The Number of Meiotic Double-strand Breaks Influences Crossover Distribution in Arabidopsis

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TPC2017-00273-RA	Submission received:	April 6, 2017
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TPC2018-00531-RA	Submission received:	July 13, 2018
	1st Decision:	Aug. 15, 2018 *accept with minor revisions*
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2017-00273-RA 1st Editorial decision – *declined* May 8, 2017

Thank you for choosing to send your manuscript entitled "The number of meiotic double-strand breaks influences crossover number and distribution in Arabidopsis" for consideration at *The Plant Cell*. Your submission has been evaluated by members of the editorial board as well as expert reviewers in your field, and we regret to inform you that we are not able to recommend publication of this manuscript. We have not made this decision lightly. We have had input from multiple scientists, and have solicited post-review comments as well. Our present policy is to offer streamlined decisions and to not advise on the direction of the work by requesting extensive modifications or substantial additional experiments.

That said, we would be willing to re-consider a new manuscript that fully addressed the concerns raised during this review process. This would be treated as a new submission, but we would attempt to use at least some of the same reviewers. Nevertheless, reviewers will be asked to assess as a new manuscript (i.e. are the claims fully supported by the data; do the results presented move the field forward), and not only whether previous reviewer comments have been addressed.

The reviewers appreciated the use of a partial loss of function mutation to approach this difficult problem but thought that additional replicates would be a valuable addition to ensure that the one example was not representative for some reason. Also, the reviewers note that male and female differences in recombination rate should be assessed. It was also questioned how a reduction in pollen viability could occur if, as claimed, there were no effects on pairing or synapsis or meiotic recombination. In this regard, it was also questioned whether the determination of chromosome pairing was sufficiently assayed.

We thank you for your interest in and support of *The Plant Cell*. We wish you good luck with your research and we look forward to seeing future submissions of your work.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00531-RA Submission received July 13, 2018

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

1. A transgenic plant with 30% reduction in DSBs was obtained, that behaved otherwise apparently normally re meiosis, and used in this work. The effect on crossover rate was determined using various methods: the number of chiasmata per meiosis and the number of MLH1 foci were both reduced by 13%, which was shown to be significantly lower than WT. A reduction of 13% in crossover-should it be compared to the WT (as done here) or to the expected value or preferably both. The question is what should we expect? A proportional decrease of 30%, or a decrease after obligatory chiasmata are deduced? Please elaborate and relate to statistical significance.

This is a very good point. We believe that relating the CO numbers in the hypomorphic lines directly to wild type, as we have done, will be less confusing to non-specialist readers. However, we have also calculated the expected CO numbers given the DSB number decrease, as the reviewer suggests, and have noted they are not significantly different from the observed CO numbers. We have elaborated on this issue in the Discussion section of the revised manuscript.

2. The use of a single transgenic line is problematic-one never knows if it is stably expressed or if transgenesis *per se* has induced other mutations. Why not two? As replica-- if many lines were already made.

In response to reviewers' comments, we have examined a second independently-derived transgenic line harboring a hypomorphic *SPO11-1* allele. The results, included in the revised manuscript, are consistent with the results of the previously analyzed line.

3. Line 62-do you mean a small fraction of recombination or DSB events?

We meant a small fraction of recombination products (which include COs and NCOs).

4. Line 97-mentioning the NCO in the bottom line of the introduction is strange as nothing was done on NCO analysis.

We agree and have revised the last section of Introduction accordingly.

5. The 13% overall value suggesting "partial homeostastis" has not much meaning-indeed this effect on overall rate is rather modest and in fact not very informative as it is a rough average of two opposite effects as seen from the localized analysis (discussed below).

We agree that the term "partial homeostastis" was vague. In the current manuscript version, we have thoroughly revised the section dealing with homeostasis to make it clearer and more streamlined, following suggestions of the reviewer. In the new version, we refrain from using the abovementioned term.

