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

Pedro Crevillen, Cagla Sonmez, Zhe Wu and Caroline Dean

Corresponding author: Caroline Dean, John Innes Centre

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 July 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see below, the referees find the analysis interesting albeit a bit preliminary as we gain limited insight into mechanism of loop formation/disruption or function of the loop. Despite the preliminary nature of the paper, the referees do support publication in the EMBO Journal. Given the referees’ comments, I would like to invite you to submit a suitably revised manuscript, addressing the comments of all three reviewers. Some further data is needed to support the 3C analysis. It would also be good if you could add some mechanistic insight into loop disruption. Referee #3 suggests to look if loop disruption is linked to COOLAIR transcription. I don't know if you have data on hand to address this issue, but anything along those lines will clearly strengthen the paper.

When preparing your letter of response to the referees’ comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
The EMBO Journal

REFEREE REPORTS

Referee #1

This manuscript by Crevillen et al., described a gene loop that is formed within the floral repressor gene FLC. The main method that the authors employed in this study is the 3C (chromosome conformation capture) technique. The data of 3C is presented in the form of histograms. It would be better if the raw data from qPCR assays is provided, and the underlying calculations that led to the individual data points in the graphs are presented, in addition to the generalized statements in the Method section. This will make it easier for the readers to understand the approach and the reviewers to evaluate the results. Furthermore, in addition to controls used in qPCR, it would be better if the formation of gene loop in the FLC locus can be validated by another approach (maybe as easy as using a couple of different restriction enzymes in the 3C experiment?). The major findings of this manuscript are: 1) 5' and 3' flanking regions of FLC interact with each other; 2) this short-range interaction, which can be referred to as gene loop, can be disrupted by vernalization; and 3) the formation of this gene loop is only dependent on the sequences of the FLC locus but not on the genomic contexts that the endogenous FLC locus resides in. Besides, the authors also tested several other intriguing hypotheses, such as whether gene loop formation is affected by the expression levels of FLC, whether alternative gene loop can be induced by the transcription of a non-coding RNA specific for the FLC locus, and whether gene loop disruption is dependent on Polycomb repression. However, the results from these experiments are negative. A minor comment here is that, for Figures 2 and 3, it is better to show FLC expression data in the respective genotypes. Although FLC expression data in these lines can be found in earlier studies, it is still necessary to confirm the effect on FLC expression under the conditions used in the current study. Overall, this work seemed a bit preliminary, as it only describes an interesting phenomenon (see three major findings above), and the attempts to reveal its mechanism, function, or relationship with other regulatory systems at work at FLC locus, have not yet borne fruit. Nonetheless, this report is interesting and I support publication.

Referee #2

Comments for transmission to authors

Gene looping is the physical interaction of the promoter and terminator regions of a gene in a transcription-dependent manner. It is a newly discovered transcription regulatory mechanism that has been demonstrated for several genes in budding yeast and a few genes in mammalian systems. The physiological significance of gene looping is, however, not entirely clear. It is also not known how prevalent gene looping is in eukaryotic systems. The manuscript by Crevillen et al., extends the concept of gene looping to plants. Using 3C analysis, the authors demonstrated that the floral repressor gene FLC of Arabidopsis exists in a looped configuration during transcription. Gene looping of FLC was not affected appreciably by the factors that modulate transcriptional efficiency of the gene. The vernalization-induced repression of FLC, however, resulted in an almost complete abolition of gene looping. The authors' conclusions suggest that gene looping serves as an ON/OFF switch for the expression of FLC gene, but is not affected by the level of transcription of the gene. While these observations make a novel and interesting scientific story, several controls and data extensions, listed below, are needed to fully validate these results.

Major points:
1) The paper describes 3C analysis of FLC gene under different conditions. None of the 3C results are, however, supported by the expression analysis of FLC. A meaningful analysis of 3C results in the context presented here requires that the expression data of FLC should accompany every 3C result. This can be done either by RT-PCR or Northern blot analysis. Referring to the published results of FLC expression is not sufficient.
2) In the 3C analysis shown in Fig. 5, FIII and FIV were used as anchor regions and it was shown
that both these regions interact with the FI region. To strengthen the specificity of these interactions, it is important to show that FII and FIV are not interacting with the region upstream of FI. The authors should therefore add another data point in their 3C analysis by choosing a site upstream of the FI region and checking its interaction with FIII and FIV.

