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A gene loop containing the floral repressor *FLC* is disrupted in the early phase of vernalization

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Gene activation in eukaryotes frequently involves interactions between chromosomal regions. We have investigated whether higher-order chromatin structures are involved in the regulation of the Arabidopsis floral repressor gene *FLC*, a target of several chromatin regulatory pathways. Here, we identify a gene loop involving the physical interaction of the 5′ and 3′ flanking regions of the *FLC* locus using chromosome conformation capture. The *FLC* loop is unaffected by mutations disrupting conserved chromatin regulatory pathways leading to very different expression states. However, the loop is disrupted during vernalization, the cold-induced, Polycomb-dependent epigenetic silencing of *FLC*. Loop disruption parallels timing of the cold-induced *FLC* transcriptional shut-down and upregulation of *FLC* antisense transcripts, but does not need a cold-induced PHD protein required for the epigenetic silencing. We suggest that gene loop disruption is an early step in the switch from an expressed to a Polycomb-silenced state.

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Introduction

The complexity of gene regulation in the chromatin environment of eukaryotic cells is enormous and frequently involves chromosomal interactions between regulatory sequences and their target genes (Li *et al*, 2007; Palstra, 2009). Chromosomal loops that bring distant regulatory elements to gene promoters (Tolhuis *et al*, 2002; Louwers *et al*, 2009a; Kagey *et al*, 2010) and co-localization of silenced genes in Polycomb bodies (Lanzuolo *et al*, 2007; Bantignies *et al*, 2011) are examples of functionally important long-range interactions. Short-range chromatin interactions also occur and are exemplified by gene loops where the promoter and terminator regions of individual genes are juxtaposed when transcribed (O'Sullivan *et al*, 2004; Ansari and Hampsey, 2005). Gene loops were first described in yeast but have now also been observed in higher eukaryotes (O'Reilly and Greaves, 2007; Perkins *et al*, 2008; Tan-Wong *et al*, 2008; Larkin *et al*, 2012). The formation of these gene loops relies on initial rounds of transcription and requires interaction of TFIIB, a general RNA polymerase II transcription factor, with 3′ end processing factors (Singh and Hampsey, 2007; Perkins *et al*, 2008; Medler *et al*, 2011). In yeast, the promoter–terminator loop conformation is proposed to promote recycling of RNA polymerase from the 3′ terminator to the promoter (Ansari and Hampsey, 2005; Lykke-Andersen *et al*, 2011). Gene looping has also been proposed to be involved in intron-mediated enhancement of transcription (Moabbi *et al*, 2012) and plays a role in maintaining 'transcriptional memory' in yeast (Laine *et al*, 2009; Tan-Wong *et al*, 2009). Recently, a comprehensive study using yeast and mammalian cells has proposed that transcriptional directionality is enhanced by loop formation (Tan-Wong *et al*, 2012). However, gene loop formation is not always associated with enhanced gene activity in higher eukaryotic cells (Tan-Wong *et al*, 2008; Larkin *et al*, 2012), suggesting that gene looping may have different functions in different biological scenarios.

We were interested to see if gene looping may play a role in the complex transcriptional regulation of the *Arabidopsis thaliana* floral repressor gene *FLOWERING LOCUS C* (*FLC*). This gene is a target of several regulatory pathways involving chromatin remodelling, co-transcriptional RNA processing and Polycomb silencing (Baurle and Dean, 2006; Crevillen and Dean, 2010; Ietswaart *et al*, 2012; Song *et al*, 2012). These pathways significantly influence the timing of the transition to reproductive development in Arabidopsis, a trait important for adaptation to different climates. In ambient temperatures, *FLC* expression is upregulated by FRIGIDA (FRI), a plant-specific protein that elevates *FLC* mRNA levels by a co-transcriptional mechanism involving direct physical interaction with the nuclear cap-binding complex (Geraldo *et al*, 2009; Crevillen and Dean, 2010). FRI function requires conserved transcriptional components such as the Paf complex and chromatin remodelling activities (Crevillen and Dean, 2010; Choi *et al*, 2011). High *FLC* expression also requires the Arabidopsis Trithorax ATX1 and ATX2 histone methyltransferases (Pien *et al*, 2008), and the SWR1 complex subunit ARP6 (Choi *et al*, 2005; Deal *et al*, 2005; Martin-Trillo *et al*, 2006). The autonomous pathway antagonizes these activation functions (Baurle and Dean, 2006) in a mechanism involving the RNA-binding proteins FCA and FPA, the 3′ processing factor FY and the histone H3 lysine 4 demethylase FLD (Liu *et al*, 2007). The RNA-binding proteins and 3′ processing factors alter processing of *FLC* antisense transcripts (Hornyik *et al*, 2010; Liu *et al*, 2010; Ietswaart *et al*, 2012), resulting in changed histone methylation levels and reduced expression of *FLC* (Liu *et al*, 2007).

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The upregulation of *FLC* levels by FRI or mutations in the autonomous pathway is suppressed by vernalization, the acceleration of flowering in response to winter. This process is an adaptation used by many plants to align flowering with favourable conditions of spring. In Arabidopsis, vernalization involves a cold-induced Polycomb-mediated epigenetic silencing of *FLC* (recently reviewed by Song *et al*, 2012). After 2–3 weeks of cold, *FLC* transcription is downregulated concomitantly with increased accumulation of antisense transcripts, named *COOLAIR* (Swiezewski *et al*, 2009). Cold also induces the quantitative accumulation of Polycomb silencing. This involves activation of the PHD protein VIN3 (Sung and Amasino, 2004), heterodimerization with a homologue VRN5 (Sung *et al*, 2006; Greb *et al*, 2007) and association with a pre-loaded polycomb repressive complex 2 (PRC2) at an internal site in *FLC* (De Lucia *et al*, 2008). The PHD–PRC2 complex causes localized and progressive increases in histone H3 lysine 27 trimethylation (H3K27me3) during the cold (Finnegan and Dennis, 2007; Angel *et al*, 2011). Upon transfer to the warm, the PHD–PRC2 complex spreads across the gene resulting in high H3K27me3 across the locus, necessary for epigenetic stability through the rest of development (Finnegan and Dennis, 2007; De Lucia *et al*, 2008; Angel *et al*, 2011). A sense non-coding RNA (ncRNA) *FLC* transcript, *COLDAIR*, is induced by cold but later than *COOLAIR* and is involved in recruitment of the PHD–PRC2 complex (Heo and Sung, 2010).

