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RESEARCH PAPER

# Acceleration of flowering in *Arabidopsis thaliana* by Cape Verde Islands alleles of *FLOWERING H* is dependent on the floral promoter *FD*

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## Abstract

**Flowering time in the model plant *Arabidopsis thaliana* is regulated by both external environmental signals and internal developmental pathways. Natural variation at the *FLOWERING H* (*FLH*) locus has previously been described, with alleles present in the Cape Verde Islands accession causing early flowering, particularly after vernalization. The mechanism of *FLH*-induced early flowering is not understood. Here, the integration of *FLH* activity into the known flowering time pathways is described using molecular and genetic approaches. The identification of molecular markers that co-segregated with the *FLH* locus allowed the generation of multiple combinations of *FLH* alleles with mutations in flowering time genes in different flowering pathways. Combining an early flowering *FLH* allele with mutations in vernalization pathway genes that regulate *FLC* expression revealed that *FLH* appears to act in parallel to *FLC*. Surprisingly, the early flowering allele of *FLH* requires the floral integrator *FD*, but not *FT*, to accelerate flowering. This suggests a model in which some alleles of *FLH* are able to affect the FD-dependent activity of the floral activator complex.**

**Key words:** *FLH*, flowering time, quantitative trait loci, vernalization.

## Introduction

The transition from a vegetative to reproductive phase of development in flowering plants is tightly regulated by a complex network of control mechanisms that sense environmental signals (Andres and Coupland, 2012). In *Arabidopsis thaliana*, four major pathways containing hundreds of genes have been identified – the autonomous, photoperiod, gibberellic acid, and vernalization pathways. A smaller number of genes function as floral integrators and respond to these multiple pathways to regulate the transition to flowering.

The difference between late and early flowering varieties of *Arabidopsis* is partly due to natural allelic variation in two genes with winter annual plants having active alleles of *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) (Clarke and Dean, 1994; Koornneef *et al.*, 1994; Lee *et al.*, 1994a; Lee and Amasino, 1995; Gazzani *et al.*, 2003; Shindo *et al.*, 2006). *FLC* is a MADS-box transcription factor which delays flowering by repressing the expression of the floral integrators *SUPRESSOR OF OVER-EXPRESSION OF CO* (*SOC1*) (Lee *et al.*, 2000; Onouchi *et al.*, 2000), the RAF kinase inhibitor-like/phosphatidylethanolamine binding family encoding gene *FLOWERING LOCUS T* (*FT*) and the bZIP transcription factor *FD* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Searle *et al.*, 2006). *FLC* expression is determined by the RNA Polymerase associated complex (Paf1C) and the coil–coil protein *FRI* via interactions with *SUPPRESSOR OF FRIGIDA4* (*SUF4*), *FRIGIDA-LIKE1*

(*FRL1*) and *FRIGIDA ESSENTIAL1* (*FES1*) (Michaels *et al.*, 2004; Schmitz *et al.*, 2005; Kim and Michaels, 2006; Kim *et al.*, 2006).

The core polycomb repressive complex 2 (PRC2) is associated with *FLC* chromatin prior to, during and after a cold exposure (De Lucia *et al.*, 2008). Vernalization accelerates flowering through stable repression of *FLC* through the increased transcription of the antisense *FLC* transcript *COOLAIR* (Swiezewski *et al.*, 2009) and the non-coding *COLDAIR* sense transcript from a cryptic promoter with the first intron of *FLC*, with COLDAIR thought to recruit the PHD proteins VNR5, VIN3, and VEL1 to form a complex with PRC2 (to produce the PHD–PRC2 complex) at the *FLC* locus (Sung and Amasino, 2004; Sung *et al.*, 2006b; Greb *et al.*, 2007; De Lucia *et al.*, 2008; Heo and Sung, 2011). These proteins induce the trimethylation of lysine 27 of histone 3 (H3K27me3) that maintain *FLC* in a repressed state upon the return to warm conditions (Bastow *et al.*, 2004). Once the vernalized state is established, it is subsequently epigenetically maintained by the activity of *VERNALIZATION 1* (*VRN1*) (Levy *et al.*, 2002) and *LIKE-HETEROCHROMATIN PROTEIN 1* (*LHP1*) (Mylne *et al.*, 2006; Sung *et al.*, 2006a).

Although *FLC* is the primary regulator of flowering in response to vernalization in winter-annual varieties of *Arabidopsis*, analysis of *flc* null mutants has demonstrated that an *FLC*-independent pathway also exists (Michaels and Amasino, 2001; Sung and Amasino, 2004; Alexandre and Hennig, 2008). Two MADS-box genes promote flowering in response to vernalization independently of *FLC*; *AGAMOUS-LIKE 19* (*AGL19*) (Schonrock *et al.*, 2006) and *AGAMOUS-LIKE 24* (*AGL24*) (Michaels *et al.*, 2003a). Similarly to *FLC*, *AGL19* is maintained in a transcriptionally repressed state by polycomb proteins (Schonrock *et al.*, 2006) and this repression is alleviated by vernalization via a mechanism requiring *VIN3*, but which is independent of *VRN2* (Schonrock *et al.*, 2006). Once activated, *AGL19* expression induces flowering by upregulating *LFY* and *APETALA11*, but not *SOC1* (Schonrock *et al.*, 2006). In contrast, *AGL24* has a complex interaction with *SOC1*, as both genes are able to positively regulate the expression of the other, and overexpression of one without the other has a minimal effect (Michaels *et al.*, 2003a). Like *AGL19*, *AGL24* is thought to promote flowering by upregulating *LFY* (Yu *et al.*, 2002).

When released from FLC repression, the FT protein is transported from the leaves to the shoot apical meristem where it interacts with the bZIP transcription factor FD (An *et al.*, 2004; Abe *et al.*, 2005; Wigge *et al.*, 2005; Corbesier *et al.*, 2007; Ikeda *et al.*, 2007). In rice, this interaction is mediated by the 14-3-3 GF14c protein (Taoka *et al.*, 2011), which forms a hexameric florigen activation complex (FAC), composed of two molecules each of Hd3a (the rice FT orthologue), FD, and GF14c (Taoka *et al.*, 2011). In this model, FD anchors the FAC to regulatory regions of FAC target genes through the bZip DNA binding domain of FD. Consistent with this model, similar pairwise interactions have been described in tomato (Pnueli *et al.*, 2001). In *Arabidopsis*, FD binds the promoters of several *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes, including *SPL3*, *SPL4*, *SPL5*, and *SOC1* (Teper-Bamnolker and Samach, 2005; Jung *et al.*, 2012). SOC1 also binds the promoters of *SPL3*, *SPL4*, and *SPL5* (Jung *et al.*, 2012), and SPL3, SPL4, and SPL5 can directly activate *LFY* expression (Yamaguchi *et al.*, 2009; Jung *et al.*, 2012). FD also, probably indirectly, activates *AP1* expression (Abe *et al.*, 2005; Wigge *et al.*, 2005; Benlloch *et al.*, 2011). The TERMINAL FLOWER 1 (TFL) protein, an FT paralogue with opposite function, normally represses flowering perhaps by competing with FT for binding to FD or the FAC (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006).

