THE EMBO JOURNAL

Arabidopsis HEAT SHOCK FACTOR BINDING PROTEIN is required to limit meiotic crossovers and *HEI10* transcription

Juhyun Kim, Jihye Park, Heejin Kim, Namil Son, Eun-Jung Kim, Jaeil Kim, Dohwan Byun, Youngkyung Lee, Yeong Park, Divyashree Nageswaran, Pallas Kuo, Teresa Rose, Tuong Vi Dang, Ildoo Hwang, Christophe Lambing, Ian Henderson, and Kyuha Choi
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1st Editorial Decision **16th Nov 2021**

Dear Dr Choi,

Thank you again for the submission of your manuscript (EMBOJ-2021-109958) entitled "HEAT SHOCK FACTOR BINDING PROTEIN limits meiotic crossovers by repressing HEI10 transcription". As you know, we have asked three experts to assess your manuscript for the EMBO Journal. We have now received reports from all of them, which I include at the bottom of this message.

As you will see, the referees appreciate the significance of your central finding and are generally supportive of your work. However, there are a number of important issues that have been raised which need to be addressed before we can progress towards publication in The EMBO Journal.

I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. Please contact me if you have any questions; I would be very happy to discuss the manuscript with you either over the phone or by video call.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve these concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Yours sincerely,

William

William Teale
Editor
The EMBO Journal

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 Checklist%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) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised

manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

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

7) 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:
http://bit.ly/EMBOPressFigurePreparationGuideline

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.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) 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). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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: .

- Additional Tables/Datasets should be labelled 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.

11) 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 .

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 14th Feb 2022:

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

Referee #1:

This manuscript describes a regulatory mechanism of meiotic crossover (CO) frequency via transcriptional repression of HEI10

by HeatShock factor Binding Protein (HSBP), encoded by the HCR2 gene. In the previous study (Nageswaran et al. 2020), the authors' group selected anti-CO factor mutants in Arabidopsis, hcr1 to hcr4, from EMS-mutagenized hemizygous 420/++ CO-reporter line, and characterized HCR1 function in meiosis. This ms is a sequel paper about HCR2 protein.
A single substitution mutation in HCR2, encoding HSBP, occurred at a minor class of splicing acceptor-donor site conserved within the fourth intron, and contributed to elevation of meiotic CO rate at euchromatic regions, but not around pericentromeric regions, in hcr2 mutants. The HCR2 function in CO repression was confirmed by the complementation test by a HSBP genome introduction and phenotypic observations of T-DNA-tagged hsbp-2 mutants and meiMIGS-HSBP lines. Next, the authors produced double mutants with several meiotic mutants involved in class-I and class-II CO pathways, and concluded that HCR2 represses class I CO formation.
The authors found that the expression of HEI10, the E3 ligase promoting the class I CO formation, increased in hcr2, hsbp-2 and meiMIGS-HSBP plants, and that CO frequency was highly correlated with HEI10 dosage. Furthermore, they performed ChIP-seq and ATAC-seq analysis, and together with previously reported DAP-seq results, revealed that HSBPs can directly associate with 5'-UTR of HEI10 gene. BS-seq and McrBC-qPCR revealed that DNA methylation level higher at the HEI10 5'-UTR in wildtype was significantly decreased in hcr2 and hsbp-2 mutants. The methylation level at HEI10 5'-UTR was anti-correlated with HEI10 expression level, and so they concluded that HCR2 HSBP epigenetically represses HEI10 transcription to maintain appropriate CO numbers. Furthermore, they confirmed that HSBP proteins load onto meiotic leptotene-pachytene chromosomes, while no loading was observed in hsbp-2 mutant.
It is widely and empirically known that plant meiosis is affected by stressful conditions including high/low temperatures, but little is known about underlying mechanisms behind susceptible properties of plant meiosis for environmental stresses. This ms clearly unveiled a part of the mechanisms underlying how CO formation is affected by temperature in plants. I endeavored to critically review this paper, but found no problem in logics, texts and figures. Thus I think this paper is worthy to be published. However, I have several comments as follows;

1. Fig. 6F, H,
I think this result is important to propose the importance of HSBP on repression of the class I COs. However, I don't think the cell numbers observed are enough to show a significant difference between Col and hcr2.

2. Fig. 5A,
The results of wildtype should be shown in addition to hcr2's.

3. Fig. 5E,
The authors should explain the meaning of red and black numbers at the bottom. There is no explanation in the legend.

4. L145,
"(Fig. 2A)" should be inserted after "I5ab".

5. L317,
"maker" --> "marker"

Referee #2:

In this manuscript, Juhyun Kim and colleagues identified the heat shock protein HSBP as a novel regulator of meiotic recombination in Arabidopsis thaliana, following a dedicated genetics screen. They claim that HSBP1 negatively regulates class I crossovers through the repression of the HEI10 gene. This is a very interesting study, but while some claims are well supported, some others are insufficiently supported.
The authors showed convincingly that the mutation of hsbp increase recombination in several genetic intervals in the Columbia background (Figure 1). The authors thus clearly show that HSBP (HCR2) limit crossover, which is an exciting discovery.

Major concerns:
1) Line 94 "aberrant shorter and longer HSBP splice variants compared to WT transcripts". Line 73 "hypomorphic allele of HSBP" . line 960 "The hcr2 mutant is a weak hsbp allele". I do not understand what supports this conclusion.
2) The hcr2 allele is disrupted in an intron splicing site, however in Figure 1E a band from RT-PCR analysis shows that the hcr2 mutant produces two mRNAs transcripts from the mutant gene, one of which corresponds to an transcript similar in size to the wt transcripts. Sequencing of the different transcripts will be required to determine the effect of the mutation in the protein. It is particularly important as figure 1I suggests that hcr2 is equally affected as hsbp-2, arguing against the conclusion that hcr2 is an hypomorphic allele.

3) It is suggested that only class I CO are increased in the hsbp mutant. This is supported by the incapacity of hcr2 mutation to restore fertility of zip4 (figure 3D). However, this lacks quantification (i.e seed count rather than simply a picture of the plant), and should be complete by meiotic chromosome spread to be able to conclude on the absence of effect of hrc2 on crossover in zip4.
4) Further, MLH1 foci count (figure 6H) shows an increase of only 11% of this marker of class I crossover. How the author explains this major discrepancy with the genetic results? As it is crucial for the conclusion, the MLH1 counts should be done in

the two mutant alleles, and in the F1 hybrid context.
5) Figure4F. The recombination profile of chromosome 3 meiMIGS-HSBP is aberrant, notably with a region of abolished recombination compared to wild type. This strongly suggest a chromosome rearrangement on chromosome3, presumably associated with the meiMIGS-HSBP transgene. On both sides of this rearrangement, recombination is dramatically increased, more than in any other region of the genome, strongly suggesting a compensatory effect of the reduced crossovers in the rearranged central regions of chromosome 3. The effect of increased recombination on the other chromosome is probably still valid. However, all result on chromosome 3 should be taken very cautiously. Notably, the figure 4E should be done excluding the chromosome 3. More importantly, the result shown on figure 2E, with a massive effect of meiMIGS-HSBP on both male and female recombination on the 420 interval (chromosome 3), is probably artefactual. Analysis of another independent meiMIGS-HSBPline may help to resolve this artefact.
6) In addition, it appears that the wild type control of Figure 4F are taken from a previous dataset, rather than from an internal wild type control. This lowers the confidence in the conclusions as growth conditions can affect crossover frequencies.