To address whether higher-order chromatin structure is involved in any of these regulatory pathways, we used quantitative chromosome conformation capture (3C) (Dekker *et al*, 2002) in a range of Arabidopsis genotypes. We detected a robust gene loop at the *FLC* locus reflecting interaction between sequences in the *FLC* 5′ flanking region with sequences in the 3′ flanking region. Analysis of loop formation in a range of mutants suggested that loop formation was not dependent on *FLC* expression level. However, it was efficiently disrupted within the first 2 weeks of cold exposure during vernalization and did not reform after transfer of plants back to warm conditions. Disruption did not require the cold-induced PHD protein VIN3, important for nucleation of the epigenetic silencing of *FLC*. Similar results were observed with an *FLC* transgene, indicating that gene loop formation and disruption are independent of the genomic context of the locus. Thus, as in many other higher eukaryotes, gene loop formation occurs in plant genes perhaps aiding forms of transcriptional regulation. We propose that for *FLC* and maybe other Polycomb regulated genes, loop disruption is an early step during the switch to an epigenetically silent state.

Results

The 5′ and 3′ flanking regions of FLC interact creating a gene loop

We performed 3C experiments to explore whether a higher-order chromatin structure may contribute to the complexity of *FLC* regulation. This technique relies on formaldehyde crosslinking to detect interacting chromatin fragments in intact cells (Dekker *et al*, 2002; Hagege *et al*, 2007; Louwers *et al*, 2009b). We divided the *FLC* locus into different fragments using double digest with *Bgl*II and *Bam*HI restriction enzymes, schematically shown in Figure 1, and searched for chromosome interactions. Tandem primers were designed to ensure specific amplification of 3C ligated products only and chromatin interactions were estimated by calculating the ligation frequencies in the 3C DNA preparations by real-time quantitative PCR (Q-PCR) (see Materials and methods and Hagege *et al*, 2007). Fragment I, hereinafter referred to as FI, which includes the *FLC* promoter, first exon and key regulatory elements in intron 1 (Finnegan and Dennis, 2007; De Lucia *et al*, 2008; Angel *et al*, 2011), was used as an anchor region. Using young Columbia (Col) wild-type (WT) seedlings high interaction frequencies were found between the anchor region and FV (Figure 2A), a region ∼355–970 bp downstream of the *FLC* poly (A) sites (see Figure 1). The interaction frequencies observed between FI and FV were higher than expected by random collisions, indicating that the 5′ and 3′ flanking regions of the *FLC* locus are frequently in physical contact *in vivo* (Dekker *et al*, 2002; Louwers *et al*, 2009b). We also found significant interaction frequencies between FI and FIV (Figure 2A), a fragment starting downstream of the *FLC* poly (A) sites (see Figure 1). This loop seemed similar to promoter–terminator loops described in yeast, but in the case of *FLC* the gene loop extends beyond the poly (A) site. FIV is a region we previously identified with homology to small RNAs (Swiezewski *et al*, 2007) and contains the major transcriptional start site of the *COOLAIR* antisense transcripts. FV region is further downstream of *FLC* and probably contains promoter elements necessary for *COOLAIR* antisense RNA transcription (Swiezewski *et al*, 2009), although these have still to be defined. The *FLC* loop was also detected when the 3′ regions FIV and FV were used as anchor fragments in the 3C experiments (see Figure 5).

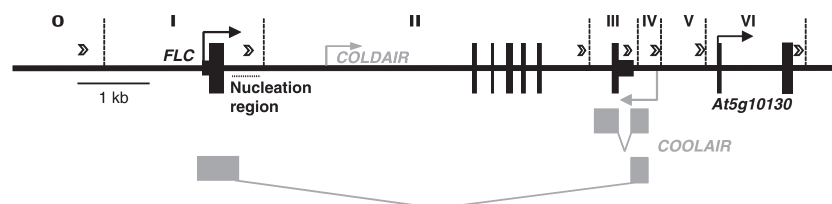

Figure 1 Schematic representation of the *FLC* locus and the 5′ region of the downstream gene (*At5g10130*) showing the regions analysed in this study. *Bam*HI and *Bgl*II restriction sites are indicated with vertical dotted lines. Primer positions are represented with open arrows. Cold-induced ncRNA transcripts *COLDAIR* (Heo and Sung, 2010) and *COOLAIR* (Swiezewski *et al*, 2009) are represented in grey. *COOLAIR* has alternatively processed transcripts but only the two main spliced forms present in warm-grown tissue are represented. The H3K27me3 nucleation region is also represented (Angel *et al*, 2011).