Although several studies have investigated natural variation in vernalization response in *Arabidopsis*, several of these studies have revealed variation at *FRI*, *FLC*, or loci interacting with these loci accounted for much of the observed variation (Shindo *et al.*, 2006; Strange *et al.*, 2011; Coustham *et al.*, 2012; Sanchez-Bermejo *et al.*, 2012). While some additional loci have been identified (Sanchez-Bermejo *et al.*, 2012), little is known about the identity or function of other loci or how these loci function in the context of existing vernalization pathways. One such locus *FLOWERING H* (*FLH*) was identified in recombinant inbred lines (RILs) derived from crosses of the Landsberg *erecta* (L*er*) accession with the Cape Verdi Islands (CVI) accession (Alonso-Blanco *et al.*, 1998). Plants carrying CVI alleles of *FLH* flower earlier than L*er*, particularly after vernalization, but responded similarly to photoperiod (Alonso-Blanco *et al.*, 1998).

This study further characterizes the *FLH* flowering time locus and shows that *FLH* is likely to represent a novel flowering time gene. Furthermore, the ability of CVI alleles of *FLH* to confer early flowering after vernalization operates in parallel to the PHD-PRC2 mediated repression of *FLC*. This study also demonstrates that the earliness conferred by CVI alleles of *FLH* is dependent on the presence of FD, but does not require FT, suggesting that FLH may act as a modifier of the FD-specific activity of the FAC.

## Materials and methods

### *Plant growth conditions, vernalization treatment, and flowering time analysis*

Seeds of Landsberg *erecta* (L*er,* NW20), *ft-1* and *fd-1* (Koornneef *et al.*, 1991), *ap1-1* (Mandel *et al.*, 1992), and *ap1-1*/*cal1-1* (Ditta *et al.*, 2004), all in the L*er* background, were obtained from the *Arabidopsis* Biological Resource Collection (ABRC, Columbus, Ohio, USA). Seeds of near-isogenic line 1 (NIL1) containing the CVI allele at *FLH* (Alonso-Blanco *et al.*, 1998) were provided by C. Alonso-Blanco. Seeds of *flc-5*, *vrn5-1*, *vin3-7* (Greb *et al.*, 2007), and *vrn1-2* (Levy *et al.*, 2002) in the L*er* background were provided by C. Dean. Seeds of *soc1-2* in L*er* (Melzer *et al.*, 2008) were provided by G. Coupland.

Seeds were sown on moist soil (Debco seed raising mix/vermiculite, 4:1) before being vernalized in the dark for 3 weeks in the dark at 4 °C and then transferred to a controlled-environment growth room at 22 °C under an 8/16 light/dark regime (short day condition, SD) with cool white fluorescent light (Sylvania Luxline Plus F36W/840). Control, non-vernalized plants were stratified at in the dark at 4 °C for 2 days prior to transfer to SD or long day (LD) conditions. For plants grown in LD, the photoperiod was 16/8 light/dark under the same illumination conditions. Plant were grown for 21 days (SD) or 7 days (LD) before being transferred to individual wells of a 48-well

tray. Flowering time was measured by counting the total number of rosette leaves and cauline leaves present on the main stem.

### *Genetic and physical mapping of* FLH

The *FLH* gene was initially mapped by genotyping F2 plants from a cross of L*er* and *FLH*-CVI (NIL1). Genomic DNA was extracted from 648 plants according to the method described by Klimyuk *et al.* (1993), and plants with recombination events between the markers CER455033 (www.arabidopsis.org; last accessed 23 April 2013) and a CAPS marker derived from the AFLP marker SM78–320 marker (Peters *et al.*, 2001) were selected. The *FLH* genotype of recombinant plants was determined by analysing the flowering time of 24–48 plants in the F3 generation. Subsequent fine mapping was performed using a selection of new markers (Supplementary Table S1, available at *JXB* online) on high-quality genomic DNA extracted from recombinants according to the method by Dellaporta *et al.* (1983).

Mutant alleles were detected as previously described for *flc-5*, *vrn5-1* (Greb *et al.*, 2007), and *vrn1-1* (Levy *et al.*, 2002) or using the markers described in Supplementary Table S2.

### *Reverse-transcription PCR analysis*

For semi-quantitative reverse-transcription (RT)-PCR, whole seedlings were harvested at 10 days post germination or vernalization every 10 days for 40 days after being germinated on MS plates without sucrose. Total RNA was extracted from pooled seedlings at each stage with and without vernalization using the RNeasy Plant Kit (Qiagen) as per the manufacturer's instructions. The optional on-column DNAse step was also included. The yield and RNA purity was determined spectrophotometrically (NanoDrop ND-1000) and visualized by gel electrophoresis. cDNA was synthesized using Superscript III (Invitrogen) as per the manufacturer's instructions. Total RNA (500 ng) was used per cDNA synthesis reaction in an Eppendorf Mastercycler. cDNA concentration across samples was normalized using ACTIN7 as an internal control and visualized on a 1% (w/v) agarose gel using ethidium bromide staining. The primer sequences are described in Supplementary Table S3.

For quantitative RT (qRT) PCR, total RNA was extracted from material enriched in shoot apices (Lopez-Juez *et al.*, 2008) collected from pools of 24 plants each of *FLH-CVI* and L*er* grown for at 15–20 short days post germination/vernalization (i.e. at the same developmental stage). Three biological replicates of each genotype and treatment were used. RNA was purified using the Qiagen RNeasy Kit as per the manufacturer's instructions. Total RNA (2 µg) was DNase treated with the Promega RQ1 DNase Kit as per the manufacturer's instructions to remove any genomic DNA contamination. Quantitative real-time PCR was performed using the Bio-RAD iCycler and the iScript One Step RT-PCR Kit with SYBR Green as per the manufacturer's instructions. PCR conditions consisted of a reverse-transcription step at 50 °C for 10 minutes, a reverse transcription inactivation step at 95 °C for 5 minutes, and 40 cycles of 95 °C for 10 seconds followed by 64.5 °C for 30 seconds. Results were visualized using the BioRad iQ5 Optical System Software. Expression of *FD* and *FT* was normalized relative to the expression of *UBC_21* previously validated as a reference gene for qRT-PCR (Czechowski *et al.*, 2005). Details of qRT-PCR primers are described in Supplementary Table S4. Transcript abundance was calculated using the Pfaffl model for relative quantification with efficiency correction (Pfaffl, 2001), and statistical analysis was performed using a Student's t-test.