7) Figure 5A represents a rather arbitrary selection of genes. Many other key factors of the recombination machinery should be shown, including the DSB machinery (SPO11s, PRDs, MTOPVI...), DMC1, RAD51, HOP2, MH4, MH5, MER3; ZYP1, SHOC1, ASY3, ASY4, RECQ4s, FANCM...

8) HEI10 appears to be expressed differentially in hcr2 compared to wild type. But this is even stronger for ASY1, and is probably the case for many other genes. The conclusion that HSBP regulates crossover by directly repressing HEI10 expression requires further support. This hypothesis is mainly based on the occurrence of Heat Shock Elements present in the promoter of HEI10 and the HSBP binding to this element. Altogether, it is not clear if HRC2 indeed regulates CO formation via HEI10, and even less than HCR2 regulates CO exclusively via HEI10. The title and the abstract go thus beyond what it is actually demonstrated in the manuscript, and should be modified. The model presented in figure 6J appears thus highly speculative, and should be presented as such or deleted.

9) Line 236-238. "We chose HSFA1a and HSFA7a among the class A HSF activator family, because they are highly expressed in meiotic buds (Supplemental Fig. S6A)". It is not clear what was the selection criteria as most of the genes are highly expressed in meiotic buds. Did the author made a preselection based on qRT-PCR?

10) Although the authors saw a reduction on DNA methylation on the 5' UTR of HEI10 in hcr2, the connection of HSBP with methylation is not completely clear. Is HCR2 a regulator of DNA methylation on a global scale or is this only affecting the HEI10 loci? Are among the miss expressed genes in hcr2, DNA methylation regulators?

11) Based on public expression data, HCR2 shows a broad expression pattern, then one would expect that its product will inhibit the activity of HSF on a more general level. An explanation on the difference between meiotic vs somatic cell activity for HCR2 will help to understand better the proposed model on Figure 6J.

12) The proposed HCR2 mode of action imply that wtils type and hcr2 mutants start meiosis with similar HEI10 transcript levels and that those will get reduced during pachytene/Diplotene in wt, whereas in the hcr2, those levels stay high or even increase. However, this data is not provided in the current form of the manuscript.

Minor comments

It will be better to stick to the use of HCR2 or HSBP throughout the text as the indiscriminate use of both lead to confusion.

Figure 5E it is not clear the meaning of the axes. I understood that the black line is the endogenous HEI10 (thus 0 means homozygous mutant), while the rec line is a HEI10 transgene (0 meaning wild type). This should be clarified. Was the transgene copy number per insertion determined?

The section "hcr2 shows more MLH1 foci and HSBP localizes to the nucleus during meiosis" is presented rather late. Better move it early in the result section

Referee #3:

Improving crossover rates is of utmost importance as this contributes to a better reshuffling efficiency of alleles in the progenies. Several factors affecting crossover rate have been identified including Hei10, an E3 ligase that increases crossover rate when overexpressed. In their study, the authors isolated a new heat-shock binding protein in Arabidopsis that repressed the transcription of Hei10, reducing therefore the number of crossovers. They conducted an in-depth approach combining RNA sequencing, DNA methylome analysis and chromatin immuno-precipitation assay to decipher how this protein binds with heat-

shock factors at the promoter of Hei10 and how this maintains DNA methylation on the promoter. The genetics using mutants (single, double, heterozygotes) and wild type is rigorous. All the results found are well analysed with regular controls and appropriate statistics. All the conclusions are sounded are the results found will be of main interest for researchers working on meiosis and recombination, especially with regards to environmental factors such as heat stress. I just have minor comments that may improve the manuscript.
L74: which type of mutants? (EMS?) Even if you refer to a publication in the M&Ms, it could be useful to have the information without reviewing the literature.
L84: specify the distance in the WT
L190: for chromosome 3 (Fig.4F), this is the only one for which, crossovers are reduced in the mutant background compared to WT close to the centromere while for the others, this is equal in WT and mutant. This is not pointed out in the results. Do you have any explanation for that?
L328: It is surprising that the discussion does not refer to a paper in barley (Higgins et al. The Plant Cell, Vol. 24: 4096-4109, October 2012) showing that increasing temperature from 22{degree sign}C to 30{degree sign}C modifies the number of interstitial chiasmata, which perfectly fits with the results found here. I suggest to add a short section on this.
L351-354: I don't see any reason why ROS could be the main activator of HSBP and HSF during meiosis? I agree that ROS can activate HSF but this is probably not the only signal affecting its expression. And I am not aware of anything regarding HSBP. You either have evidence or remove. Same for ASY1. Why this one would be more affected by ROS than other meiotic genes?
L394: Precise what type of mutant hcr2 is.

Dear Dr Teale,

Thank you for the overall positive reviews of our manuscript entitled 'HEAT SHOCK FACTOR BINDING PROTEIN limits meiotic crossovers by repressing *HEI10* transcription'.

We now submit our revised manuscript and a detailed point-by-point response that addresses the comments and concerns raised by reviewers. We have now added new data and analyses to the manuscript according to these comments, as listed below:

- We have characterized *HSBP* expression and HSBP abundance in the *hcr2* and *hsbp* T-DNA alleles by RT-PCR and immunoblot analyses. We thus determined that *hcr2* (renamed *hsbp-3* thereafter), an EMS-derived point mutation close to a splicing donor site, leads to lower *HSBP* transcript and HSBP protein levels. We show that the two intronic T-DNA insertion mutants *hsbp-1* and *hsbp-2* also have lower HSBP protein levels. These results thus indicate that *hsbp-3*, *hsbp-2* and *hsbp-1* are not null alleles, but rather weak (*hsbp-3*, *hsbp-1*) or strong (*hsbp-2*) alleles with different low HSPB levels (Fig 1E and F, Fig EV1).
- We have quantified MLH1 foci in Col, *hcr2* (*hsbp-3*), *hsbp-2*, Col/Ler and *meiMIGS-HSBP* Col/Ler. We counted more MLH1 foci in *hsbp-3*, *hsbp-2* and *meiMIGS-HSBP*, compared to Col or the Col/L*er* hybrids (Fig 6G and H).
- We have quantified the number of immunostained HEI10 foci in the wild type and *hsbp-3*, which revealed the greater number of HEI10 foci in *hsbp-3*, in agreement with our MLH1 data (Fig 6I and J).
- We have performed immunoblot analysis of HEI10 in the wild type, *hsbp-3*, *HEI10-myc*, and *HEI10-myc hsbp-3* using an anti-HEI10 antibody or a myc antibody for the HEI10-myc transgenic lines. This analysis showed that HEI10 abundance is higher in *hsbp-3* relative to Col-0, which is consistent with the increased *HEI10* transcript levels measured in *hsbp-2* (Fig 6K).
- We have provided additional GBS datasets for 96 Col/L*er* and 96 *meiMIGS-HSBP* Col/L*er* F_2 individuals derived from F_1 plants that were grown under the same growth conditions as the previous Col control. These GBS datasets revealed increased crossover numbers in *meiMIGS-HSBP* Col/L*er*, which is consistent with the results presented in the original version of this manuscript. (Fig 4).
- We have performed meiotic chromosome spreads of the wild type, *hsbp-3*, *zip4* and *zip4 hcr2*. We now show that bivalents are not significantly increased in *zip4 hsbp-3*compared to *zip4*. This observation is consistent with fertility results (seed number per silique), which supports the role of HSBP in limiting class I crossovers (Fig 3).
- We have drawn new heatmap representations of transcript levels quantified by RNA-seq in the wild type and *hsbp-3* for meiotic, epigenetic and *HSF* family genes (Fig 5A and Fig EV3A). These heatmaps show that *HEI10* transcript levels are increased in *hsbp-3* buds, while *hsbp-3* does not affect transcription of other meiotic genes except *ASY1*. We have also included heatmaps illustrating the changes in transcript levels for epigenetic genes in *hsbp-3*, which explains the observed lower number of crossovers in pericentromeres and centromeres.
- We now show that HSBP translocates from the cytosol to the nucleus in Arabidopsis protoplasts upon treatment with hydrogen peroxide (H_2O_2) in addition to high temperature (Fig EV3I). We also show the nuclear localization of HSBP-YFP in male meiocytes of *HSBPpro:HSBP-YFP* plants (Fig EV3J).

We have also accepted the reviewers concerns about the scope of the role played by HSBP in limiting crossovers via *HEI10* transcription and DNA methylation. We have therefore modified the manuscript title, abstract, results and discussion sections accordingly, including updating the model for HSBP (Fig EV5). We hope this presents a more balanced presentation of our data.

We thank the reviewers for their detailed comments and hope these revisions will fully address the points raised.

Sincerely,

Kyuha Choi

Referee #1:

This manuscript describes a regulatory mechanism of meiotic crossover (CO) frequency via transcriptional repression of HEI10 by HeatShock factor Binding Protein (HSBP), encoded by the HCR2 gene. In the previous study (Nageswaran et al. 2020), the authors' group selected anti-CO factor mutants in Arabidopsis, hcr1 to hcr4, from EMS-mutagenized hemizygous 420/++ CO-reporter line, and characterized HCR1 function in meiosis. This ms is a sequel paper about HCR2 protein.
A single substitution mutation in HCR2, encoding HSBP, occurred at a minor class of splicing acceptor-donor site conserved within the fourth intron, and contributed to elevation of meiotic CO rate at euchromatic regions, but not around pericentromeric regions, in hcr2 mutants. The HCR2 function in CO repression was confirmed by the complementation test by a HSBP genome introduction and phenotypic observations of T-DNA-tagged hsbp-2 mutants and meiMIGS-HSBP lines. Next, the authors produced double mutants with several meiotic mutants involved in class-I and class-II CO pathways, and concluded that HCR2 represses class I CO formation.
The authors found that the expression of HEI10, the E3 ligase promoting the class I CO formation, increased in hcr2, hsbp-2 and meiMIGS-HSBP plants, and that CO frequency was highly correlated with HEI10 dosage. Furthermore, they performed ChIP-seq and ATAC-seq analysis, and together with previously reported DAP-seq results, revealed that HSBPs can directly associate with 5'-UTR of HEI10 gene. BS-seq and McrBC-qPCR revealed that DNA methylation level higher at the HEI10 5'-UTR in wildtype was significantly decreased in hcr2 and hsbp-2 mutants. The methylation level at HEI10 5'-UTR was anti-correlated with HEI10 expression level, and so they concluded that HCR2 HSBP epigenetically represses HEI10 transcription to maintain appropriate CO numbers. Furthermore, they confirmed that HSBP proteins load onto meiotic leptotene-pachytene chromosomes, while no loading was observed in hsbp-2 mutant.
It is widely and empirically known that plant meiosis is affected by stressful conditions including high/low temperatures, but little is known about underlying mechanisms behind susceptible properties of plant meiosis for environmental stresses. This ms clearly unveiled a part of the mechanisms underlying how CO formation is affected by temperature in plants. I endeavored to critically review this paper, but found no problem in logics, texts and figures. Thus I think this paper is worthy to be published. However, I have several comments as follows;

RESPONSE: We are pleased that the reviewer appreciated our results and recognized the importance of our findings.

1. Fig. 6F, H,
I think this result is important to propose the importance of HSBP on repression of the class I COs. However, I don't think the cell numbers observed are enough to show a significant difference between Col and hcr2.

RESPONSE: To address this concern, we now provide cytological data from additional cells for RAD51 and MLH1 foci (Fig 6F, H), as well as new results of MLH1 foci counts in Col, *hcr2* (*hsbp-3*), *hsbp-2*, Col/L*er* and Col-0/L*er* *meiMIGS-HSBP* F_1 hybrid plants (Fig 6H), as reviewer 2 requested. We show that the number of RAD51 foci is unchanged. The MLH1 dataset shows more MLH1 foci in *hsbp* alleles and the *meiMIGS-HSBP* line compared to Col.

2. Fig. 5A,
The results of wildtype should be shown in addition to hcr2's.

RESPONSE: Thank you for providing this useful comment. We now provide a heatmap representation of RNA-seq data for meiotic genes in Col and *hsbp-3*, along with a heatmap of fold-changes in seedlings and buds (Fig. 5A). We have also added an RNA-seq analysis of other meiotic recombination genes, as requested by reviewer 2.

3. Fig. 5E,
The authors should explain the meaning of red and black numbers at the bottom. There is no explanation in the legend.

RESPONSE: We apologize for this omission. We have now added an explanation in the legend. Black numbers represent *HEI10* and the endogenous *HEI10* genotype (0, *hei10*; 1, *hei10/HEI10*; 2, *HEI10/HEI10*). Red numbers represent *HEI10* and *HEI10-myc* transgene copy number using *HEI10* or other meiotic gene promoter.

4. L145,
"(Fig. 2A)" should be inserted after "I5ab".

RESPONSE: Thank you for pointing this out. Done.

5. L317,
"maker" --> "marker"

RESPONSE: Now corrected

Referee #2:

In this manuscript, Juhyun Kim and colleagues identified the heat shock protein HSBP as a novel regulator of meiotic recombination in Arabidopsis thaliana, following a dedicated genetics screen. They claim that HSBP1 negatively regulates class I crossovers through the repression of the HEI10 gene. This is a very interesting study, but while some claims are well supported, some others are insufficiently supported.
The authors showed convincingly that the mutation of hsbp increase recombination in several genetic intervals in the Columbia background (Figure 1). The authors thus clearly show that HSBP (HCR2) limit crossover, which is an exciting discovery.