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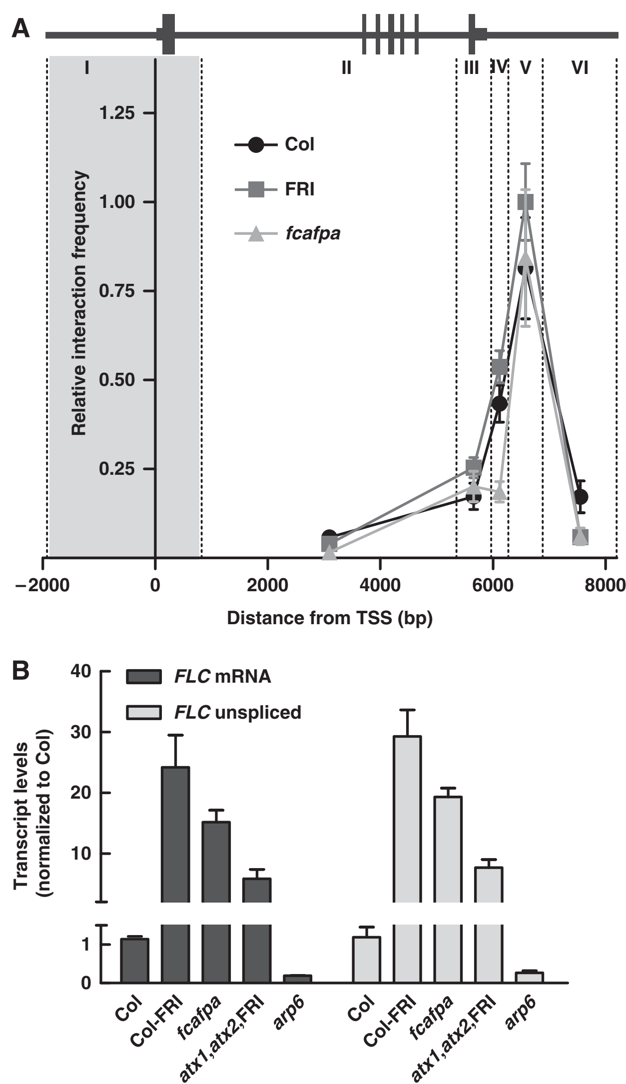

Figure 2 *FLC* loop formation in Columbia seedlings and genotypes with high *FLC* expression. (**A**) Quantitative 3C of the *FLC* locus using FI as the anchor region in 10-day-old WT Col, Col-FRI and *fcafpa* seedlings. Relative interaction frequencies were calculated as described in Materials and methods. The data are the average of four biological replicates each with two technical replicates. The error bars indicate s.e.m. ($n = 8$). In the graph, *Bam*HI and *Bgl*II restriction sites are indicated with vertical dotted lines, the analysed *FLC* regions are numbered with Roman numerals and the anchor region is highlighted with a grey-shadowed area. A schematic representation of the *FLC* locus is shown above. (**B**) RNA expression analysis of 10-day non-vernalized seedlings from different genotypes. The graph represents the ratio *FLC*/*UBC* normalized to Col WT. The error bars indicate s.e.m. ($n = 3$).

There was no PCR amplification when plant material was not crosslinked or ligase was not included in the 3C reactions, indicating that the observed chromosomal interactions are not PCR artefacts. The discovery of a chromosomal loop containing the *FLC* gene raises the question of whether the loop plays a role in the different regulatory pathways that target the *FLC* locus.

High FLC expression levels do not enhance loop formation

The FRI complex is associated with increased *FLC* transcriptional activation and 5′ mRNA capping (Geraldo *et al*, 2009; Crevillen and Dean, 2010; Choi *et al*, 2011). Common laboratory accessions like Columbia (Col) carry naturally mutated versions of FRI, so we tested whether the *FLC* gene loop was influenced by the FRI-dependent increase in expression using Col-FRI seedlings—Col plants into which an active *FRI Sant Feliu* allele had been introgressed (Michaels and Amasino, 1999). Col-FRI has 30- and 25-fold increase in *FLC* RNA unspliced and mRNA levels, respectively (Figure 2B) but no major difference in chromosome interaction was found compared with Col (Figure 2A).

In yeast, gene loop formation requires an interaction between general transcription factors and 3′ RNA processing factors (Ansari and Hampsey, 2005; Medler *et al*, 2011; Tan-Wong *et al*, 2012). However, mutations in Arabidopsis polyadenylation factors FCA and FPA did not disrupt the FI–FV interaction (Figure 2A) despite increasing *FLC* transcript levels 20-fold (Figure 2B). As well as influencing polyadenylation of the *FLC* antisense transcript FCA and FPA promote the use of proximal poly (A) sites in many other transcripts in the Arabidopsis genome (Sonmez *et al*, 2011). We found that loss of FCA and FPA leads to a small but significant reduction in use of the *FLC* major sense poly A sites (Feng *et al*, 2011; Supplementary Figure 1). A reduced interaction frequency between FI and FIV was observed in *fcafpa* compared with Col and Col-FRI (Figure 2A), suggesting the increased transcriptional read-through influenced the FI–FIV interaction.

FLC loop is not disrupted in mutants with low FLC expression levels

We asked if mutations in core chromatin remodelling components that impair *FLC* expression (Figure 2B) would perturb *FLC* loop formation. The deposition of histone H3 lysine 4 methylation at *FLC* promoter by the Arabidopsis Trithorax proteins ATX1 and ATX2 is required for high *FLC* expression (Pien *et al*, 2008). 3C analysis on seedlings of a Col-FRI *atx1-2 atx2-1* (*atx1atx2*) double mutant (Pien *et al*, 2008) showed that the *FLC* loop was not reduced compared with Col-FRI seedlings (Figure 3A). Mutations in Arabidopsis *ARP6* greatly reduce *FLC* transcript levels by affecting H2A.Z variant deposition at promoter and terminator regions of the gene (Choi *et al*, 2005; Deal *et al*, 2005; Martin-Trillo *et al*, 2006; Deal *et al*, 2007). We found that *FLC* RNA levels in *arp6-1* were reduced more than 5- and 100-fold compared with Col and Col-FRI, respectively (Figure 2B). Nevertheless, the interaction between the *FLC* 5′ and 3′ flanking regions was only moderately reduced in *arp6-1* seedlings (Figure 3B).

