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LncRNA *FLAIL* affects alternative splicing and represses flowering in Arabidopsis

Yu Jin, Maxim Ivanov, Anna Dittrich, Andrew Nelson, and Sebastian Marquardt
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Corresponding author(s): Sebastian Marquardt (sebastian.marquardt@plen.ku.dk)

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Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Cannot tell / Not applicable

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

The authors characterized a new lncRNA locus named FLAIL that controls flowering time in Arabidopsis thaliana. The functional validation of this locus is strongly supported by the use of several different tools (CRISPR-Cas9 deletions, T-DNA insertion, amiRNA gene silencing, and transgene complementation of KO lines).
It is also suggested that FLAIL lncRNA works in trans but not in cis. There are strong observations supporting that FLAIL works in trans.

Moreover, it is suggested that FLAIL regulates gene expression by interacting with distant chromatin loci. This was assessed using RNA-Seq and ChIRP-Seq. Yet, the overlap between DEGs in the flail mutant and FLAIL binding sites at the chromatin is very small, with only 12 genes. From those, only 2 flowering genes' expression was rescued by FLAIL transgene complementation. The final conclusion that FLAIL lncRNA represses flowering by direct inhibition of the 2 flowering genes expression is correlative, and lacks genetic validation.
In addition inspection of the supplementary file shows that the ChIRP analysis was done without filtering for the FDR so that some of the positive hits have an FDR of 0,232. In addition, many of the peaks land in intergenic regions with is not mentioned in the text a graph with the position of the peaks in respect to nearby genes would help.

In one sentence, the authors used the right model system and methodology, including advanced techniques, to characterize a new trans-acting lncRNA important for controlling the flowering time in Arabidopsis but lack evidence supporting a

mechanism of action that goes beyond the interaction with several chromatin loci.

minor points:

line#63-64 the authors say the COLDAIR and ASL work on FLC in cis in my view the original papers suggested/showed they work in trans.

Fig 1B please add some more protein-coding RNAs for the bio-info analysis for comparison

Order of Supplementary Fig citation is mixed with S2 coming before S1B

It would help the reader to have a schematic of the crisper deletions, T-DNA insertion, and position of primers used for the RT-qPCR.

In the supplementary PDF file, some of the text is missing on page 3 beginning and end of lines.

3. Significance:

Significance (Required)

The use of several different tools to validate the biological function of FLAIL locus is a major strength of this work.

The authors propose that flowering time and its gene regulation are controlled by sense FLAIL lncRNAs. However, the sense transcription of FLAIL locus is not detected in wild-type plants by TSS-Seq, TIF-Seq, or plaNET-Seq. If the authors would have explored further the expression of FLAIL transcripts in different stages of development (vegetative and non-vegetative) and in response to different conditions, it would make their claims on the function of FLAIL lncRNAs more convincing. Additionally, flail mutants could have been obtained in the hen-2 background, since it's there where we can observe FLAIL transcription.

FLAIL locus lays on the proximal promoter region of PORCUPINE (PCP), an important regulator of plant development. As flail mutants, pcp mutants display an early flowering phenotype. The authors show no link between FLAIL and PCP from

the overlap between re-analysis of published RNA-Seq data for pcp and RNA-Seq and ChIRP-Seq from the authors. This analysis is not enough to exclude the involvement of PCP from the FLAIL function. PCP expression using RT-qPCR should be performed in flail mutants to further support that FLAIL works independently from PCP.

This work does not hypothesize any molecular mechanism besides the interaction of FLAIL lncRNAs with several chromatin loci. It was recently proposed in Arabidopsis that a trans-acting lncRNA interacts with distant loci via the formation of R-loops. The authors do not comment on that. This work would benefit in correlating FLAIL binding sites with R-loop-forming regions mapped in Arabidopsis, regardless of the results from this analysis. Additionally, the authors could attempt to look for a motif responsible for FLAIL binding.

Most of the key conclusions are convincing, except for the flowering time control directly through CIR1 and LAC8, which should be mentioned as speculative

The words locus and loci are latin and they should be written in italic. The word Brassicaceae, referring to the family should be in italic, and should not be "Brassicaceaes". The word analysis has the wrong spelling.

I was asked "How much time do you estimate the authors will need to complete the suggested revisions:
this is difficult to answer as it depends to which level the author would like to take their work. In my view, if all new experiments would have to be started from scratch it is too far away to be estimated.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this ms, the authors identified the FLAIL lncRNA that represses flowering in Arabidopsis from a locus producing sense and antisense transcripts. They use an allelic series involving T-DNA insertions, CRISPR/Cas9 and artificial miRNAs to study the role of FLAIL in flowering. A complementation series of constructs of the flail3 allele allowed them to show that the sense FLAIL lncRNA can act in trans. RNAseq revealed a small group of genes linked to the regulation of flowering whose expression is affected in the mutant and restored in the complementation line. To gain further insight into FLAIL function, the authors used a ChIRPeq approach to test whether the lncRNA can recognize potential target genes along the genome and they could show that FLAIL binds specific genomic regions. Clearly, this paper shows very nice evidence that the FLAIL lncRNA can act in trans to regulate gene expression. Nevertheless, there are certain points that need to be clarified to further support the action of the sense FLAIL transcript.

1.According to Fig. 1 A, the antisense FLAIL is "internal" to the DNA genomic area spanning the sense FLAIL. Hence, with direct RT-qPCR is very difficult to distinguish between these molecules as a minor "RT" activity of the Taq polymerase may lead to detection of low levels of antisense, idem if RDRs may generate low antisense levels. Although I think that the plaNET seq brings strong evidence about the start and ends of these molecules, to measure them by RT-qPCR is not trivial and requires the use of strand-specific RT-PCR using a 5' extension of the oligo and amplification with one oligo of the FLAIL sequence (sense or antisense) and the added oligo. It is not clear how they could distinguish precisely sense and antisense particularly when both RNAs correlate as it is the case here in all alleles (Fig. 1 C and 1D). This should be more explicitly mentioned in the materials and methods section.

2.In Fig. 2, what are the levels of antisense in the complementing lines with the sense transcript? And reciprocally sense levels in antisense constructs? This will definitively demonstrate the assumption that the T-NOS termination will not allow any expression on the other strand. At present, only one of the lncRNAs is measured in each experiment?

3.In Fig. 3, it will be important to also show the FLAIL locus in the flail3 mutants (in comparison to the wt) as well as the transgene locus. Here the reads will be strand specific and furthermore this will allow to show that the transgene is not generating

antisense transcripts (through RDRs for gene silencing?) and confirm that the sense FLAIL is required for the complementation.

4.In Fig. S5, the expression of FLAIL is shown in the artificial miRNA lines. Is the antisense FLAIL affected "indirectly" by the cleavage of the amiRNA or remains constant? This is likely the case but should be shown.

5.The ChIRPseq data adds major novelty to the ms and brings new ideas about the way of action of FLAIL. However, are there any common epigenetic states between ChIRP targets (e.g. histone modifications, antisense RNA production, homologies "detected" in the conserved regions between Camelina and Arabidopsis and the target loci? Or others) that may highlight potential mechanisms leading to repression mediated by FLAIL of these loci? There are many databases that could be explored (even during flowering) to search for potential relationships. Although precise description of the mechanism is out of the scope of this ms, this can be discussed in more detail to further expand on the nice data obtained.

Minor comments:

6.In Fig. S3, a global alignment between FLAIL and two loci in Arabidopsis and Camelina is sown. What is the extent of homology? How conserved is this sequence at nucleotide level (small or very long?) to support the conservation of this lncRNA. Are there potential structures conserved among these lncRNAs?

7.In Fig. S4B, arrows may help to understand which seeds were selected.

3. Significance:

Significance (Required)

This paper is a very nice piece of work and demonstrate the action of a long non-coding RNA (lncRNA) in trans on specific targets involved in the regulation of a developmental process, flowering. There is growing evidences that the non-coding genome hides large number of lncRNAs and there is little detailed genetic support for the action of lncRNAs globally. In contrast to many descriptive papers in the field, this ms demonstrates genetically, through an allelic series and complementation experiments, that this lncRNA locus is involved in flowering regulation and that its sense lncRNA recognizes target loci genome-wide, bringing interesting perspectives on potential new mechanisms of transcriptional regulation mediated by non-coding RNAs.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

More than 6 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In the manuscript by Jin et al authors characterize the FLAIL DNA locus in Arabidopsis (using a wide array of publicly available datasets), which produces a set of sense and anti-sense lncRNAs. Authors determined that the sense FLAIL lncRNA (or a set of sense lncRNAs, which isn't fully clear from the way the data are presented) is involved in flowering time in Arabidopsis based on the fact that the several flail mutants lead to the early flowering phenotype and this flowering defect is complemented by transgenic FLAIL DNA, meaning that FLAIL lncRNA acts in trans. The T-DNA flail3 mutant results in expression changes (up or down) of 1221 genes, including twenty genes linked to flowering in various ways. Expression of a group of these flowering-related genes could be either fully (for eight genes) or partially (for five genes) rescued by transgenic FLAIL. Authors also conducted the ChIRP-seq to determine which genes are physically bound by FLAIL lncRNA genome-wide. It was found that 210 genes in the genome are bound by FLAIL lncRNA. Comparison of the dataset of differentially expressed genes in the T-DNA flail3 mutant with the ChIRP-seq dataset of genes that are bound by FLAIL lncRNA revealed the 12 overlapping genes.

Among these twelve overlapping genes, four were found to be functionally connected to flowering with expression of these four genes being down in flail3 T-DNA mutant. Two out of these four genes were ruled out from being involved in the regulation of flowering by FLAIL. Authors conclude that the two other genes (Cir1 and Lac8) are

responsible for the late flowering phenotype of flail mutants based on the three lines of evidence: (i) these genes expression is reduced in the flail mutant, (ii) FLAIL lncRNA directly interacts with these genes chromatin, (iii) the mutants of these genes were previously reported by others to display early flowering phenotypes too.
While I find many of the findings reported in the manuscript very interesting, building a good foundation on which to expand the study and providing a very good leads for follow up experiments, I also have serious concerns about the manuscript in its current form. Most importantly, this reviewer doesn't think that the mechanism of FLAIL lncRNA action was convincingly demonstrated. The main question would be how FLAIL lncRNA works and this question wasn't fully answered. It is great that FLAIL lncRNA binds directly to the two flowering-related genes, but what does it mean? Does it change any chromatin context of these genes quantitatively or qualitatively to affect the transcription? Or does it bind any components of transcriptional machinery and thus controls the transcriptional output? Additionally, flail3 T-DNA mutant affects the expression of 1221 genes and FLAIL lncRNA physically interact with 210 genes, so how can authors be fully sure that FLAIL lncRNA has only direct effect on these two genes and doesn't also contribute to the regulation of the upstream to Cir1 and Lac8 genes or even components of the transcriptional machinery that regulate these genes? Theoretically, doing RNA-seq in the amiR-FLAIL sense lncRNA mutant might have a chance of reducing the number of affected DEGs, making it easier to analyze the FLAIL targets, even if the allele can't be used for complementation experiments. Also, authors totally neglect putting the Cir1 and Lac8 genes into the context of flowering regulation, but it is something that needs to be done.
Lastly, the paper needs to be totally rewritten to be even properly evaluated. In its current state it reads like a very short draft. The Abstract is weak, the Introduction is written in a such telegraphic style that it is barely readable, in many places there is no connections between sentences leading to the information appear to be presented as random, even if it isn't. The Results section is written rather rudimentary with information not being sufficiently provided to describe the results but rather scattered between the Results and Figure legends. The Discussion is the best written part of the manuscript. The Conclusion section carries no specific information and reads more like a little summary suitable for a review article rather than experimental paper.

Therefore, this reviewer thinks that regardless of how authors will choose to proceed with the current experimental version of the manuscript, it'd be in the authors' best interests to at least fully revise the paper before resubmitting anywhere. I'd also advise authors to seek professional editorial help specifically using an editor with the background in the plant sciences. Authors might also want to consider moving Fig.3 into the Suppl. as it doesn't carry much weight or significance and perhaps make existing figures more meaningful and comprehensive and by including a better diagram of the locus (e.g., Fig. S1), etc.

It's not practical to list all issues with the writing as the paper requires total re-writing, so I can just make a few suggestions without any specific order to help authors improve the paper:

--There is no statement anywhere that states the goal of the study.

--No rational is provided on why authors decided to examine this specific genomic locus.

--Typically, the significance is in studying the function of lncRNA or a group of lncRNAs produced from a genomic locus, I don't think I ever encountered the instances when it was exciting to study just a specific genomic locus. If the locus does indeed have any significance for initiating the study, it needs to be explained.

--The locus can produce lncRNAs, but it can't harbor them.

--No length of FLAIL lncRNAs or their range is provided in the first section of Results.

--On many occasions authors don't state rational for doing experiments, which leads to information often flowing as random.

--What do authors mean by the subtitle "FLAIL characterizes a trans-acting lncRNA repressing flowering"? How can lncRNA FLAIL or FLAIL locus characterize lncRNA?

--Check all figures. E.g., Fig. 3B-E mentions only accession numbers for the genes.

--It is not clear where exactly the T-DNA insertion is located relative to sense FLAIL in flail3 mutant (Fig. S4). --- What is the length of the complementing sense FLAIL lncRNA?

--Check the description of each and every construct used and provide explanation for each in the Results. E.g., the pFLAIL:gFLAIL18/88 and pasFLAIL:gasFLAIL18/39 constructs aren't explained in Results, and can only be found in Fig. 2 legends.

3. Significance:

Significance (Required)

Tens of thousands of lncRNAs have been identified in various eukaryotes, but their biological roles have been shown only for a small fraction of them, and the mechanisms of their action are delineated for only a very few of them. Most of the advances on the field of lncRNAs are reported in metazoan, while the field of lncRNAs in plants is lagging far behind in terms of knowledge about lncRNAs with assigned biological functions or lncRNAs with delineated mechanisms of action. From this point of view, this reviewer is always excited to see any new functional plant lncRNAs for which either biological or mechanistic functions have been determined, and deems the information on this subject significant. The manuscript's findings are potentially very interesting and present a decent body of work that lays a very solid groundwork for future experiments. My main concern about the manuscript's significance in its current form is the fact that no real solid mechanism of action for the described lncRNA or a set of lncRNAs (?) has been demonstrated. The best mechanistically studied lncRNAs in Arabidopsis are involved in the regulation of flowering time, particularly those that function in the vernalization flowering pathway and to lesser extent in autonomous pathway. The new FLAIL lncRNA or lncRNAs (?) described in this manuscript also appear to regulate the flowering time in Arabidopsis, however more experiments would be needed to provide a definite conclusion about how direct FLAIL's effect is and how exactly it functions. That unfortunately obviously diminishes the significance of the manuscript and makes it potentially interesting only to researches studying flowering in Arabidopsis and even then the manuscript results would be incomplete to make solid conclusion. Additionally, the manuscript requires complete re-writing.

Reviewer comments are in black and **our response in blue**.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:

The authors characterized a new lncRNA locus named FLAIL that controls flowering time in Arabidopsis thaliana. The functional validation of this locus is strongly supported by the use of several different tools (CRISPR-Cas9 deletions, T-DNA insertion, amiRNA gene silencing, and transgene complementation of KO lines).
It is also suggested that FLAIL lncRNA works in trans but not in cis. There are strong observations supporting that FLAIL works in trans.

Moreover, it is suggested that FLAIL regulates gene expression by interacting with distant chromatin loci. This was assessed using RNA-Seq and ChIRP-Seq. Yet, the overlap between DEGs in the flail mutant and FLAIL binding sites at the chromatin is very small, with only 12 genes. From those, only 2 flowering genes' expression was rescued by FLAIL transgene complementation. The final conclusion that FLAIL lncRNA represses flowering by direct inhibition of the 2 flowering genes expression is correlative, and lacks genetic validation.

#1.1 We plan to support the conclusions in the manuscript genetically as the reviewer suggests. We started these experiments yet they will require the timeframe of the full revision.

In addition inspection of the supplementary file shows that the ChIRP analysis was done without filtering for the FDR so that some of the positive hits have an FDR of 0,232.

#1.2 We strengthened the manuscript by implementing and FDR filter of ChIRP-seq results. The distribution of *FLAIL* binding sites in Fig. S7B and Table S4, and overlapping numbers between DEGs and *FLAIL*-ChIRP in Fig. S8A were correspondingly updated.

