Vernalization-Repression of Arabidopsis FLC Requires Promoter Sequences but Not Antisense Transcripts

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Abstract

The repression of Arabidopsis FLC expression by vernalization (extended cold) has become a model for understanding polycomb-associated epigenetic regulation in plants. Antisense and sense non-coding RNAs have been respectively implicated in initiation and maintenance of FLC repression by vernalization. We show that the promoter and first exon of the FLC gene are sufficient to initiate repression during vernalization; this initial repression of FLC does not require antisense transcription. Long-term maintenance of FLC repression requires additional regions of the gene body, including those encoding sense non-coding transcripts.

Introduction

The Arabidopsis FLC gene is a repressor of flowering that confers a requirement for vernalization (a long period of cold, such as occurs during winter) to promote flowering in spring [1,2]. FLC encodes a MADS box protein that binds to and represses expression of the floral promoting genes FT and SOC1 [3,4] in addition to regulating other developmental pathways [5].

Vernalization leads to the stable repression of FLC by a plant homeodomain-polycomb repressive complex 2 (PHD-PRC2) mechanism [6,7] that results in increased abundance of H3K27me3 at the FLC locus. Detailed studies of the timing of changes in FLC mRNA expression and H3K27me3 levels showed that transcription of FLC is repressed and H3K27me3 increases at a region around the transcription start site during the cold [8]. After plants are returned to warm conditions, the level of H3K27me3 increases across the gene body and this is associated with the maintenance of repression of FLC transcription [8]. Plants with reduced PRC2 function have increased FLC expression and reduced repression of FLC after vernalization [6,9], suggesting that the presence of H3K27me3 at the FLC locus is important in down-regulating its expression pre- and post-vernalization. Experiments to define the parts of the FLC gene mediating the different phases of the vernalization response showed that the promoter and first exon are sufficient to confer repression of a reporter gene during vernalization, but maintenance of repression after return to warm conditions requires the first intron in addition to the promoter and exon 1 [10]. An FLC gene fragment including approximately 1.8 kb of FLC intron 1 and the remainder of the 3′ end of the gene recruits PRC2 and H3K27me3 in the absence of transcription [11]; it is suggested that this property is responsible for maintaining FLC repression after vernalization.

A complex array of non-coding RNAs is transcribed from eukaryotic genomes, the majority of which are of unknown function. Some long non-coding RNAs (lncRNAs) associate with and target protein complexes to regulate gene expression [12,13]; these include the well-characterised lncRNA, HOTAIR, which targets PRC2 to the HoxD locus, and is associated with HoxD silencing in humans [14,15]. Study of lncRNAs in plants is still in its infancy, but two classes of lncRNAs produced from the FLC locus have been identified. The COOLAIR antisense transcripts originate from a promoter adjacent to the FLC 3′ untranslated region and consist of two classes, terminating at proximal or distal sites (Figure 1) [16,17,18]. Antisense transcript levels increase during vernalization prior to the decrease of FLC mRNA abundance. COOLAIR promoter-driven antisense transcription of a reporter gene confers transient cold-induced repression [19]. This led to the suggestion that induction of antisense transcription is an early event in the mechanism causing vernalization-induced repression of FLC, acting upstream of PHD-PRC2 [19]. The second class of lncRNAs are sense transcripts (termed COLDAIR) originating from a region within the first intron of FLC [20]. The COLDAIR transcript has been shown to interact with PRC2 and its abundance also increases during vernalization. Reduction of COLDAIR transcript levels by RNAi showed that it is not required for the initial repression of FLC but is required for subsequent maintenance of repression.

As there are apparent contradictions in the proposed role of the COOLAIR antisense transcripts in the initial vernalization-induced repression of FLC and the results of reporter gene studies we used FLC insertion mutants to test the role of these transcripts in FLC repression.

Results and Discussion

As the FLC promoter and first intron are required for the stable vernalization-mediated down-regulation of a reporter gene we tested the effect of a Ds insertion of approximately 6 kb (flc-20) that...
separates these regions in the endogenous FLC gene [21]. Using the abundance of FLC $5'$ unsliced transcript as an approximation of transcription rate [18] we showed that vernalization leads to a reduction of transcription in the flc-20 mutant that was not maintained after plants were returned to warm conditions (Figure 1A). The results of physically separating the FLC promoter and first intron thus mirror those for reporter constructs showing that the intron and promoter together are required for the stable repression of FLC expression by vernalization. Examination of antisense transcripts in these lines showed that transcription from the COOLAIR promoter was greatly reduced in flc-20 and was only weakly induced during the cold compared to the C24 control (Figures 1B and 1C). An antisense transcript was detected $5'$ of the Ds insertion in flc-20 that we speculate originates from within the Ds element; in contrast to the situation in C24 this transcript was repressed by cold. There is an increase of H3K27me3 across the first exon of FLC (amplicon 5b) in both C24 and flc-20 at the end of cold, but this increase is not...
maintained in flc-20 after return to warm temperatures (Figure 1E), suggesting that sequences in intron 1 are required to maintain a repressed chromatin state in this region. Sites 3′ of the Ds element are marked with H3K27me3 before vernalization [11] and there is no increase following vernalization (Figures 1F and S1).