(3) The paper demonstrates the vernalization-induced loss of FLC looping signal. This result could be further strengthened by showing that looping of another gene, probably a housekeeping gene whose expression is not regulated by vernalization, remained intact under these conditions. Since FLC is the first plant gene shown to form a loop, a demonstration of transcription-dependent looping of another gene could also help to reinforce the concept in plants.

Minor points:
(1) Since the authors are showing the first reported case of gene looping in plants, they should include appropriate 3C controls in the Supplementary Information. These include the 'ligation control' to show that 3C signals are ligation dependent, and the 'formaldehyde crosslinking control' to show that the observed signals are due to random interaction of chromosomal regions.
(2) There was no effect of fcafpa double mutation on gene looping of FLC (Fig. 2). The double mutation has the potential to affect 3' end processing and termination of transcription. The 3' end processing/termination factors have been shown to be essential for gene looping in yeast and mammalian cells. Northern blot analysis of FLC in fcafpa double mutant may therefore help address the issue if 3' end processing/termination of transcription are required for gene looping in plants.
(3) The authors show that the anti-sense transcription of COOLAIR RNA initiating from the 3' end of FLC was not accompanied by gene looping. A similar observation has been reported in yeast. The transcription of GAL10 is accompanied by gene looping (Laine et al., 2009). The GAL10 gene loop is completely disrupted upon transcriptional repression of the gene. This is despite 3' end initiated anti-sense transcription during the repressed state of the gene (Murray et al., 2011, NAR). The authors should cite this to strengthen their results with COOLAIR.

Referee #3

The authors of this manuscript address the question whether regulation of the Arabidopsis thaliana floral repressor FLC involves changes in the three-dimensional chromatin configuration. By applying quantitative Chromosome Conformation Capture (3C) they identify a chromosomal loop between the 5' and 3' flanking regions of FLC. By analyzing loop formation in different mutants they conclude that loop formation is independent of the FLC transcriptional status. Instead, they found loop disruption after vernalization and hypothesize that loop disruption might involve transcription of the antisense RNA COOLAIR. They furthermore show that loop formation and vernalization-dependent loop disruption occur in an FLC transgene as well; revealing that loop formation is not dependent on the native genomic context.

Thus far, the importance of three-dimensional changes in chromatin conformation for gene regulation has been rather neglected and the data shown in this manuscript revealing the presence of such chromatin conformational changes at the FLC locus is definitely of interest. The manuscript could be strengthened by a deeper investigation of the underlying mechanism causing loop disruption. The authors hypothesize that this might be connected with COOLAIR transcription. It would be interesting to see evidence for this hypothesis. One possibility would be to test whether transgenic lines that contain deletions of the COOLAIR promoter maintain the loop upon exposure to cold. Alternatively, mutants that interfere with COOLAIR transcription could be tested whether they affect loop disruption.

Other comments:
1. The developmental stage of vernalized and non-vernalized plants differs and it is possible that loop disruption is a consequence of increasing age, rather than of cold exposure. One possibility to exclude developmentally caused effects is to vernalize seeds instead of seedlings, allowing comparison of vernalized and non-vernalized plants of similar developmental stage.

2. The interaction frequency between different experiments differ (whereas interaction in wild type for fragment V is 0.4 in Fig. 2, it is only 0.15 in Fig. 3B, which is even less than the interaction value after vernalization shown in Fig. 6A. This requires explanation.
Response to referees: Crevillén et al, EMBO J

Referee #1:

“It would be better if the raw data from qPCR assays is provided, and the underlying calculations that led to the individual data points in the graphs are presented, in addition to the generalized statements in the Method section.”

We have edited the Methods section and added an example of how the calculations were performed in the Supplementary Information.

“It would be better if the formation of gene loop in the FLC locus can be validated by another approach (maybe as easy as using a couple of different restriction enzymes in the 3C experiment?)”.

In addition to BglII and BamHI we tried ScaI and AvaII but they did not digest efficiently in our experimental conditions. Unfortunately, not all restriction enzymes digest crosslinked chromatin and not all suitable 3C enzymes cut FLC in a way that is compatible with our promoter - 3’region analysis.