In addition, many of the peaks land in intergenic regions with is not mentioned in the text a graph with the position of the peaks in respect to nearby genes would help.

#1.3 Thank you for the suggestion, we strengthened the manuscript with the requested analysis. We implemented the FDR filter, then we used "tssRegion" in ChIPseeker to set distance to the nearest TSS as (-1000, 1000), then most peaks were located in promoter regions (67.24%) and in intergenic regions with 16.38%. Since many papers present the position of the peaks by ChIPseeker (PMID: 32338596, PMID: 28221134, PMID: 31081251, PMID: 32012197, PMID: 31649032, PMID: 32633672) we also applied a similar method to display a distribution of *FLAIL* binding loci relative to distance from the nearest TSS in Fig. S7C.

In one sentence, the authors used the right model system and methodology, including advanced techniques, to characterize a new trans-acting lncRNA important for controlling the flowering time in Arabidopsis but lack evidence supporting a mechanism of action that goes beyond the interaction with several chromatin loci.

minor points:

line#63-64 the authors say the COLDAIR and ASL work on FLC in cis in my view the original papers suggested/showed they work in trans.

#1.4 We increased precision by changing this sentence to 'Vernalization-induced flowering associates with several lncRNAs such as *COOLAIR*, *COLDAIR*, *ANTISENSE LONG* (*ASL*), and *COLDWRAP* that in *cis* or in *trans* locally repress gene expression of *FLOWERING LOCUS C* (*FLC*), a key flowering repressor at different stages of vernalization'.

Fig 1B please add some more protein-coding RNAs for the bio-info analysis for comparison

#1.5 done.

Order of Supplementary Fig citation is mixed with S2 coming before S1B

#1.6 Thank you, we ordered all figures by appearance in the text.

It would help the reader to have a schematic of the crisper deletions, T-DNA insertion, and position of primers used for the RT-qPCR.

#1.7 We enhanced our presentation of Fig. 1A. It shows a schematic of them as well as positions of primers.

In the supplementary PDF file, some of the text is missing on page 3 beginning and end of lines.

#1.8 we ensured all text in new submission.

Reviewer #1 (Significance (Required)):

The use of several different tools to validate the biological function of FLAIL locus is a major

strength of this work.

The authors propose that flowering time and its gene regulation are controlled by sense FLAIL lncRNAs. However, the sense transcription of FLAIL locus is not detected in wild-type plants by TSS-Seq, TIF-Seq, or plaNET-Seq.

#1.9.1 There appears to be some confusions. Transcription of sense *FLAIL* can be observed in chr-DRS, TSS-seq, TIF-seq in wild type and even in plaNET-seq in *NRPB2-FLAG nrpb2-1* plant. We enhanced presentation of Fig. 1 and provided a more clear description in Line 81-99.

If the authors would have explored further the expression of FLAIL transcripts in different stages of development (vegetative and non-vegetative) and in response to different conditions, it would make their claims on the function of FLAIL lncRNAs more convincing. Additionally, flail mutants could have been obtained in the hen-2 background, since it's there where we can observe FLAIL transcription.

#1.9.2 Thank you for the suggestion. We included additional analyses in Fig. S2 for *FLAIL* transcription level in different tissues and different abiotic stress conditions base on 20,000 publicly available RNA-seq libraries (PMID: 32768600). Although many libraries are non-stranded, this analysis determined that sense *FLAIL* or total *FLAIL* (including sense and antisense) is broadly expressed over many tissues and induced in response to many abiotic stresses (Fig. S2A-B), therefore suggesting that *FLAIL* may be needed broadly in *Arabidopsis*.

FLAIL locus lays on the proximal promoter region of PORCUPINE (PCP), an important regulator of plant development. As flail mutants, pcp mutants display an early flowering phenotype. The authors show no link between FLAIL and PCP from the overlap between re-analysis of published RNA-Seq data for pcp and RNA-Seq and ChIRP-Seq from the authors. This analysis is not enough to exclude the involvement of PCP from the FLAIL function. PCP expression using RT-qPCR should be performed in flail mutants to further support that FLAIL works independently from PCP.

#1.10 We strengthened this conclusion by adding the requested experiment. *PCP* transcription level in *flail3* mutant was provided by RT-qPCR and RNA-seq in Fig. S11A-B.

This work does not hypothesize any molecular mechanism besides the interaction of FLAIL lncRNAs with several chromatin loci. It was recently proposed in Arabidopsis that a trans-acting lncRNA interacts with distant loci via the formation of R-loops. The authors do not comment on that. This work would benefit in correlating FLAIL binding sites with R-loop-forming regions

mapped in Arabidopsis, regardless of the results from this analysis. Additionally, the authors could attempt to look for a motif responsible for FLAIL binding.
Check R-loop forming data R-loops (Santos-Pereira and Aguilera, 2015) in Arabidopsis, determined by DRIP-seq (Xu et al., 2017).

#1.11 Thanks very much for this excellent suggestions.
First, we searched for a consensus DNA motif on *FLAIL* binding regions by Homer. We determined four commonly enriched DNA sequence motifs among *FLAIL* target genes (Fig. 4G). Notably, the target genes *CIR1* and *LAC8* contained consensus sequences that matched to all *FLAIL* binding motifs (Fig. 4G). These data are consistent with a model where *FLAIL* binds DNA targets through a sequence complementary mechanism. Functionally important sequences are frequently conserved among evolutionarily distant species, we observed three motifs that appeared to cross-species conserved (Fig. S9), suggesting a potential evolutionarily constrained role.

Second, we indeed identified R-loops peaks on several of *FLAIL* binding sites by DRIP-seq (Xu et al., 2017). For example, we observed R-loop formation over three *FLAIL* binding motifs at *CIR1* locus and one at *LAC8* (Fig. R1), indicating that R-loop formation may also be a factor determining *FLAIL* binding. Even though R-loop peaks are present at several *FLAIL* targets, full elucidation if R-loop formation determines *FLAIL* targeting requires further experimental evidence is beyond the scope of the current manuscript.

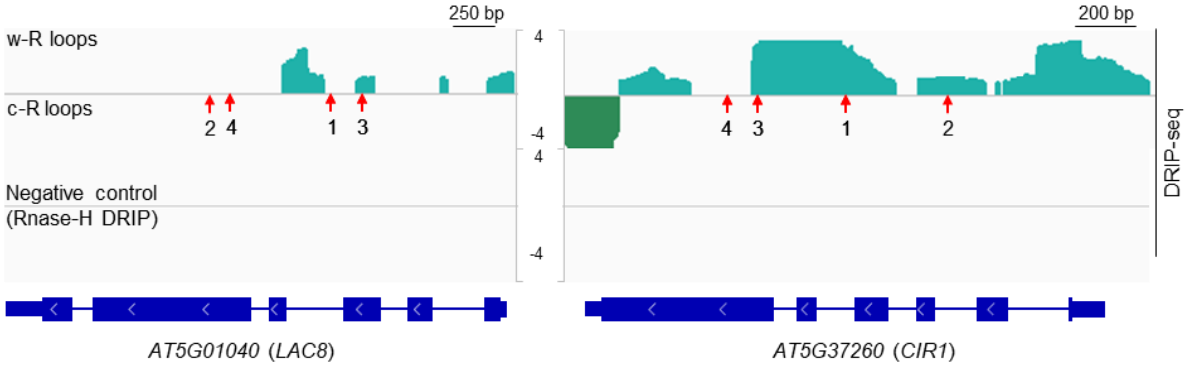

Fig. R1 Representative tracks at LAC8 and CIR1 showing R-loop formation by DRIP-seq on Watson strand (w-R loops), Crick strand (c-R loops). Undetectable R-loops after RNAse-H treatment was shown as negative control. Four conserved sequence regions of FLAIL binding motifs were indicated by red arrows at LAC8 and CIR1 loci. Gene annotation was shown at the bottom.

Most of the key conclusions are convincing, except for the flowering time control directly through CIR1 and LAC8, which should be mentioned as speculative

#1.12 Thank you for finding most key conclusions convincing. We plan strengthen the manuscript with additional genetic evidence to as part of the full revision.

The words locus and loci are latin and they should be written in italic. The word Brassicaceae, referring to the family should be in italic, and should not be "Brassicaceaes". The word analysis has the wrong spelling.

#1.13 We follow conventions given in Scientific Style and Format: The CBE Manual for Authors, Editors and Publishers (1994) Cambridge University Press, Cambridge, UK, 6th edn. The words locus and loci are common Latin terms and should not be italicized. However, should the format of the final prefer these words in italics we will change it later. We improved consistency of using italics. "Brassicaceaes" was changed to "*Brassicaceae*".

"How much time do you estimate the authors will need to complete the suggested revisions: this is difficult to answer as it depends to which level the author would like to take their work. In my view, if all new experiments would have to be started from scratch it is too far away to be estimated.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this ms, the authors identified the FLAIL lncRNA that represses flowering in Arabidopsis from a locus producing sense and antisense transcripts. They use an allelic series involving T-DNA insertions, CRISPR/Cas9 and artificial miRNAs to study the role of FLAIL in flowering. A complementation series of constructs of the flail3 allele allowed them to show that the sense FLAIL lncRNA can act in trans. RNAseq revealed a small group of genes linked to the regulation of flowering whose expression is affected in the mutant and restored in the complementation line. To gain further insight into FLAIL function, the authors used a ChIRPeq approach to test whether the lncRNA can recognize potential target genes along the genome and they could show that FLAIL binds specific genomic regions. Clearly, this paper shows very nice evidence that the FLAIL lncRNA can act in trans to regulate gene expression. Nevertheless, there are certain points that need to be clarified to further support the action of the sense FLAIL transcript.

1.According to Fig. 1 A, the antisense FLAIL is "internal" to the DNA genomic area spanning the sense FLAIL. Hence, with direct RT-qPCR is very difficult to distinguish between these molecules as a minor "RT" activity of the Taq polymerase may lead to detection of low levels of antisense, idem if RDRs may generate low antisense levels. Although I think that the plaNET seq brings strong evidence about the start and ends of these molecules, to measure them by RT-qPCR is not trivial and requires the use of strand-specific RT-PCR using a 5' extension of the oligo and amplification with one oligo of the FLAIL sequence (sense or antisense) and the added oligo.

#2.1.1 Thanks for this good suggestion. We tested both sense and antisense FLAIL transcription using oligo linked gene specific reverse primers for RT and a pair of the linked oligo and gene specific forward primer for qPCR. Primer locations were shown in Fig. 1A and new data were in Fig. 1C-D, Fig. 2B-C, and Fig. S4B-C.

It is not clear how they could distinguish precisely sense and antisense particularly when both RNAs correlate as it is the case here in all alleles (Fig. 1 C and 1D). This should be more explicitly mentioned in the materials and methods section.

#2.1.2 We gave a description of strand specific RT-qPCR method in detail in Line 397-402.

2.In Fig. 2, what are the levels of antisense in the complementing lines with the sense transcript? And reciprocally sense levels in antisense constructs?

#2.2 We added this data in Fig. 2B-C and described in Line 136-143. We indeed observed that sense *FLAIL* transcripts in the transformed *asFLAIL* construct or *asFLAIL* transcripts in the transformed sense *FLAIL* construct was similar to the control *35S:GUS* (Fig. 2B-C), validating that NOS terminator inhibits antisense transcripts. We also noted that the transformed *35S:GUS* and sense *FLAIL* construct expressed higher *asFLAIL*

compared to the flail3 mutant (Fig. 2C). This may be caused by a T-DNA insertion of the resulting transgenic plants.

This will definitively demonstrate the assumption that the T-NOS termination will not allow any expression on the other strand. At present, only one of the lncRNAs is measured in each experiment?

#2.3 We appreciate the next-level reflection of this reviewer, with so many regions initiating cryptic antisense transcription it is an interesting challenge to identify a 3´- terminator that initiates no or poor antisense transcription.
First, previous published data argue that the NOS terminator is largely abolishing initiation of antisense transcription (PMID: 33985972, PMID: 30385760, PMID: 27856735). All these studies address roles of antisense transcription by generating mutations abolishing antisense lncRNA transcription using the NOS terminator sequences.
Second, to satisfy the curiosity of this reviewer, we provide data below that from another manuscript of the lab in preparation. It's a screenshot of plaNET-seq in *fas2-4 NRPB2-FLAG nrpb2-1* mutant carrying a pROK2 construct. The pROK2 T-DNA coincidentally carries a NOS terminator. We mapped plaNET-seq reads to the pROK2 scaffold to display the reads. In pROK2, a NOS promoter activates NPTII expression (red) with NOS terminator as a terminator sequence. No antisense transcription (blue) is detectable by this sensitive method to detect nascent transcripts. Taken together, the selection of the NOS terminator as a region suppressing initiation of antisense transcription represents a valid choice.

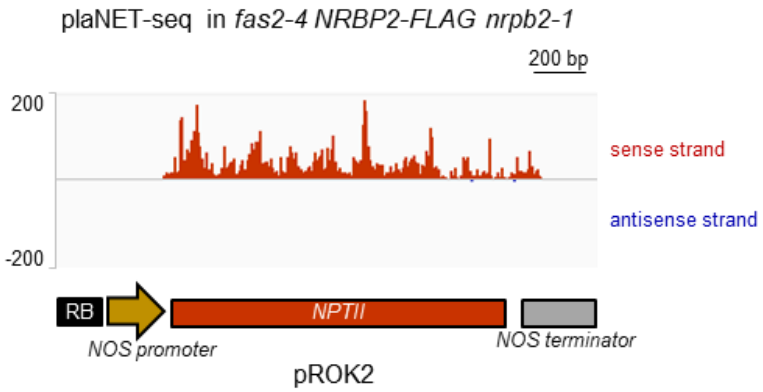

Fig. R2 Genome browser screenshot of plaNET-seq at NPTII locus of pROK2 T-DNA vector in fas2-4 NRBP2-FLAG nrpb2-1 mutant. This mutant carries a pROK2 construct, in which a NOS promoter activates NPTII expression with NOS terminator a terminator sequence. Sense strand was shown in red and antisense strand in blue. pROK2 annotation was shown at the bottom.

3.In Fig. 3, it will be important to also show the FLAIL locus in the flail3 mutants (in comparison to the wt) as well as the transgene locus. Here the reads will be strand specific and furthermore this will allow to show that the transgene is not generating antisense transcripts (through RDRs for gene silencing?) and confirm that the sense FLAIL is required for the complementation.

#2.4 Thank you very much for this suggestion. NGS reads for endogenous *FLAIL* and transgenic *FLAIL* both map to the *FLAIL* locus, so we show the *FLAIL* locus in Fig 3B. This representation shows that sense *FLAIL* transcripts were significantly reduced in *flail3* and rescued in complementation line comparing to wild type. These data argue against the idea of gene silencing and linked antisense production from the transgene. However, RNA-seq suggests that an isoform of *asFLAIL* appears to accumulate in *flail3*. Since we fail to identify this accumulation by strand specific RT-qPCR result in *flail3* and in CRISPR-deletion lines, this may be an *asFLAIL* isoform resulting from the T-DNA insertion.

4.In Fig. S5, the expression of FLAIL is shown in the artificial miRNA lines. Is the antisense FLAIL affected "indirectly" by the cleavage of the amiRNA or remains constant? This is likely the case but should be shown.

#2.5 We added this result in Fig. S4C and expression level of *asFLAIL* remains constant compared to the transformed empty vector control.

5.The ChIRPseq data adds major novelty to the ms and brings new ideas about the way of action of *FLAIL*. However, are there any common epigenetic states between ChIRP targets (e.g. histone modifications, antisense RNA production, homologies "detected" in the conserved regions between Camelina and Arabidopsis and the target loci? Or others) that may highlight potential mechanisms leading to repression mediated by *FLAIL* of these loci? There are many databases that could be explored (even during flowering) to search for potential relationships. Although precise description of the mechanism is out of the scope of this ms, this can be discussed in more detail to further expand on the nice data obtained.

#2.6 We searched for a consensus DNA motif on *FLAIL* binding regions by Homer. We determined four commonly enriched DNA sequence motifs in target genes. Notably, the target genes *CIR1* and *LAC8* contained consensus sequences that matched to all *FLAIL* binding motifs (Fig. 4G). These data are consistent with a model where *FLAIL* binds DNA targets through a sequence complementarity mechanism. Functionally important sequences are frequently conserved among evolutionarily distant species, we observed three motifs that appeared to cross-species conserved (Fig. S9), suggesting a potential evolutionarily constrained role.

