THE EMBO JOURNAL

Manuscript EMBO-2010-73952

Growth habit determination by the balance of histone methylation activities in Arabidopsis

Jong-Hyun Ko, Irina Mitina, Yosuke Tamada, Youbong Hyun, Yeonhee Choi, Richard M. Amasino, Bosl Noh and Yoo-Sun Noh

Corresponding author: Yoo-Sun Noh, Seoul National University

Review timeline:

Submission date:	15 February 2010
1st Editorial Decision:	22 March 2010
1st Revision received:	05 June 2010
2nd Editorial Decision:	05 July 2010
2nd Revision received:	14 July 2010
3rd Editorial Decision:	15 July 2010
3rd Revision received:	19 July 2010
Accepted:	20 July 2010

Transaction Report:

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

1st Editorial Decision 22 March 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see below, the referees find the analysis interesting and appropriate for the journal. However, they also bring up some technical issues, as detailed below, that have to be addressed before further consideration here. Given the comments provided, I would like to invite you to submit a revised version should you be able to address the concerns raised in full. I would like to add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to address the concerns raised at this stage.

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

I thank you for the opportunity to consider your work for publication. I look forward to seeing the revised version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The paper looks at the control of histone methylation in respect to flowering in Arabidopsis. There are three main advances:

1) they show that the FRIGIDA (FRI) gene, which is required to confer a vernalization requirement, interacts in yeast two hybrid assay with the histone methyltransferase (HMTase) EFS, coIP's with EFS, and stimulates the HMT activity of EFS in in vitro assay. Given that the mechanism of FRI action has been obscure, this seems an important step forward.

2) They show that EFS acts as a H3K4 and H3K36 HMTase based on in vitro assays and in vivo analysis of histone methylation levels in mutants

3) They show that REF6 and FLD have histone demethylase (HDM) activity based on in vitro assays and in vivo analysis of histone methylation levels in mutants.

The in vitro assays are important as these have not been much done for plant HDM and HMTases. Overall then I think the paper is an important advance in the plant epigenetics/flowering time area and is very suitable for publication in EMBO J.

I have some comments on the data below. I think the m/s would be improved if these were addressed but don't see them as critical for acceptance.

1) The ChIP assays are mostly done by gel PCR using single primer sets (i.e. with no internal control as in a multiplex reaction, and with less accurate quantification than in a real time PCR assay. I am not a big fan of this kind of assay as I don't think there is adequate control for variation in the overall amount of DNA that is present in each IP. Nonetheless, there are innumerable papers published with the same assay, so it is unreasonable to insist on alternative assays. The differences in some of the figures are quite subtle (Fig 3A and B for example) and more accurate quantification by real time PCR might be helpful.

2) The ChIP data indicating that EFS-FLAG is associated with FLC is modest, i.e. the maximum enrichment is two fold relative to the control (non transgenic line), which seems of questionable significance given that ChIP assays can be somewhat variable.

3) although FRI and EFS are postulated to be in the same complex, they show enrichment at different regions of FLC, for example FRI looks to be enriched at region D, whereas there is no enrichment for EFS in this region.

4) on page 7 EFS is described as a novel HMT with dual substrate (H3K4me1/me2 and H3K36me1/me2) specificity. I think this is a typo and me2/me3 is intended.

5) In materials and methods, it would help to clarify which antibodies were used for GUS and FLAG in the ChIP section (the information is present elsewhere in methods, but it would be clearer to have this in the ChIP methods section).

Referee #2 (Remarks to the Author):

The manuscript by Ko et al. elaborates on the interplay between FLC transcription regulation, histone modifications and histone modifying enzymes (methylases and de-methylases) that have previously been shown to participate in the regulation of FLC. The main point of the manuscript is the attempt to integrate into the model a biochemical function of FRI, an upstream activator of FLC in winter-annual accessions of Arabidopsis. The work also describes the effect of two FLC repressors on histone mark profiles and their interaction with FRI and efs. The authors show

recruitment of the FLC activating protein FRI to the first third of the FLC gene. Genetic analysis shows the recruitment to be dependent on the histone methylase EFS/SDG8. In contrast, EFS recruitment to the FLC promoter is shown to be independent of FRI. The authors suggest that a re-enforcing feed-back loop exists between EFS and FRI, where EFS is required for FRI recruitment and FRI, in turn activates the HMT-activity of EFS. This model is based on the finding that FRI and EFS interact in plant extracts and that addition of recombinant FRI enhances the HMT-activity of EFS in vitro. This model is interesting and suggests a biochemical function for FRI that has so far been elusive.