RESPONSE: We are glad that the reviewer appreciated the significance of our findings.

Major concerns:
1) Line 94 "aberrant shorter and longer HSBP splice variants compared to WT transcripts". Line 73 "hypomorphic allele of HSBP" . line 960 "The hcr2 mutant is a weak hsbp allele". I do not understand what supports this conclusion.

RESPONSE: Thank you for raising this important point. To test if *hsbp-3* is a hypomorphic or a null allele, compared to other *hsbp* alleles (*hsbp-1* and *hsbp-2*), we performed RT-PCR in wild type Col, *hsbp-3*, *hsbp-2*, and another T-DNA allele, *hsbp-1*. We found that *hsbp-3* accumulates aberrant longer and shorter *HSBP* transcripts, while fully spliced *HSBP* transcripts accumulated to levels about 53% of those seen in Col. Both *hsbp-2* and *hsbp-1* intronic T-DNA alleles also produced fully spliced *HSBP* transcripts but to much lower levels than Col (9% of wild-type levels in *hsbp-2*, 70% of wild-type levels in *hsbp-1*) (Fig 1E and Fig EV1B).

Next, we performed an immunoblot analysis of HSBP in Col, *hspb-3*, *hsbp-2*, and *hsbp-1* buds using an anti-HSBP antibody (Fig 1F and Fig EV1C).We observed that HSBP protein levels drop to about 58% in *hsbp-3* compared to the wild type. We also detected lower HSBP abundance in *hsbp-1* (77% of the wild type) and *hsbp-2* (17% of the wild type), consistent with the RT-PCR data. These RT-PCR and immunoblot analyses indicate that *hsbp-3*, *hsbp-2*, and *hsbp-1* are not null mutants, but weak (*hsbp-1*, *hsbp-3*) or strong (*hsbp-2*) alleles, with different reduced protein levels of HSBP. *hsbp-2* is likely a stronger allele than *hsbp-3*, at least in terms of seed development (Fig 6C). We also performed the same analysis in *meiMIGS-HSBP* lines (Fig EV2D-E and G). We observed a negative correlation between *HSBP* expression levels and crossover frequencies in the *meiMIGS-HSBP* lines (Fig EV2E).

Although there is no null mutant for *HSBP*, our data indicate that reduced HSBP levels in *hsbp* alleles and *meiMIGS-HSBP* lines (approximately 20-60% of the wild type) lead to increased crossover frequency (Fig 1 and Fig EV2) and *HEI10* transcript levels (Fig 5 and Appendix Fig S4). The HSF-HSBP transcriptional module is known to act in a negative feedback manner. HSBP attenuates HSF activity during the heat shock response. Once HSFs are activated by temperature or developmental signals, the activated HSFs induce transcription of *HSPs* (heat shock responsive genes) that include *HSBP* and *HSP70* genes. Then, both HSBP and HSP70 proteins act as inhibitors or attenuators of HSF activity, contributing to dissociation of HSF trimers. Thus, it is likely that the reduced HSBP levels (20-60% of the wild type) in *hsbp-3*, *hsbp* T-DNA allele (*hsbp-2*) and *meiMIGS-HSBP* plants are sufficient to disrupt the HSF-HSBP cycle, which may lead to higher HSF activity, *HEI10* transcription and crossover frequency. A null allele in *HSBP* and its effect on crossover frequency and development will need to be explored in a future study.

2) The hcr2 allele is disrupted in an intron splicing site, however in Figure 1E a band from RT-PCR analysis shows that the hcr2 mutant produces two mRNAs transcripts from the mutant gene, one of which corresponds to an transcript similar in size to the wt transcripts. Sequencing of the different transcripts will be required to determine the effect of the mutation in the protein. It is particularly important as figure 1I suggests that hcr2 is equally affected as hsbp-2, arguing against the conclusion that hcr2 is an hypomorphic allele.

RESPONSE: Thank you for pointing this out. We examined the RT-PCR products in detail and have added new pictures of agarose gel electrophoresis to Fig. 1E and Fig EV1B. S3B. We gel-purified the longer, shorter and normal PCR bands from *hsbp-3* and performed Sanger sequencing on all PCR products. We confirmed that the normal-sized PCR band corresponds to the properly spliced mRNA sequence, encoding full-length, wild-type HSBP. By contrast, aberrant shorter and longer *HSBP* splice variants introduced premature stop codons (Fig EV1A and B). Our immunoblot analysis of HSBP and Sanger sequencing data of *HSBP* transcripts indicate that *hsbp-3* leads to abnormal splicing, reduced functional *HSBP* transcript levels (53% of the wild type), and decreased HSBP protein (~58% of the wild type); this drop in *HSPB* transcript and HSBP protein levels appears to be sufficient to increase *HEI10* transcription and crossover frequency, similar to *hsbp-2* (~17% of wild-type HSBP abundance) and several *meiMIGS-HSBP* lines (~20-60% of wild-type HSBP abundance) (Fig 1I, Fig EV2D and E).

3) It is suggested that only class I CO are increased in the hsbp mutant. This is supported by the incapacity of hcr2 mutation to restore fertility of zip4 (figure 3D). However, this lacks quantification (i.e seed count rather than simply a picture of the plant), and should be complete by meiotic chromosome spread to be able to conclude on the absence of effect of hrc2 on crossover in zip4.

RESPONSE: To address this concern, we quantified the number of seeds per silique in *zip4* (3.03 seeds/fruit) and *zip4 hsbp-3* (2.94 seeds/fruit) (Fig 3D, Appendix Table S15). The average seed number in *zip4* did not differ significantly from that in *zip4 hsbp-3* (Wilcoxon test, P=0.11). As the reviewer suggested, we also quantified the number of bivalents (blue) and pairs of univalents (red) per male meiocyte at meiotic metaphase I in *zip4* and *zip4 hsbp-3* (Fig 3E–F, Appendix Table S16). We observed no significant difference in the number of bi/univalents between *zip4* and *zip4 hsbp-2* (Wilcoxon test, P=0.17). These results indicate that the additional crossovers present in *hsbp-3* depend on ZIP4 activity.