The data from the flc-20 mutant together with previous data from FLC reporter constructs raised the question of whether
antisense transcription from the COOLAIR promoter is a requirement for vernalization-induced repression of FLC. To test this we carried out further experiments with T-DNA insertions close to the 3’ end of the FLC gene that disrupt the distal (SALK_092716) or both proximal and distal (SALK_140021) antisense transcripts (Figures 2A and 2B). The T-DNA lines were crossed to ColFRI [22] to introduce an active allele of FRI to activate FLC transcription; plants homozygous for the T-DNA insertion and the active FRI allele were used in subsequent experiments. Sense transcription of FLC is stably repressed by cold when the distal antisense or both distal and proximal antisense transcripts are absent (Figure 2C). In addition, plants in which antisense transcript levels do not increase during cold (SALK_131491) showed normal repression of FLC. The changes in H3K27me3 in the T-DNA insertion lines mirrored those of the wildtype FLC allele in ColFRI (Figures 2D–G and S2), with the exception of regions 3’ of the SALK_092716 insertion which had high H3K27me3 under all conditions as seen previously for non-transcribed parts of FLC [11]. The remaining two insertion lines, SALK_140021 and SALK_131491, generally had lower levels of H3K27me3 at amplicon 11 than wild-type plants (Figure 2G).

These data show that the production of COOLAIR transcripts is not an essential component of vernalization-induced repression of FLC. The observations presented here for these SALK lines are in agreement with previous reports showing that sequences consisting of the promoter, exon 1 and intron 1 but not the COOLAIR promoter, are sufficient to confer stable repression on a reporter gene [10]. While the COOLAIR lncRNAs may play a redundant role in regulating FLC expression, our data shows that they are not required for the vernalization response.

Our data are consistent with the COOLAIR lncRNA acting to maintain repression of FLC by recruiting the PRC2 machinery [23]. None of the T-DNA insertions tested interrupt the COOLAIR transcript and all show maintenance of repression after vernalization.

To further investigate factors required for the initial repression of FLC, we measured 5’ unspliced FLC transcripts in the swn7clf28 double mutant and in the vin3-4 mutant. CLF and SWN encode histone methyl transferases components of PRC2; loss of function of these genes leads to a genome-wide loss of H3K27me3 [24]. The swn7clf28 plants have increased 5’ unspliced FLC transcript in non-vernalized plants and show a similar fold-repression as ColFRI during 4 weeks of cold exposure (Figure 3A). However this repression is not maintained after plants are returned to warm conditions in agreement with previous reports that the PRC2 complex functions in the maintenance of repression rather than initiation [9,25]. The vin3-4 mutant showed a different pattern of FLC repression with an initial reduction in transcription after 1 week in the cold that was not maintained during subsequent weeks in the cold (Figure 3A). Previous reports showed no decrease in mature FLC mRNA [26] in vin3 during vernalization which we confirmed (Figure 3B). These data suggest that although VIN3 interacts with the PRC2 complex it may have an additional role in establishing repression of FLC before the addition of H3K27me3 to FLC by PRC2.

The mechanism of the initial repression of FLC remains unknown, with none of the genes or other factors identified as being involved in the vernalization-induced repression of FLC to date being required for the initial repression by cold. Our data suggest that under our growth conditions the reduction of FLC transcription activity is saturated after 1 week of cold. However this treatment is not sufficient to saturate the vernalization response in ColFRI suggesting that subsequent events in the cold are required to establish a repressed chromatin state at FLC that is
reinforced by the addition of H3K27me3 across the whole gene body after return to warm growing conditions.

**Materials and Methods**

Salk insertion mutants were obtained from the Arabidopsis Stock Center (www.arabidopsis.org) and crossed with ColFRI, a Col line with an active FRI allele [22]. The presence of an active FRI allele activates expression of FLC. PCR was used to identify F2 plants in which the active FRI allele and the T-DNA insertions were homozygous. The flc-20 mutant contains a modified Ds element inserted in the first intron of FLC [21].

Plants were grown on MS agar plates in a 16 h light:8 h dark period under fluorescent light at 22°C. Vernalization was at 4°C.

RNA was extracted using Qiagen Plant RNeasy Mini columns with an on-column DNase treatment according to the manufacturer's protocol. cDNA was synthesized using Superscript III (Invitrogen), primed with oligo dT (distal and proximal COOLAIR transcripts) or with gene specific primers for the FLC 5’ unspliced transcript. Quantitative real-time PCR was carried out using an Applied Biosystems 7900HT instrument. Reactions were carried out in a total volume of 10 μl with Platinum Taq DNA polymerase (Invitrogen). Primers used are listed in Table S1. PCR were reactions carried out in quadruplicate, quantified using a standard curve of diluted cDNA and normalized to At4g26410 [27].

Chromatin immunoprecipitation was carried out and amplicons for ChiP-qPCR are as described [11]. Primer sequences are given in Table S1.

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