6. The reorganization of the landscape in the line with a reduced amount of breaks is the real story. The author failed to provide evidence for or to propose a satisfactory mechanism. Did the author map the breaks? Maybe it is the breaks which were redistributed in this line? Another even more plausible possibility is that the DSB reduction affected differentially the male and female meiocytes. Indeed, as shown by the Mezard lab, female meiocytes show more recombination in the pericentric region than in the distal regions, while it is the opposite for the males. If there was a reduction of crossovers mostly in the female, the result would indeed show an effect on the pericentric region. In this study, the cytogenetic analyses were done only on the male while the measurements with the genetic markers are an average of both the male and female recombination. The "gender-specific" model is easy to test experimentally.

The CO landscape analyses were performed in such a way to only examine male meiosis, as hybrid plants were crossed as males to create the BC_1 progeny. We have revised the relevant section of the manuscript to make this point clear. In the revised manuscript, we also present a more-refined model for mechanistic events that could result in reduced CO numbers in pericentromeric regions. While mapping DSBs in Arabidopsis is possible, and has been recently reported, it is far from trivial, and the existing method does not allow examination of the dynamics of DSB formation and repair, which are likely be at the heart of the phenomenon we observe.

7. To summarize, this work shows novel and interesting data. The experiments have no major flaws (except maybe for the need for at least 2 independent lines showing the redistribution effect). The way the story is told with long discussions on homeostasis should be reconsidered because the real action is on the redistribution of crossovers. Moreover the interpretations provided here do not provide much mechanistic insight: saying that the redistribution seen here resembles the effect of *met1* and *ddm1* does not tell much as the works on these mutants themselves have no clue on the redistribution mechanism.

We appreciated reviewer's interest in our work. In the revised version of the manuscript, we added an analysis of a second transgenic line and streamlined the Discussion section, shortening the divagations on homeostasis. We have also strived to present a more cohesive model to explain the changes in CO patterns in the hypomorphic lines. The fact that CO landscape in *met1* and *ddm1* remains unexplained, as the reviewer points out, likely attests to the complexity of mechanisms affecting recombination dynamics. We believe that several more studies will be needed to elucidate these issues.

Reviewer #2:

1. Additional lines with various levels of SPO11-1 expression should be included in the analysis. It is especially important when chromosomal aberrations potentially derived from T-DNA integration events are a concern, as mentioned by the authors. By checking plants with different levels of DSBs, the possibility of CO alteration resulting from chromosomal aberration can be better excluded. This would also provide novel information on the minimal requirement of DSB for CO assurance, and perhaps reveal a linear correlation between DSB and CO numbers.

We have examined a second independently-derived transgenic line during the last year. The results, included in the revised manuscript, are consistent with the results of the previously analyzed line.

2. Figure 1 nicely shows a correlation between DSB number, SPO11-1 expression and pollen viability. It is conceivable that DSB numbers are correlated with SPO11-1 RNA expression and pollen viability. However, it is surprising to know that a moderate reduction of DSB numbers in the *spo11-1-w2* line resulted in no defects in meiotic recombination, homologous pairing or synapsis, but this line did exhibit reduced pollen viability. Therefore, the authors should explain the decreased pollen viability in the hypomorphic mutant *spo11-1-w2*. Were any univalents or multivalents observed? If meiotic recombination and chromosome segregation are normal in *spo11-1-w2*, how does DSB reduction affect pollen viability? Is it possible that a mild phenotypic aberration in terms of meiotic recombination was missed? These questions reinforce the need to examine additional lines with different SPO11-1 expression levels.

We agree that given the lack of obvious defects in chromosome segregation in *spo11-1-w2* (as well as in *spo11-1-w3*), one would expect lower frequencies of inviable pollen. We hypothesize that while we do not observe major defects in chromosome segregation, minor defects, which are more difficult to detect, may still be present and result in reduced pollen viability. The other possibility is that the slight reduction of CO number has a negative role in pollen viability. This is reminiscent of our previous finding through quantitative analysis of pollen viability between WT, *atmus81*, *atrfc1* and *atmus81 atrfc1* mutants (Wang et al., 2012, PLOS Genetics). Although the plant fertility seems normal between WT and *atmus81*, the pollen viability was significantly reduced in *atmus81* with ~350 compared with WT with ~500. In the new version of the manuscript, we have revised our conclusions on the lack of chromosome segregation defects to make them less categorical. As the reviewer suggested, we have also analyzed a second hypomorphic line.

