-1* plants that were vernalised (8V, yellow) or non-

vernalised (NV, grey). Statistical comparisons are between genotypes at following 29 h

under constant light and between 29 and 33 hours under constant light, both determined

using ANOVA with post-hoc Tukey's HSD tests for pairwise comparisons. **(A-I)** Transcript

levels calculated relative to *PP2AA3*, using RT-qPCR. Data are mean ± s.e.m, n = 3.

Supplementary Datasets

Supplementary Dataset 1. Quantification of oscillations of circadian clock transcripts before

and after vernalisation**.** Estimates of rhythmic parameters of circadian oscillator components

in non-vernalised (NV) and vernalised (8V) Col *FRI* plants entrained under 19 ˚C 12L:12D

released into free-running conditions.

Supplementary Dataset 2: Potential candidate genes that may mediate the vernalisation

dependent phase shift of *PRR7* transcripts in Col *FRI*.

Supplementary Dataset 3: Source data for this study.

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A *Col FRI*
B Col-0 *fri*
C *FLClean*

NV - 25 h under LL
NV - 35 h under LL

8V - 25 h under LL
8V - 35 h under LL

Relative transcript abundance

D *vin3-4 FRI*
E *vrn5-8 FRI*
F *NTL8-D FRI*
G *vrn2-1 FRI*
H *clf-81 FRI*

I *PRR7*

Relative transcript abundance

Time under LL (hours)

Between
29 h and 33 h

29 h

Col *FRI* 8V   ***
vrn2-1 FRI 8V   **   n.s.
vrn2-1 FRI NV   ***   **   n.s.