The paper is interesting, and identifying a biochemical function for FRI is of major interest. Unfortunately, the experimental set-ups for two key experiments appear unsatisfactory. The pull-down assay (Figure 1E) suffers from a lack of controls, such as a mock-pull down from GUS EFS:FLAG plants with anti-GUS antibodies or a similar experiment that convincingly shows the detected FLAG:EFS signal to be specific for the FRI interaction. Therefore the direct interaction is not convincingly shown (in addition it is not clear whether the material for the pull-down was cross-linked as suggested by the wording in the MM section).

In addition, the HMT assays were carried out using an excess of recombinant protein over the substrate-nucleosomes and very long incubation times (14h at 30˚). It is unclear whether the slight increase in HMT-activity observed after the addition of FRI is due to an enhanced activity or a better survival of the HMT enzyme. The assay does not have a resolution to compare affinities or turn-over rates and therefore leaves it unclear whether the EFS is specific for H3K36me2/3 or also active as an H3K4me3 transferase. The dual specificity, which is important for the proposed model, is somewhat in contradiction to the previous finding by Xu et al. 2008 that H3K36me2/3 but not H3K4me2/3 levels are globally affected in efs/sdg8 mutant plants.

Ko et al. accompany the data by a detailed analysis of histone modifications H3K4me2/me3 and H3K36me2 (why not H3K36me3?) at the FLC locus in various mutants and double mutants (efs, ref6, fld, fri, FRI). However, the observed histone profiles and changes, all based on semi-quantitative PCR are difficult to judge for their significance. As previously shown, presence of FRI is correlated with an increase in H3K4me3 over the locus but the loss of increase in FRI efs of this mark may be caused by decreased FLC transcription and not by a loss of EFS stimulation. Using qPCR would generate more convincing quantitative data.

The authors also show the direct recruitment of two FLC repressors to the locus. Biochemical data indicate that REF6 acts as H3K4me2-demethylase. In contrast, there is no convincing difference in the occurrence of this histone-modification throughout the locus (REF6 seems to target the promoter of FLC), which leaves the mechanistic connection to FLC unresolved. Changes in H3K4me3 are observed in the ref6 mutants at the first third of the FLC locus, but again, FLC is increased in transcription. The autonomous pathway component FLD is targeted to the transcriptional start site, downstream of the position where REF6 is found. The histone profile changes observed in the fld mutant are different from those observed in ref6 and taken together with the genetic data it is likely that these proteins act independently to repress FLC. The conclusion from this part is somewhat imprecise and this is also reflected in the manuscript title.

Referee #3 (Remarks to the Author):

This manuscript investigates the roles of histone methylation and demethylation of chromatin associated with the floral repressor, FLC, in regulating the summer- versus winter-annual growth habit of Arabidopsis. The authors present evidence that the histone methyltransferase, EFS, has a novel, dual specificity for histone H3 lysines 4 and 36. The activity of EFS is counterbalanced by the demethylases FLD and REF6, the latter also being shown to have dual specificity. FRI, a positive regulator of FLC, is recruited to FLC chromatin through an interaction with EFS; recruitment of FRI tips the balance in favour of histone methylation at FLC chromatin by enhancing EFS activity, leading to up-regulation of FLC and a winter-annual growth habit. Similarly, loss of histone demethylase activity, particularly that of FLD, leads to increased histone methyltransferase activity and winter-annual habit, even in the absence of FRI.

The data presented are novel and for most part the conclusions are well supported by the data. The finding the EFS has a dual specificity is interesting and makes sense of some previous reports that have implicated EFS in either K4 or K36 trimethylation. Overall this is a nice piece of work that adds another piece of knowledge to our understanding of FLC regulation, but like most good research raises a new set of questions. I have outlined these and some other criticisms of the manuscript below:

1. Figure 1 shows that recruitment of FRI to FLC chromatin is dependent on both EFS and SUF4, and yet the text (bottom line p5) states "that EFS is crucial for the recruitment of FRI to FLC chromatin" implying that this is all that is needed, when clearly SUF4 is important too. In fact the yeast 2 hybrid data suggest a stronger interaction between SUF4 and EFS than between EFS and FRI. Perhaps SUF4 is strongly recruited to FLC by EFS, or SUF4 recruits EFS to FLC?

2. This raises the question of what happens to EFS recruitment to FLC chromatin in a suf4 mutant background? Similarly, what happens to SUF4 recruitment in en efs background?

3. efs mutants have a pleiotropic phenotype and quite a number of genes are downregulated in these plants (for example see Cazzonelli et al., 2009 Plant Cell 21:39-). Given that FRI appears to be specific for FLC how does this specificity occur, if EFS is indeed important for recruiting FRI to FLC as the data suggest? I feel that some discussion of this point is warranted. This is an important point given the demonstrated enhancement of EFS activity on an in vitro oligonucleosome template (ie where there is no specificity) (Fig 1H).