## Results

### *CVI alleles of* FLH *accelerate flowering compared to* L*er alleles*

The presence of the alleles at *FLH* originating from the CVI accession in an otherwise L*er* near-isogenic line reduced flowering time when grown in a SD photoperiod, particularly in response to vernalization (Fig. 1A; Alonso-Blanco *et al.*, 1998). *FLH*-CVI plants appeared otherwise normal and did not exhibit any additional other phenotypes associated with early flowering, and produced normal flowers and cauline leaves (Fig. 1A). In the absence of a vernalization treatment, *FLH*-CVI plants flowered with approximately five fewer leaves than L*er* in SD conditions (Fig. 1B). CVI alleles of *FLH* also conferred slightly earlier flowering in LD conditions, with *FLH*-CVI plants flowering with approximately three fewer leaves in the absence of vernalization and with four fewer leaves than L*er* after a vernalization treatment (Fig. 1C).

In order to further analyse the function of *FLH* in response to vernalization, this study measured the flowering

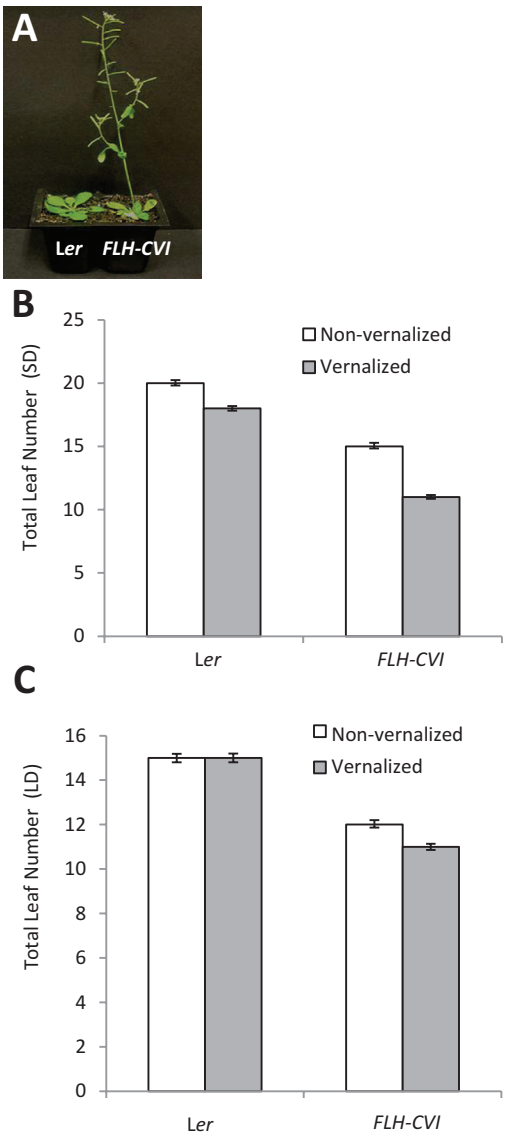


**Fig. 1.** CVI alleles of *FLH* accelerate flowering compared to L*er* alleles. (A) Comparison of L*er* and *FLH*-CVI (NIL1) grown for 30 short days after 3 weeks of vernalization. (B, C) Flowering time of L*er* and *FLH*-CVI in short days (B) and long days (C) as measured by total leaf number in response to 3 weeks of vernalization at 4 °C ($n$ = 48). Bars are standard errors.

time of *FLH*-CVI and L*er* after exposure to different durations of vernalization as measured by total leaf number after growth in SD conditions (Fig. 2). A vernalization response was detected in both *FLH*-CVI and L*er* after 3-week vernalization, with vernalized *FLH*-CVI plants flowering significantly earlier than L*er*. Exposure to a longer vernalization treatment of 6 weeks did not lead to significantly earlier flowering, suggesting that saturation of the vernalization response occurs at or before 3 weeks in plants with a CVI allele of *FLH*.

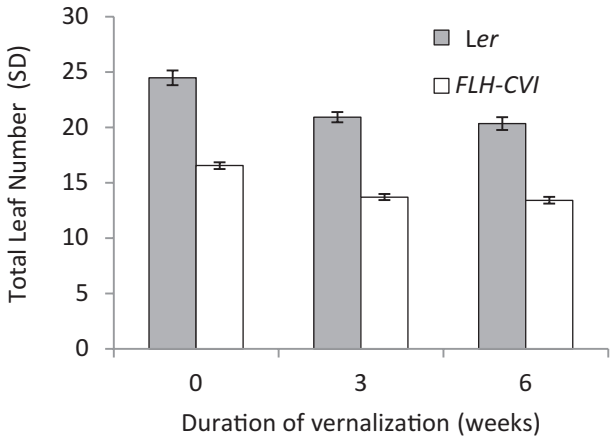


**Fig. 2.** Dosage sensitivity of *FLH* alleles to increasing durations of vernalization. Flowering time of L*er* and *FLH*-CVI as measured by total leaf number in response to various durations of vernalization ($n$ = 24). Bars are standard errors.

### *CVI of* FLH *alleles differentially affects gene expression*

In order to gain an understanding of where *FLH* may fit into the current model of floral induction in *Arabidopsis*, semi-quantitative RT-PCR was performed on genes located throughout the vernalization-induced flowering pathway (*FLC*, *AGL24*, and *AGL19*), floral integrator genes (*FT*, *FD*, *SOC1*, and *LFY*), and the meristem identify gene *AP1*, all of which are known to be regulated either directly or indirectly by vernalization. The transcript levels of these genes were examined in L*er* and *FLH*-CVI plants every 10 days for 40 days in both control (non-vernalized) plants and in plants exposed to 3-week vernalization (Fig. 3). Transcript levels in vernalized and non-vernalized samples were then compared to identify those transcripts with altered abundance in *FLH*-CVI relative to L*er*. After applying these criteria, only *AP1* appeared to show any significant change in abundance in *FLH*-CVI compared to L*er*, relative to its levels when non-vernalized at the same stages of development. In the *FLH*-CVI early-flowering plant line, *AP1* transcript abundance was increased, particularly at 40 days of growth. This increase in *AP1* expression was not accompanied with an increase in *SOC1*, *LFY*, *FT*, or *FD* expression, implying that CVI alleles of *FLH* may be capable of upregulating *AP1* independently of these genes. The semi-quantitative RT-PCR data also implied that *FLH* does not regulate any of the other vernalization-induced