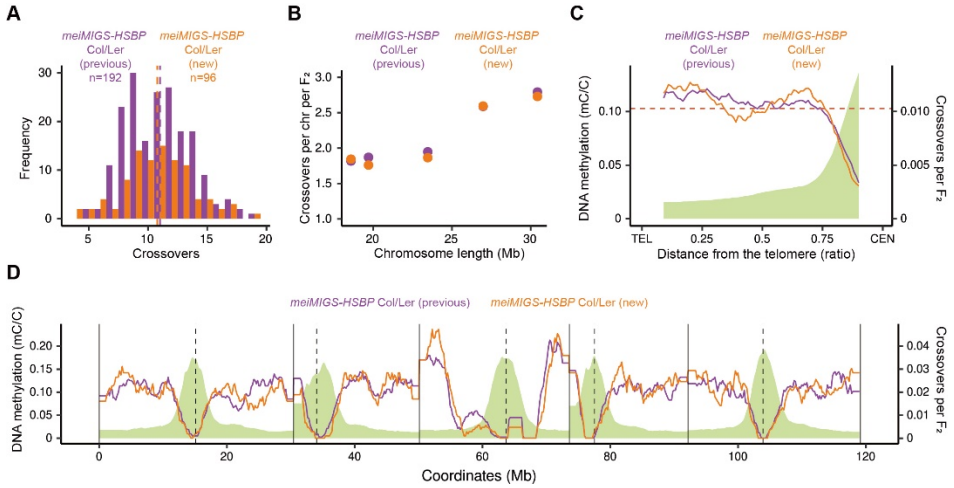
4) Further, MLH1 foci count (figure 6H) shows an increase of only 11% of this marker of class I crossover. How the author explains this major discrepancy with the genetic results? As it is crucial for the conclusion, the MLH1 counts should be done in the two mutant alleles, and in the F1 hybrid context.

RESPONSE: We thank the reviewer for pointing this out. We performed MLH1 immunostaining and counted the number of MLH1 foci in Col-0, *hsbp-3*, *hsbp-2*, Col/L*er* and Col-0/L*er* *meiMIGS-HSBP* F_1 hybrid plants that

were grown simultaneously in the same growth room (Fig 6G andH). We replaced the previous data with a new dataset of MLH1 foci counts (Fig 6H). These results show a significant increase of class I crossovers in *hsbp-3* (~48% increase), *hsbp-2* (~71%) and *meiMIGS-HSBP* Col-0/L*er* (~57%) in male meiosis, which is similar to the genetic results (Fig. 2) (*hsbp-3*, FTLs-male [~54%], *hsbp-3*, CTLs-sex average [~30%], *meiMIGS-HSBP*, GBS-sex average [~42%]).

5) Figure4F. The recombination profile of chromosome 3 meiMIGS-HSBP is aberrant, notably with a region of abolished recombination compared to wild type. This strongly suggest a chromosome rearrangement on chromosome3, presumably associated with the meiMIGS-HSBP transgene. On both sides of this rearrangement, recombination is dramatically increased, more than in any other region of the genome, strongly suggesting a compensatory effect of the reduced crossovers in the rearranged central regions of chromosome 3. The effect of increased recombination on the other chromosome is probably still valid. However, all result on chromosome 3 should be taken very cautiously. Notably, the figure 4E should be done excluding the chromosome 3. More importantly, the result shown on figure 2E, with a massive effect of meiMIGS-HSBP on both male and female recombination on the 420 interval (chromosome 3), is probably artefactual. Analysis of another independent meiMIGS-HSBPline may help to resolve this artefact.

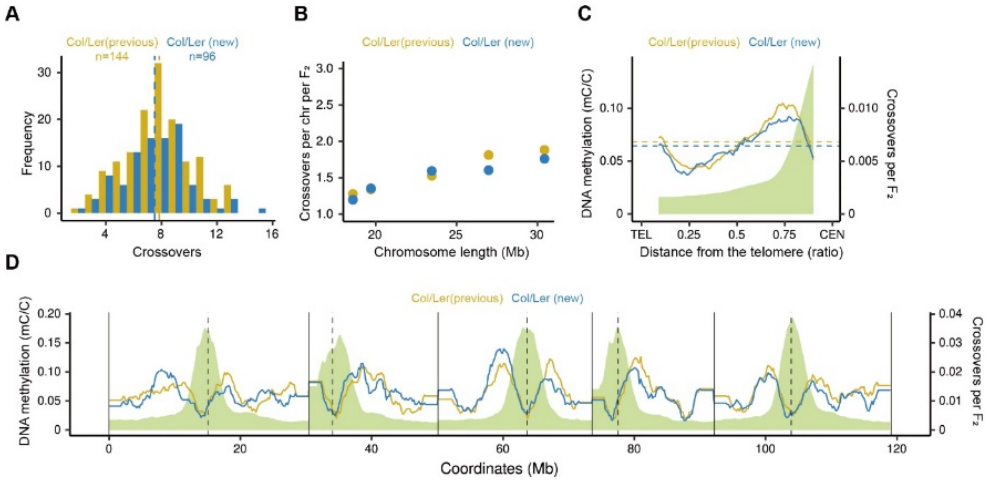
RESPONSE: We agree with the reviewer that a chromosomal rearrangement in *meiMIGS-HSBP* might have caused the reshaping of crossover patterns along chromosome 3 (Fig. 4F). In this revised version, we have replotted the TEL-CEN graph by excluding the crossovers on chromosome 3 in Fig. 4E. We have also removed the *meiMIGS-HSBP* data in Fig. 2E. For the new TEL-CEN graph excluding chromosome 3, we provide an additional set of GBS (96 F_2 individuals) from the same *meiMIGS-HSBP* Col/L*er* F_2 population. The new set of GBS (n=96, mean=10.79) showed a similar crossover number (Wilcoxon test, P=0.93) and distribution as the previous set (n=192, mean=11.01), although they also have the same chromosome rearrangement as the previous GBS set. However, the combined GBS data set (n=288) supports increased crossovers along the chromosome arms of the remaining four chromosomes as well as along the chromosomes and across the genome (Fig 4A and E).



6) In addition, it appears that the wild type control of Figure 4F are taken from a previous dataset, rather than from an internal wild type control. This lowers the confidence in the conclusions as growth conditions can affect crossover frequencies.

RESPONSE: For the GBS dataset used in this study, we grew all GBS F_1 hybrid plants for the wild-type control, *meiMIGS-HSBP* and *meiMIGS-PPX1-PPX2* (Kim et. al., 2021) in the same growth conditions at POSTECH (Korea), but not in Cambridge (UK). We did not use the previous dataset generated in the UK. However, we have provided an additional GBS set of 96 F_2 individuals from a Col/Ler F_1 hybrid plant that was grown with *meiMIGS-*

HSBP Col-0/Ler F_1 plants at the same growth room (in Korea). The average CO number of the new GBS set (n=96, average=7.51) did not differ from the previous set (n=144, average=7.84) (Wilcoxon test, *P*=0.29). Their genomic CO landscapes were also similar. Thus, we have combined these two datasets and added them to Fig. 4 (n=240 for Col/Ler F_2, n=288 for *meiMIGS-HSBP* Col/Ler F_2).



7) Figure 5A represents a rather arbitrary selection of genes. Many other key factors of the recombination machinery should be shown, including the DSB machinery (SPO11s, PRDs, MTOPVI...), DMC1, RAD51, HOP2, MH4, MH5, MER3; ZYP1, SHOC1, ASY3, ASY4, RECQ4s, FANCM...

RESPONSE: We appreciate this useful suggestion. We have generated new heatmap representations of the normalized RNA seq data for most meiotic recombination genes in both Col-0 and *hsbp-3*, along with a heatmap for fold-change in seedlings and buds between *hsbp-3* and Col-0 (Fig 5A). Information on additional key meiotic genes was also included, as the reviewer suggested.