4. Figure 1 shows two peaks of binding of EFS to FLC chromatin - is there any sequence similarity in the underlying DNA that might account for EFS binding to these two regions?

5. Figure 2; the in vitro assays look good, but I wonder if one might expect that to see an increase in the level of H3K4me1 on Western blots given that REF6 can efficiently demethylate both H3K4me3 and H3K4me2?

6. I think that the data presented in Supplementary Fig 3 should appear in the main body of the paper.

7. I found the title to be a little cryptic, especially for a more general audience. I'd suggest spelling out what is meant by "growth habit determination", perhaps "The balance of histone methylation activities determines the choice between winter- or summer-annual growth".

1st Revision - authors' response 05 June 2010

We thank the editor and reviewers for their valuable comments and hope we have satisfactorily addressed all the issues raised.

Response to Reviewers' comments

Referee #1: *"I have some comments on the data below. I think the m/s would be improved if these were addressed but don't see them as critical for acceptance."*

1) The ChIP assays are mostly done by gel PCR using single primer sets (i.e. with no internal control as in a multiplex reaction, and with less accurate quantification than in a real time PCR assay. I am not a big fan of this kind of assay as I don't think there is adequate control for variation in the overall amount of DNA that is present in each IP. Nonetheless, there are innumerable papers published with the same assay, so it is unreasonable to insist on alternative assays. The differences in some of the figures are quite subtle (Fig 3A and B for example) and more accurate quantification by real time PCR might be helpful.

Response: Following the reviewer's suggestion, we have used three independent biological replicates, quantified the differences in the histone methylation levels by ChIP-qPCR, and replaced

all the ChIP-gel PCR data with new ChIP-qPCR data (Figures 1B, 2A, 3A, and 3B). Our new ChIP-qPCR data are fully consistent with the previously presented ChIP-gel PCR data.

2) The ChIP data indicating that EFS-FLAG is associated with FLC is modest, i.e. the maximum enrichment is two fold relative to the control (non transgenic line), which seems of questionable significance given that ChIP assays can be somewhat variable.

Response: We tested the significance of the fold enrichment for EFS:FLAG by the Student's t-test. Because the P values were less than 0.05 for the regions FLC A and FLC F, the association of EFS:FLAG with these regions was considered significant. We indicated the test results in Figure 1D and in the figure legend (page 23). One thing should be noted here is that EFS is not a DNA-binding-motif containing protein. Thus its binding with chromatin must be mediated by a protein complex, and many times this type of chromatin association results in low binding signals.

3) although FRI and EFS are postulated to be in the same complex, they show enrichment at different regions of FLC, for example FRI looks to be enriched at region D, whereas there is no enrichment for EFS in this region.

Response: This is an interesting point and should be clarified at some point in the future. One possible explanation for this issue might be related with the role of FRI in enhancing the activity of EFS. The methyltransferase activity of EFS might be enhanced by the initial contact with FRI through an unknown mechanism rather than by continuous complex formation with FRI. In this case, the initial recruitment of FRI by EFS at the transcription initiation region of *FLC* might be sufficient for enhancement of the methyltransferase activity of EFS, and after this both FRI and EFS might move on *FLC* chromatin independently. This and other possibilities might be tested in future studies.

4) on page 7 EFS is described as a novel HMT with dual substrate (H3K4me1/me2 and H3K36me1/me2) specificity. I think this is a typo and me2/me3 is intended.

Response: Our *in vitro* histone methyltransferase activity assay in Figure 1I showed that EFS can increase the levels of H3K4me2/H3K4me3 and H3K36me2/H3K36me3. These results indicate that the actual substrates of EFS are H3K4me1/me2 and H3K36me1/me2 (thus we can see increased H3K4me2/H3K4me3 and H3K36me2/H3K36me3 levels after the reaction).

5) In materials and methods, it would help to clarify which antibodies were used for GUS and FLAG in the ChIP section (the information is present elsewhere in methods, but it would be clearer to have this in the ChIP methods section).

Response: As the reviewer suggested, we included the information for GUS and FLAG antibodies in the "ChIP assay" section as "For the ChIP assays using *FRI:GUS* and *EFS:FLAG*, GUS ab (Invitrogen A5790) and FLAG ab (Sigma A8592) were used, respectively." (page 12).