Minor comments:

6.In Fig. S3, a global alignment between FLAIL and two loci in Arabidopsis and Camelina is sown. What is the extent of homology? How conserved is this sequence at nucleotide level (small or very long?) to support the conservation of this lncRNA. Are there potential structures conserved among these lncRNAs?

#2.7 Two consensus regions of *FLAIL* sequences among eleven disparate *Brassicaceae*

genomes were shown in Fig. S9. *Camelina sativa* shared 98-nucleotide conserved sequences with *Arabidopsis thaliana*. In the future, it will be interesting to explore evolutional conserved structures among *Brassicaceae* genomes. However, these analyses are beyond the scope of the current manuscript.

7.In Fig. S4B, arrows may help to understand which seeds were selected.

#2.8 Thanks. Arrows were included.

Reviewer #2 (Significance (Required)):

This paper is a very nice piece of work and demonstrate the action of a long non-coding RNA (lncRNA) in trans on specific targets involved in the regulation of a developmental process, flowering. There is growing evidences that the non-coding genome hides large number of lncRNAs and there is little detailed genetic support for the action of lncRNAs globally. In contrast to many descriptive papers in the field, this ms demonstrates genetically, through an allelic series and complementation experiments, that this lncRNA locus is involved in flowering regulation and that its sense lncRNA recognizes target loci genome-wide, bringing interesting perspectives on potential new mechanisms of transcriptional regulation mediated by non-coding RNAs.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In the manuscript by Jin et al authors characterize the *FLAIL* DNA locus in Arabidopsis (using a wide array of publicly available datasets), which produces a set of sense and anti-sense lncRNAs.

While our work on the FLAIL manuscript was ongoing we published the manuscripts where we presented these novel genomics methods and related data to capture nascent transcription and cryptic isoforms. We shared most data with TAIR, so we are happy to hear that these data are considered publically available.

Authors determined that the sense FLAIL lncRNA (or a set of sense lncRNAs, which isn't fully clear from the way the data are presented) is involved in flowering time in Arabidopsis based on the fact that the several flail mutants lead to the early flowering phenotype and this flowering defect is complemented by transgenic FLAIL DNA, meaning that FLAIL lncRNA acts in trans.

A series of experiments lead us to conclude that the sense isoform of *FLAIL* is responsible for the effect. We improved the data representation and writing of the manuscript to enhance accessibility.

The T DNA flail3 - mutant results in expression changes (up or down) of 1221 genes, including twenty genes linked to flowering in various ways. Expression of a group of these flowering-related genes could be either fully (for eight genes) or partially (for five genes) rescued by transgenic *FLAIL*. Authors also conducted the ChIRP-seq to determine which genes are physically bound by *FLAIL* lncRNA genome-wide. It was found that 210 genes in the genome are bound by *FLAIL* lncRNA. Comparison of the dataset of differentially expressed genes in the T-DNA *flail3* mutant with the ChIRP-seq dataset of genes that are bound by *FLAIL* lncRNA revealed the 12 overlapping genes.

Among these twelve overlapping genes, four were found to be functionally connected to flowering with expression of these four genes being down in flail3 T-DNA mutant. Two out of these four genes were ruled out from being involved in the regulation of flowering by FLAIL. Authors conclude that the two other genes (Cir1 and Lac8) are responsible for the late flowering phenotype of flail mutants based on the three lines of evidence: (i) these genes expression is reduced in the flail mutant, (ii) FLAIL lncRNA directly interacts with these genes chromatin, (iii) the mutants of these genes were previously reported by others to display early flowering phenotypes too.
While I find many of the findings reported in the manuscript very interesting, building a good foundation on which to expand the study and providing a very good leads for follow up experiments, I also have serious concerns about the manuscript in its current form.

Most importantly, this reviewer doesn't think that the mechanism of FLAIL lncRNA action was convincingly demonstrated. The main question would be how FLAIL lncRNA works and this question wasn't fully answered. It is great that FLAIL lncRNA binds directly to the two flowering-related genes, but what does it mean? Does it change any chromatin context of these

genes quantitatively or qualitatively to affect the transcription? Or does it bind any components of transcriptional machinery and thus controls the transcriptional output?

#3.1 This manuscript addresses an important question in the field question: what is the evidence for functional elements in non-coding regions of genomes? Despite many efforts, convincing genetic support for these functions often remained limited. In addition to our strong genetic data, we provided new evidence that *FLAIL* recognizes targets with evolutionally conserved sequence motifs as part of the revision in Fig 4F and Fig. S9. Additionally, we plan to do ChIP-qPCR to identify histone modifications on *FLAIL* targets.

Additionally, flail3 T-DNA mutant affects the expression of 1221 genes and FLAIL lncRNA physically interact with 210 genes, so how can authors be fully sure that FLAIL lncRNA has only direct effect on these two genes and doesn't also contribute to the regulation of the upstream to Cir1 and Lac8 genes or even components of the transcriptional machinery that regulate these genes?

#3.2 We agree with this opinion. It is the reason why we felt stating this exact conclusion in our previous manuscript was justified. We improved accessibility of our manuscript in the revision, these clarify our model, that the *trans*-acting lncRNA sense *FLAIL* can interact with the chromatin regions of its target genes to directly or indirectly regulate gene expression changes involving flowering (Line 274).

Theoretically, doing RNA-seq in the amiR-FLAIL sense lncRNA mutant might have a chance of reducing the number of affected DEGs, making it easier to analyze the FLAIL targets, even if the allele can't be used for complementation experiments.

#3.3 Thanks for this suggestion. We plan to confirm key gene expression changes using amiRNA-*FLAIL* in full revision.

Also, auhors totally neglect putting the Cir1 and Lac8 genes into the context of flowering regulation, but it is something that needs to be done.

#3.4 We discussed roles of *CIR1* and *LAC8* in flowering regulation in Line 260-272. Flowering is fine-tuned to maximize reproductive success and seed production and by endogenous genetic cues and external environmental stimuli such as photoperiod. Nevertheless, many details of the flowering pathways and their integration remain to be investigated. *CIR1* is a circadian clock gene, induced by light and involved in a regulatory feedback loop that controls a subset of the circadian outputs and thus determines flowering time. Our GO analysis supports that a subset of DEGs are connected to the response to red or far red light that contains among other key flowering genes such as *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) and *CONSTANS* (*CO*). *FLAIL* also binds the chromatin region of *LAC8*. *LAC8* is a laccase family member that mainly modulates phenylpropanoid pathway for lignin biosynthesis. Similar to *flail*, *lac8* mutants flower early. While

intermediates in this pathway or dysregulation of lignin-related genes could promote flowering in plants, the molecular connections of reduced *LAC8* expression to effects on flowering time will require further investigation.

Lastly, the paper needs to be totally rewritten to be even properly evaluated. In its current state it reads like a very short draft.

#3.5 We reorganized the structure of manuscript, improved clarity and provided new mechanistic evidence in Fig. 4G and Fig. S9 to present a more complete manuscript.

The Abstract is weak, the Introduction is written in a such telegraphic style that it is barely readable, in many places there is no connections between sentences leading to the information appear to be presented as random, even if it isn't.

#3.6- We strengthened the Abstract by providing new evidence and improved for the Introduction.

The Results section is written rather rudimentary with information not being sufficiently provided to describe the results but rather scattered between the Results and Figure legends.

#3.7 Thanks for your suggestions, we described each *FLAIL* length and all constructs in detail in Results, put a schematic of T-DNA and CRISPR mutants in Fig. 1A, moved comparative genomics data to the end of Results and ensured all figures in order.

The Discussion is the best written part of the manuscript.

Thanks for your appreciation of the Discussion.

The Conclusion section carries no specific information and reads more like a little summary suitable for a review article rather than experimental paper.

#3.8 We agree this opinion, this paragraph fits Discussion better and Conclusion was removed.

Therefore, this reviewer thinks that regardless of how authors will choose to proceed with the current experimental version of the manuscript, it'd be in the authors' best interests to at least fully revise the paper before resubmitting anywhere. I'd also advise authors to seek professional editorial help specifically using an editor with the background in the plant sciences.
Authors might also want to consider moving Fig.3 into the Suppl. as it doesn't carry much weight or significance and perhaps make existing figures more meaningful and comprehensive and by including a better diagram of the locus (e.g., Fig. S1), etc.

#3.9 We thank this helpful suggestion. Fig.3 represents the RNA-seq data. In combination with supporting data in the supplementary material, it gives an easy visual readout of the reproducibility of the findings in replicates of stranded RNA-seq. In a new submission, we moved it to Fig. S5B and highlighted 13 differentially expressed flowering genes as well as sense *FLAIL* in *flail3* that were rescued in complementation line in Fig. 3A. Moreover, we gave screenshots of *FLAIL* itself and four flowering related *FLAIL* targets in RNA-seq with a clear schematic representation of each locus. We believe these revisions improve Fig. 3.

It's not practical to list all issues with the writing as the paper requires total re-writing, so I can just make a few suggestions without any specific order to help authors improve the paper:

We are happy to improve our manuscript with the help of the reviewers. We addressed all comments including from reviewer #3 with a constructive spirit. However, since colleagues and reviewers #1 and #2 found the manuscript comprehensible to the point where they could make expert-level comments that illustrate understanding of the manuscript, a total re-writing did not feel like the most constructive suggestion to improve the manuscript.

--There is no statement anywhere that states the goal of the study.

#3.10 We stated the goal of the study in line 50-69 and we think this is a misunderstanding. We summarized three issues currently exist in characterization of functional lncRNA in the last sentence of the first three paragraphs in Background: 1 in Line 50, the broad range of candidate hypotheses by which lncRNA loci may play functional roles call for multiple approaches to distinguish alternative molecular mechanisms. 2 in Line 59, functional characterization of *trans*-acting lncRNAs remains a key knowledge gap to understand the regulatory contributions of the non-coding genome. 3 in Line 69, the contribution of *trans*-acting lncRNAs to the regulation of distant flowering genes is currently unclear. So in the last paragraph of the background, we claimed that our goals are to address these questions through characterization of functional *FLAIL* lncRNA in flowering repression using multiple genetic approaches and various genomic data.

--No rational is provided on why authors decided to examine this specific genomic locus.

#3.11 For several years, our lab studies the rules and roles of non-coding transcription. We characterized and are characterizing several loci with evidence of non-coding transcription in a range of species. Early experiments suggested that *FLAIL* functioned in flowering, this manuscript clarifies that the function is executed as trans-acting lncRNA of the sense *FLAIL* isoform.

--Typically, the significance is in studying the function of lncRNA or a group of lncRNAs produced from a genomic locus, I don't think I ever encountered the instances when it was

exciting to study just a specific genomic locus. If the locus does indeed have any significance for initiating the study, it needs to be explained.

#3.12 This study is remarkable in many aspects. We fully discuss key strengths in the discussion. First, we exhibit a *trans*-acting lncRNA *FLAIL* that represses flowering by promoting the expression of floral repressor genes as discussed in Line 247-281; Second, in Line 284-306, we informed that this study provide a compelling model about how to apply series of convincing genetic data to functionally characterize lncRNA loci. Third, in Line 307-312, evolutionary conserved *FLAIL* sequences across species is key to characterize the functional microhomology in other *Brassicaceae.*

--The locus can produce lncRNAs, but it can't harbor them.

#3.13 We clarified this confusion by enhancing presentation of Fig. 1 and providing a more clear description of each sequencing method and results in Line 81-99. Although we provided evidence that transcription of both sense and antisense *FLAIL* are more stable in *hen2-2*, they were clearly observed in chr-DRS in wild type and plaNET-seq in *NRBP2-FLAG nrpb2-1* and sense *FLAIL* was even detected in TSS-seq and TIF-seq in wild type.

--No length of *FLAIL* lncRNAs or their range is provided in the first section of Results.

#3.14 We gave the length of sense *FLAIL* in Line 82 and antisense *FLAIL* in Line 86.

--On many occasions authors don't state rational for doing experiments, which leads to information often flowing as random.

#3.15 we enhanced clarity of the rational for each experiment and made some connections between sentences to make more fluent. For example, in sentences in Line 99, Line 113, Line 126, Line 159, Line 183, Line 214, and Line 219.

--What do authors mean by the subtitle "FLAIL characterizes a trans-acting lncRNA repressing flowering"? How can lncRNA FLAIL or FLAIL locus characterize lncRNA?

#3.16 We changed it to "*FLAIL* represses flowering as *trans*-acting lncRNA" in Line 112.

--Check all figures. E.g., Fig. 3B-E mentions only accession numbers for the genes.

#3.17 The systematic gene IDs are a valid way to represent data, in particular for genomics data since it facilitates cross-comparisons. To make it more accessible we also show systematic names of each gene in Fig. 3A-F, Fig. S6 and Table S3.

--It is not clear where exactly the T-DNA insertion is located relative to sense FLAIL in flail3 mutant (Fig. S4).

#3.18 We moved the schematic to clarify this to revised Fig. 1A and the exact T-DNA insertion site is mentioned in the legend.

--- What is the length of the complementing sense FLAIL lncRNA?

#3.19 We now include the length of the complementing sense and antisense *FLAIL*s in Line 351-352.

--Check the description of each and every construct used and provide explanation for each in the Results. E.g., the p*FLAIL*:g*FLAIL*18/88 and pas*FLAIL*:gas*FLAIL*18/39 constructs aren't explained in Results, and can only be found in Fig. 2 legends.

#3.20 We described each construct including *pFLAIL:gFLAIL18/88* and *pasFLAIL:gasFLAIL18/39* constructs in Line 133, *amiR-FLAIL-11* and *amiR-FLAIL-11* in Line 149.

Reviewer #3 (Significance (Required)):

Tens of thousands of lncRNAs have been identified in various eukaryotes, but their biological roles have been shown only for a small fraction of them, and the mechanisms of their action are delineated for only a very few of them. Most of the advances on the field of lncRNAs are reported in metazoan, while the field of lncRNAs in plants is lagging far behind in terms of knowledge about lncRNAs with assigned biological functions or lncRNAs with delineated mechanisms of action. From this point of view, this reviewer is always excited to see any new functional plant lncRNAs for which either biological or mechanistic functions have been determined, and deems the information on this subject significant. The manuscript's findings are potentially very interesting and present a decent body of work that lays a very solid groundwork for future experiments. My main concern about the manuscript's significance in its current form is the fact that no real solid mechanism of action for the described lncRNA or a set of lncRNAs (?) has been demonstrated. The best mechanistically studied lncRNAs in Arabidopsis are involved in the regulation of flowering time, particularly those that function in the vernalization flowering pathway and to lesser extent in autonomous pathway. The new FLAIL lncRNA or lncRNAs (?) described in this manuscript also appear to regulate the flowering time in Arabidopsis, however more experiments would be needed to provide a definite conclusion about how direct FLAIL's effect is and how exactly it functions. That unfortunately obviously diminishes the significance of the manuscript and makes it potentially interesting only to researches studying flowering in Arabidopsis and even then the manuscript results would be incomplete to make solid conclusion.
Lots of functional phenotype have
Additionally, the manuscript requires complete re-writing.

We thank this reviewer for the appreciation of a decent body of work and a very solid groundwork for future experiments. We are confident that our revisions make the manuscript more comprehensible to highlight the qualities of our manuscript more accessibly.

Dear Sebastian,

Thank you for transferring your revised manuscript from Review Commons (EMBOJ-2022-110921) to The EMBO Journal. I have now read your study carefully, looked at the Review Commons referee reports, as well as your response, and discussed everything with the other members of the editorial team. In addition, following this discussion, I also consulted with two of the initial referees on the revised manuscript. However, taking everything into consideration, we have unfortunately decided that we will not pursue publication of this manuscript in The EMBO Journal.

We appreciate that you further characterize the FLAIL lncRNA locus and identify a sense transcript that acts in trans to repress flowering. In addition, you assess genome-wide binding of FLAIL lncRNA and propose an evolutionarily conserved common motif in potential target genes, including two candidates involved in regulating flowering in Arabidopsis CIR1 and LAC8. We recognize that the report of a trans-acting lncRNA with a function in regulating flowering in Arabidopsis, will be of interest to the field. We also realize that you have, or are planning to, address a number of the concerns raised by the referees. However, in our view, for further consideration here, the mechanism how FLAIL functions would need to be defined in further detail, for example which other factors are involved, what occurs upon FLAIL binding to certain loci and/or how FLAIL is regulated.