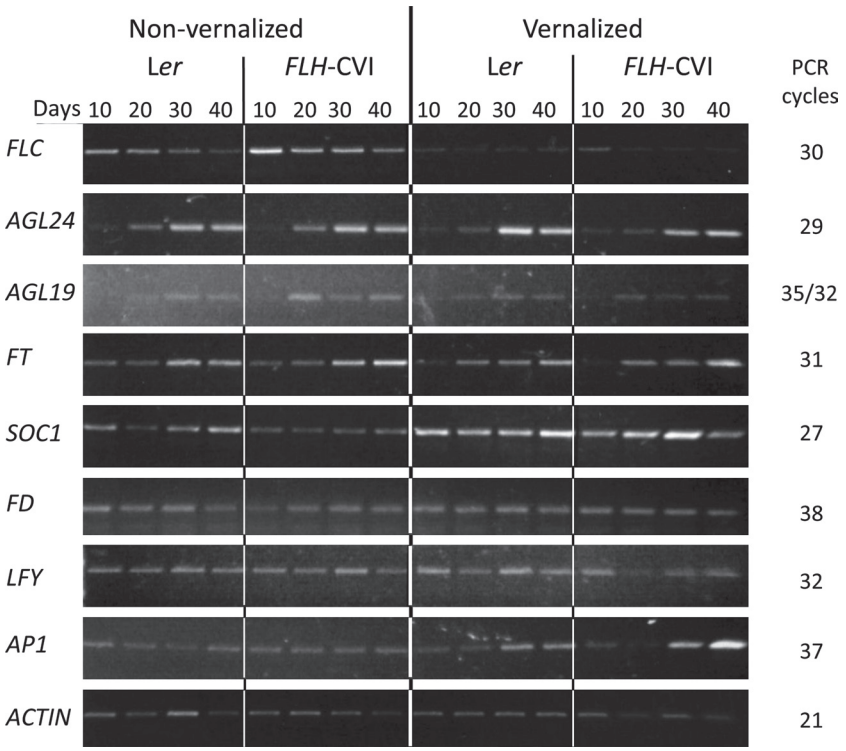


**Fig. 3.** Semi-quantitative RT-PCR analysis of flowering time genes in the presence of CVI alleles of *FLH*, showing transcript levels of flowering time genes in seedlings of L*er* relative to *FLH*-CVI at 10-day increments with and without vernalization. Days is days of growth in SD conditions post stratification or post vernalization.

flowering time genes investigated, including the major repressor *FLC*.

### FLH *is a novel flowering time locus*

To further investigate the mechanism by which CVI alleles of *FLH* confer early flowering, combinations of CVI alleles of *FLH* with mutants in other flowering time genes were generated. In order to accurately determine which progeny of various crosses contained the CVI alleles of *FLH*, this study sought to identify a molecular marker closely linked to *FLH*. This approach also allowed a more precise determination of the chromosomal location of the *FLH* locus, as the first step towards cloning the *FLH* quantitative trait locus.

The *FLH* locus was originally identified by Alonso-Blanco *et al.* (1998) and further characterized in a *FLH*-CVI near-isogenic line derived from introgression of CVI alleles into L*er*. The *FLH*-CVI line contains an approximately 10 centimorgan region of CVI-derived DNA at the bottom of the long arm of chromosome 5 in a L*er* background (Fig. 4A; Alonso-Blanco *et al.*, 1998). The L*er*-CVI polymorphic marker G2368 was previously used as a marker for *FLH* in the *FLH* NIL1. G2368 maps to 109 cM on the L*er*-CVI genetic map (Alonso-Blanco *et al.*, 1998) and is located at approximately 25.8 Mb on chromosome 5 of the Columbia accession reference genome, close to the *MAF2–MAF5* genes (Ratcliffe *et al.*, 2003). NIL1 has previously been shown to contain L*er* alleles at EG7F2 (24.6 Mb) and CVI alleles at markers T6B16 (24.8 Mb) and MQB2 (25.2 Mb) (Swarup *et al.*, 1999) thus delimiting containing *FLH* to a region of approximately 2.4 Mb between 24.6 Mb and the end of chromosome 5 at 27.0 Mb.

To determine the degree of linkage between G2368 and *FLH*, NIL1 was first crossed to L*er* and the F2 progeny were analysed for flowering time, which confirmed that early flowering conferred by the CVI alleles of *FLH* was dominant (Fig. 4B). Plants carrying recombination events between markers CER455033 and PLOP3 were identified, and the F3 progeny were tested for segregation of late flowering, indicating that the F2 parent was homozygous for the L*er* allele of *FLH*. F2 plants with recombinants events between CER455033 and SM78-320 were then further analysed using additional polymorphic markers (Fig. 4C). In a population of 648 F2 plants, 11 recombinants between G2368 and *FLH* could be detected. Furthermore, *FLH* co-segregated with the marker 2662, and thus the region containing *FLH* was delimited by markers K1F13D and 2672. Therefore, marker 2662 was used for all further genotyping of *FLH* alleles. Interestingly, a reduction in recombination was detected distal to *FLH* (i.e. towards the telomere), suggesting that recombination is suppressed in this region in the L*er* × NIL1 cross.