The lack of an association of loop formation with the expression state of the *FLC* locus is striking when one compares the expression differences of the genotypes used (Figures 2 and 3). There is a >10-fold increase in *fcafpa* or FRI compared with Col in *FLC* mRNA and unspliced RNA levels, the latter used as a proxy for transcriptional level; and a >5-fold reduction in *atx1,atx2*-FRI or *arp6* compared with Col-FRI or Col, respectively (Figure 2B).

FLC loop is disrupted as an early step during vernalization

We then investigated the relationship between the physical and transcriptional state of the *FLC* locus during vernalization, where *FLC* expression is downregulated by prolonged cold and then epigenetically silenced by the Polycomb

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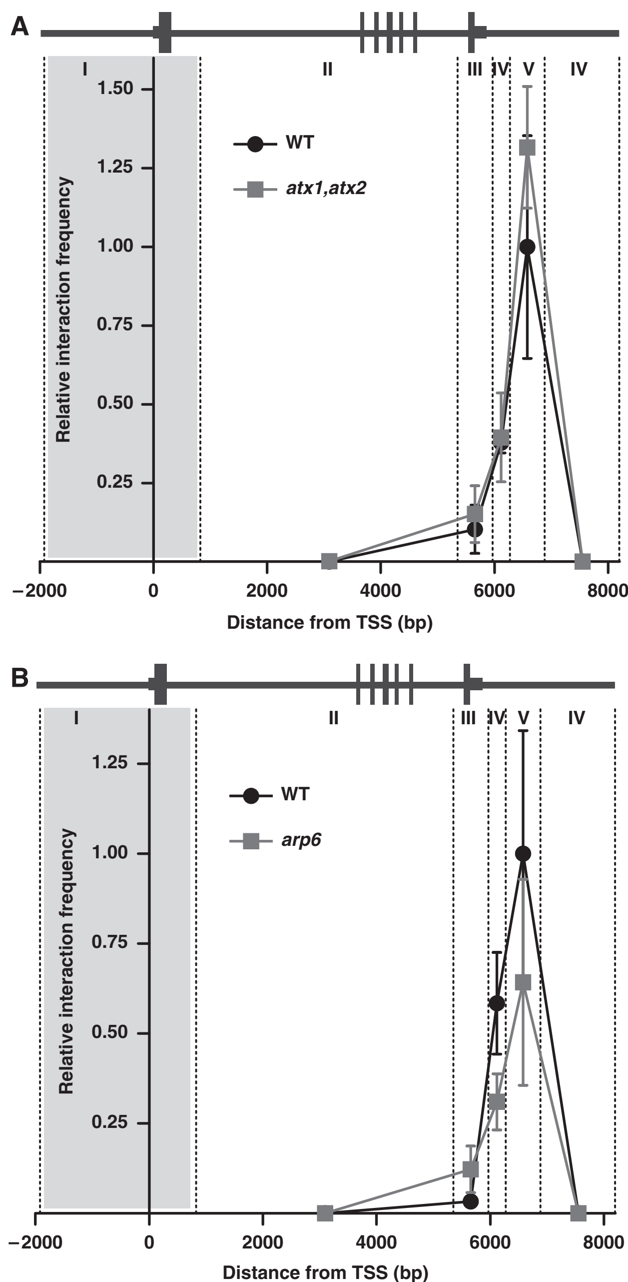

Figure 3 *FLC* loop formation in mutants with low *FLC* expression. Quantitative 3C of the *FLC* locus fragment FI as the anchor region. Relative interaction frequencies were calculated as described in Materials and methods. (**A**) Chromatin interactions in 10-day-old Col-FRI (WT) and *atx1atx2*-FRI seedlings. The data are the average of two biological replicates each with two technical replicates. The error bars indicate s.e.m. ($n = 4$). (**B**) Chromatin interactions in Col (WT) compared with *arp6-1* mutant 10-day-old seedlings. The data are the average of two biological replicates each with two technical replicates. The bars in the graphs indicate s.e.m. ($n = 4$). In the graphs, *Bam*HI and *Bgl*II restriction sites are indicated with vertical dotted lines, the analysed *FLC* regions are numbered with Roman numerals and the anchor region is highlighted with a grey-shadowed area. A schematic representation of the *FLC* locus is shown above each graph.

machinery (Song *et al*, 2012). In Arabidopsis, this epigenetic silencing overrides the high *FLC* levels induced by FRI activity or *fcafpa* mutations (Baurle and Dean, 2006). 3C experiments were undertaken on Col-FRI seedlings given 2 weeks of cold and then either harvested immediately (2WT0) when *FLC* transcription is shut-down (Swiezewski *et al*, 2009) and a PHD–Polycomb complex accumulates at the nucleation site in *FLC*; or 7 days later after further growth in the warm (2WT7) when H3K27me3 and the PHD–Polycomb complex spreads across *FLC* and the locus is epigenetically silenced (Finnegan and Dennis, 2007; De Lucia *et al*, 2008; Angel *et al*, 2011; Song *et al*, 2012). When 3C experiments were performed on Col-FRI vernalized seedlings, we found that the FI interaction with the 5′ flanking region of *FLC* locus was substantially reduced in plants grown for 2 weeks in the cold (Figure 4A). Interestingly, this non-looped conformation was maintained in the absence of cold during the subsequent growth of the plant in warm when *FLC* expression remains epigenetically silenced (Figure 4A). *FLC* gene loop disruption is not an immediate response to chilling temperatures as we could not detect reduced interaction frequencies after 5 days in the cold (Supplementary Figure 2). It is also not a genome-wide response to the cold because intragenic interactions detected within the housekeeping *UBC* locus (*At5g25760*; Czechowski *et al*, 2005) are not disrupted during the cold (Supplementary Figure 3). Interestingly, loop disruption parallels the timing of downregulation of *FLC* expression in the cold (Figure 4B).