As mentioned, I also contacted initial referees for their expert opinion on the revised version for publication in The EMBO Journal. They too appreciated that many of the issues raised will likely be largely addressed and acknowledged the identification of the trans-acting lncRNA, but also noted that the mechanism for the function remains more unclear and found that the role in the biological context was not yet sufficiently strong. Overall, without additional experimental definition of the mechanism, the referees did not see the manuscript as a strong candidate for publication in The EMBO Journal. I will copy an excerpt of one of the referees' comments below, hoping this may be helpful. Taken together, in light of the comments and our concerns regarding the degree of mechanistic insight, we have decided that we unfortunately cannot offer publication of the manuscript here.

Thank you for again giving us the opportunity to consider your work, I am very sorry that we cannot be more positive this time.

Kind regards,

Stefanie

Stefanie Boehm
Editor
The EMBO Journal

Referee comments

"I'm fully convinced by the authors that FLAIL is a trans-acting lncRNA and according to authors data actually most likely works even more broadly rather than being involved in flowering only, so the flowering time phenotype might be just one of the additional biological functions of FLAIL.[...]However, in my view it is usually a mechanism of action that would be of the most value, particularly for generalist journal like The EMBO J.
[...]
It is a large and very interesting body of work, the revised text and the new version appear much improved. I really liked the author's addition of using Homer in finding the consensus DNA motif enrichment on FLAIL binding regions. I think it was a nice step towards better understanding how FLAIL finds its targets. However, it doesn't explain how FLAIL actually acts in controlling gene expression. Authors' mention plans to do ChIP-qPCR to identify histone modifications on FLAIL targets (which should be not just identifications but rather examining any potential qualitative and/or quantitative changes in them due to FLAIL binding), and it'd be one of good steps towards trying to elucidate one of potential mechanisms of FLAIL action, but that is still a question and not a guaranteed answer since no one can predict what the results will show. The mechanism could as well be different from causing changes in epigenetic landscape and might be even more interesting, so in my opinion, the large body of work in the manuscript has a great potential to answer a very interesting question in the future."

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Rev_Com_number: RC-2021-01159
New_manu_number: EMBOJ-2022-110921
Corr_author: Marquardt
Title: A trans-acting long non-coding RNA represses flowering in Arabidopsis

Reviewer comments are in black and **our response in blue**.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:

The authors characterized a new lncRNA locus named *FLAIL* that controls flowering time in Arabidopsis thaliana. The functional validation of this locus is strongly supported by the use of several different tools (CRISPR-Cas9 deletions, T-DNA insertion, amiRNA gene silencing, and transgene complementation of KO lines).
It is also suggested that *FLAIL* lncRNA works in trans but not in cis. There are strong observations supporting that *FLAIL* works in trans.

Moreover, it is suggested that *FLAIL* regulates gene expression by interacting with distant chromatin loci. This was assessed using RNA-Seq and ChIRP-Seq. Yet, the overlap between DEGs in the *flail* mutant and *FLAIL* binding sites at the chromatin is very small, with only 12 genes. From those, only 2 flowering genes' expression was rescued by *FLAIL* transgene complementation. The final conclusion that *FLAIL* lncRNA represses flowering by direct inhibition of the 2 flowering genes expression is correlative, and lacks genetic validation.

#1.1 We provided genetic evidence by generating *lac8* single and *lac8 flail3* double mutant by CRISPR/Cas9 in Fig 5I-J and Appendix Fig S8. We observed that *lac8* single mutants accelerated flowering to a similar extent as *flail3* single mutants. Importantly, *lac8 flail3* double mutants flowered as early as *lac8* and *flail* single mutants (Fig 5I-J), supporting the conclusion that *FLAIL* and *LAC8* function in the same pathway for flowering suppression.

In addition inspection of the supplementary file shows that the ChIRP analysis was done without filtering for the FDR so that some of the positive hits have an FDR of 0,232.

#1.2 We strengthened the manuscript by implementing and FDR filter of ChIRP-seq results. The distribution of *FLAIL* binding sites in Appendix Fig S5B and Dataset EV3, and overlapping numbers between DEGs and *FLAIL*-ChIRP in Fig EV3 were correspondingly updated.

In addition, many of the peaks land in intergenic regions with is not mentioned in the text a graph with the position of the peaks in respect to nearby genes would help.

#1.3 Thank you for the suggestion, we strengthened the manuscript with the requested analysis. We implemented the FDR filter, then we used "tssRegion" in ChIPseeker to set distance to the nearest TSS as (-1000, 1000), then most peaks were located in promoter regions (67.24%) and in intergenic regions with 16.38%. Since many papers present the position of the peaks by ChIPseeker (PMID: 32338596, PMID: 28221134, PMID: 31081251,

PMID: 32012197, PMID: 31649032, PMID: 32633672) we also applied a similar method to display a distribution of *FLAIL* binding loci relative to distance from the nearest TSS in Appendix Fig S5C.

In one sentence, the authors used the right model system and methodology, including advanced techniques, to characterize a new trans-acting lncRNA important for controlling the flowering time in Arabidopsis but lack evidence supporting a mechanism of action that goes beyond the interaction with several chromatin loci.

minor points:

line#63-64 the authors say the COLDAIR and ASL work on FLC in cis in my view the original papers suggested/showed they work in trans.

#1.4 We increased precision by changing this sentence to 'Vernalization-induced flowering associates with several lncRNAs such as *COOLAIR*, *COLDAIR*, *ANTISENSE LONG* (*ASL*), and *COLDWRAP* that in *cis* or in *trans* locally repress gene expression of *FLOWERING LOCUS C* (*FLC*), a key flowering repressor at different stages of vernalization'.

Fig 1B please add some more protein-coding RNAs for the bio-info analysis for comparison

#1.5 done.

Order of Supplementary Fig citation is mixed with S2 coming before S1B

#1.6 Thank you, we ordered all figures by appearance in the text.

It would help the reader to have a schematic of the crisper deletions, T-DNA insertion, and position of primers used for the RT-qPCR.

#1.7 We enhanced our presentation of Fig 1A. It shows a schematic of them as well as positions of primers.

In the supplementary PDF file, some of the text is missing on page 3 beginning and end of lines.

#1.8 we ensured all text in new submission.

Reviewer #1 (Significance (Required)):

The use of several different tools to validate the biological function of *FLAIL* locus is a major strength of this work.

The authors propose that flowering time and its gene regulation are controlled by sense *FLAIL* lncRNAs. However, the sense transcription of *FLAIL* locus is not detected in wild-type plants by TSS-Seq, TIF-Seq, or plaNET-Seq.

#1.9.1 There appears to be some confusions. Transcription of sense *FLAIL* can be observed in chr-DRS, TSS-seq, TIF-seq in wild type and even in plaNET-seq in *NRPB2-FLAG nrpb2-1* plant. We enhanced presentation of Fig 1 and provided a more clear description in Line 123-140.

If the authors would have explored further the expression of *FLAIL* transcripts in different stages of development (vegetative and non-vegetative) and in response to different conditions, it would make their claims on the function of *FLAIL* lncRNAs more convincing. Additionally, *flail mutant*s could have been obtained in the hen-2 background, since it's there where we can observe *FLAIL* transcription.

#1.9.2 Thank you for the suggestion. We included additional analyses in Appendix Fig S2 for *FLAIL* transcription level in different tissues and different abiotic stress conditions base on 20,000 publicly available RNA-seq libraries (PMID: 32768600). Although many libraries are non-stranded, this analysis determined that sense *FLAIL* or total *FLAIL* (including sense and antisense) is broadly expressed over many tissues and induced in response to many abiotic stresses (Appendix Fig S2A-B), therefore suggesting that *FLAIL* may be needed broadly in *Arabidopsis*.

FLAIL locus lays on the proximal promoter region of PORCUPINE (PCP), an important regulator of plant development. As *flail mutant*s, pcp mutants display an early flowering phenotype. The authors show no link between *FLAIL* and PCP from the overlap between re-analysis of published RNA-Seq data for pcp and RNA-Seq and ChIRP-Seq from the authors. This analysis is not enough to exclude the involvement of PCP from the *FLAIL* function. PCP expression using RT-qPCR should be performed in *flail mutant*s to further support that *FLAIL* works independently from PCP.

#1.10 We strengthened this conclusion by adding the requested experiment. *PCP* transcription level in *flail3* mutant was provided by RT-qPCR and RNA-seq in Fig R1.

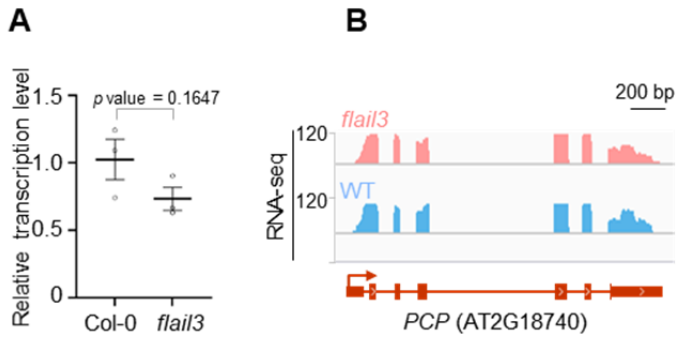

Fig R1 FLAIL doesn't regulate PCP expression. ***A*** *Detection of PCP gene expression in Col-0 and flail mutant by RT-qPCR. Bars represented average ± s.e.m (n = 3 independent 14-d seedling pools). p value was determined by Student's t-test.* ***B*** *Genome browser screenshots illustrating PCP transcription in RNA-seq.*

This work does not hypothesize any molecular mechanism besides the interaction of *FLAIL* lncRNAs with several chromatin loci. It was recently proposed in Arabidopsis that a trans-acting lncRNA interacts with distant loci via the formation of R-loops. The authors do not comment on that. This work would benefit in correlating *FLAIL* binding sites with R-loop-forming regions mapped in Arabidopsis, regardless of the results from this analysis. Additionally, the authors could attempt to look for a motif responsible for *FLAIL* binding.
Check R-loop forming data R-loops (Santos-Pereira and Aguilera, 2015) in Arabidopsis, determined by DRIP-seq (Xu et al., 2017).

#1.11 Thanks very much for this excellent suggestions.
First, we searched for a consensus DNA motif on FLAIL binding regions by Homer. We determined four commonly enriched DNA sequence motifs among FLAIL target genes (Fig 4G). Notably, the target genes *CIR1* and *LAC8* contained consensus sequences that matched to all FLAIL binding motifs (Fig 4G). These data are consistent with a model where FLAIL binds DNA targets through a sequence complementary mechanism. Functionally important sequences are frequently conserved among evolutionarily distant species, we observed three motifs that appeared to cross-species conserved (Fig EV4), suggesting a potential evolutionarily constrained role.

Second, we indeed identified R-loops peaks on several of *FLAIL* binding sites by DRIP-seq (Xu et al., 2017). For example, we observed R-loop formation over three *FLAIL* binding motifs at *CIR1* locus and one at *LAC8* (Fig R2), indicating that R-loop formation may also be a factor determining *FLAIL* binding. Even though R-loop peaks are present at several *FLAIL* targets, full elucidation if R-loop formation determines *FLAIL* targeting requires further experimental evidence is beyond the scope of the current manuscript.

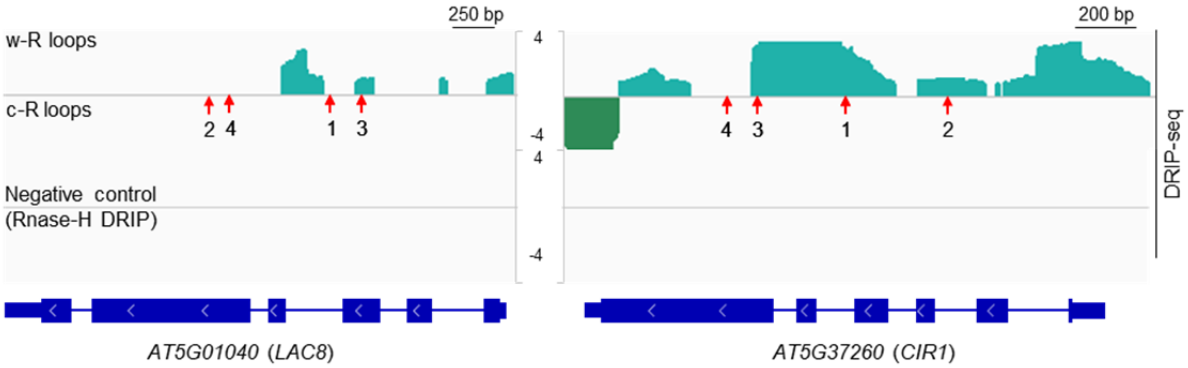

Fig R2 Representative tracks at LAC8 and CIR1 showing R-loop formation by DRIP-seq on Watson strand (w-R loops), Crick strand (c-R loops). Undetectable R-loops after RNAse-H treatment was shown as negative control. Four conserved sequence regions of FLAIL binding motifs were indicated by red arrows at LAC8 and CIR1 loci. Gene annotation was shown at the bottom.

Most of the key conclusions are convincing, except for the flowering time control directly through CIR1 and LAC8, which should be mentioned as speculative

#1.12 Thank you for finding most key conclusions convincing. We strengthened the manuscript with additional genetic evidence as shown in 1.1.

The words locus and loci are latin and they should be written in italic. The word Brassicaceae, referring to the family should be in italic, and should not be "Brassicaceaes". The word analysis has the wrong spelling.

#1.13 We follow conventions given in Scientific Style and Format: The CBE Manual for Authors, Editors and Publishers (1994) Cambridge University Press, Cambridge, UK, 6th edn. The words locus and loci are common Latin terms and should not be italicized. However, should the final copy editor prefer these words in italics we will change it later. We improved consistency of using italics. "Brassicaceaes" was changed to "*Brassicaceae*".

"How much time do you estimate the authors will need to complete the suggested revisions: this is difficult to answer as it depends to which level the author would like to take their work. In my view, if all new experiments would have to be started from scratch it is too far away to be estimated.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this ms, the authors identified the *FLAIL* lncRNA that represses flowering in Arabidopsis from a locus producing sense and antisense transcripts. They use an allelic series involving T-DNA insertions, CRISPR/Cas9 and artificial miRNAs to study the role of *FLAIL* in flowering. A complementation series of constructs of the *flail3* allele allowed them to show that the sense *FLAIL* lncRNA can act in trans. RNAseq revealed a small group of genes linked to the regulation of flowering whose expression is affected in the mutant and restored in the complementation line. To gain further insight into *FLAIL* function, the authors used a ChIRPeq approach to test whether the lncRNA can recognize potential target genes along the genome and they could show that *FLAIL* binds specific genomic regions. Clearly, this paper shows very nice evidence that the *FLAIL* lncRNA can act in trans to regulate gene expression. Nevertheless, there are certain points that need to be clarified to further support the action of the sense *FLAIL* transcript.

1.According to Fig. 1 A, the antisense *FLAIL* is "internal" to the DNA genomic area spanning the sense *FLAIL*. Hence, with direct RT-qPCR is very difficult to distinguish between these molecules as a minor "RT" activity of the Taq polymerase may lead to detection of low levels of antisense, idem if RDRs may generate low antisense levels. Although I think that the plaNET seq brings strong evidence about the start and ends of these molecules, to measure them by RT-qPCR is not trivial and requires the use of strand-specific RT-PCR using a 5' extension of the oligo and amplification with one oligo of the *FLAIL* sequence (sense or antisense) and the added oligo.

#2.1.1 Thanks for this good suggestion. We tested both sense and antisense FLAIL transcription using oligo linked gene specific reverse primers for RT and a pair of the linked oligo and gene specific forward primer for qPCR. Primer locations were shown in Fig 1A and new data were in Fig 1C-D, Fig 2B-C, and Fig EV2B-C.

It is not clear how they could distinguish precisely sense and antisense particularly when both RNAs correlate as it is the case here in all alleles (Fig. 1 C and 1D). This should be more explicitly mentioned in the materials and methods section.

#2.1.2 We gave a description of strand specific RT-qPCR method in detail in Line 538-546.