“A minor comment here is that, for Figures 2 and 3, it is better to show FLC expression data in the respective genotypes”.

We performed a new RNA expression analysis of all the genotypes used in this work and added two figures to the manuscript (Figures 2B and 4B).

Referee #2:

Major points

(1) “A meaningful analysis of 3C results in the context presented here requires that the expression data of FLC should accompany every 3C result.”

As mention before, we have included new data regarding FLC RNA expression.

(2) “In the 3C analysis shown in Fig. 5, FIII and FIV were used as anchor regions and it was shown that both these regions interact with the FI region. To strengthen the specificity of these interactions, it is important to show that FIII and FIV are not interacting with the region upstream of FI. The authors should therefore add another data point in their 3C analysis by choosing a site upstream of the FI region and checking its interaction with FIII and FIV.” (We understand that the referee meant FIV and FV which are the fragments used as anchor points in Figure 5)

We designed a new primer downstream FLC promoter; in a region we called F0. This primer was used to check the interaction between F0 to FIV or FV and we found a very low interaction frequency. This new data was added to Figure 5.

(3) “The paper demonstrates the vernalization-induced loss of FLC looping signal. This result could be further strengthened by showing that looping of another gene, probably a housekeeping gene whose expression is not regulated by vernalization, remained intact under these conditions.”

We detected some new chromosomal interactions between the promoter region of the UBC locus and some internal regions of this gene. These chromosomal interactions do not change with vernalization. A new graph showing these data has been included in the supplementary information of paper. These data reinforce our idea that FLC loop disruption it is not only a response to cold but to vernalization.
Minor points:

(1) “Since the authors are showing the first reported case of gene looping in plants, they should include appropriate 3C controls in the Supplementary Information. These include the ‘ligation control’ to show that 3C signals are ligation dependent, and the ‘formaldehyde crosslinking control’ to show that the observed signals are not due to random interaction of chromosomal regions.”

In our previous version, we indicated that “chromosomal interactions were not observed without formaldehyde crosslinking” (line 9, page 5). What we mean is that without ligase or without crosslinking we are not able to amplify any DNA at all by standard PCR or Q-PCR, indicating that our PCR reactions are specific for 3C products. We have edited the text to make this observation clear to the reader.

(2) “There was no effect of fcafpa double mutation on gene looping of FLC (Fig. 2). The double mutation has the potential to effect 3’ end processing and termination of transcription. The 3’ end processing/termination factors have been shown to be essential for gene looping in yeast and mammalian cells. Northern blot analysis of FLC in fcafpa double mutant may therefore help address the issue if 3’ end processing/termination of transcription are required for gene looping in plants.”

We added extra data to the manuscript addressing this fact (Supplementary Figure 1).

(3) “The authors show that the anti-sense transcription of COOLAIR RNA initiating from the 3’ end of FLC was not accompanied by gene looping. A similar observation has been reported in yeast. The transcription of GAL10 is accompanied by gene looping (Laine et al., 2009). The GAL10 gene loop is completely disrupted upon transcriptional repression of the gene. This is despite 3’ end initiated anti-sense transcription during the repressed state of the gene (Murray et al., 2011, NAR). The authors should cite this to strengthen their results with COOLAIR”.

We are grateful to the referee for this suggestion and have included these references in the new version.

Referee #3:

“The authors hypothesize that this might be connected with COOLAIR transcription. It would be interesting to see evidence for this hypothesis. One possibility would be to test whether transgenic lines that contain deletions of the COOLAIR promoter maintain the loop upon exposure to cold. Alternatively, mutants that interfere with COOLAIR transcription could be tested whether they affect loop disruption”

We have generated transgenic lines where the COOLAIR promoter (and corresponding sense 3’ region) is swapped with another plant terminator. However, as recently found in yeast, this did not result in complete disruption of the antisense transcription, preventing us addressing whether antisense transcription is required for disruption of the loop. We also do not have mutations that reduce COOLAIR transcription. We actually favour the opposite scenario to the referee due to the timing of the observed changes, namely that loop disruption enables antisense transcription; the loop is disrupted at ten days in the cold (Supplementary Figure 2), whereas the antisense transcription takes more than two weeks to be maximal. We have modified the paragraph in the Discussion to reflect this.