3. The title "chromosome interactions under reduced DSB numbers are not affected" is ambiguous. I presume the authors mean "homologous chromosome pairing" when they refer to "chromosome interactions"? In this section, the authors examined rH2AX and RAD51 (for DSB formation and recombination progression), and used FISH with a centromere probe (for pairing dynamics; line 159), as well as ASY1/ZYP1 immuno-staining (for SC formation). This section is problematic. Firstly, the centromere probe is not a good marker for meiotic chromosome pairing since the centromere is a special chromosome region that often exhibits coupling activity in many organisms. It is not yet clear that centromere coupling in plants absolutely requires DSB-dependent recombination. It will be necessary to examine

homologous pairing of multiple locations for examination of pairing dynamics. Perhaps, lower numbers of DSBs delay pairing of interstitial regions (not necessarily the centromere region), and that in turn results in a redistribution of CO. Secondly, immunostaining of ASY1 and ZYP1 only indicates normal loading of SC but, without BrdU incorporation to monitor meiotic progression (as shown in Sanchez-Moran et al., Gene Dev. 21:2220), it would seem that the authors' conclusion of normal meiotic progression is overstated. Thus, I disagree with the strong conclusion in the Discussion (lines 278-280) saying "the reduction of the DSB number by 33% did not result in any defects in pairing and synapsis, or in any other obvious changes in chromosome interaction dynamics".

We agree that this section, as it was written, was confusing and problematic. We have thoroughly revised this part of the Results to correct the errors. To specifically respond to reviewer's questions, we agree that to assess homologous chromosome pairing we should have used other FISH probes. However, our intent was to specifically examine chromosome interactions at centromeres, also sometimes referred to as centromere pairing or coupling. To avoid confusion, we refrain from using the term "chromosome pairing" in the revised version of the manuscript. For the same reason, we have separated the centromere FISH analyses into their own section in Results. Our goal of using immunostaining with the anti-ASY1 and anti-ZYP1 antibodies was to examine SC formation. We agree that these observations may not be sufficient to assess meiosis progression. To avoid confusion, we have removed the term "meiosis progression" from the revised manuscript.

4. It appears that the reference style used here is not for *The Plant Cell*.

We have corrected the reference list in the revised manuscript.

5. Fig 1A and 1B may have the same factor for the X axis. And it would be more informative if the authors provide a supplemental table that contains relative expression of SPO11-1, number of γH2Ax foci, and pollen viability for each transgenic line, so that the reader can know whether there is correlation between number of γH2Ax foci and pollen viability.

We have corrected Figure 1 and added the Supplemental Table1 containing detailed data used to create this figure.

6. Page 2, line 37, studies in some species. Please specify what species.

This sentence has been removed from the revised manuscript.

7. Page 2, line 39. It is not clear, however, "is". Change to "whether".

This sentence has been removed from the revised manuscript.

8. Page 2, line 47, "These results indicate that the DSB dynamics has a major impact on the CO/NCO decision". CO/NCO decision doesn't seem to be the theme of this research. Perhaps consider a different closing sentence.

The statement has been removed from the revised manuscript.

9. Page 3, line 76: "as distribution, of COs" should modify to "as distribution of COs"

This issue has been corrected.

10. Page 5, line 112: "These phenotypes were very stable, ...", which phenotype it is? "viable pollen ranged from 20% to 100% ?". It should be specified with presented results. In addition, "ranging from producing from 20%..." needs to be rephrased.

We have rephrased these sentences to correct them.

11. Line 133, SC appears for the first time, so spell out.

We have corrected this issue in the revised manuscript.

12. Figure 2D, in the bottom panel, the RAD51 signals look brighter and higher in numbers in the merged image.

We have corrected this issue in the revised manuscript.

13. In figure 2, ND does not appear on the boxplot.

We have corrected this issue in the revised manuscript.

14. Figure 2: No zygotene marker and pachytene marker (such as FISH probes indicating pairing) were shown. I wonder how the authors determine the stage of each meiocytes. The authors should explain this in lines 137-145.