2.In Fig. 2, what are the levels of antisense in the complementing lines with the sense transcript? And reciprocally sense levels in antisense constructs?

#2.2 We added this data in Fig 2B-C and described in Line 178-182, compared to *flail3 35S:GUS*, the transcription levels of both *asFLAIL* in sense *FLAIL* complementation lines and sense *FLAIL* in *asFLAIL* complementation lines were unchanged, indicating that NOS terminator inhibits antisense transcripts. However, we also noted that *asFLAIL* was increased in *flail3 35S:GUS* compared to wild type. This may be caused by a T-DNA insertion of the resulting transgenic plants. *asFLAIL* complementation lines maintained the same low level of sense *FLAIL* transcripts as *flail3* mutant compared to the control *flail3*

35S:GUS (Fig 2B), validating NOS inhibition of any expression on the other strand in _asFLAIL_ transformed construct. However, sense _FLAIL_ complementation lines expressed _asFLAIL_ transcripts was not significantly different from the control _flail3 35S:GUS_ (Fig 2C), indicating that NOS terminator in sense _FLAIL_ construct also inhibits _asFLAIL_ transcription.

This will definitively demonstrate the assumption that the T-NOS termination will not allow any expression on the other strand. At present, only one of the lncRNAs is measured in each experiment?

#2.3 We appreciate the high-level reflection of this reviewer, with so many regions initiating cryptic antisense transcription it is an interesting challenge to identify a 3´-terminator that initiates no or poor antisense transcription.
First, previous published data argue that the NOS terminator is largely abolishing initiation of antisense transcription (PMID: 33985972, PMID: 30385760, PMID: 27856735). All these studies address roles of antisense transcription by generating mutations abolishing antisense lncRNA transcription using the NOS terminator sequences.
Second, to satisfy the curiosity of this reviewer, we provide data below that from another manuscript from the lab in preparation. It's a screenshot of plaNET-seq in _fas2-4 NRPB2-FLAG nrpb2-1_ mutant carrying a pROK2 construct. The pROK2 T-DNA coincidentally carries a NOS terminator. We mapped plaNET-seq reads to the pROK2 scaffold to display the reads. In pROK2, a NOS promoter activates NPTII expression (red) with NOS terminator as a terminator sequence. No antisense transcription (blue) is detectable by this sensitive method to detect nascent transcripts. Taken together, the selection of the NOS terminator as a region suppressing initiation of antisense transcription represents a valid choice.

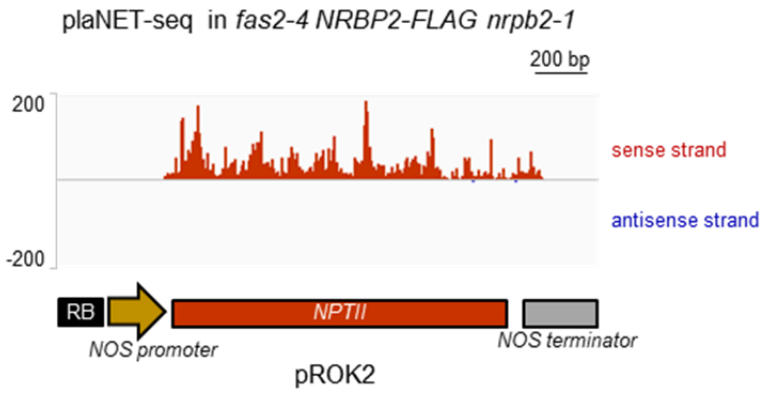

Fig R3 Genome browser screenshot of plaNET-seq at NPTII locus of pROK2 T-DNA vector in fas2-4 NRBP2-FLAG nrpb2-1 mutant. This mutant carries a pROK2 construct, in which a NOS promoter activates NPTII expression with NOS terminator a terminator sequence. Sense strand was shown in red and antisense strand in blue. pROK2 annotation was shown at the bottom.

3.In Fig. 3, it will be important to also show the *FLAIL* locus in the *flail3* mutants (in comparison to the wt) as well as the transgene locus. Here the reads will be strand specific and furthermore

this will allow to show that the transgene is not generating antisense transcripts (through RDRs for gene silencing?) and confirm that the sense *FLAIL* is required for the complementation.

#2.4 Thank you very much for this suggestion. NGS reads for endogenous *FLAIL* and transgenic *FLAIL* both map to the *FLAIL* locus, so we show the *FLAIL* locus in Fig 3B. This representation shows that sense *FLAIL* transcripts were significantly reduced in *flail3* and rescued in complementation line comparing to wild type. These data argue against the idea of gene silencing and linked antisense production from the transgene. However, RNA-seq suggests that an isoform of *asFLAIL* appears to accumulate in *flail3*. Since we fail to identify this accumulation by strand-specific RT-qPCR result in *flail3* and in CRISPR-deletion lines, this may be an *asFLAIL* isoform resulting from the T-DNA insertion.

4.In Fig. S5, the expression of *FLAIL* is shown in the artificial miRNA lines. Is the antisense *FLAIL* affected "indirectly" by the cleavage of the amiRNA or remains constant? This is likely the case but should be shown.

#2.5 We added this result in Fig EV2C and expression level of *asFLAIL* remains constant compared to the transformed empty vector control.

5.The ChIRPseq data adds major novelty to the ms and brings new ideas about the way of action of *FLAIL*. However, are there any common epigenetic states between ChIRP targets (e.g. histone modifications, antisense RNA production, homologies "detected" in the conserved regions between Camelina and Arabidopsis and the target loci? Or others) that may highlight potential mechanisms leading to repression mediated by *FLAIL* of these loci? There are many databases that could be explored (even during flowering) to search for potential relationships. Although precise description of the mechanism is out of the scope of this ms, this can be discussed in more detail to further expand on the nice data obtained.

#2.6 We searched for a consensus DNA motif on *FLAIL* binding regions by Homer. We determined four commonly enriched DNA sequence motifs in target genes. Notably, the target genes *CIR1* and *LAC8* contained consensus sequences that matched to all *FLAIL* binding motifs (Fig 4G). These data are consistent with a model where *FLAIL* binds DNA targets through a sequence complementary mechanism. Functionally important sequences are frequently conserved among evolutionarily distant species, we observed three motifs that appeared to cross-species conserved (Fig EV4), suggesting a potential evolutionarily constrained role. In addition, we identified that *FLAIL* interacts with RBPs (NSRa and GRP7) (Fig 5A). Computational analysis indicates that *FLAIL* and RBPs partially share a common effect on splicing (Appendix Fig S7B-C). We also revealed the interaction between *FLAIL* and spliceosomal RNAs (*U1*, *U2*, *U5*) (Fig 5F-H, Appendix Fig S7D). We further detected splicing defects in *flail3* at the direct *FLAIL* target flowering gene *LAC8* correlated with reduced mRNA expression (Fig 5B-E). Moreover, we analyzed the levels and profiles of key active histone tail modifications that are positively correlated with *FLAIL*-mediated mRNA expression at the spliced intron regions of *LAC8* and *CIR1* loci

(Fig EV5), indicating that *FLAIL* binding effects target gene expression through epigenetic regulation. Taken together, these data suggest that the *trans*-acting lncRNA *FLAIL* affects mRNA expression by alternative splicing associated and co-transcriptional chromatin signaling.

Minor comments:

6.In Fig. S3, a global alignment between *FLAIL* and two loci in Arabidopsis and Camelina is sown. What is the extent of homology? How conserved is this sequence at nucleotide level (small or very long?) to support the conservation of this lncRNA. Are there potential structures conserved among these lncRNAs?

#2.7 Two consensus regions of *FLAIL* sequences among eleven disparate *Brassicaceae* genomes were shown in Fig EV4. *Camelina sativa* shared 98-nucleotide conserved sequences with *Arabidopsis thaliana*. In the future, it will be interesting to explore evolutional conserved structures among *Brassicaceae* genomes. However, these analyses are beyond the scope of the current manuscript.

7.In Fig. S4B, arrows may help to understand which seeds were selected.

#2.8 Thanks. Arrows were included.

Reviewer #2 (Significance (Required)):

This paper is a very nice piece of work and demonstrate the action of a long non-coding RNA (lncRNA) in trans on specific targets involved in the regulation of a developmental process, flowering. There is growing evidences that the non-coding genome hides large number of lncRNAs and there is little detailed genetic support for the action of lncRNAs globally. In contrast to many descriptive papers in the field, this ms demonstrates genetically, through an allelic series and complementation experiments, that this lncRNA locus is involved in flowering regulation and that its sense lncRNA recognizes target loci genome-wide, bringing interesting perspectives on potential new mechanisms of transcriptional regulation mediated by non-coding RNAs.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In the manuscript by Jin et al authors characterize the *FLAIL* DNA locus in Arabidopsis (using a wide array of publicly available datasets), which produces a set of sense and anti-sense lncRNAs.

While our work on the FLAIL manuscript was ongoing we published the manuscripts where we presented these novel genomics methods and related data to capture nascent transcription and cryptic isoforms. We shared most data with TAIR, so we happy to hear that these data are considered publically available.

Authors determined that the sense *FLAIL* lncRNA (or a set of sense lncRNAs, which isn't fully clear from the way the data are presented) is involved in flowering time in Arabidopsis based on the fact that the several *flail* mutants lead to the early flowering phenotype and this flowering defect is complemented by transgenic *FLAIL* DNA, meaning that *FLAIL* lncRNA acts in trans.

A series of experiments lead us to conclude that the sense isoform of FLAIL is responsible for the effect. We improved the data representation and writing of the manuscript to enhance accessibility.

The T-DNA *flail3* mutant results in expression changes (up or down) of 1221 genes, including twenty genes linked to flowering in various ways. Expression of a group of these flowering-related genes could be either fully (for eight genes) or partially (for five genes) rescued by transgenic *FLAIL*. Authors also conducted the ChIRP-seq to determine which genes are physically bound by *FLAIL* lncRNA genome-wide. It was found that 210 genes in the genome are bound by *FLAIL* lncRNA. Comparison of the dataset of differentially expressed genes in the T-DNA *flail3* mutant with the ChIRP-seq dataset of genes that are bound by *FLAIL* lncRNA revealed the 12 overlapping genes.

Among these twelve overlapping genes, four were found to be functionally connected to flowering with expression of these four genes being down in *flail3* T-DNA mutant. Two out of these four genes were ruled out from being involved in the regulation of flowering by *FLAIL*. Authors conclude that the two other genes (Cir1 and Lac8) are responsible for the late flowering phenotype of *flail* mutants based on the three lines of evidence: (i) these genes expression is reduced in the *flail* mutant, (ii) *FLAIL* lncRNA directly interacts with these genes chromatin, (iii) the mutants of these genes were previously reported by others to display early flowering phenotypes too.
While I find many of the findings reported in the manuscript very interesting, building a good foundation on which to expand the study and providing a very good leads for follow up experiments, I also have serious concerns about the manuscript in its current form.

Most importantly, this reviewer doesn't think that the mechanism of *FLAIL* lncRNA action was convincingly demonstrated. The main question would be how *FLAIL* lncRNA works and this question wasn't fully answered. It is great that *FLAIL* lncRNA binds directly to the two flowering-related genes, but what does it mean? Does it change any chromatin context of these

genes quantitatively or qualitatively to affect the transcription? Or does it bind any components of transcriptional machinery and thus controls the transcriptional output?

#3.1 This manuscript addresses an important question in the field question: what is the evidence for functional elements in non-coding regions of genomes? Despite many efforts, convincing genetic support for these functions often remained limited. In addition to our strong genetic data, we provided new evidence that *FLAIL* recognizes targets through an evolutionally conserved sequence complementary mechanism as part of the revision in Fig 4G and Fig EV4. Moreover, we identified that *FLAIL* interacts with protein and RNA components of the spliceosome to affect target mRNA expression by alternative splicing associated and co-transcriptional chromatin signaling in Fig 5 and Appendix Fig S7-8. We finally provided genetic evidence by generating *lac8 flail3* double mutant that supports a model where *FLAIL*-mediated splicing of *LAC8* promotes mRNA expression and represses flowering in Fig 6 and Appendix Fig S8.

Additionally, *flail3* T-DNA mutant affects the expression of 1221 genes and *FLAIL* lncRNA physically interact with 210 genes, so how can authors be fully sure that *FLAIL* lncRNA has only direct effect on these two genes and doesn't also contribute to the regulation of the upstream to Cir1 and Lac8 genes or even components of the transcriptional machinery that regulate these genes?

#3.2 We agree with this opinion. It is the reason why we felt stating this exact conclusion in our previous manuscript was justified. To test the hypothesis that the effect of *FLAIL* on flowering was caused by reduced *LAC8* mRNA expression, we generated *lac8* single and *lac8 flail3* double mutant using CRISPR/Cas9 (Appendix Fig S8). We observed that *lac8* single mutants accelerated flowering to a similar extent as *flail3* single mutants. Importantly, *lac8 flail3* double mutants flowered as early as *lac8* and *flail* single mutants (Fig 5I-J), supporting the conclusion that *FLAIL* and *LAC8* function in the same pathway for flowering suppression. Our analyses thus far are consistent with a model where the functional effect of *FLAIL* on flowering could be explained by direct effects on *LAC8* mRNA expression.

Theoretically, doing RNA-seq in the amiR-*FLAIL* sense lncRNA mutant might have a chance of reducing the number of affected DEGs, making it easier to analyze the *FLAIL* targets, even if the allele can't be used for complementation experiments.

#3.3 Thanks for this suggestion. We confirmed key gene expression changes using amiRNA-*FLAIL* in Fig EV3C-D, both *CIR1* and *LAC8* showing reduced transcripts upon *FLAIL* knock-down.

Also, auhors totally neglect putting the Cir1 and Lac8 genes into the context of flowering regulation, but it is something that needs to be done.

#3.4 We discussed roles of *CIR1* and *LAC8* in flowering regulation in Line 392-416. Flowering is fine-tuned to maximize reproductive success by endogenous genetic cues and external environmental stimuli such as photoperiod, yet many molecular details of flowering regulation remain unclear (Cho *et al.*, 2017). *CIR1* is a circadian clock gene, induced by light and involved in a regulatory feedback loop that controls a subset of the circadian outputs and thus determines flowering time (Zhang *et al.*, 2007). Our GO analysis supports that a subset of DEGs in *flail3* are connected to the response to red or far red light that contains other key flowering genes such as *phytochrome interacting factor 4* (*PIF4*) and *CONSTANS* (*CO*) (Table EV3). *FLAIL* represses flowering by directly binding the chromatin region of *LAC8* to promote *LAC8* mRNA expression. We observed non-additive genetic interactions between single mutants of *flail*, *lac8*, and *lac8 flail3* double mutants. These genetic data argue for a model where *FLAIL* and the direct target *LAC8* act in one linear pathway to repress flowering. *LAC8* is a laccase family member that mainly modulates phenylpropanoid pathway for lignin biosynthesis (Cai *et al.*, 2006; Wang *et al*, 2014). While intermediates in this pathway (Hatayama & Takeno, 2003; Nakanishi *et al*, 1995) or dysregulation of lignin-related genes (Shi *et al.*, 2011; Wang *et al*, 2018; Xu *et al.*, 2012) could promote flowering in plants, the molecular connections of reduced *LAC8* expression that mediate the effects on flowering time will require further investigation. Similar to *flail* and *lac8* mutants, *nsrab* mutant and transgenic plants ectopically overexpressing circadian clock-regulated *GRP7* also show early flowering, these effects correlate with effects on RNA processing of the *FLC/COOLAIR* module (Bazin *et al.*, 2018; Streitner *et al*, 2008; Xiao *et al*, 2015). NSRs and GRP7 interact with *FLAIL* RNA and DAS events in *nsrab* or *grp7* mutant are partially shared with those in *flail3*, offering the attractive interpretation that the effects of NSRs and GRP7 on flowering time could include *FLAIL*-modulated AS of *LAC8*. Nevertheless, our observations of reduced expression and altered AS of *LAC8* in *flail*, combined with restored expression upon re-introduction of *FLAIL*, and no additive early flowering phenotype in double mutants, suggested that direct activation of *LAC8* mRNA expression by FLAIL as key molecular effect on flowering time.

Lastly, the paper needs to be totally rewritten to be even properly evaluated. In its current state it reads like a very short draft.

#3.5 We reorganized the structure of manuscript, improved clarity and provided new mechanistic evidence in Fig 4G, Fig 5, Fig EV4, and Appendix Fig S7-8 to present a more complete manuscript.