8) HEI10 appears to be expressed differentially in hcr2 compared to wild type. But this is even stronger for ASY1, and is probably the case for many other genes. The conclusion that HSBP regulates crossover by directly repressing HEI10 expression requires further support. This hypothesis is mainly based on the occurrence of Heat Shock Elements present in the promoter of HEI10 and the HSBP binding to this element. Altogether, it is not clear if HRC2 indeed regulates CO formation via HEI10, and even less than HCR2 regulates CO exclusively via HEI10. The title and the abstract go thus beyond what it is actually demonstrated in the manuscript, and should be modified. The model presented in figure 6J appears thus highly speculative, and should be presented as such or deleted.

RESPONSE: We agree with the reviewer's point that HSBP can control the transcription of not only *HEI10* but many other genes including *ASY1* by inhibiting HSFs during meiosis. Based on the reviewer's suggestion, we have modified the model for HSBP action during meiosis by adding other HSBP target genes and moved the model to Fig EV5. We have also modified the title and abstract. New title: HSBP is required to limit meiotic crossovers and *HEI10* transcription.

However, to address the reviewer's concern about the role of HSBP in restricting crossovers via *HEI10*, in this revised version we now provide substantial results including HEI10 foci counts and immunoblots in support of the model that HSBP regulates crossovers at least via *HEI10*;

1) We provided the results of HSBP ChIP-qPCR experiments in both Arabidopsis buds and seedlings (Fig 5K and Fig EV3F), which supports the notion that HSBP binds to the *HEI10* promoter (HSE) as the reviewer

also mentioned.

2) We showed direct binding of HSFs to the *HEI10* promoter (HSE) in protoplasts using ChIP-qPCR (Fig EV3E).

3) The transient protoplast expression assays revealed that HSFs increase *HEI10* transcription, and co-expression of both *HSBP* and *HSF* reduces HSF-mediated *HEI10* transcription (Fig EV3B and C), which indicates that HSFs bind to the *HEI10* promoter for *HEI10* transcriptional activation and HSBP can attenuate HSF activity, probably via dissociating HSF trimers.

4) We showed the protein interaction and co-localization of HSFs and HSBP in the nucleus using co-immunoprecipitation and co-localization assays (Fig EV3H, I, K and L).

5) We showed that HSBP translocates from the cytosol to the nucleus of protoplasts upon hydrogen peroxide treatment as well as after exposure to high temperature (Fig EV3I)and that HSBP is abundant and localized in the nucleus in male meiocytes using immunoblot analysis, immunocytology and *HSBPpro:HSBP-YFP* transgenic plants (Fig 6K and L, Fig EV3J and K).

6) We showed that meiotic *HSBP* overexpression in transgenic plants leads to lower crossover frequency in *420* (Fig 5I).

7) We provide data that *HEI10* transcript levels are reduced in *HSBP* overexpression plants, compared to wild-type plants (Fig 5J).

8) RNA-seq and RT-qPCR analyses revealed that *HEI10* transcript levels are increased in *hsbp* alleles and *meiMIGS-HSBP* plants (Fig 5A, B and M, Appendix Fig S4A).

9) Genetic analysis of *hsbp-3* with *zip4* and *hei10* showed that the extra crossovers in *hsbp-3* depend on HEI10 and ZIP4 (Fig 3 and Fig 5D).

10) We showed that both HEI10 foci number per cell and HEI10 protein levels are increased in *hsbp-3* (Fig 6J and K).

11) Finally, we detected more MLH1 foci in *hsbp* alleles and *meiMIGS-HSBP* (Fig 6H).

Together, our results demonstrate that HSBP inhibits *HEI10* transcription by binding and attenuating HSFs at the *HEI10* promoter and is required to limit class I crossovers, in addition to controlling temperature-responsive and other genes. However, the mechanisms controlling *HEI10* transcription remain elusive. Future studies will need to be conducted to elucidate which HSFs and specific transcription factors activate *HEI10* transcription.

9) Line 236-238. "We chose HSFA1a and HSFA7a among the class A HSF activator family, because they are highly expressed in meiotic buds (Supplemental Fig. S6A)". It is not clear what was the selection criteria as most of the genes are highly expressed in meiotic buds. Did the author made a preselection based on qRT-PCR?

RESPONSE: We agree with the reviewer that many class A *HSF* genes are highly expressed in buds, suggesting that they may redundantly contribute to gene expression during meiosis. We did not preselect these genes via RT-qPCR. Instead, we provided our RNA-seq data of seedlings and buds for the HSF family, which are shown as a heatmap in Fig EV3A. We sought to investigate whether HSFs might directly activate *HEI10* transcription by binding the *HSE* (heat shock element) at the *HEI10* promoter or other regulatory regions using protoplast transient expression and ChIP-qPCR assays. HSFA1a was chosen as a representative of the class A1 group of HSF activators because A1a-d are well known and characterized as key HSFs acting redundantly in heat shock response. HSFA7a was selected because it is one of several highly induced *HSF* genes by high temperature and also increased in *hsbp-3* buds (our heatmap) (Fig EV3A).

Since HSBP is known to inhibit HSF activity, we examined whether HSFs can induce *HEI10* transcription directly.

Using the protoplast transient assay, we observed that HSFA1a and HSFA7a lead to an increase in *HEI10* transcription, which allowed us to determine *in vivo* which HSE at the *HEI10* promoter is bound using HSFA7a ChIP-qPCR. We also demonstrate here that HSBP binds to the same HSE at the *HEI10* promoter in heat-treated seedlings and buds using ChIP-qPCR and anti-HSBP antibody. Although which HSF(s) acts and induces *HEI10* transcription during meiosis will need to be investigated with *hsf* mutants or *HSF* overexpressing lines, our findings suggest that some HSFs may activate *HEI10* transcription as well as many other genes.

10) Although the authors saw a reduction on DNA methylation on the 5' UTR of HEI10 in hcr2, the connection of HSBP with methylation is not completely clear. Is HCR2 a regulator of DNA methylation on a global scale or is this only affecting the HEI10 loci? Are among the miss expressed genes in hcr2, DNA methylation regulators?

RESPONSE: Thank you for asking this important question. We agree that the mechanisms by which *hsbp* mutations cause a significant reduction in DNA methylation at the 5' UTR of *HEI10* remain elusive. To address the reviewer's questions, we have analyzed RNA-seq and bisulfite (BS)-seq data from *hsbp-3* in more detail and show the results in Appendix Fig S6.

Our RNA-seq analysis showed significant changes in gene expression for DNA methylation regulators in *hsbp-3*. We observed that *hsbp-3* leads to increased transcript levels for genes involved in transposon-associated small RNA production and DNA methylation (Appendix Fig S6F). Consistently, we found that DNA methylation levels are moderately increased in heterochromatin regions and transposons in *hsbp-3* (Appendix Fig S6A–C).