Other comments:

1. “The developmental stage of vernalized and non-vernalized plants differs and it is possible that loop disruption is a consequence of increasing age, rather than of cold exposure. One possibility to exclude developmentally caused effects is to vernalize seeds instead of seedlings, allowing comparison of vernalized and non-vernalized plants of similar developmental stage.”
We have determined that in our growth conditions one week of vernalization equals one day of growth at warm temperatures. In our experiments we therefore compare plants of similar developmental stage: NV 10 days old seedlings are similar to 2WT0 vernalized plants because the vernalized plants are pre-grown for 7 days in the warm.

2. “The interaction frequency between different experiments differ (whereas interaction in wild type for fragment V is 0.4 in Fig. 2, it is only 0.15 in Fig. 3B, which is even less than the interaction value after vernalization shown in Fig. 6A. This requires explanation”

Each graph in the manuscript represents the average of a number of experiments performed in parallel. All experiments have the same growing conditions, crosslinking, digestion, ligation and Q-PCR analysis. As the referee comments the actual interaction frequencies determined by 3C changes between experiments. This is a standard feature of these kind experiments, but the relative change is always constant. To make it easier for the reader to compare the different graphs we represent all data relative to the interaction frequency of Fl-FV in non-vernalized Col-FRI or Col in the new version of the manuscript.

Referee #2
The authors have constructively addressed the issues raised in my previous communication. In the revised version, the major conclusions of the paper are in agreement with the reported experimental findings. There are, however, a few points of concern that needs to be taken care.

They are as follows:
(1) In Fig. 2, there is a marginal decrease in the transcript level of FLC in fcafpa mutant, which is accompanied by a slight decrease in the FLC looping signal as well. This should be accordingly described in the manuscript.
(2) In Fig. 3A, there is >20% decrease in 3C looping signal of FLC in atx1atx2 mutant. Authors have however stated in the manuscript that there is no affect of this mutation on gene looping of FLC. The decrease in looping signal should be described accurately.
(3) Similarly in Fig. 3B, there is about 40% decrease in looping signal in arp6-1 mutant. Inexplicably, authors again concluded that there is no affect of arp6 on the gene looping of FLC. The decrease in looping signal in arp6-1 mutant is substantial and should be described accordingly in the manuscript.

Referee #3
The authors have successfully addressed my major concerns and revised the manuscript accordingly.
Response to referees: Crevillén et al, EMBO J

Referee #2

“The authors have constructively addressed the issues raised in my previous communication. In the revised version, the major conclusions of the paper are in agreement with the reported experimental findings. There are, however, a few points of concern that need to be taken care.

They are as follows:

(1) In Fig. 2, there is a marginal decrease in the transcript level of FLC in fcafpa mutant, which is accompanied by a slight decrease in the FLC looping signal as well. This should be accordingly described in the manuscript.”

We have edited the text in the revised manuscript (last paragraph, page 5) to explain our results better and make the conclusions clearer.

“(2) In Fig. 3A, there is >20% decrease in 3C looping signal of FLC in atx1atx2 mutant. Authors have however stated in the manuscript that there is no affect of this mutation on gene looping of FLC. The decrease in looping signal should be described accurately. “

Despite having low FLC RNA levels, atx1atx2 showed on average a higher 3C interaction frequency. However, we consider this difference is unlikely to be significant due to the variation in experimental values (large error bars) (Figure 3A). We have changed the sentence “FLC loop was not affected” for “FLC loop was not reduced” (line 14, page 6) in the revised manuscript.

“(3) Similarly in Fig. 3B, there is about 40% decrease in looping signal in arp6-1 mutant. Inexplicably, authors again concluded that there is no affect of arp6 on the gene looping of FLC. The decrease in looping signal in arp6-1 mutant is substantial and should be described accordingly in the manuscript. “

We have edited the parts concerning arp6 in page 6 and page 9 in the revised manuscript to reflect that there is a moderate reduction on the FLC gene loop in arp6 mutant.

Referee #3

The authors have successfully addressed my major concerns and revised the manuscript accordingly.”