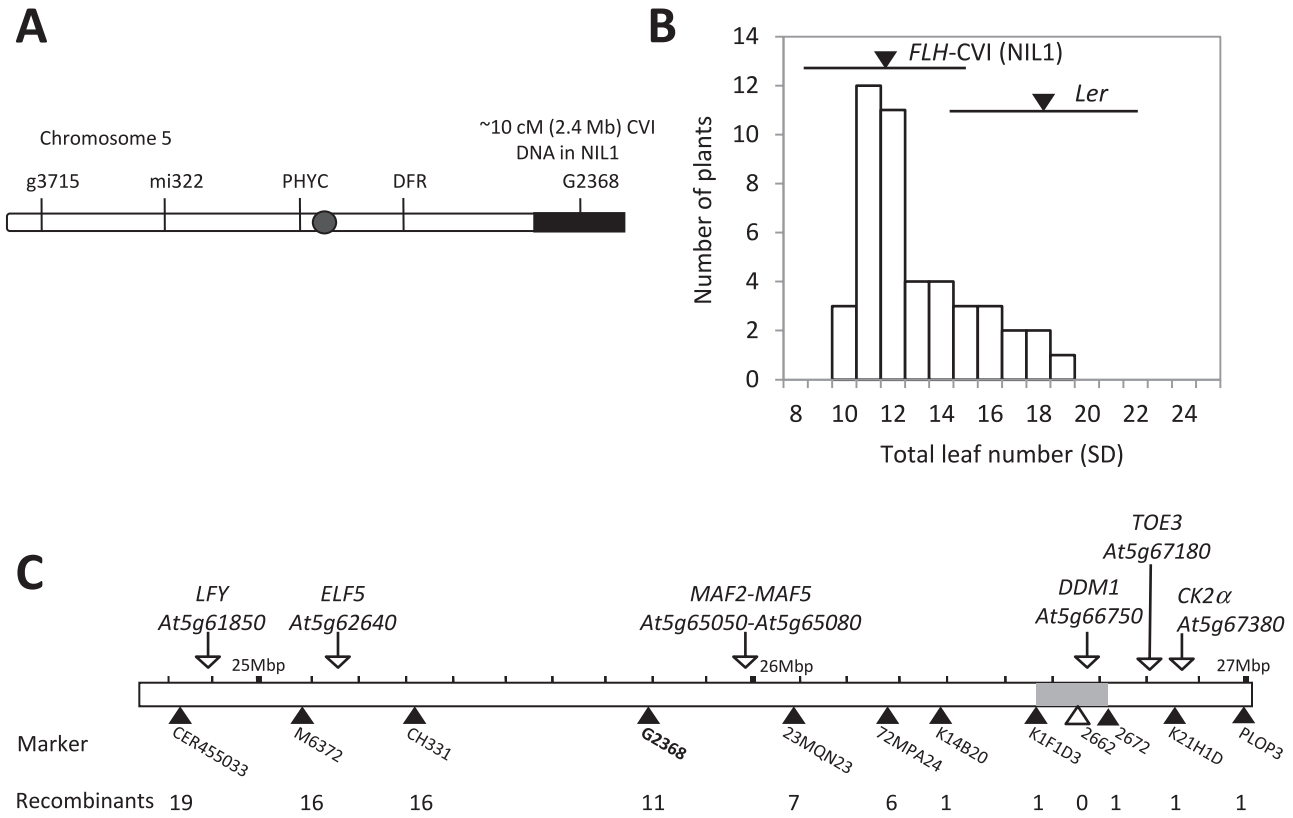


**Fig. 4.** Identification of markers that co-segregate with *FLH*. (A) Location of CVI-derived DNA on chromosome 5 in the *FLH*-CVI NIL is indicated by the filled area. (B) Segregation of flowering time in the F2 progeny of a cross between L*er* and the *FLH*-CVI NIL ($n$ = 48); filled triangles indicate the mean flowering time of each of the parents and horizontal lines indicate the range of flowering times ($n \geq 35$). (C) Mapping of *FLH*; filled triangles indicate the positions of markers used to map *FLH*. Recombinants were identified in 648 F2 progeny of a cross between L*er* and the *FLH*-CVI NIL.

This rough mapping of *FLH* also suggests that *FLH* is likely to represent a new flowering time locus, as only a single gene with a flowering time effect, *DDM1*, is localized to this region (Jeddeloh *et al.*, 1999). However, it seems unlikely that *FLH* is an allele of *DDM1*, as mutations in *DDM1*, which encodes a SWI-SNF2-like protein, produces plants with exhibit unstable pleiotropic phenotypes that become progressively more severe with each generation (Kakutani *et al.*, 1996) – phenotypes which are not shared with plants carrying CVI alleles of *FLH*.

### *CVI alleles of* FLH *act independently of VIN3, VNR5, and FLC to accelerate flowering*

In order to identify the role and interaction that CVI alleles of *FLH* have with known vernalization genes within the vernalization flowering time pathway, the *FLH*-CVI NIL1 was crossed with various mutants that regulate *FLC* expression. In order to avoid any effects on flowering time due to mixing of genetic backgrounds, this study only used mutations that were in the L*er* genetic background, which contains a weakly active allele of *FLC* (Koornneef *et al.*, 1994; Lee *et al.*, 1994b; Gazzani *et al.*, 2003; Michaels *et al.*, 2003b). F2 progeny were genotyped at *FLH* using the 2662 marker, and F3 plants doubly homozygous for CVI alleles at *FLH* and the mutation of interest were subjected to flowering time analysis.

Mutations in *VIN3* lead to late flowering, particularly after vernalization, as upregulation of *VIN3* during the cold is required for the initial repression of *FLC* (Sung and Amasino, 2004; Greb *et al.*, 2007). *vin3* mutant plants were slightly late flowering in the absence of a vernalization treatment and were completely unresponsive to a vernalization response and subsequent growth in SD conditions (Fig. 5A and B). When CVI alleles of *FLH* were combined with a *vin3* mutation, the resulting *FLH*-CVI *vin3* plants flowered earlier than the *vin3* mutant but later than *FLH*-CVI plants and did not respond to vernalization, suggesting that the earliness conferred by CVI alleles of *FLH* does not require VIN3 activity. Combinations of CVI alleles of *FLH* with mutations in VRN5, which forms a dimer with VIN3, behaved similarly. *FLH*-CVI *vrn5* plants flowered earlier than *vrn5* mutants, but not as early as *FLH*-CVI plants, and *FLH*-CVI *vrn5* plants retained a weak response to vernalization (Fig. 5A and B). *FLH*-CVI *vrn1* plants flowered almost as early as *FLH*-CVI plants, but did not exhibit a vernalization response (Fig. 5A and B).

To determine if there is an interaction between CVI alleles of *FLH* and active alleles of *FLC*, the CVI allele of *FLH* was introgressed into Columbia and selected plants with CVI alleles at *FLH* using the molecular marker 2662. CVI alleles of *FLH* were able to confer earlier flowering, particularly after vernalization in the Columbia background; however, the flowering time was more similar to Columbia (Fig. 5C). This suggests that CVI alleles of *FLH* confer earlier flowering than Col alleles of *FLH* and that CVI alleles of FLH are not able to completely overcome the lateness caused by active alleles of *FLC*.

### *CVI alleles of* FLH *require* FD *but not* FT *to accelerate flowering*

To establish how CVI alleles of *FLH* may be effecting the transcript levels of *AP1* through the activitivy of floral integrators, CVI alleles of *FLH* were introduced by crossing into floral integrator mutants in a L*er* background. Combinations of CVI alleles of FLH with *ap1* and *ap1/cal* mutations produced plants with a flowering time that was intermediate between the two parents, suggesting FLH is able to promote flowering partially independently of *AP1* and *CAL* (Fig. 6B and C). When CVI alleles of *FLH* were combined with mutations in *soc1*, the resulting *FLH*-CVI *soc1* plants were slightly earlier flowering than *soc1* plants (Fig. 6B), suggesting that the earlinesss conferred by CVI allels of *FLH* is partially dependent on, or acts additively with, *SOC1*.