We also determined that the genes in DNA demethylation pathways (*DEMETER*, *ROS1*, *DML2*, and *IDM1*) are upregulated in *hsbp-3* buds, which is consistent with hypo-methylation in a subset of genes including *HEI10* (Appendix Fig S6D–F). We looked for the overlap between DMRs (differentially methylated regions) and DEGs (differentially expressed genes) in *hsbp-3* seedlings and buds, which revealed 61 and 132 overlapped genes in seedlings and buds, respectively. The overlapping analysis revealed that *HEI10* appears in both DMRs and DEGs in *hsbp-3* seedlings and buds. We added this explanation to Results and Discussion.

11) Based on public expression data, HCR2 shows a broad expression pattern, then one would expect that its product will inhibit the activity of HSF on a more general level. An explanation on the difference between meiotic vs somatic cell activity for HCR2 will help to understand better the proposed model on Figure 6J.

RESPONSE: We thank the reviewer for this useful suggestion. To explain the difference between somatic and meiotic cell activity for HSBP, we performed immunoblot analysis of HSBP to check its abundance and nuclear translocation in seedlings and buds. In plant somatic cells, HSBP is known to show a basal expression level at room temperature; *HSBP* transcription is induced and HSBP protein translocates from the cytosol to the nucleus at high temperatures, which inhibits HSF activity in plants. Our transient protoplast assay showed here that both high temperature and H_2O_2 induce the nuclear translocation of HSBP (Fig EV3H and I). We also observed that nuclear HSBP levels in Arabidopsis seedlings treated at 37ºC for 3 h are higher than those of seedling grown at 20ºC (Fig EV3K), indicating that somatic cells require high temperature or stress signals for promoting the translocation of HSBP from the cytosol to the nucleus. Our immunoblot analysis of HSBP showed higher HSBP levels in buds, compared to seedlings (Fig EV1C). We also provided nuclear localization and enrichment of HSBP-YFP fusion protein in meiocytes using *HSBPpro:HSBP-YFP* transgenic plants, in addition to immunostained HSBP in male meiocytes (Fig 6L, Figs EV4J and EV4). These results indicate that meiotic cells may have intrinsic developmental signals to induce abundant HSBP levels and nuclear localization at early meiosis. We added this explanation on the difference between meiotic and somatic cells for HSBP activity to the proposed model (Fig EV5).

12) The proposed HCR2 mode of action imply that wtils type and hcr2 mutants start meiosis with similar HEI10 transcript levels and that those will get reduced during pachytene/Diplotene in wt, whereas in the hcr2, those levels stay high or even increase. However, this data is not provided in the current form of the manuscript.

RESPONSE: Thank you for pointing this out. To address the reviewer's point and clarify the model for HSBP during meiosis, we performed quantification of HEI10 immunostaining at the zygotene-mid-pachytene stages and immunoblot analysis of HEI10 in *hsbp-3*. We observed that the number of HEI10 foci increases at the zygotene/pachytene stages in *hspb-3* (Fig 6J), and HEI10 protein levels increased in *hsbp-3* (Fig 6K). We propose a model according to our new data showing HSBP foci counts, HEI10 protein abundance and localization in the wild type and *hsbp* in somatic and meiotic (Fig EV5). We showed that *hsbp* mutations lead to a loss of DNA methylation at the *HEI10 5′* UTR and increased *HEI10* transcripts in somatic cells, which likely contributes to higher *HEI10* transcription in early meiosis, in addition to the lower attenuation of HSF activity at early prophase I. Thus, in the model, we propose that *hsbp-3* mutants start meiosis with higher *HEI10* transcript and HSBP protein levels than the wild type. The levels of HSBP and HSFs are likely important for controlling *HEI10* transcript level during early meiosis.

Based on the known HSF-HSBP model (cycle of activation and attenuation) in heat shock response in somatic tissues, HSFs are activated by high temperature, ROS and other developmental signals. The activated HSFs induce the transcription of heat shock protein genes (*HSP*s) including *HSBP* and *HSP70*, both acting as HSF inhibitors. During the heat shock, HSBP proteins immediately translocate from the cytosol to the nucleus, which contributes to attenuating HSF trimeric activity by dissociating HSF trimers. In meiotic cells, HSBP is highly abundant and located in the nucleus at leptotene/zygotene stages and may bind with the active HSFs at the *HEI10* promoter, which HSBP is proposed to reduce *HEI10* transcription during meiosis by dissociating HSFs. We demonstrated the interaction between HSFs and HSBP and that HSBP can inhibit HSF activity and *HEI10* transcription when it is co-expressed with HSF in protoplasts (Fig EV3). *HSBP* overexpression can decrease *HEI10* expression and crossovers using transgenic plants expressing *HSBP* during meiosis (Fig 5I and J). Thus, it is likely that the protein abundance and activity between HSFs and HSBP are important for determining *HEI10* transcript levels during early meiosis (Fig EV5).

Minor comments

It will be better to stick to the use of HCR2 or HSBP throughout the text as the indiscriminate use of both lead to confusion.

RESPONSE: Thank you for this suggestion. We have edited the text to first mention the *hcr2* mutant, which we then renamed *hsbp-3* thereafter, since *hsbp-1* and *hsbp-2* were already defined in a previous report (Hsu et al., Plant Physiology, 2010)

Figure 5E it is not clear the meaning of the axes. I understood that the black line is the endogenous HEI10 (thus 0 means homozygous mutant), while the rec line is a HEI10 transgene (0 meaning wild type). This should be clarified. Was the transgene copy number per insertion determined?

RESPONSE: Thank you for pointing out this. We have clarified the red and black numbers in the legend. We have determined the transgene copy number using the ratio of resistance to antibiotics and found that all transgenic plants tested in Fig 5E (Appendix Table S11) harbor a single T-DNA insertion (Chi-square test)

Measurement plant number	Genotype	*P* value
	HEI10pro:HEI10-myc (sensitive/resistant)	
1	39/82	0.303
		Not significant at $p < 0.01$
	ASY1pro:HEI10-myc (sensitive/resistant)	
1	26/66	0.728

2	15/80	0.039
3	46/170	0.556
4	42/145	0.735
5	41/130	0.966
6	20/126	0.037
		Not significant at $p < 0.01$
	REC8pro:HEI10-myc (sensitive/resistant)	
1	28/71	0.716
2	28/74	0.813
3	46/140	0.926
4	48/127	0.767
5	44/125	0.965
6	47/146	0.983
		Not significant at $p < 0.01$
	DMC1pro:HEI10-myc (sensitive/resistant)	
1	21/70	0.888
2	40/61	0.039
3	32/159	0.127
4	49/112	0.420
5	45/133	0.927
6	26/145	0.067
		Not significant at $p < 0.01$

The section "hcr2 shows more MLH1 foci and HSBP localizes to the nucleus during meiosis" is presented rather late. Better move it early in the result section

RESPONSE: Thank you for this suggestion. In the revised version we have added the results of HEI10 immunoblot and HEI10 foci counts, and MLH1 foci data from *hsbp* alleles and *meiMIGS-HSBP* to Figure 6. Therefore, we think that it is reasonable to place these cytological datasets (HEI10 and MLH1) and HEI10 protein abundance data in Fig 6 after the RNA-seq and RT-qPCR showing *HEI10* transcript levels in Fig 5.