Referee #2: *"The paper is interesting, and identifying a biochemical function for FRI is of major interest. Unfortunately, the experimental set-ups for two key experiments appear unsatisfactory."*

1) The pull-down assay (Figure 1E) suffers from a lack of controls, such as a mock-pull down from GUS EFS:FLAG plants with anti-GUS antibodies or a similar experiment that convincingly shows the detected FLAG:EFS signal to be specific for the FRI interaction. Therefore the direct interaction is not convincingly shown (in addition it is not clear whether the material for the pull-down was cross-linked as suggested by the wording in the MM section).

Response: Because a *GUS EFS:FLAG* transgenic line is not available, we performed a mock pull-down from *FRI:GUS* and *FRI:GUS EFS:FLAG* nuclear extracts without antibody (Mock IP; the second panel in Figure 1E) and compared the FLAG western signal with that of immunoprecipitated with GUS antibody (the third panel in Figure 1E). The results show that the western signal for EFS:FLAG is specific to FRI:GUS immunoprecipitated with GUS antibody. Therefore, we believe an appropriate control is now included in the revised manuscript.

Nuclear extracts used for the Co-IP were cross linked as the reviewer mentioned. To make this fact clearer, we have corrected the corresponding sentence in the Materials and Methods to "Cross-linked nuclear proteins were extracted…" (page 12).

2) In addition, the HMT assays were carried out using an excess of recombinant protein over the substrate-nucleosomes and very long incubation times (14h at 30˚). It is unclear whether the slight increase in HMT-activity observed after the addition of FRI is due to an enhanced activity or a better survival of the HMT enzyme. The assay does not have a resolution to compare affinities or turn-over rates and therefore leaves it unclear whether the EFS is specific for H3K36me2/3 or also active as an H3K4me3 transferase. The dual specificity, which is important for the proposed model, is somewhat in contradiction to the previous finding by Xu et al. 2008 that H3K36me2/3 but not H3K4me2/3 levels are globally affected in efs/sdg8 mutant plants.

Response: In our pilot *in vitro* assays, we had tested the HMT activity of EFS using nucleosomes with various incubation times and temperatures, for example 2 hrs/37°C or 13 hrs/30°C, following the protocol published (Rea et al, 2000). In these assays, we had obtained similar FRI-dependent increased EFS activities in all conditions tested. Therefore, we selected a condition of longer incubation time (13 hrs) with lower temperature (30°C) which had resulted in more significant FRI-dependent H3K4 and H3K36 methylation changes than conditions of shorter incubation times. Although these pilot assays with various incubation times do not completely rule out the possibility of "better survival of the HMT enzyme by FRI", the fact that shorter incubations also had resulted in enhanced HMT activity suggests that chance for the longer survival of EFS by FRI is not high. Furthermore, our results in Figures 1B and 1C clearly demonstrate that both EFS and FRI are required for elevated H3K4 and H3K36 methylations at *FLC* and EFS is crucial for FRI recruitment into *FLC* chromatin. These *in vivo* results is consistent with our interpretation that FRI enhances the HMT activity of EFS but cannot be easily explained by longer survival of EFS by FRI.

Contrary to H3K36me2/3 levels, H3K4me2/3 levels in *efs/sdg8* mutants are not affected globally (Xu et al, 2008). It might be possibly because of a locus-specific H3K4 HMT activity of EFS. Zhao et al (2005) reported that mutations in *efs/sdg8* result in reduced H3K36me but not H3K4me at *FLC* locus in a *fri* mutant (which is Col) background. However, as we presented in Figure 1B, mutations in *efs/sdg8* result in reduced H3K4me as well as H3K36me at *FLC* in a functional *FRI* background. Therefore, at *FLC* locus, the H3K4 HMT activity of EFS is obvious only with functional FRI unlike the H3K36 HMT activity of EFS which is obvious with or without FRI. In contrast, Cazzonelli et al (Plant Cell 21: 39-53, 2009) reported that mutations in *efs/sdg8* result in reduced H3K4me at *CRTISO* locus in a *fri* mutant (Col) background. In summary, the H3K36 HMT activity of EFS might be obvious *in vivo* independently of FRI, and this might result in globally affected H3K36me2/3 levels in *efs/sdg8*. In contrast, the H3K4 HMT activity of EFS might be regulated in a locus-specific manner *in vivo*, for example it becomes obvious only with FRI at *FLC* but not at *CRTISO*. Thus, the levels of H3K4me2/3 might not be affected globally by *efs/sdg8* mutations. We should also emphasize that our *in vitro* HMT activity assays in Figure 1I clearly show that EFS possesses intrinsic HMT activity for both H3K4 and H3K36.