The most striking results were observed when CVI alleles of *FLH* were combined with mutations in *FD* and *FT*. *FLH*-CVI *ft* plants flowered with an average of 13 fewer leaves compared to the *ft* single mutant control in both vernalized and non-vernalized treatments (Fig. 6), which was similar to the number of leaves in wildtype L*er* plants. CVI alleles of *FLH* were thus able to completely overcome the loss of *FT* and to restore flowering time comparable to that of wild type L*er*. However, *FLH*-CVI *ft* plants did not flower as early as *FLH*-CVI plants, suggesting that the ability of *FLH* to accelerate flowering is partially dependent on *FT*.

In contrast, CVI alleles of *FLH* were unable to overcome the late flowering due to the loss of *FD,* in both non-vernalized and vernalized treatments. *FLH*-CVI *fd* plants exhibiting a flowering time similar to that of the *fd* single mutant in both treatments, suggesting that *fd* mutations are completely epistatic to CVI alleles of *FLH* (Fig. 6A and B). This suggests that the ability of CVI alleles of *FLH* to accelerate flowering is completely dependent on *FD* activity.

To determine if CVI alleles of *FLH* can accelerate flowering when the photoperiod pathway is active, the flowering time measurements were repeated using plants grown in LD conditions. CVI alleles of *FLH* behaved similarly in combination with most mutations in long days as they did in short days. Mutations in *ap1* and *fd* prevented the vernalization response of CVI alleles of *FLH* (Fig. 6C), and mutation of *soc1* reduced the ability of CVI alleles of FLH to confer earliness.

### FD *expression is increased by CVI alleles of* FLH *in the absence of vernalization*

As the results of the genetic analysis suggested that *FLH* requires *FD* to confer early flowering, this study examined the possibility that CVI alleles of *FLH* may lead to the increased expression of *FD* in the shoot meristem. Therefore, material enriched in shoot apices were collected from L*er* and *FLH*-CVI plants grown in SD conditions and *FD* expression was examined by qRT-PCR (Fig. 7A). Consistent with previous reports, the levels of *FD* expression was higher in plants that had been vernalized (Searle and Coupland, 2004). *FD* mRNA levels were slightly elevated (by approximately 2-fold)

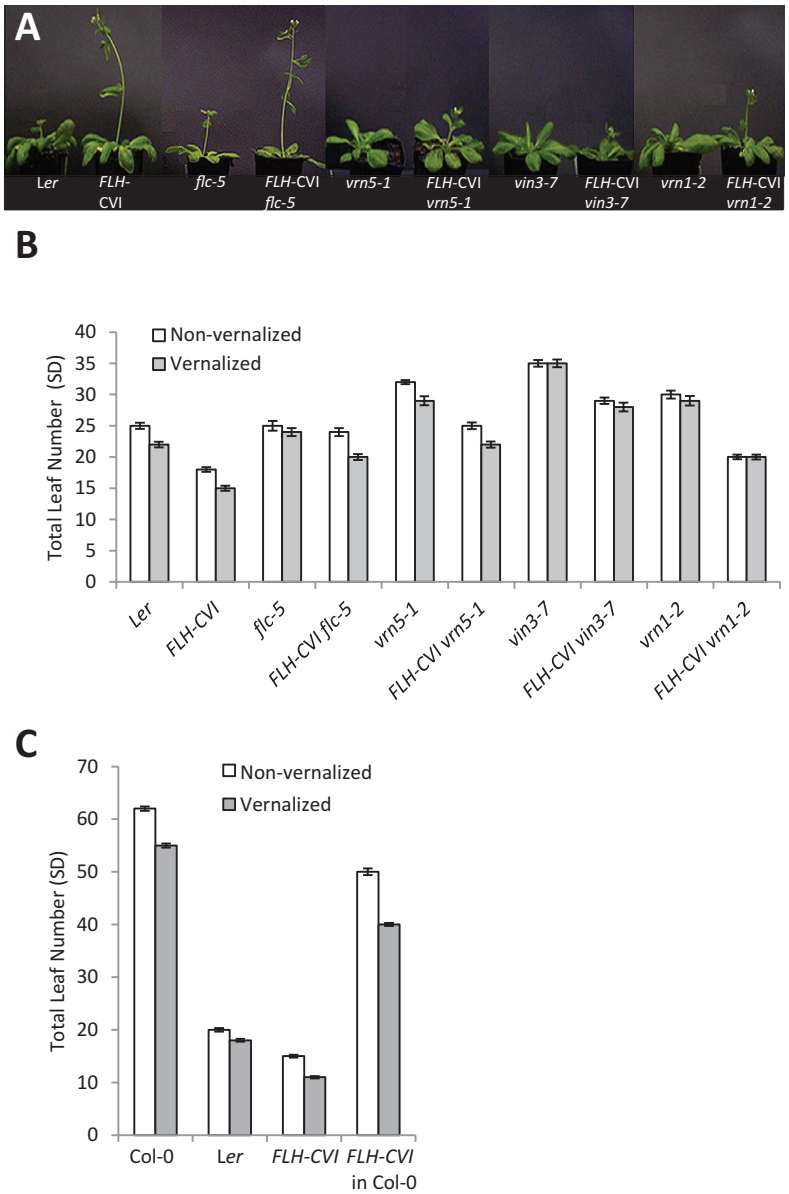


**Fig. 5.** Genetic analysis of *FLH*-CVI interaction with vernalization pathway genes. (A) Vernalized wildtype L*er*, *flc-5*, *vrn5-1*, *vin3-7*, and *vrn1-2* mutants and *FLH*-CVI combinations grown in for 21 short days after 3-week vernalization. (B) Comparison of flowering time between vernalized treatments of wild-type L*er*, *FLH*-CVI, various known vernalization mutants *flc-5*, *vin5–1*, *vin3-7*, and *vrn1-2*, and FLH-CVI in the mutant backgrounds grown in short days ($n \geq 48$). (C) Comparison of flowering time between vernalized treatments of wild-type Columbia and L*er*, *FLH*-CVI, and *FLH*-CVI introgressed into Col (*FLH*-CVI in Col) grown in short days ($n \geq 48$). Bars are standard errors.

in *FLH*-CVI apices compared to L*er* plants in non-vernalized control plants, but *FD* expression was not different between vernalized L*er* and *FLH*-CVI plants. This suggests that CVI alleles of *FLH* can increase *FD* expression under some conditions. Consistent with the genetic analysis, no substantial differences in *FT* expression between *FLH*-CVI and L*er* plants were detected (Fig. 7B).