Transcription of ncRNA can result in novel chromatin interactions as recently shown for the *Hox* genes and *Igf2/H19* locus in animal cells (Court *et al*, 2011; Wang *et al*, 2011). We considered the possibility that the induction of ncRNAs at *FLC* during the cold could generate alternative loops. At least two ncRNA are produced from *FLC*: *COLDAIR* (Heo and Sung, 2010) and *COOLAIR* (Swiezewski *et al*, 2009). *COLDAIR* sense ncRNA production does not overlap with either FI or FV and is induced after longer vernalization periods (∼3 weeks) (Heo and Sung, 2010), but FV likely contains *COOLAIR* promoter elements, so we reasoned that during the cold it could promote an alternative loop disrupting the FI–FV loop. We tested this model by looking for the appearance of novel chromosomal interactions during the cold of FV and FIV with FIII, FII or downstream *FLC* promoter (F0; see Figure 1). These fragments had very high interaction frequencies with nearby regions (Figure 5), as expected, due to random collisions of such fragments (Dekker *et al*, 2002). FV and FIV also showed high interaction frequency with FI confirming the previously detected *FLC* loop (Figures 2–4). We also confirmed that the FI–FIV and FI–FV interactions were disrupted by vernalization but no additional non-random interactions were detected between the fragments analysed (Figure 5). Thus, *COOLAIR* induction does not appear to promote new chromatin interactions that could disrupt the *FLC* gene loop formed in warm conditions.

FLC loop disruption is independent of Polycomb repression

Cold causes downregulation of *FLC* transcription independently of the cold-induced accumulation of VIN3 (Swiezewski *et al,* 2009). The dynamics of these cold-induced processes vary in different Arabidopsis accessions but in Col-FRI seedlings 2 weeks cold is sufficient to trigger both processes (Swiezewski *et al*, 2009). In order to analyse which cold-induced process was involved in loop disruption, we undertook 3C experiments on the *vin3* mutant. Cold-induced transcriptional downregulation occurs in this

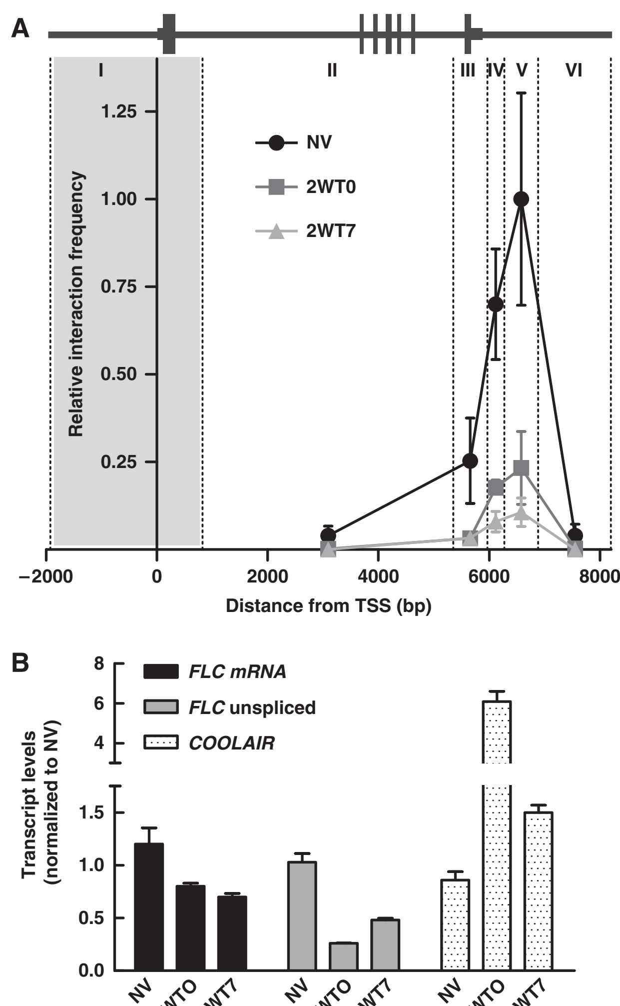

Figure 4 *FLC* loop is disrupted during and after cold exposure. (**A**) Quantitative 3C of the *FLC* the locus using FI as the anchor region in Col-FRI seedlings harvested after 10 days of growth in standard conditions (NV), immediately after 2 weeks in the cold (2WT0) and after 2 weeks in the cold and 7 days of further growth in the warm (2WT7). Relative interaction frequencies were calculated as described in Materials and methods. The data are the average of three biological replicates each with two technical replicates. The error bars indicate s.e.m. ($n=6$). In the graph, *Bam*HI and *Bgl*II restriction sites are indicated with vertical dotted lines, the analysed *FLC* regions are numbered with Roman numerals and the anchor region is highlighted with a grey-shadowed area. A schematic representation of the *FLC* locus is shown above. (**B**) RNA expression analysis of Col-FRI during vernalization. The graph represents the ratio *FLC*/*UBC* or *COOLAIR*/*UBC* normalized to Col-FRI non-vernalized. The error bars indicate s.e.m. ($n=3$).