To distinguish between zygotene and pachytene meiocytes we used two criteria. First, careful examination of DAPI-stained meiocytes allows the determination whether chromosomes are predominantly present as univalent or bivalents. Second, FISH with a centromere probe helps identify paired centromere regions. Please refer to lines 189–192 of the revised manuscript.

15. Fig 4, red signals look blurred on white chromosomes.

We have corrected this problem in the revised manuscript.

16. Fig 4A, one arrowhead is mislabeled.

We have corrected this problem in the revised manuscript.

17. Fig 4B, perhaps include % change and p-values in the table (Fig 4B) since the numbers look similar. Legend: indicate what the yellow arrows are.

In the revised manuscript, we have replaced this table with a graph, included P values, and corrected the figure legend.

18. Line 175, delete "in we".

This sentence has been corrected.

19. Line 183, Figure 4A and B. Should be only Figure 4B?

This sentence has been corrected.

20. Line 200, "*spo11-1-w2* line was used to backcrossed to L*er*". But in the M&M, it says that" *spo11-1-3* mutant was used to backcrossed to L*er*, and then the resulting *SPO11-1-3/spo11-1-3* in L*er* was crossed with *spo11-1-w2*. Please clarify.

This sentence has been corrected.

21. Page 10, line 202, the resulting *spo11-1-w2* (L*er*) plants were crossed to *spo11-1-w2* (Col). This should be "The resulting *spo11-1-3* (L*er*) plants ..."??

This sentence has been corrected.

22. Should the "*spo11-1-w2* allele" be written as "*Spo11-1-w2* allele"? since this allele is reduced expression of gene product instead of mutated gene product.

Since *spo11-1-w2* is still a mutant (i.e. not wild-type) allele, we believe that *spo11-1-w2* is more correct. In general, the same nomenclature is used to designate mutant alleles in Arabidopsis, regardless of whether it is a mutant gene product or a change in the gene expression level. Please note that *Spo11-1-w2* would not follow the established nomenclature in Arabidopsis, as mutant alleles should be designated with all small letters whereas wild-type alleles should be in all caps.

23. Lines 211-222. Authors should state more clearly that the wild-type population is not a simple BC1 population of COl x L*er*. Instead, *SPO11-1/spo11-1 spo11-1-w2/+* was used for the CO mapping as control.

We have included this information in the Materials and Methods section of the revised manuscript.

24. It is not appropriate to use "CO mapping" here, since the analysis only scored recombination frequency in intervals.

We have refrained from using the term "CO mapping" in the revised manuscript.

25. If *spo11-1-w2* (in the *spo11-1* homo mutant) showed a 30% reduction of *spo11* RNA level, CO number and pollen viability are reduced. How about *spo11-1* heterogeneous plants (may have a 50% reduction of *spo11* RNA level)?

Assuming that the reviewer meant *spo11-1* heterozygous plants, the suggested analyses on plants heterozygous for *spo11-1-3*. However, using qRT-PCR we did not detect significant changes in the transcript level of *SPO11-1*

compared to *SPO11-1*/*SPO11-1* wild-type plants. Therefore, the *SPO11-1* transcript level reductions in *spo11-1-3*/*spo11-1-3 spo11-1-w2*/+ and *spo11-1-3*/*spo11-1-3 spo11-1-w3*/+ plants appear to be results of lower transcription level of the *spo11-1-w2* and *spo11-1-w3* transgenes compared to the endogenous wild-type *SPO11-1* gene. We hypothesize that lower expression levels are caused by transgene position effects.

26. Figure 5, figure legend does not indicate A and B.

This issue has been corrected in the revised manuscript.

27. Line 252, hemizygouse is a typo?

This issue has been corrected in the revised manuscript.

28. Lines 256-258, the sentence is not clear.

This issue has been rewritten in the revised manuscript.

29. Lines 331-334. The sentence is not clear.

This section has been thoroughly rewritten and the confusing sentence has been deleted.

30. Lines 351-356, the sentences are not clear. Perhaps elaborate on how DSB repair speed can affect CO formation in this case.

This section has been thoroughly rewritten to make it clearer and the confusing sentence has been deleted.