## Discussion

Natural variation in vernalization responses in *Arabidopsis* has been investigated using a variety of approaches, and several QTLs have been identified (Lempe *et al.*, 2005; Sanchez-Bermejo *et al.*, 2012). In some cases, genes responsible for these QTLs have also been identified (Shindo *et al.*, 2006). This study characterized the activity of the *FLOWERING H* locus and demonstrated that alleles from the Cape Verde Islands accession accelerate flowering in an *FD*-dependent manner.

*FLH* was identified in the L*er*/CVI recombinant inbred line population, as a locus which conferred early flowering, particularly in response to vernalization, with CVI alleles at *FLH* conferring early flowering compared to L*er* alleles (Alonso-Blanco *et al.*, 1998). This suggests that the CVI allele of *FLH* may be a gain-of-function polymorphism, or that the L*er* allele

of *FLH* may represent a loss-of-function allele. The direct comparison of CVI and Columbia alleles of *FLH* (Fig. 5) revealed that Columbia appeared to have a similar allele of *FLH* as L*er*, as plants with CVI alleles of *FLH* were earlier flowering than those with Col alleles. Flowering time analysis of RILs derived from crosses of L*er* to Col did not identify a flowering time QTL at the *FLH* locus, suggesting that L*er* and Col have similar *FLH* alleles (Jansen *et al.*, 1995). The present analysis of CVI alleles of *FLH* introgressed into Columbia is also consistent with a previous QTL analysis performed on a Col × CVI RIL population when a weak flowering time QTL was detected close to *FLH* in the Col × CVI RIL, with Columbia alleles conferring later flowering than CVI alleles (Simon *et al.*, 2008).

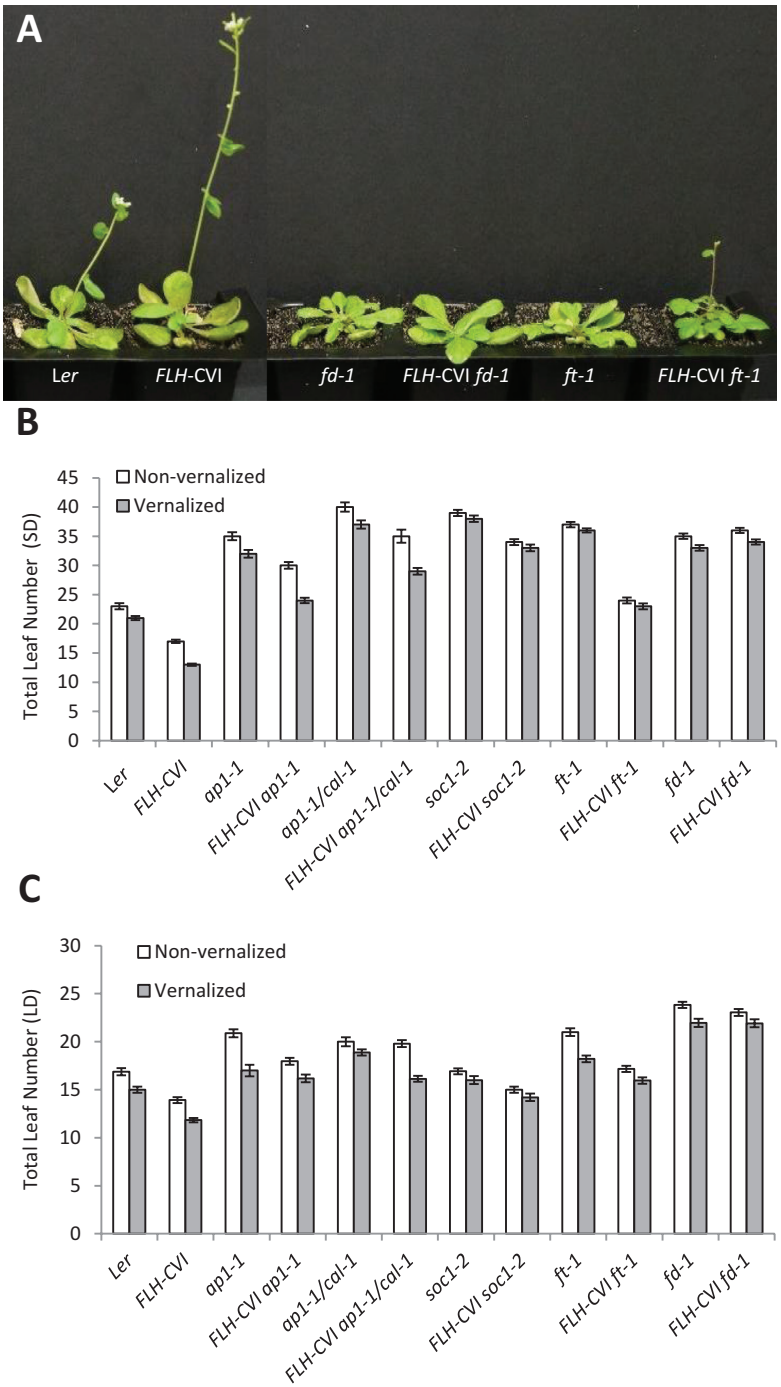


**Fig. 6.** Genetic analysis of the interaction of *FLH*-CVI with floral integrator genes. (A) Vernalized wildtype L*er*, *FLH*-CVI, *fd-1*, *ft-1*, and *FLH*-CVI combinations grown in short days for 30 days after 3-week vernalization. (B) Comparison of flowering time between vernalized treatments of wild-type L*er*, *FLH*-CVI, and various mutants and *FLH*-CVI combinations grown in SD conditions ($n \geq 48$). (C) Comparison of flowering time between vernalized treatments of wild-type L*er*, *FLH*-CVI, and various mutants and *FLH*-CVI combinations grown in LD conditions ($n \geq 48$). Bars are standard errors.