Referee #3:

Improving crossover rates is of utmost importance as this contributes to a better reshuffling efficiency of alleles in the progenies. Several factors affecting crossover rate have been identified including Hei10, an E3 ligase that increases crossover rate when overexpressed. In their study, the authors isolated a new heat-shock binding protein in Arabidopsis that repressed the transcription of Hei10, reducing therefore the number of crossovers. They conducted an in-depth approach combining RNA sequencing, DNA methylome analysis and chromatin immuno-precipitation assay to decipher how this protein binds with heat-shock factors at the promoter of Hei10 and how this maintains DNA methylation on the promoter. The genetics using mutants (single, double, heterozygotes) and wild type is rigorous. All the results found are well analysed with regular controls and appropriate statistics. All the conclusions are sounded are the results found will be of main interest for researchers working on meiosis and recombination, especially with regards to environmental factors such as heat stress. I just have minor comments that may improve the manuscript.

L74: which type of mutants? (EMS?) Even if you refer to a publication in the M&Ms, it could be useful to

have the information without reviewing the literature.

RESPONSE: We thank the reviewer for pointing this out. We now make it clear that *hsbp-3* is derived from ethyl methanesulfonate (EMS) mutagenesis and indicate the EMS point mutation (C-to-T) in the main text, materials and methods.

L84: specify the distance in the WT

RESPONSE: Done.

L190: for chromosome 3 (Fig.4F), this is the only one for which, crossovers are reduced in the mutant background compared to WT close to the centromere while for the others, this is equal in WT and mutant. This is not pointed out in the results. Do you have any explanation for that?

RESPONSE: As mentioned in our responses to reviewer 2, we think that a T-DNA insertion-mediated chromosomal rearrangement may have occurred close to the peri-centromere along chromosome 3 in the *meiMIGS-HSBP* line, which might explain the crossover landscape along chromosome 3, which is higher at the chromosome arms and lower around the pericentromere. In this revised version, we have thus excluded chromosome 3 from genome-wide analyses and from the TEL-CEN plot.

L328: It is surprising that the discussion does not refer to a paper in barley (Higgins et al. The Plant Cell, Vol. 24: 4096-4109, October 2012) showing that increasing temperature from 22{degree sign}C to 30{degree sign}C modifies the number of interstitial chiasmata, which perfectly fits with the results found here. I suggest to add a short section on this.

RESPONSE: Thank you for this suggestion. We have cited the article and added a sentence to the Discussion.

L351-354: I don't see any reason why ROS could be the main activator of HSBP and HSF during meiosis? I agree that ROS can activate HSF but this is probably not the only signal affecting its expression. And I am not aware of anything regarding HSBP. You either have evidence or remove. Same for ASY1. Why this one would be more affected by ROS than other meiotic genes?

RESPONSE: We agree with the reviewer's point that many transcriptional regulators and developmental signals such as ROS and calcium concentration may contribute to modulating the expression and activity of the HSF-HSBP module during meiosis. In addition, the HSF-HSBP transcriptional module may control not only *HEI10* transcription but also many other genes during meiosis. However, based on our results (nuclear localization and interactions of HSBP with HSFs) in this study and the known critical roles for ROS in stress responses and development, we propose that ROS is one of potential signals that facilitates the HSF-HSBP regulatory module for *HEI10* transcription during meiosis.

To address the reviewer's concern regarding the relationship between HSBP and ROS, we examined the effect of ROS (H_2O_2) on nuclear translocation of HSBP itself using protoplast transient expression assay (Fig EV3I). We observed that as with high temperature, ROS (H_2O_2) were able to induce the translocation of HSBP from the cytosol to the nucleus in protoplasts (Fig EV3I). Meiotic cells are surrounded by multiple cell layers and thick callose cell walls in plants, which may induce hypoxic conditions and ROS accumulation. We observed the nuclear localization of HSBP in meiotic cells using immunocytology in wild-type plants. In this revised version, we also

included analysis from *HSBPpro:HSBP-YFP* plants that shows the nuclear localization of HSBP-YFP in meiocytes (Fig EV3J).

L394: Precise what type of mutant hcr2 is.

RESPONSE: We now clearly state that *hsbp-3* is an EMS-derived point mutation.

Dear Kyuha,

I have now heard back from three referees and they are all satisfied with the revised version of your manuscript (the comments are pasted below). Before I send you a formal acceptance letter, please attend to the following editorial points:

1. Rewrite the Conflict of Interest statement according to the guidelines on the EMBO Journal website.
2. Assign author contributions to Heelin Kim and Eun-Jung Kim. Re-label the two JKs as JaK and JuK.
3. Please complete the author checklist.
4. In the manuscript text, figure callouts are missing for Figures 6L, EV2G, EV3K and L, EV5A-C and appendix figure S6A-C.
5. In Appendix 1 (table of contents), please add page numbers.

If you have any questions, or need any further clarification, please do not hesitate to contact me.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
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Referee #1:

In the revised manuscript, the authors rephased descriptions in Results and Discussion sections appropriately according to previous comments of reviewers. The revisions successfully figured the HSBP role in epigenome control of HEI10 transcription out more clearly compared to the previous ms. So I think this paper whorthy to be published.

Referee #2:

The authors satisfactory addressed my concerns in the revised manuscript.

Referee #3:

This paper is a revised version of a previously-submitted manuscript that shows that a HEAT SHOCK FACTOR BINDING PROTEIN affect the transcription of HEI10 which results in a reduction of the crossover rate. I found that the authors made a lot of effort to answer all the concerns the different reviewers had regarding their study. They provide additional evidences by doing more experiments. I think that this is a nice study that will bring interesting results regarding the way meiosis is affected by temperature in plants.

The authors have made all requested editorial changes.

ACCEPTED **27th Apr 2022**

Dear Kyoha,

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

Congratulations on putting together a really compelling story!

Yours sincerely,

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

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods; no restrictions

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods: α-ASY1 α-MLH1 α-RAD51 (Higgins et al, 2005; Chelysheva et al, 2010; Sanchez-Morgan et al 2007), α-HEI10 (Mathilde Grelon), α-HSBP (in this study)

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table S30

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Meterials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Meterials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Not applicable

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	Not applicable
Please detail **housing and husbandry conditions**.	Not Applicable	Not applicable

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Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	Not applicable

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and Methods, Data Availability

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	Not applicable

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	Not applicable

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Mehods, Figures, AppendixTables
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	Not applicable
Include a statement about **blinding** even if no blinding was done.	Not Applicable	Not applicable
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	Not applicable
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Mehods, Figures, AppendixTables

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
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Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	Not applicable
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	Not applicable
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Reporting

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For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	Not applicable

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Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
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