3) Ko et al. accompany the data by a detailed analysis of histone modifications H3K4me2/me3 and H3K36me2 (why not H3K36me3?) at the FLC locus in various mutants and double mutants (efs, ref6, fld, fri, FRI). However, the observed histone profiles and changes, all based on semi-quantitative PCR are difficult to judge for their significance. As previously shown, presence of FRI is correlated with an increase in H3K4me3 over the locus but the loss of increase in FRI efs of this mark may be caused by decreased FLC transcription and not by a loss of EFS stimulation. Using qPCR would generate more convincing quantitative data.

Response: As suggested, we have used three independent biological replicates, quantified the differences in the histone methylation levels by ChIP-qPCR, and replaced all the ChIP-gel PCR data with new ChIP-qPCR data (Figures 1B, 2A, 3A, and 3B). Our new ChIP-qPCR data are fully consistent with the previously presented ChIP-gel PCR data and allow quantitative comparisons for histone methylation levels.

4) The authors also show the direct recruitment of two FLC repressors to the locus. Biochemical data indicate that REF6 acts as H3K4me2-demethylase. In contrast, there is no convincing difference in the occurrence of this histone-modification throughout the locus (REF6 seems to target the promoter of FLC), which leaves the mechanistic connection to FLC unresolved. Changes in

H3K4me3 are observed in the ref6 mutants at the first third of the FLC locus, but again, FLC is increased in transcription. The autonomous pathway component FLD is targeted to the transcriptional start site, downstream of the position where REF6 is found. The histone profile changes observed in the fld mutant are different from those observed in ref6 and taken together with the genetic data it is likely that these proteins act independently to repress FLC. The conclusion from this part is somewhat imprecise and this is also reflected in the manuscript title.

Response: Our revised ChIP-qPCR data in Figure 2A show that H3K4me2 levels are also moderately increased in both *ref6* and *fld* mutants consistent with the intrinsic histone demethylase activities of REF6 in Figure 1G. At this point, we do not completely understand the reasons for the slight difference in the localization pattern between REF6 and FLD at around the transcription start site of *FLC* and for the difference between the localization pattern of REF6 and the profile of H3K4me3 levels in various *FLC* regions. One possibility is that a histone modifying protein might be recruited into a region of chromatin and stalled there temporally until a full protein complex (which might allow full catalytic activity and rapid movement along the target chromatin) is assembled. We believe the demonstration of the above or other hypotheses is beyond the scope of this manuscript and might be better to be handled by follow-up studies.

As the reviewer pointed out, the difference in the localization pattern between REF6 (Figure 2E) and FLD (Figure 2F) and the genetic data in Figures 1C and 1D indicate that REF6 and FLD have independent roles in *FLC* repression. To make this point clearer, we changed the text on page 8 to "…, also supporting their independent repressive roles.".

Referee #3: *"Overall this is a nice piece of work that adds another piece of knowledge to our understanding of FLC regulation, but like most good research raises a new set of questions. I have outlined these and some other criticisms of the manuscript below."*

1) Figure 1 shows that recruitment of FRI to FLC chromatin is dependent on both EFS and SUF4, and yet the text (bottom line p5) states "that EFS is crucial for the recruitment of FRI to FLC chromatin" implying that this is all that is needed, when clearly SUF4 is important too. In fact the yeast 2 hybrid data suggest a stronger interaction between SUF4 and EFS than between EFS and FRI. Perhaps SUF4 is strongly recruited to FLC by EFS, or SUF4 recruits EFS to FLC?

2) This raises the question of what happens to EFS recruitment to FLC chromatin in a suf4 mutant background? Similarly, what happens to SUF4 recruitment in en efs background?

Response: The above 2 questions are related each other, so we will try to answer for them together. In the beginning of the second paragraph on page 5, we wrote "Surprisingly, the association of FRI:GUS with *FLC* chromatin was abolished in the absence of EFS or SUPPRESSOR OF FRIGIDA 4 (SUF4; Figure 1C), a FRI-interacting nuclear protein (Kim *et al*, 2006).", clearly indicating that both EFS and SUF4 are required for the recruitment of FRI to *FLC* chromatin.

The reviewer's question regarding the relationship between EFS and SUF4 would be interesting. Because the question can only be tested properly using transgenic lines containing EFS:FLAG in *suf4* mutants and an epitope-tagged SUF4 in *efs* mutants, the work might be better to be a part of our next paper. In this paper, we have tried to focus on the relationship between FRI and EFS.

3) efs mutants have a pleiotropic phenotype and quite a number of genes are downregulated in these plants (for example see Cazzonelli et al., 2009 Plant Cell 21:39-). Given that FRI appears to be specific for FLC how does this specificity occur, if EFS is indeed important for recruiting FRI to FLC as the data suggest? I feel that some discussion of this point is warranted. This is an important point given the demonstrated enhancement of EFS activity on an in vitro oligonucleosome template (ie where there is no specificity) (Fig 1H).