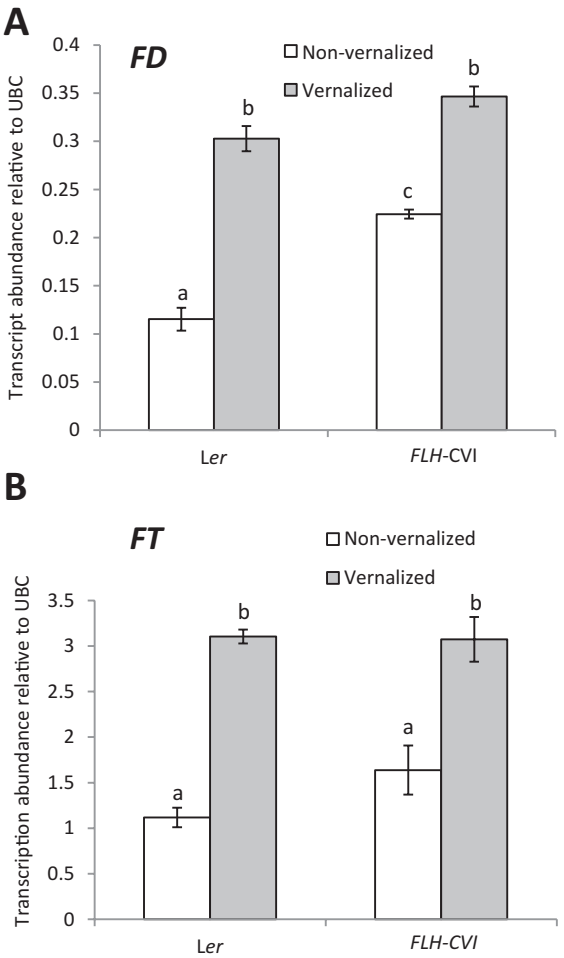


**Fig. 7.** *FT* and *FD* expression in *FLH*-CVI. Expression of *FD* (A) and *FT* (B) in shoot apical meristem-enriched tissues, as measured by quantitative RT-PCR relative to the internal control *UBC*. Data are means and standard errors of three biological replicates. Different letters indicate significant differences in relative expression levels ($P \leq 0.05$).

This situation is similar to that observed in an analysis of natural variation of *CRYPTOCHROME 2* (*CRY2*). The allele of *CRY2* present in CVI was identified as the underlying cause of the *EARLY DAYLENGTH INSENSITIVE* (*EDI*) QTL in the L*er*/CVI RIL population (Alonso-Blanco *et al.*, 1998; El-Din El-Assal *et al.*, 2001). *CRY2*-CVI confers dominant, day-length-insensitive early flowering due to the increased accumulation of *CRY2*-CVI (El-Din El-Assal *et al.*, 2001). Although the CVI allele of *CRY2* appears to be unique, and other functional variants in *CRY2* have not been described, this example highlights the use of natural variation to uncover novel alleles that shed light on gene function. Similarly, identification of natural variation in an advanced multiparent population uncovered a previously unknown function in the control of short architecture for the *AGAMOUS-LIKE6* gene (Huang *et al.*, 2012).

As several genes are located close to the previously reported location of *FLH*, it was important to be able to exclude these genes as *FLH*. The fine mapping of *FLH* confirms that *FLH* is not a novel allele of *MAF2*, *MAF3*, *MAF4*, or *MAF5*, which lie close to *FLH*. *MAF2* is involved in vernalization response (Ratcliffe *et al.*, 2003) and natural variation in *MAF2* and *MAF3* has been described (Rosloski *et al.*, 2010). One *FLH* candidate with a role in flowering time that could not be eliminated by fine mapping has been described, the SWI/SNF2-like protein *DECREASED DNA METHYLATION* (*DDM1*) (Jeddeloh *et al.*, 1999). The role of *DDM1* in maintenance of 'global' chromatin methylation is also not consistent with the subtle effect of *FLH* on flowering time. *ddm1* mutants exhibit pleiotropic defects and are unstable (Jeddeloh *et al.*, 1999), phenotypes that are not observed in multiple generations of crosses and propagation of the *FLH*-CVI NIL. Furthermore, preliminary sequence analysis does not support the hypothesis that *FLH* is *DDM1* (A. Dinsdale, unpublished).

CVI alleles of *FLH* did not affect the expression of any of the tested genes differently compared to L*er* alleles of *FLH*, except *AP1* and *FD*. This suggests that *FLH* may be acting very late in a floral promoting pathway to activate transcription or is acting post-transcriptionally. CVI alleles of *FLH* can still accelerate flowering in the absence of *AP1*, particularly in short days, suggesting at least a partial independence from *AP1*. The observation that CVI alleles of *FLH* do not affect the expression of *FT*, and only weakly increase *FD* expression in the absence of vernalization, suggests that the CVI allele of *FLH* may act as a weak transcriptional activator of *FD* expression.

One flowering time gene with a similar phenotype to *FLH*-CVI is *TERMINAL FLOWER1* (*TFL1*) (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992). The *FLH*-CVI phenotype is similar to that conferred by the overexpression of a TFL1-VP16 fusion protein, in which the TFL repressor is converted to a strong transcriptional activator. The early flowering of 35S::TFL-VP16 is strongly dependent on *FD* (Hanano and Goto, 2011), similar to that observed with CVI alleles of *FLH*. Furthermore, the early flowering *tfl* mutant phenotype is suppressed by a *fd* mutation, similarly to the phenotype observed in the *FLH*-CVI *fd* plants. The dominance and activity of CVI alleles of *FLH* has some similarity to that of *FWA*, but dominant late flowering alleles of *FWA* are due to the misexpression of *FWA* (Soppe *et al.*, 2000), which interacts with FT, and FWA may therefore be competing for the FT-binding site on the 14-3-3 molecule in the FAC (Ikeda *et al.*, 2007).

Taken together, this suggests several models for *FLH* activity. CVI alleles of *FLH* may increase the expression or widen the expression domain of *FD*, resulting in earlier flowering. However, the increase in *FD* expression in *FLH*-CVI plants is subtle and only occurs prior to vernalization. In wild-type plants, *FD* expression dramatically increases by approximately 10-fold in the shoot apex upon the transition to flowering (Wigge *et al.*, 2005), so it seems unlikely that the small increase in *FD* expression observed in *FLH*-CVI plants could entirely account for the observed earliness. Alternatively, CVI alleles of *FLH* may result in the misexpression, either by expression in a wider range of cells or by increased levels of expression, of a protein that can interact and cooperate directly with FD, perhaps as a transcriptional co-activator as part of the FAC. Alternatively, the protein encoded by CVI

alleles of *FLH* may itself interact with the FAC, with FLH proteins encoded by the L*er* and Columbia alleles of *FLH* may be unable to bind to the FAC or may bind at reduced affinity compared to the *FLH* protein encoded by CVI alleles. The ability of CVI alleles of *FLH* to rescue the late flowering of *ft* mutants also supports the notion that FLH may be able to substitute for FT as part of the FAC. However, none of the predicted genes in the *FLH* region of the genome encodes an *FT*- or *TFL*-like protein suggesting that if FLH does interact with the FAC, it may represent a novel interaction.

## Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Mapping primers

Supplementary Table S2. Genotyping primers

Supplementary Table S3. Semi-quantitative RT-PCR primers

Supplementary Table S4. Quantitative RT-PCR primers

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