The Abstract is weak, the Introduction is written in a such telegraphic style that it is barely readable, in many places there is no connections between sentences leading to the information appear to be presented as random, even if it isn't.

#3.6- We strengthened the Abstract by providing new evidence and improved for the Introduction.

The Results section is written rather rudimentary with information not being sufficiently provided to describe the results but rather scattered between the Results and Figure legends.

#3.7 Thanks for your suggestions, we described each *FLAIL* length and all constructs in detail in Results, put a schematic of T-DNA and CRISPR mutants in Fig 1A, moved comparative genomics data to the end of Results and ensured all figures in order.

The Discussion is the best written part of the manuscript.

Thanks for your appreciation of the Discussion.

The Conclusion section carries no specific information and reads more like a little summary suitable for a review article rather than experimental paper.

#3.8 We agree this opinion, this paragraph fits Discussion better and Conclusion was removed.

Therefore, this reviewer thinks that regardless of how authors will choose to proceed with the current experimental version of the manuscript, it'd be in the authors' best interests to at least fully revise the paper before resubmitting anywhere. I'd also advise authors to seek professional editorial help specifically using an editor with the background in the plant sciences.
Authors might also want to consider moving Fig.3 into the Suppl. as it doesn't carry much weight or significance and perhaps make existing figures more meaningful and comprehensive and by including a better diagram of the locus (e.g., Fig. S1), etc.

#3.9 We thank this helpful suggestion. Fig 3 represents the RNA-seq data. In combination with supporting data in the supplementary material, it gives an easy visual readout of the reproducibility of the findings in replicates of stranded RNA-seq. In a new submission, we moved it to Appendix Fig S3B and highlighted 13 differentially expressed flowering genes as well as sense *FLAIL* in *flail3* that were rescued in complementation line in Fig 3A. Moreover, we gave screenshots of *FLAIL* itself and four flowering related *FLAIL* targets in RNA-seq with a clear schematic representation of each locus. We believe these revisions improve Fig 3.

It's not practical to list all issues with the writing as the paper requires total re-writing, so I can just make a few suggestions without any specific order to help authors improve the paper:

We are happy to improve our manuscript with the help of the reviewers. We addressed all comments including from reviewer #3 with a constructive spirit. However, since colleagues and reviewers #1 and #2 found the manuscript comprehensible to the point where they could make expert-level comments that illustrate understanding of the manuscript, a total re-writing did not feel like the most constructive suggestion to improve the manuscript.

--There is no statement anywhere that states the goal of the study.

#3.10 We stated the goal of the study in line 28-108 and we think this is a misunderstanding. We summarized three issues currently exist in characterization of functional lncRNA in the last sentence of the first three paragraphs in Background: 1 in Line 45, the broad range of candidate hypotheses by which lncRNA loci may play functional roles call for multiple approaches to distinguish alternative molecular mechanisms. 2 in Line 96, how *trans*-acting lncRNAs are directly involved in AS of target genes to control plant growth and development is largely unclear. 3 in Line 107, the contribution of *trans*-acting lncRNAs to the regulation of distant flowering genes is currently unclear. So in the last paragraph of the background, we claimed that our goals are to address these questions through characterization of functional *FLAIL* lncRNA in flowering repression using multiple genetic approaches and various genomic data.

--No rational is provided on why authors decided to examine this specific genomic locus.

#3.11 For several years, our lab studies the rules and roles of non-coding transcription. We characterized and are characterizing several loci with evidence of non-coding transcription in a range of species. Early experiments suggested that *FLAIL* functioned in flowering, this manuscript clarifies that the function is executed as trans-acting lncRNA of the sense *FLAIL* isoform.

--Typically, the significance is in studying the function of lncRNA or a group of lncRNAs produced from a genomic locus, I don't think I ever encountered the instances when it was exciting to study just a specific genomic locus. If the locus does indeed have any significance for initiating the study, it needs to be explained.

#3.12 This study is remarkable in many aspects. We fully discuss key strengths in the discussion. First, in Line 342-365, we informed that this study provide a compelling model about how to apply series of convincing genetic data to functionally characterize lncRNA loci. Second, in Line 365-372, evolutionary conserved *FLAIL* sequences across species is key to characterize the functional microhomology in other *Brassicaceae*. Third, we exhibit a *trans*-acting lncRNA *FLAIL* that represses flowering by promoting the expression of floral repressor genes as discussed in Line 375-416; Forth, in Line 419-448, we discuss how *FLAIL* serves as an accessory lncRNA component of the spliceosome to shape the outcome of gene transcription for flowering repression.

--The locus can produce lncRNAs, but it can't harbor them.

#3.13 We clarified this confusion by enhancing presentation of Fig 1 and providing a more

clear description of each sequencing method and results in Line 123-140. Although we provided evidence that transcription of both sense and antisense *FLAIL* are more stable in *hen2-2*, they were clearly observed in chr-DRS in wild type and plaNET-seq in *NRBP2-FLAG nrpb2-1* and sense *FLAIL* was even detected in TSS-seq and TIF-seq in wild type.

--No length of *FLAIL* lncRNAs or their range is provided in the first section of Results.

#3.14 We gave the length of sense *FLAIL* in Line 121 and antisense *FLAIL* in Line 126.

--On many occasions authors don't state rational for doing experiments, which leads to information often flowing as random.

#3.15 we enhanced clarity of the rational for each experiment and made some connections between sentences to make more fluent. For example, in sentences in Line 140, Line 155, Line 169, Line 191, Line 203, Line 228, and Line 262.

--What do authors mean by the subtitle "*FLAIL* characterizes a trans-acting lncRNA repressing flowering"? How can lncRNA *FLAIL* or *FLAIL* locus characterize lncRNA?

#3.16 We changed it to "*FLAIL* represses flowering as a *trans*-acting lncRNA" in Line 154.

--Check all figures. E.g., Fig. 3B-E mentions only accession numbers for the genes.

#3.17 The systematic gene IDs are a valid way to represent data, in particular for genomics data since it facilitates cross-comparisons. To make it more accessible we also show systematic names of each gene in Fig 3A-D, Appendix Fig S4 and Table EV1.

--It is not clear where exactly the T-DNA insertion is located relative to sense *FLAIL* in *flail3* mutant (Fig. S4).

#3.18 We moved the schematic to clarify this to revised Fig 1A and the exact T-DNA insertion site is mentioned in the legend.

--- What is the length of the complementing sense *FLAIL* lncRNA?

#3.19 We now include the length of the complementing sense and antisense *FLAIL*s in Line 492-493.

--Check the description of each and every construct used and provide explanation for each in the Results. E.g., the p*FLAIL*:g*FLAIL*18/88 and pas*FLAIL*:gas*FLAIL*18/39 constructs aren't explained in Results, and can only be found in Fig. 2 legends.

#3.20 We described each construct including *pFLAIL:gFLAIL18/88* and *pasFLAIL:gasFLAIL18/39* constructs in Line 177, *amiR-FLAIL-11* and *amiR-FLAIL-12* in Line 193.

Reviewer #3 (Significance (Required)):

Tens of thousands of lncRNAs have been identified in various eukaryotes, but their biological roles have been shown only for a small fraction of them, and the mechanisms of their action are delineated for only a very few of them. Most of the advances on the field of lncRNAs are reported in metazoan, while the field of lncRNAs in plants is lagging far behind in terms of knowledge about lncRNAs with assigned biological functions or lncRNAs with delineated mechanisms of action. From this point of view, this reviewer is always excited to see any new functional plant lncRNAs for which either biological or mechanistic functions have been determined, and deems the information on this subject significant. The manuscript's findings are potentially very interesting and present a decent body of work that lays a very solid groundwork for future experiments. My main concern about the manuscript's significance in its current form is the fact that no real solid mechanism of action for the described lncRNA or a set of lncRNAs (?) has been demonstrated. The best mechanistically studied lncRNAs in Arabidopsis are involved in the regulation of flowering time, particularly those that function in the vernalization flowering pathway and to lesser extent in autonomous pathway. The new *FLAIL* lncRNA or lncRNAs (?) described in this manuscript also appear to regulate the flowering time in Arabidopsis, however more experiments would be needed to provide a definite conclusion about how direct *FLAIL*'s effect is and how exactly it functions. That unfortunately obviously diminishes the significance of the manuscript and makes it potentially interesting only to researches studying flowering in Arabidopsis and even then the manuscript results would be incomplete to make solid conclusion.
Lots of functional phenotype have
Additionally, the manuscript requires complete re-writing.

We thank this reviewer for the appreciation of a decent body of work a very solid groundwork for future experiments. We are confident that our revisions make the manuscript more comprehensible to highlight the qualities of our manuscript more accessibly.

Referee comments

"I'm fully convinced by the authors that FLAIL is a trans-acting lncRNA and according to authors data actually most likely works even more broadly rather than being involved in flowering only, so the flowering time phenotype might be just one of the additional biological functions of FLAIL.[...]However, in my view it is usually a mechanism of action that would be of the most value, particularly for generalist journal like The EMBO J.
[...]
It is a large and very interesting body of work, the revised text and the new version appear much

improved. I really liked the author's addition of using Homer in finding the consensus DNA motif enrichment on FLAIL binding regions. I think it was a nice step towards better understanding how FLAIL finds its targets. However, it doesn't explain how FLAIL actually acts in controlling gene expression. Authors' mention plans to do ChIP-qPCR to identify histone modifications on FLAIL targets (which should be not just identifications but rather examining any potential qualitative and/or quantitative changes in them due to FLAIL binding), and it'd be one of good steps towards trying to elucidate one of potential mechanisms of FLAIL action, but that is still a question and not a guaranteed answer since no one can predict what the results will show. The mechanism could as well be different from causing changes in epigenetic landscape and might be even more interesting, so in my opinion, the large body of work in the manuscript has a great potential to answer a very interesting question in the future."

In this new version, we identified that *FLAIL* interacts with RBPs (NSRa and GRP7) (Fig 5A). Computational analysis indicates that *FLAIL* and RBPs partially share a common effect on splicing (Appendix Fig S7B-C). We also revealed the interaction between *FLAIL* and spliceosomal RNAs (*U1*, *U2*, *U5*) (Fig 5F-H, Appendix Fig S7D). We further detected splicing defects in *flail3* at the direct *FLAIL* target flowering gene *LAC8* correlated with reduced mRNA expression (Fig 5B-E). Moreover, we analyzed the levels and profiles key active histone tail modifications that are positively correlated with *FLAIL*-mediated mRNA expression at the spliced intron regions of *LAC8* and *CIR1* loci (Fig EV5), indicating that *FLAIL* binding effects target gene expression through epigenetic regulation. Taken together, these data suggest that the *trans*-acting lncRNA *FLAIL* affects mRNA expression by alternative splicing associated and co-transcriptional chromatin signaling.

Dear Sebastian,

Thank you again for submitting a further revised version of your manuscript EMBOJ-2022-1110921, which we had rejected after receiving editorial advice on the revision plan for the Review Commons transferred manuscript. Given the additional experimental data and extent of revision, we decided to send it back to the three initial referees, and have now received their comments (please see below). Given their overall positive feedback, I am happy to say that we have decided to consider the study further for publication in The EMBO Journal, pending a final round of revision to respond to the remaining concerns. In our view, these issues can be addressed by textual revision and/or expanding the discussion to include the referees' concerns, as well as further explanations or discussion in the point-by-point response. Therefore, please revise the manuscript accordingly and provide the point-by-point response when resubmitting the study. Please also remember that the manuscript should then fulfill all journal-specific formatting guidelines (please also see below and https://www.embopress.org/page/journal/14602075/authorguide#submissionofrevisions). At EMBO Press we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

Please contact me if you have any questions about this revision or are uncertain about specific points. Otherwise, I look forward to receiving your revised manuscript.

Kind regards,
Stefanie

Stefanie Boehm
Editor
The EMBO Journal

***IMPORTANT NOTE: we now perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author%20Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) All corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database. Please remember to provide a reviewer password if the datasets are not yet public.

Please see the instructions below on how to format the Data Availability section (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

[data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at < https://www.embopress.org/page/journal/14602075/authorguide#referencesformat >.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel).

Please note that source data should be uploaded in the following format:
a) For main figures: please upload the source data as one zip file per figure and label it as source_data_figX.
b) For EV figures please compile individual (zip) files for each figure, then combine these and upload one zip file for all figures. Please label this as source_data_EVfigures.
c) For Appendix figures please also make one file per figure, combine all Appendix files into one final zip file for all figures and label it as source_data_Appendix.

Additional information on source data and instruction on how to label the files are available at < https://www.embopress.org/page/journal/14602075/authorguide#sourcedata >.

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in http://msb.embopress.org/content/11/6/812). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc.. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: < https://www.embopress.org/page/journal/14602075/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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****
Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 9th Apr 2023:

Link Not Available
****

Referee #1:

This is a resubmission of an original manuscript that describes the discovery and characterisation of FLAIL a lncRNA transcript.

Looking at the revised version I believe there are some strong points of the work including:
- comprehensive description of the FLAIL lncRNA, RNAseq, 5'3'RACE, strand specific RT-qPCR,
- analysis that shows the effect of FLAIL on flowering based on multiple mutants, complementation as well as genetic analysis in the double FLAILxLAC8 mutant.

This part has been strengthened in the revision, and the genetic data that is now included in the manuscript in my view convincingly shows that FLAIL controls flowering through LAC8.

I however still have concerns about the mechanism. The authors say "our data thus suggest that FLAIL affects flowering through reducing mRNA expression of its direct target LAC8, mechanistically linked to AS and associated co-transcriptional chromatin changes"

To support this view they present:
- reanalysis of published RIP experiments with two splicing factors GRP7 and NSRa,
- splicing defects of LAC8 in flail mutant
- H3K4me1 and 3 change by ChIP on LAC8 and CIR1 in flail

Major points:

1. First, my concern is still that FLAIL RNAseq and ChIRP seq show a marginal overlap of 8 out of 1222 and 116 respectively targets. And some of those targets are probably not direct as their expression is not reversed in the complemented line.
Here I wonder if this could not be a consequence of the direct but unintended binding of the probes to the targets DNA? One could test it by selecting a set of targets and doing ChIRP in a flail KO background.
One can say that the authors can focus on the LAC8 target and somehow downplay the genome-wide data. But in my view, the low overlap between RNAseq and ChIRP puts in doubt the subsequent reasoning whenever the authors refer back to genome-wide data like in Fig 4G when they analyse the overrepresented motifs in the ChIRP data set.

2 The role of splicing in LAC8 regulation by FLAIL.

2a
It is not clear to me if the splicing defects observed are not a consequence of LAC8 expression changes in the flail mutant. It would help if the authors reconsidered the splicing as the fraction of transcripts with intron 1 alternatively spliced compared to the total RNA level of LAC8. Or affected the LAC8 expression by some other means/mutants and analysed the splicing. Clearly, a splicing defects analysis in grp8 and nsra mutants would also help.

2b
The observed defect in the termination of LAC8 in the flail could be a consequence of expression change or could be the main driver behind the FLAIL action. This is not clear.

2c
In the Figures 5F, G, H the authors show that FIAL is bound by U1, U2 and U5. A control with a not-protein coding, not-spliced RNA like FLAIR would help to establish if the observed binding is specific, but I agree it may be difficult to find a control here.

3. "FLAIL binding regions" identification is presented in Figure 4G.
3a
Identified overrepresented motifs named by the authors "FLAIL binding regions" are not located in LAC8 intron 1 that shows defects in splicing, or close by. One would need deletion analysis of the intron and putative "FLAIL binding regions" to confirm the model.

3b
I like to ask what was the background model for the motif discovery. Looking at for example motif 3 - highlighted in red as ATTTTT, I wonder what is the chance of encountering the discovered motifs at random? It is also not clear to me if the motifs were analysed based on peaks or target genes.

3c

If the proposed motifs are imported for FLAIL mode of action one would predict that LAC8 target should also be conserved including the putative FLAIL binding sites in it.