Response: This is really an interesting point for us too. One possibility we currently imagine is that FRI and other FRI family members might be recruited by EFS to different loci. We already discussed about this issue in the last paragraph on page 10.

4) Figure 1 shows two peaks of binding of EFS to FLC chromatin - is there any sequence similarity in the underlying DNA that might account for EFS binding to these two regions?

Response: The two EFS-associated regions have 46% sequence identity. However, it should be noted that EFS is not a DNA-binding-motif containing protein, thus its chromatin binding should be indirect. We are not sure is the same DNA-binding protein mediates the localization of EFS in the two regions of *FLC*. The association of EFS with the transcription initiation region of *FLC* might be related with its H3K4 methyltransferase activity while its association with the transcription elongation region is more likely related with its H3K36 methyltransferase activity. We do not know if these two distinctive methyltransferase activities are mediated by the same or different DNA-binding proteins.

5) Figure 2; the in vitro assays look good, but I wonder if one might expect that to see an increase in the level of H3K4me1 on Western blots given that REF6 can efficiently demethylate both H3K4me3 and H3K4me2?

Response: Based on our western blot, H3K4me1 is a lot more abundant than H3K4me2 and H3K4me3 in the substrate (calf thymus histone) as evidenced by the much stronger signal (Figure 2G). Thus, what we believe is that the slightly increased H3K4me1 pool from the demethylation reaction is buffered by the already existing highly abundant H3K4me1 pool in the substrate such that the increase is not easily detected by western blot.

6) I think that the data presented in Supplementary Fig 3 should appear in the main body of the paper.

Response: Following the reviewer's suggestion, Supplementary Figure S3 has been incorporated into Figure 1 as a panel (Figure 1F) in the revised manuscript.

7) I found the title to be a little cryptic, especially for a more general audience. I'd suggest spelling out what is meant by "growth habit determination" perhaps "The balance of histone methylation activities determines the choice between winter- or summer-annual growth."

Response: Thanks for the suggestion! We agree that the title might need to be more specific. However, EMBO J. restricts the length of the tile to less than 100 characters (including spaces). Thus, there is no room to spell out the meaning of "growth habit" in the title. Your kind understanding would be appreciated.

2nd Editorial Decision 05 July 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees #2 and 3 to review the revised version and I have now heard back from both referees. As you can see below both referees appreciate that the revisions carried out have improved the manuscripts. However, there are still a few issues that have to be attended to before final acceptance here. One concerns the Co-IP analysis in figure 1E where a negative control is missing (check if GUS interacts with EFS-FLAG). Is it possible to carry out this negative control? If not, then referee #2 suggests removing this panel from the manuscript. I would like to see if we could sort this out. We can discuss it further if that is helpful. The are some other issues raised as well among these that the materials and methods section needs to be updated to indicate how the ChIP data has been quantified. Given the comments provided, I would like to ask you to respond to the remaining concerns in a final revision. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

This manuscript is a revised version of one reviewed earlier. The authors have made revisions to address my earlier criticisms and have improved the manuscript. My earlier criticisms were:

1. The control for the co-immunoprecipitation shown in Figure 1E was poor. This has not been improved in the revision. The authors argue that EFS and FRI proteins interact in vivo. The evidence is based on the interaction between FRI-GUS and EFS-FLAG. The authors show that immunoprecipitating with the GUS antibody they can detect EFS-FLG in the precipitate. However, it remains possible that EFS-FLAG actually interacts with GUS. A negative control should have been done using GUS without FRI attached. Nevertheless, the authors do have an independent confirmation with the yeast two hybrid interaction between FRI and EFS. In the absence of proper controls we would recommend deleting this panel.

2. Imprecise biochemistry in the histone methylation assay. Here the authors have stressed that this experiment was repeated.

3. Difficult quantification of gel based ChIP. This point was addressed.

4. Discussion that confused cause and effect. This was improved.

Based on these improvements, I recommend acceptance of the manuscript which does make an important contribution in determining the function of FRIGIDA.

Referee #3 (Remarks to the Author):

The major change in the revised version of this manuscript has been in the presentation of the ChIP data, which is now been quantified, rather than being presented as gel images of PCR reactions. This is a vast improvement as it makes it easier for the reader to assess the changes in histone modifications associated with the different mutants discussed. However, the Materials and Methods section describing the ChIP experiments has not been updated to reflect this. So, there is no indication of how the quantification has been done - by realtime PCR or by quantifying the gel images. There is also no indication of what has been used to normalise these values - are they compared to no antibody control? Clearly one sample (fri EFS in Figure 1, Col in Figures 2 and 3) has been set as 1 and all others are expressed relative to this.