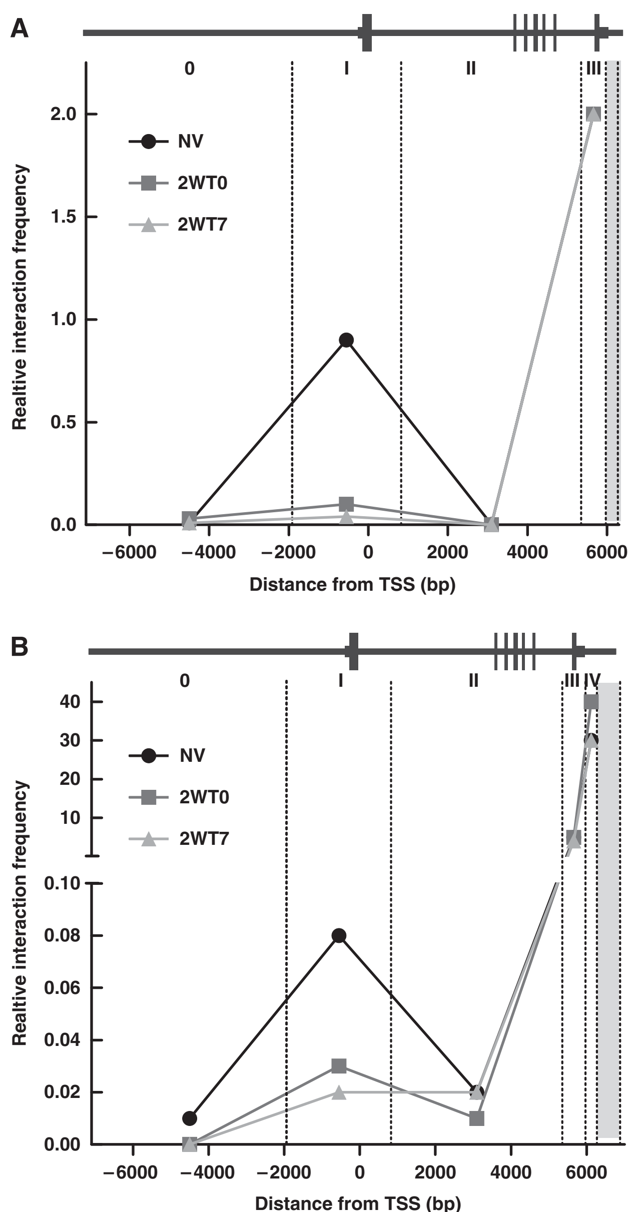

Figure 5 Search for alternative *FLC* gene loops induced by vernalization. (**A**) Quantitative 3C analysis using FIV as the anchor region. (**B**) Quantitative 3C analysis using FV as the anchor region. Col-FRI seedlings were harvested after 10 days of growth in standard conditions (NV), immediately after 2 weeks in the cold (2WT0) and after 2 weeks in the cold and 7 days of further growth in the warm (2WT7). Relative interaction frequencies were calculated as described in Materials and methods. The data are the average of two biological replicates. In the graphs, *Bam*HI and *Bgl*II restriction sites are indicated with vertical dotted lines, the analysed *FLC* regions are numbered with Roman numerals and the anchor region is highlighted with a grey-shadowed area. A schematic representation of the *FLC* locus is shown above each graph.

mutant but epigenetic silencing is impaired (Sung and Amasino, 2004; Greb *et al*, 2007). The *FLC* loop was similarly disrupted by 2 weeks vernalization in Col-FRI and *vin3-4* FRI mutant seedlings (Figure 6A). These data indicate that loop disruption is independent of VIN3 function, suggesting that loop disruption is associated with the early transcriptional downregulation of *FLC*.

Altering genomic location does not influence loop formation or disruption

We wondered if the genomic context of the endogenous *FLC* locus was important for loop formation. To address this we used an *FLC*::*LUC* transgenic line (Mylne *et al*, 2004; Greb *et al*, 2007), with the transgene inserted on chromosome 3 rather than the native position on chromosome 5, in a FRIGIDA *flc-2* genetic background (Michaels and Amasino, 1999). The *FLC*::*LUC* transgene does not introduce any new restrictions site or affect the primer sets used in our study (Mylne *et al*, 2004), and *flc-2* carries a large deletion that removes a large section of the endogenous *FLC* gene (Michaels and Amasino, 1999). The data in Figure 6B show

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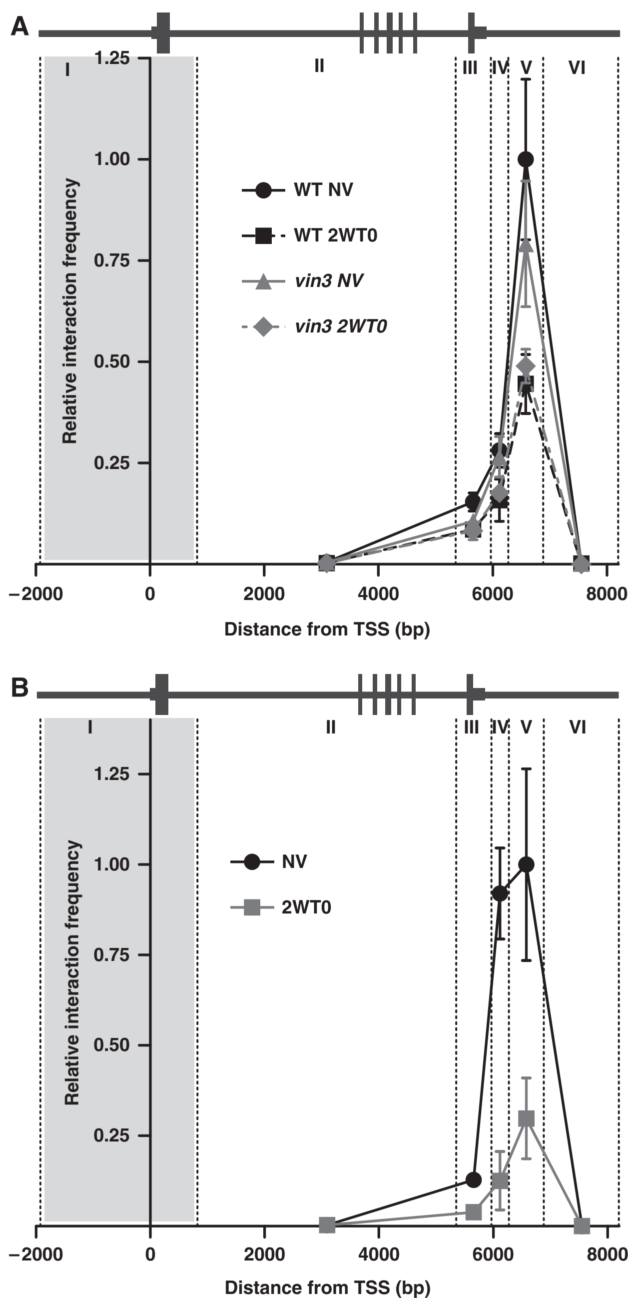