4. The alternative role of FLAIL in PCP regulation is still not fully explained. The authors provide:
- RT-qPCR quantification of PCP in flail Fig R1A where a reduction can be observed but has a p-value of 0.16
- Panel R1B shows RNAseq snapshot but I am not convinced if the height of the graphs is adjusted to include all the reads please redraw with a higher cut-off.
- In the manuscript the authors mention:
"We could not detect an effect of FLAIL on PCP mRNA expression, or a large number of gene expression changes common between flail and pcp mutants including effects on AS (Dataset EV1 and EV4) "
However, I failed to find the referred datasets. In addition, a Venne diagram with statistical analysis would help here to substantiate the claim.
In addition, a PCP mutant analysis that would not show LAC8 defects would help the logic.

Mainor points"
5. line 309 Author mention CIR1 splicing defects but do not show the data.

6. line 229
"mRNA expression of most flowering genes was fully (eight) or partially (five) rescued by the expression of pFLAIL:gFLAIL (Fig 3C-D and Appendix Fig S4A-K)."
It would help to label or name targets authors considered fully and partially rescued as it is not clear to me what they mean.

7. Appendix Fig S2B the figure needs to include the non-treated control level otherwise it is difficult to interpret the stress effect.

Referee #2:

In this ms, the authors identified the FLAIL lncRNA that represses flowering in Arabidopsis from a locus producing sense and antisense transcripts. They use an allelic series involving T-DNA insertions, CRISPR/Cas9 and artificial miRNAs to study the role of FLAIL in flowering. A complementation series of constructs of the flail3 allele allowed them to show that the sense FLAIL lncRNA can act in trans. RNAseq revealed a small group of genes linked to the regulation of flowering whose expression is affected in the mutant and restored in the complementation line. The FLAIL lncRNA was shown to interact with alternative splicing regulators NSRab and GRP7. In addition, they showed using a ChIRPseq approach, that the lncRNA recognize distant targets at chromatin level as FLAIL binds to specific genomic regions. They also identified changes in specific histone modification between the wt and flail3 mutants and how this is restored in complementation lines. These changes were also linked to a specific change in alternative splicing on the 3'UTR of LAC8, a known regulator of flowering targeted by FLAIL. Finally, they prepared the double mutant lac8flail3 to demonstrate genetically that both genes act in the same pathway (no additive effect). Interestingly, potential homologs of FLAIL were found in Camelina and other species. The amount of work is impressive and the paper is very well written.
This paper is a very piece of work and demonstrates using a combination of genomic and genetic studies that the FLAIL lncRNA can act in trans to regulate alternative splicing of specific targets and consequently control gene expression. There is growing evidences that the non-coding genome hides large number of lncRNAs and there is little detailed genetic support for the action of lncRNAs globally. In contrast to many descriptive papers in the field, this ms demonstrates genetically, through an allelic series and complementation experiments, that this lncRNA locus is involved in flowering bringing interesting perspectives on potential new mechanisms of transcriptional regulation mediated by non-coding RNAs. This paper should interest a wide audience from molecula biology to evolution.
I have only minor comments to suggest to the authors:
1. Line 18, Abstract: chromatin "signaling". Is this really a signaling mechanism? I would say regulation or similar for chromatin.
2. Lines 21-23: The last phrase is a bit complex and too general? A more direct phrase showing the non-coding genome generates lncRNAs that can regulate alternative splicing and gene expression to impact organismal development?
3. Line 119 ...through AS of genes with effects
4. Line 154 ...ribosome association does not necessarily mean translation of sORFs (e.g. Bazin et al 2017) show that well known tasiRNA precursors are associated with ribosomes. In the absence of any ATG mutant, this part can be simplified as it is not clear, for me, whether the authors think that these ORFs play any role? Clearly not in AS which is a nuclear process.
5. The expression of lac8 in the amiRNA sense lines will be a plus. There is a minor albeit significative difference in FLAIL expression (Fig. EV2) although there is a phenotype.

Referee #3:

Significance:

The significance of the work is as per my original assessment--the field of lncRNAs in plants is lagging far behind in terms of knowledge about lncRNAs with assigned biological functions or lncRNAs with delineated mechanisms of action in plants. From this point of view, any identification of new functional plant lncRNAs for which either biological or mechanistic functions have been determined, is significant and exciting.

To give authors credit, the manuscript has undergone very thorough revision and it now reads like a totally different paper, very nicely written. Additionally, way more attention was paid to address the function of FLAIL lncRNA and its potential mechanism of action, which was totally missing in the original version, so it is a huge improvement. The focus of the new version of the manuscript is mostly on the alternative splicing affected by FLAIL. On the other hand, it still looks like FLAIL has a very pleotropic phenotype- there are three totally different directions in the manuscript: regulation of flowering (on the phenotypical level), plus the role of FLAIL in regulating alternative splicing; and the role of FLAIL in regulating the chromatin landscape on the mechanistical level. I'd advise authors to tighten up the any specific direction that is easier to make the paper more mechanistically focused, be it splicing or chromatin modifications in response to flowering. Or, alternatively, downplay flowering and focus on everything FLAIL does mechanistically in terms of gene expression.
Minor:
-- p. 117. The binding doesn't argue, it only might suggest, to be on the safe side;
-- p.309. Why do authors claim that FLAIL affects transcription by affecting LAC8 and CIR1, if the described in this section finding is on alternative splicing? Transcription and alternative splicing are two different processes, so I don't understand why these two processes are confused together. Transcription is transcription, while AS is AS. Try to figure out what is FLAIL claimed to be doing in terms of producing the correct amount of correctly spliced mRNA. Is it transcription or splicing?
-- I fully agree with authors responses in rebuttal and in my opinion, authors put very serious and responsible effort to improve the manuscript. However, the answer #3.4 is a little bit far-fetched in the absence of a deeper studies on the subject.

***

Rev_Com_number: RC-2021-01159
New_manu_number: EMBOJ-2022-110921R-Q
Corr_author: Marquardt
Title: FLAIL affects alternative splicing and represses flowering in Arabidopsis

Reviewer comments are in black and **our response in blue**.

Referee #1:

This is a resubmission of an original manuscript that describes the discovery and characterisation of *FLAIL* a lncRNA transcript.

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This part has been strengthened in the revision, and the genetic data that is now included in the manuscript in my view convincingly shows that *FLAIL* controls flowering through *LAC8*.

I however still have concerns about the mechanism. The authors say "our data thus suggest that *FLAIL* affects flowering through reducing mRNA expression of its direct target *LAC8*, mechanistically linked to AS and associated co-transcriptional chromatin changes"

To support this view they present:
- reanalysis of published RIP experiments with two splicing factors GRP7 and NSRa,
- splicing defects of *LAC8* in *FLAIL* mutant
- H3K4me1 and 3 change by ChIP on *LAC8* and CIR1 in *FLAIL*

Major points:

1. First, my concern is still that *FLAIL* RNAseq and ChIRP seq show a marginal overlap of 8 out of 1222 and 116 respectively targets. And some of those targets are probably not direct as their expression is not reversed in the complemented line. Here I wonder if this could not be a consequence of the direct but unintended binding of the probes to the targets DNA?

Response #1.1a: We opted for a stringent approach in our analysis of the ChiRP-seq data; we believe this offers a likely explanation for the overlap between RNA-seq and ChIRP-seq we described. We discussed two main parameter that determine the extent of the overlap in Line 407-411: 1) we set a very strict condition for *FLAIL* binding peaks. They have to be identified by both odd and even probes, but not in at least two Luc controls. These criteria lead to a limited yet rather credible number of identified *FLAIL* targets; 2), *FLAIL* targets such as *CIR1* are transcription factors that affect expression of multiple downstream genes. We agree that RNA-seq DEGs include direct and indirect targets; we highlighted it in the revised manuscript, lines 261-264.

One could test it by selecting a set of targets and doing ChIRP in a *FLAIL* KO background.

Response #1.1b: We are grateful for the suggestion of an additional control experiment. Unfortunately, we could not perform the requested new ChIRP-seq experiments in *FLAIL* mutants due to a long period of preparing all new samples and multiple changing of datasets.

One can say that the authors can focus on the *LAC8* target and somehow downplay the genome-wide data. But in my view, the low overlap between RNAseq and ChIRP puts in doubt the

subsequent reasoning whenever the authors refer back to genome-wide data like in Fig 4G when they analyse the overrepresented motifs in the ChIRP data set.

Response #1.1c: RNA-seq and ChIRP-seq inform on different aspects of *FLAIL*. In the simplest case, all binding events change splicing and mRNA expression, we get the impression that this is in line with the expectation of the reviewer. We are not sure if this expectation is justified genome-wide since other *FLAIL* targets may rely on the effect of *FLAIL* quantitatively different, or fully agree with our analyses of *LAC8* in a different condition. We find it reasonable that scenarios that are more complex may exist where *FLAIL* binding does not necessarily result in changed mRNA expression and splicing. We revised the manuscript to draw more attention to *LAC8* through text revisions in Lines 261-264. In addition, we took the opportunity to revise our motif analyses in the manuscript to focus on conserved motifs at the *LAC8* locus in *Brassicaceae* (see Figure R2). We present three conserved motifs in revised Fig. 4G.

2 The role of splicing in *LAC8* regulation by *FLAIL*.

2a

It is not clear to me if the splicing defects observed are not a consequence of *LAC8* expression changes in the *FLAIL* mutant. It would help if the authors reconsidered the splicing as the fraction of transcripts with intron 1 alternatively spliced compared to the total RNA level of *LAC8*. Or affected the *LAC8* expression by some other means/mutants and analysed the splicing. Clearly, a splicing defects analysis in grp8 and nsra mutants would also help.

#1.2a We presented a new panel with the ratio of each *LAC8* splicing isoform to the total RNA level of *LAC8* in revised Fig. 5E. These data suggest reduced levels of *LAC8*.1/8.2 with intron 1 isoforms but increased *LAC8*.3 in *FLAIL3* compared to WT. These data argue against the idea that the *LAC8* mRNA expression level (reduced in *flail* mutants) strictly correlates the expression of the *LAC8.3* isoform we observed. These data raise tantalizing new future questions, yet resolving these fully is that out of scope for a revision.

We present the RNA-seq screenshots at *LAC8* in *nsra* and *grp7* mutant to satisfy the curiosity of the reviewer. The *FLAIL*, *nsra* and *grp7* mutants appear to display less intron 1 and higher 3'UTR, respectively. It is experimentally challenging to disentangle cause and consequence at this stage since transcription and splicing are coupled. We favor the interpretation of *FLAIL*-induced *LAC8* expression through a co-transcriptional AS process (revised Line 18).

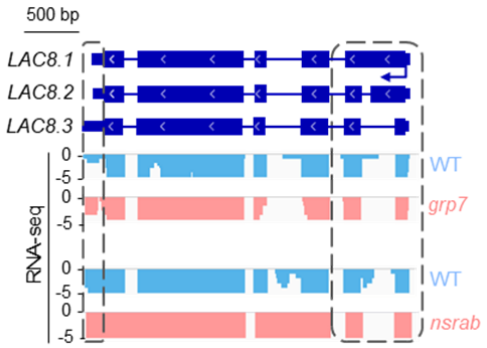


Figure R1 – Genome browser screenshots of RNA-seq at LAC8 locus in grp7 and nsrab mutants. *Upper panel: LAC8 isoforms are shown with large blue boxes indicating exons, narrow blue boxes indicating UTRs, and blue lines indicating introns. Different 3'UTR lengths and intron retention are labeled in dashed box. Bar = 500 bp.*

2b

The observed defect in the termination of *LAC8* in the *FLAIL* could be a consequence of expression change or could be the main driver behind the *FLAIL* action. This is not clear.

#1.3 We agree that fully resolving the causality would be very exciting. We are planning to dissect this at a deeper level in follow-up experiments by generating suitable *lac8* mutants focusing on the 3'UTR, ideally knock-ins. However, these experiments will take a significant amount of work and would form the basis of a different future manuscript.

2c

In the Figures 5F, G, H the authors show that FIAL is bound by U1, U2 and U5. A control with a not-protein coding, not-spliced RNA like FLAIR would help to establish if the observed binding is specific, but I agree it may be difficult to find a control here.

#1.4 Thank you very much for the suggestion. We performed new experiments to test additional *Arabidopsis* non-coding and unspliced RNAs as negative controls. We revised Fig. S7D by including the requested additional control of a non-coding, unspliced *lncCOBRA1* (*AT1G05913*) (Kramer et al 2022). The new data showed no difference of immunoprecipitated RNA quantities between WT and *flail3* mutant, indicating specific binding of U1, U2 and U5 by *FLAIL*. We revised the text (Lines 327-8) to reference the additional control data.

Kramer M C, Kim H J, Palos K R, et al. A conserved long intergenic non-coding RNA containing snoRNA sequences, lncCOBRA1, affects Arabidopsis germination and development. Frontiers in Plant Science, 2022: 1703.

3. "*FLAIL* binding regions" identification is presented in Figure 4G.

3a

Identified overrepresented motifs named by the authors "*FLAIL* binding regions" are not located in *LAC8* intron 1 that shows defects in splicing, or close by. One would need deletion analysis of the intron and putative "*FLAIL* binding regions" to confirm the model.

#1.5 The reviewer makes a valid point. We appreciate the experimental suggestion that is fully in line with our future research interests in this area, unfortunately, these experiments are out of scope of a revision. Even more so, we need to consider that deletion of the intron may also result in additional effects on *LAC8* expression, so many control strains would be necessary to allow an appropriate interpretation. We believe that *FLAIL* binding regions not located in intron1 may still affect AS of intron1. For example, the process of gene expression in 3D is likely very different from the linear piece of DNA that we depict in Fig.4. It remains plausible that the cluster of enriched motifs in the body of *LAC8* may connect to intron 1 through looping. *FLAIL* also occupies *LAC8* over intron 1, so spreading of *FLAIL* to intron 1 from a "*FLAIL* binding region" dense gene body could also be consistent with our experimental data. Of course, additional factors are likely involved that may make splicing of intron 1 particularly sensitive to *FLAIL*. We plan to resolve these questions in our future research.

3b

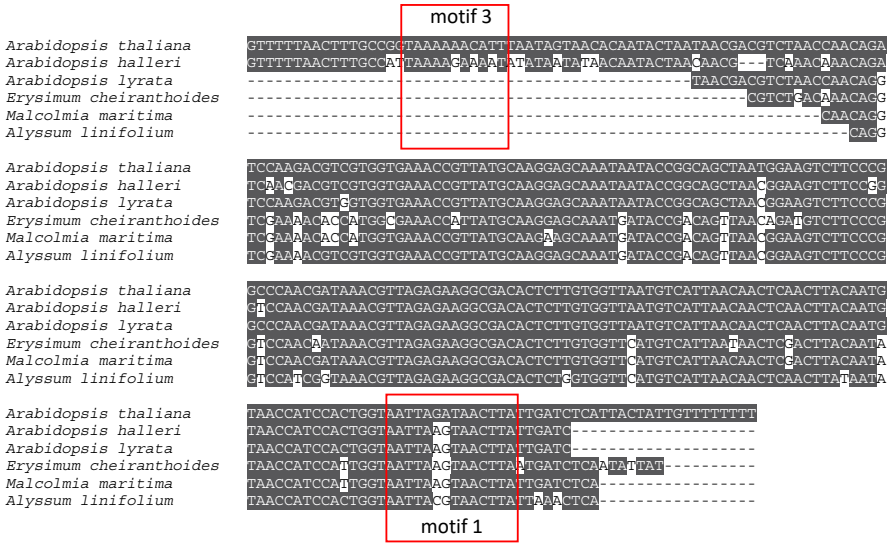
I like to ask what was the background model for the motif discovery. Looking at for example motif 3 - highlighted in red as ATTTTT, I wonder what is the chance of encountering the discovered motifs at random? It is also not clear to me if the motifs were analysed based on peaks or target genes.

#1.6 We enhanced our description of the motif discovery in the revised manuscript in Line 670-2. Motifs among the identified *FLAIL* binding peaks were discovered based on the random background selection model generated by HOMER's findMotifsGenome.pl program that allows background regions match the GC-content distribution of the peak sequence regions. In principle, we considered motifs containing ATTTTT… sequences as plausible when we examined the collective *FLAIL* binding regions. *FLAIL* peaks are not specific to exon gene bodies as for *LAC8*, we find them in non-coding and regulatory regions including promoters, intergenic regions, introns and 3'-UTRs (Appendix Fig. S5). Imbalanced preferential sequences in these regions may include AT-rich stretches. Nevertheless, we removed this motif based on the conservation result in Figure R2. In conclusion, we addressed this comment with a text revision and a Figure revision.