I have outlined my other criticisms below:

1. Abstract; I don't believe that the conclusion that FRI is recruited to FLC chrmatin bt EFS is adequately supported by the data. For example, FRI could be recruited by SUF4, which interacts with EFS at FLC. It is still a puzzle as to why FRI is present only at the 5' end of the FLC whereas EFS is both at the 5' end and within the gene body.

2. p7 line 4; refers to Figure 1H - I think this should be Figure 1I

3. Figure 2; one piece of data that seems odd to me is that there is apparently no increase in H3K36me2 within the coding region of FLC in an fld mutant. As K36me2 is generally deposited on chromatin during transcription, and as FLC expression is elevated in fld relative to Col (see Fig 2C) this seems very strange. Compare this to the data presented in Fig 1B where K36me2 is elevated in the FRI background where FLC is also expressed. This is relevant as EFS is required for FLC expression in both FRI and autonmous pathway mutants (see Kim et al., 2007). This needs to be discussed

4. p 14 line 14; Johnson, L.M., Cao, X.F. and Jacobsen, S.E. (2002). Interplay between two

epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. 16, 1360-1367. is a more apporpriate reference than Song et al., 2009 as this former is the paper where the Actin primers wer first described.

5. Figure legends; there is some inconsistency as to whether the error bars represent standard error or standard deviation. I suggest that the authors stick to one or the other.

2nd Revision - authors' response 14 July 2010

We thank the editor and reviewers for their valuable comments and hope we have satisfactorily addressed all the issues raised.

Response to Reviewers' comments

Referee #2:

1) However, it remains possible that EFS-FLAG actually interacts with GUS. A negative control should have been done using GUS without FRI attached. Nevertheless, the authors do have an independent confirmation with the yeast two hybrid interaction between FRI and EFS. In the absence of proper controls we would recommend deleting this panel.

Response: Regarding the Co-IP issue the Referee #2 pointed, we do acknowledge that the Referee's opinion is correct and a true negative control would be a double transgenic line containing *EFS:FLAG* and *GUS*. Unfortunately, we do not currently have this transgenic line and, as you might easily imagine, it will take several months to construct the line. As the Referee also pointed, the data in Figure 1E is complementary to the Y2H data in Figure 1F. Therefore, instead of deleting Figure 1E, we have handled this matter by correcting the text on page 5 describing Figure 1E: from "Coimmunoprecipitation (CoIP) of FRI:GUS and EFS:FLAG (Figure 1E) indicated that FRI and EFS are present in the same complex" to "Coimmunoprecipitation (CoIP) of FRI:GUS and EFS:FLAG (Figure 1E) suggested that FRI and EFS might be present in the same complex, although the data did not exclude the possibility of interaction between the FLAG-tagged EFS and GUS instead of FRI". This change is also consistent with the following sentence "Consistent with a possible *in vivo* interaction, the C-terminal region of EFS interacted with a functional FRI (FRI^{Sf-2}) and SUF4 but not with a nonfunctional FRI (FRI^{Col}) in yeast two-hybrid assays (Figure 1E)".

Referee #3:

1) However, the Materials and Methods section describing the ChIP experiments has not been updated to reflect this. So, there is no indication of how the quantification has been done - by realtime PCR or by quantifying the gel images. There is also no indication of what has been used to normalise these values - are they compared to no antibody control? Clearly one sample (fri EFS in Figure 1, Col in Figures 2 and 3) has been set as 1 and all others are expressed relative to this.

Response: We have updated the corresponding sections in the Materials and Methods. First, in the ChIP assay section on page 13, we have written "ChIP DNA was analyzed by qPCR as described in the RT-PCR and qPCR section using *FLC* genomic primers FLC A to H (Liu *et al*, 2007) and U1 (Sung and Amasino 2004)". Second, in the RT-PCR and qPCR section on page 15, we have written "Control levels were set to 1 after normalization and others were expressed as relative values to the control levels".

2) Abstract; I don't believe that the conclusion that FRI is recruited to FLC chromatin by EFS is adequately supported by the data. For example, FRI could be recruited by SUF4, which interacts with EFS at FLC.

Response: We have changed the text in the Abstract from "FRI is recruited into *FLC* chromatin by EFS…" to "FRI is recruited into *FLC* chromatin through EFS…". We wished to write "FRI is

recruited into *FLC* chromatin either directly or indirectly by EFS…". However, the EMBO Journal limits the length of Abstract to 175 words, and our current version has already 175 words in full. Therefore, your kind understanding on this would be appreciated!