Figure 6 *FLC* loop is disrupted independently of Polycomb complexes or the genomic context of the locus. Seedlings were harvested after 10 days of growth in normal conditions (NV) and immediately after 2 weeks in the cold (2WT0). (**A**) Quantitative 3C of the *FLC* locus using FI as the anchor region in Col-FRI (WT) and *vin3*-FRI (**B**) Quantitative 3C of the *FLC::LUC* transgene in *flc*-2 background. Relative interaction frequencies were calculated as described in Materials and methods. The data are the average of two biological replicates each with two technical replicates. The error bars indicate s.e.m. ($n = 4$). In the graph, *Bam*HI and *Bgl*II restriction sites are indicated with vertical dotted lines, the analysed *FLC* regions are numbered with Roman numerals and the anchor region is highlighted with a grey-shadowed area. A schematic representation of the *FLC* locus is shown above each graph.

that the *FLC::LUC* transgene forms a gene loop and that, as detected with the endogenous *FLC* locus, the loop is disrupted by vernalization. Thus, formation and disruption of the *FLC* loop does not depend on the chromosomal position.

Discussion

We have demonstrated a gene loop involving the 5′ and 3′ flanking regions of the *FLC* locus in Arabidopsis seedlings. Thus, we can now extend the concept of gene looping, previously described for yeast and mammalian genes, to plant gene expression. We found that *FLC* loop formation is independent of expression level but is disrupted in the early phase of vernalization (Figure 4A). This coincides with downregulation of transcription and accumulation of antisense transcripts to *FLC* (Figure 4B). Loop disruption occurs independently of VIN3, the cold-induced PHD protein involved in triggering the cold-induced epigenetic silencing at *FLC*. We suggest that gene loop disruption occurs as an early step associated with cold-induced transcriptional shut-down of *FLC* but preceding and independently of the Polycomb-mediated accumulation of epigenetic silencing.

Gene looping is tightly associated with transcriptional regulation in yeast. In the case of *FLC*, we cannot rule out that the formation of the loop requires initial rounds of transcription but the presence of the loop is not affected by mutations that significantly increase or reduce *FLC* gene expression (Figures 2 and 3). All these data suggest that gene looping and *FLC* transcription have different dynamics. In yeast, gene looping plays a role in ‘transcriptional memory’—where rapid gene reactivation depends on the persistence of the loop in non-induced conditions (Laine *et al*, 2009; Tan-Wong *et al*, 2009). Loop function in transcriptional memory in yeast has been associated with locus-specific H2A.Z-mediated localization at the nuclear periphery (Brickner *et al*, 2007; Tan-Wong *et al*, 2009). However, *FLC* gene loop is still present in *arp6* mutant (Figure 3A), which argues against this association for Arabidopsis *FLC*.

The genomic region defined by the FI–FV *FLC* gene loop (Figure 1) corresponds exactly to the genomic region accumulating very high levels of H3K27me3 during vernalization (Angel *et al*, 2011). This feature and the relatively high frequency of interaction of FI–FV suggest that the loop could delimit a functional chromosomal domain. Factors defining such chromosomal domains are unclear but topologically associated domains have been proposed to delimit genomic regions in which genes are co-regulated and distinctly regulated from adjacent domains (Splinter *et al*, 2011). Given the complex regulation of *FLC* and the relatively compact Arabidopsis genome, some kind of physical isolation of this important developmental regulator may be functionally important. This domain is independent of the genomic context of *FLC* as loop formation and normal vernalization response are observed when *FLC* is integrated into random locations in the genome (Figure 6B).

We asked whether alternative chromatin interactions would form at different phases of the cold-silencing process, perhaps stimulated by expression of the *COOLAIR* antisense transcripts. However, no alternative loops were identified during *COOLAIR* antisense induction (Figure 5). This is similar to what has been reported for the yeast *GAL10* locus where a gene loop is disrupted despite the presence of antisense transcription (Laine *et al*, 2009; Murray *et al*, 2012). A recent report has shown gene loops prevent divergent transcription from otherwise bidirectional promoters (Tan-Wong *et al*, 2012). We have not observed any divergent transcription from either *FLC* or *COOLAIR*

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promoters in the cold based on our custom tiling array analysis (Swiezewski *et al*, 2009; Angel and Dean, unpublished results); however, these analyses did not include Arabidopsis genotypes deficient in exosome function.