3c

If the proposed motifs are imported for *FLAIL* mode of action one would predict that *LAC8* target should also be conserved including the putative *FLAIL* binding sites in it.

#1.7 We are grateful for this suggestion. To address this concern we aligned DNA sequences containing motif regions of *LAC8* among ten *Brassicaceae* genomes. We focused on *Brassicaceae* genomes where we could identify putative *FLAIL* homologs. We found that motif 3 (in the previous manuscript version) is not conserved. We revised the manuscript by removing the AT rich motif3 from our revised manuscript on the basis of conservation. The other three motifs at *LAC8* locus are highly conserved. One interpretation of the conservation of these motifs is that these regions are conserved to maintain the putative *FLAIL* binding sites.



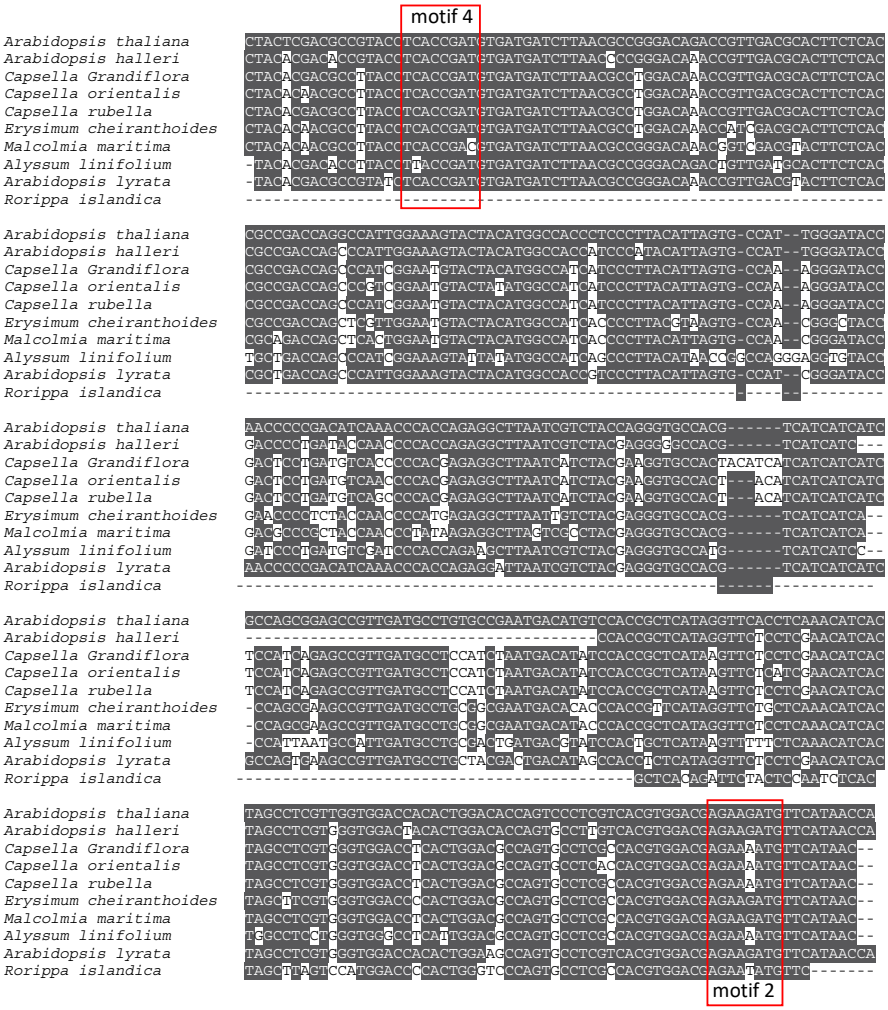


Figure R2 – LAC8 is conserved in Brassicaceae. *The sequences of LAC8 in Arabidopsis thaliana was blasted against nine disparate Brassicaceae genomes in CoGe BLAST with an e-value of 10^{-5}. Multiple Alignment of two consensus regions of LAC8 sequences containing conserved motif regions across species in Brassicaceae were highlighted in black using Geneious Prime software. Sequences in red box indicated consensus FLAIL binding motifs shown in Figure 4G.*

4. The alternative role of *FLAIL* in PCP regulation is still not fully explained. The authors provide:
- RT-qPCR quantification of PCP in *FLAIL* Fig R1A where a reduction can be observed but has a p-value of 0.16
- Panel R1B shows RNAseq snapshot but I am not convinced if the height of the graphs is adjusted to include all the reads please redraw with a higher cut-off.

#1.8 We addressed this comment by revising the panel. We added a screenshot of RNA-seq at *PCP* locus with a higher cut-off and a box plot for TPM value in Appendix Fig. S9B-C. These data fail to indicate changed *PCP* expression in *flail3*.

- In the manuscript the authors mention:
"We could not detect an effect of *FLAIL* on PCP mRNA expression, or a large number of gene

expression changes common between *FLAIL* and pcp mutants including effects on AS (Dataset EV1 and EV4) "
However, I failed to find the referred datasets. In addition, a Venne diagram with statistical analysis would help here to substantiate the claim.
In addition, a PCP mutant analysis that would not show *LAC8* defects would help the logic.
#1.9 We thank the reviewer for pointing this out. We revised the manuscript by including Venn diagrams of gene expression and AS changes between *FLAIL3* and *pcp* RNA-seq in Appendix Fig S9D and Dataset EV1, EV4, EV7, and EV8, which shared few common DEGs or DAS events. *pcp* mutation does not affect gene expression (Appendix Fig. S9E) or AS of *LAC8* (Dataset EV8).

Mainor points"
5. line 309 Author mention CIR1 splicing defects but do not show the data.
#1.10 The text mistake has been corrected in Line317.

6. line 229
"mRNA expression of most flowering genes was fully (eight) or partially (five) rescued by the expression of p*FLAIL*:g*FLAIL* (Fig 3C-D and Appendix Fig S4A-K)."
It would help to label or name targets authors considered fully and partially rescued as it is not clear to me what they mean.

#1.11 All gene names are included in the revised manuscript, shown in Line231-235 and Fig 3.

7. Appendix Fig S2B the figure needs to include the non-treated control level otherwise it is difficult to interpret the stress effect.
#1.12 Thank you for this suggestion, we now included the non-treated controls in our revision. We re-analyzed *FLAIL* expression in wild type under mock and treatment conditions and significantly dysregulated *FLAIL* expression was shown in Appendix Fig S2B.

Referee #2:

In this ms, the authors identified the *FLAIL* lncRNA that represses flowering in Arabidopsis from a locus producing sense and antisense transcripts. They use an allelic series involving T-DNA insertions, CRISPR/Cas9 and artificial miRNAs to study the role of *FLAIL* in flowering. A complementation series of constructs of the *FLAIL3* allele allowed them to show that the sense *FLAIL* lncRNA can act in trans. RNAseq revealed a small group of genes linked to the regulation of flowering whose expression is affected in the mutant and restored in the complementation line. The *FLAIL* lncRNA was shown to interact with alternative splicing regulators NSRab and GRP7. In addition, they showed using a ChIRPseq approach, that the lncRNA recognize distant targets at chromatin level as *FLAIL* binds to specific genomic regions. They also identified changes in specific histone modification between the wt and *FLAIL3* mutants and how this is restored in complementation lines. These changes were also linked to a specific change in alternative splicing on the 3'UTR of *LAC8*, a known regulator of flowering targeted by *FLAIL*. Finally, they prepared the double mutant *LAC8FLAIL3* to demonstrate genetically that both genes act in the same pathway (no additive effect). Interestingly, potential homologs of *FLAIL* were found in Camelina and other species. The amount of work is impressive and the paper is very well written.
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Thank you, we are grateful for this positive assessment.

I have only minor comments to suggest to the authors:
1. Line 18, Abstract: chromatin "signaling". Is this really a signaling mechanism? I would say regulation or similar for chromatin.

#2.1 We agree that using "chromatin regulation" captures it more accurately that "chromatin signaling" and have revised the text in line 18.

2. Lines 21-23: The last phrase is a bit complex and too general? A more direct phrase showing the non-coding genome generates lncRNAs that can regulate alternative splicing and gene expression to impact organismal development?

#2.2 Thank you for this suggestion. We phrased line 21-23 more directly to address this comment.

3. Line 119 ...through AS of genes with effects

#2.3 The text mistake has been edited as "AS of genes" in Line 120.

4. Line 154 ...ribosome association does not necessarily mean translation of sORFs (e.g. Bazin et al 2017) show that well known tasiRNA precursors are associated with ribosomes. In the absence of any ATG mutant, this part can be simplified as it is not clear, for me, whether the authors think that these ORFs play any role? Clearly not in AS which is a nuclear process.

#2.4 We thank the reviewer for pointing this out. We agree that ribosome association does not necessarily mean translation of sORFs and cited this paper in Line 47, 157, 377. We clarify sORFs do not play any role for *FLAIL* in the discussion in Line 377.

5. The expression of *LAC8* in the amiRNA sense lines will be a plus. There is a minor albeit significative difference in *FLAIL* expression (Fig. EV2) although there is a phenotype.

#2.5 We understand the confusion in that the reduction of *FLAIL* in amiRNA mutants is lower than with other approaches to perturb *FLAIL* expression. We consider it a likely explanation that these changes nevertheless trigger a robust repression of the downstream genes such as *LAC8* or *CIR1*. RT-qPCR data clearly showed that the expression *of CIR1* and *LAC8* were highly reduced in amiR-*FLAIL* mutants (Fig. EV3C-D), suggesting that *LAC8* and *CIR1* are potential direct *FLAIL* targets responsible for flowering repression. Notably, *FLAIL* is broadly expressed throughout the plant yet to different levels in various tissues (Appendix Fig. S2A). Our measurement of *FLAIL* expression from seedlings tissues with amiRNA empty vector transformed Col-0 plant as control may also explain the minor *FLAIL* reduction in amiRNA mutants. A long lasting decrease of *FLAIL* from seedling to flowering stage in various tissues together likely contributes to an early flowering phenotype of amiRNA mutants.

Referee #3:

Significance:
The significance of the work is as per my original assessment--the field of lncRNAs in plants is lagging far behind in terms of knowledge about lncRNAs with assigned biological functions or lncRNAs with delineated mechanisms of action in plants. From this point of view, any identification of new functional plant lncRNAs for which either biological or mechanistic functions have been determined, is significant and exciting.

To give authors credit, the manuscript has undergone very thorough revision and it now reads like a totally different paper, very nicely written. Additionally, way more attention was paid to address the function of *FLAIL* lncRNA and its potential mechanism of action, which was totally missing in the original version, so it is a huge improvement. The focus of the new version of the manuscript is mostly on the alternative splicing affected by *FLAIL*. On the other hand, it still looks like *FLAIL* has a very pleotropic phenotype- there are three totally different directions in the manuscript: regulation of flowering (on the phenotypical level), plus the role of *FLAIL* in regulating alternative splicing; and the role of *FLAIL* in regulating the chromatin landscape on the mechanistical level. I'd advise authors to tighten up the any specific direction that is easier to make the paper more mechanistically focused, be it splicing or chromatin modifications in response to flowering. Or, alternatively, downplay flowering and focus on everything *FLAIL* does mechanistically in terms of gene expression.

We thank reviewer #3 for acknowledging the huge improvements. We find that the complexity inherent to the connections between splicing, chromatin and *LAC8* expression challenge the idea that these process can be addressed in isolation.

Minor:
-- p. 117. The binding doesn't argue, it only might suggest, to be on the safe side;

#3.1 "argue" has been changed to "suggest" in Line 120.

-- p.309. Why do authors claim that *FLAIL* affects transcription by affecting *LAC8* and CIR1, if the described in this section finding is on alternative splicing? Transcription and alternative splicing are two different processes, so I don't understand why these two processes are confused together. Transcription is transcription, while AS is AS. Try to figure out what is *FLAIL* claimed to be doing in terms of producing the correct amount of correctly spliced mRNA. Is it transcription or splicing?

#3.2 We believe that the concept of co-transcriptional splicing is well supported, and hence that splicing, transcription and chromatin are coupled. There is growing evidence indicating that AS represents a co-transcriptional process, where splicing, transcription are in close-proximity and influence each other. For instance, in yeast, a co-transcriptional process of splicing occurs as soon as the intron is transcribed and before transcription termination (Alexander et al. 2010); Transcription rate strongly affects co-transcriptional splicing in budding yeast (Aslanzadeh et al, 2018). In mammalian cells, pri-miRNA processing is achieved through co-transcriptional cleavage by an RNase III like enzyme Drosha (Morlando et al, 2008) and further mammalian NET-seq reveals a dynamic and detailed information on nascent transcription and co-transcriptional RNA processing in mammalian cells (Nojima et al, 2015). Similarly, the genome-wide co-transcriptional splicing of both coding and noncoding RNAs and its regulation are shown in plant cells (Zhu et al,

2020; Li et al, 2020; Marquardt et al, 2022). We addressed this comment by illustrating these connections more clearly in lines 329-324.

Alexander RD, Innocente SA, Barrass JD, Beggs JD. 2010. Splicing-dependent RNA polymerase pausing in yeast. Mol Cell 40: 582–593
Aslanzadeh V, Huang Y, Sanguinetti G, Beggs JD (2018) Transcription rate strongly affects splicing fidelity and cotranscriptionality in budding yeast. Genome Res 28: 203–213
Morlando M, Ballarino M, Gromak N, Pagano F, Bozzoni I, Proudfoot NJ (2008) Primary microRNA transcripts are processed cotranscriptionally. Nat Struct Mol Biol 15: 902–909
Nojima T, Gomes T, Grosso ARF, Kimura H, Dye MJ, Dhir S, Carmo-Fonseca M, Proudfoot NJ (2015) Mammalian NET-seq reveals genome-wide nascent transcription coupled to RNA processing. Cell 161: 526–540
Li S, Wang Y, Zhao Y, Zhao X, Chen X, Gong Z (2020) Global cotranscriptional splicing in Arabidopsis and the correlation with splicing regulation in mature RNAs. Mol Plant 13: 266–277
Kindgren P, Ivanov M, Marquardt S (2020) Native elongation transcript sequencing reveals temperature dependent dynamics of nascent Pol II transcription in Arabidopsis. Nucleic Acids Res 48: 2332–2347
Zhu D, Mao F, Tian Y, Lin X, Gu L, Gu H, Qu LJ, Wu Y, Wu Z (2020) The features and regulation of cotranscriptional splicing in Arabidopsis. Mol Plant 13: 278–294
Marquardt S, Petrillo E, Manavella PA (2022) Cotranscriptional RNA processing and modification in plants. Plant Cell: koac309

-- I fully agree with authors responses in rebuttal and in my opinion, authors put very serious and responsible effort to improve the manuscript. However, the answer #3.4 is a little bit far-fetched in the absence of a deeper studies on the subject.

#3.3 We are grateful for the reviewer for this assessment. To address this comment we downplayed conclusions as shown in Line 404-432.

2nd Revision - Editorial Decision **6th Mar 2023**

Dear Sebastian,

We have now received re-review reports from the three referees who initially appraised your manuscript for Review Commons. As you will see, all appreciated the effort you invested in the revision process and agree that the manuscript is significantly stronger. Referee 1 has some residual concerns over mechanistic detail, but, in my opinion, your clear answers to these points (available to readers in the accompanying RPF file) faithfully summarise the scientific question which is being discussed.

Before I can finally accept the manuscript though, there are some remaining editorial points which need to be addressed. In this regard would you please:

- make sure that the final text file (.doc) contains no differently coloured or highlighted text,
- ensure acknowledgement of grant number nnf21oc0066776 is included in the manuscript file,
- rename the conflict of interest statement as the "DISCLOSURE AND COMPETING INTERESTS STATEMENT",
- remove the AC/CrediT section from the text,
- complete the "Adherence to community standards" section of the author checklist,
- add page numbers to the table of contents, and include the figure legends below the appendix figures,
- provide a general summary image, and
- ensure all Legends for Datasets, EV Tables and Appendix figures are removed from the manuscript file,

EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

All editorial and formatting issues were resolved by the authors.

3rd Revision - Editorial Decision **9th Mar 2023**

Dear Sebastian,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Congratulations on an elegant series of experiments, and an exciting piece of work (also on behalf of Stefanie).

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Yours sincerely,

William

William Teale, PhD
Editor
The EMBO Journal
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