3) p7 line 4; refers to Figure 1H - I think this should be Figure 1I.

Response: The Referee's point is right, and we have corrected our mistake on page 7 as pointed.

4) Figure 2; one piece of data that seems odd to me is that there is apparently no increase in H3K36me2 within the coding region of FLC in an fld mutant. As K36me2 is generally deposited on chromatin during transcription, and as FLC expression is elevated in fld relative to Col (see Fig 2C) this seems very strange. Compare this to the data presented in Fig 1B where K36me2 is elevated in the FRI background where FLC is also expressed. This is relevant as EFS is required for FLC expression in both FRI and autonmous pathway mutants (see Kim et al., 2007). This needs to be discussed.

Response: Now we have discussed the point by creating a sentence "The elevated level of *FLC* mRNA but not H3K36me2 at the *FLC* locus in *fld* mutants suggests that the deposition of H3K36me2 can be uncoupled from transcriptional activity" on page 8. Based on the model (Figure 4) supported by our work, the deposition of H3K36me2 at *FLC* is caused by the presence of both functional *FRI* and *EFS*. However, in *fld* mutants in which only a non-functional *fri* allele exists, the reduced EFS activity is believed not to be sufficient for the high level deposition of H3K36me2. One more thing we should empathize here is that H3K36me does not always act as a positive marker for transcriptional activity. Sometimes it has negative effects on transcriptional activity and reduces the efficiency of aberrant transcription initiation (for example, reviewed in Li et al., 2007).

5) p 14 line 14; Johnson, L.M., Cao, X.F. and Jacobsen, S.E. (2002). Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. 16, 1360-1367. is a more appropriate reference than Song et al., 2009 as this former is the paper where the Actin primers wer first described.

Response: We have changed the citation to Johnson *et al*, 2002 on page 15 and also included the reference in the list (page 21). Thanks for pointing the original reference!

6) Figure legends; there is some inconsistency as to whether the error bars represent standard error or standard deviation. I suggest that the authors stick to one or the other.

Response: We used sd for leaf number counting (Figures 2D, 3E, and 3F) and RT-qPCR analyses (Figure 2C) for which three technical replicates were used. Se was used throughout the manuscript for all ChIP assays (Figures 1B-1D, 2A, 2E, 2F, 3A, and 3B) for which three biological instead of technical replicates were used. During this revision, we found that the legend for Figures 2E and 2F contained an error and have corrected it: "Error bars represent sd (**C** and **D**) or se (**E** and **F**)" on page 25. Now we also have clearly written which one was used for each experiment in the Materials and Methods sections (ChIP assay and RT-PCR and qPCR) as well as in the Figure legends. Sd is commonly used in measuring flowering time (i.e., leaf number counting) and gene expression analyses as we used here. However, we and others tend to use se for independent biological repeats for which larger variations between samples are normally created.

3rd Editorial Decision 15 July 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee # 3 to evaluate the revised version and I have now heard back from this referee. As you can see this referee finds the revised version has satisfactorily addressed the raised concerns. There is one remaining issue that has to be addressed before acceptance here and that concerns the use of error bars - see below. I would like to ask you to sort this last issue out. Once we received the revised version, we will proceed with its acceptance for publication in the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

The authors have now addressed my criticisms satisfactorily. My only comment is that it is not appropriate to put error bars on technical replicates for PCR (see Figure 2C and associated text in M&M)- this only measures the accuracy of pipetting, not biological variation as most readers would assume. I would like to see this stated in the figure legend, not just the M&M where most people will not see it.

I refer the authors to Cumming et al., (2007) J Cell Biol.177: 7-11 for the appropriate use of error bars.

3rd Revision - authors' response 19 July 2010

We thank for your encouraging decision on our manuscript entitled "Growth habit determination by the balance of histone methylation activities in *Arabidopsis*".

Here we are submitting a final revision of the manuscript.

In this revision, we have addressed the final point of the Referee #3 on the error bars in Figure 2C ("My only comment is that it is not appropriate to put error bars on technical replicates for PCR (see Figure 2C and associated text in M&M)- this only measures the accuracy of pipetting, not biological variation as most readers would assume. I would like to see this stated in the figure legend, not just the M&M where most people will not see it"): We have removed the error bars from Figure 2C and also mentioned that the values are the means of three technical replicates in the legend (page 24). For this correction, we have moved explanations for error bars for Figures 2D, 2E and 2F to appropriate positions within the legend.

We hope we have satisfactorily addressed all the issues raised by now and the final acceptance of the manuscript.