It has been reported that multiple Polycomb-dependent long chromosomal loops maintain human *GATA-4* gene locus in a silenced but inducible state (Tiwari *et al*, 2008). To our knowledge there are no reports linking gene looping and Polycomb silencing. The independence of the *FLC* gene loop from VIN3 suggests that it is different from changes in chromosomal conformation previously described for non-plant Polycomb targets (Delest *et al*, 2012). The gene-specific looping detected here does not exclude additional long-range chromosomal interactions occurring before, during or after vernalization. Polycomb components modulate three-dimensional genome architecture through formation of long-range loops and clustering of targets at discrete nuclear foci called Polycomb bodies (Lanzuolo *et al*, 2007; Bantignies *et al*, 2011; Delest *et al*, 2012). It is therefore possible that *FLC* loop disruption is just one of many chromosome conformation changes during vernalization (Delest *et al*, 2012; Moissiard *et al*, 2012).

From all our data we propose that the *FLC* gene loop provides a transcriptional memory that is independent of actual *FLC* mRNA expression levels. As an early step in vernalization, cold induces disruption of the loop coincident with the transcriptional downregulation. We favour a model where this loop disruption reveals *COOLAIR* promoter elements, thus contributing to higher antisense transcription that aids shut-down of *FLC* expression. The observed time course of events supports this; loop disruption happens very early in the cold (Supplementary Figure 2) before antisense transcription reaches its maximum levels (Swiezewski *et al*, 2009; Heo and Sung, 2010). However, more detailed analyses will be required to fully elaborate cause and effect. After further cold, PHD–Polycomb complexes form at the internal nucleation site and H3K27me3 quantitatively accumulates. Upon transfer back to warm conditions, H3K27me3 and PHD–Polycomb complexes spread over *FLC* locus that flips the locus into an epigenetically repressed state. This state would impede further loop formation, maybe because Polycomb silencing impairs specific chromosomal interactions or because *FLC* is sequestered into a different nuclear compartment. Our work reveals an important role for gene loops in plant gene regulation in response to environmental signals and raises the possibility that loop disruption is a general early step during the switch to an epigenetically silent state.

Materials and methods

Plant material and growth conditions

Seeds were sown on GM media plates, stratified for 2 days and grown in long-day conditions for 10 days (16-h light at 20°C, 8-h darkness at 16°C). To vernalize, seeds were pre-grown for 7 days at standard warm-growing conditions (16-h light at 20°C, 8-h darkness at 16°C) before being transferred to cold (8-h light and 16-h darkness at 5°C) for 2 weeks, and then returned to warm conditions for 7 days.

Chromosome Conformation Capture (3C)

3C assays were performed according to Louwers *et al* (2009b) with some modifications. Arabidopsis seedlings (2 g) were crosslinked with 2% formaldehyde PBS buffer at room temperature for 20 min. Nuclei were purified and treated with SDS 0.3% at 65°C for 20 min. SDS was sequestered with 1% Triton X-100. Digestions were performed overnight at 37°C with 600U *Bam*HI and *Bgl*II. Restriction enzymes were inactivated by addition of 1.6% SDS and incubation at 65°C for 10 min. After that 2% Triton X-100 was added to sequester SDS. Ligations were performed at 16°C for 5 h in 5-ml volume using 50 U of T4 DNA ligase. Reverse crosslinking was performed by overnight treatment at 65°C. DNA was recovered after Proteinase K treatment by phenol/chloroform extraction and ethanol precipitation.

3C quantification, normalization and controls

Relative interaction frequencies (Hagege *et al*, 2007) were calculated by Q-PCR on a Roche LightCycler 480 system using SYBR Green I Master mix from the same supplier. Differences in DNA concentration between samples were normalized using a loading control (LC), an *FLC* primer set that does not span any restriction site. To compensate for different primer efficiencies during PCR, we normalized to a control template (CT) DNA including all possible ligation products in equimolar amounts. The CT DNA was generated by digesting purified *FLC-15* plasmid with *Bam*HI and *Bgl*II, and subsequent random ligation without dilution. *FLC-15* carries a 20-kb genomic DNA fragment containing the whole *FLC* locus (∼6 kb). In the case of *UBC* locus, the CT was generated by mixing equimolar quantities of purified PCR products. All figures in this work are the average of at least two biologically independent samples; each 3C DNA preparation was quantified twice by independent Q–PCR experiments; and each Q–PCR experiment included triplicates of all DNA samples. The values represented on each graph are relative interaction frequencies relative to the value of FI–FV in Col-FRI or Col. The full list of primers used in this work and an example of the calculations performed can be found in the Supplementary Information.

The quality of each experiment was assessed using two controls. First, we monitored the restriction efficiency using Q–PCR on chromatin aliquots taken before and after digestion. Under our experimental conditions, we found that restriction of crosslinked chromatin was between 70–90% of total genomic DNA. Second, genomic *Bgl*II and *Bam*HI digestion resulted in fragments of the control locus *UBC,* which is unrelated to *FLC* and is routinely used to normalize mRNA expression data in our laboratory (Czechowski *et al*, 2005). The interaction frequencies of two fragments of *UBC* were quantified by quantitative 3C using the primers UBC_b2F and UBC_d2F. Experiments in which UBC 3C values were lower than the average indicated a failure in the experimental procedure (nuclei isolation, ligation, etc), and were subsequently discarded and not considered in the calculations.

RNA expression analysis

Hot-phenol total RNA extraction, DNAse I treatment, cDNA synthesis and Q-PCR analyses was performed as described by Sonmez *et al* (2011) using TURBO DNA-free (Life Technologies), Superscript III reverse transcriptase (Life Technologies) and SYBR Green I Master mix (Roche). Each data point is based on nine PCR reactions from three biological replicates.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: PC and CD designed the research; PC and CS performed chromosome conformation capture experiments; ZW performed RNA expression analysis; PC and CD wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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