31. According to the Materials and Methods, DMC1 antibody has been used in immunostaining, however no figure shows DMC1 result.

This was an inadvertent error in the Materials and Methods section that has now been corrected.

32. In the figure S5 legend, descriptions of dashed and solid lines were inverted.

We have corrected this error.

33. The method that the authors used to calculate the CO number is not provided in Materials and methods section.

We have listed the method to calculate CO number in the revised manuscript.

Reviewer #3:

1. Lines 151-152: I can see why the authors did this, but I feel that their conclusion that the "...33% reduction of DSB numbers does not affect their repair dynamics..." is too strong. This should either be toned-down or better explained in the text.

We have toned down this statement to reflect our intended meaning that we have not identified severe defects in DSB repair in the two hypomorphic lines.

2. It would be good to explain how the authors counted their foci. As seen in the images (fig 2), doing this with such precision is not a trivial undertaking and it would help readers if the approach used was laid out in the methods.

In the revised version of the manuscript, we listed the method used to count the number of RAD51 and rH2A.x foci.

3. Supplemental fig 1a is a low-resolution image? blurred?

We have corrected this problem in the revised version of the manuscript.

4. Typing error on line 175.

We have corrected this sentence in the revised manuscript.

We have received reviews of your manuscript entitled "The number of meiotic double-strand breaks influences crossover distribution in Arabidopsis." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in *The Plant Cell.* This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following: The reviewers were satisfied with the revisions and feel that the manuscript describes a nice advance. They did, however, have a few more points that need some clarification, which you should be able to do easily.

Please see the attached files for comments on the figures, which you can correct in the revision. You can download the attachment, open in acrobat and view in comment mode. please apply the comments to all figures in the paper.

Please highlight all changes and include a detailed annotation to changes to the text, with line numbers, and noting your responses to the comments

---------- Reviewer comments:

[Provided below along with author responses]

TPC2018-00531-RAR1 1st Revision received Sept. 14, 2018

Reviewer comments on previous submission and **author responses**:

Reviewer #1:
Figure S5 is labeled in the legend as S6, moreover there is a reference to asterisks that do not exist and the authors should clarify the origin of the p value (what they compared to what).

We have corrected the figure legend. The P values indicate the significance differences between CO rates in the *spo11-1-w2* and *spo11-1-w3* lines compared to wild type, which we now explain in the revised figure legend. Please see lines 495-505 of the revised manuscript.

Reviewer #2:
1. In Fig 1A and 1B, the caption of Y axis may use "pollen viability (%)", and remove all other % symbols.

We have corrected Figures 1A and Fig.1B as recommended.

2. In the Line 77, A number of factors "affects".

We are a bit confused by this comment. The correct English usage is to follow "A number of" with plural, which was the form we have used in the manuscript. Please see lines 72 of the revised manuscript.

3. In the Line 115, it may be better to describe the phenotype like "ranging from severe sterility with 20% of pollen viability to a complete fertility with 100% of pollen viability.

We have corrected the sentence. Please see lines 110-111 of the revised manuscript.

4. In the line 130, the average number of rH2AX is inconsistent with the data shown in the table S1.

We have corrected the numbers. Please see lines 126-127 of the revised manuscript.

5. It is better to state that the wild-type control used in this study was ecotype WT plants or Wt-like plants (in the complementary experiment) in the main text.

We have revised the way we refer to control plants throughout the manuscript to make it more precise.

6. In the line 141, "in in".

We have corrected the sentence. Please see lines 137 of the revised manuscript.

7. In the line 189, "pachytene number" is not clear.

We have corrected the sentence. Please see line 187-189 of the revised manuscript.

8. Lines 281-282, the sentence is not clear.

We have corrected the sentence. Please see lines 277-281 of the revised manuscript.

9. I disagree with using the example of another maize study (lines 292-295) in which different meiotic mutants were used to show that decreased RAD51 number is correlated to meiotic defects. Since authors do not know most functions of genes in these meiotic mutants and they may not influence DSB formation per se. It may be not comparable with hypomorphic *spo11* mutants in this study.

We have removed the sentence from the revised manuscript. Please see lines 293.

10. In the line 257, it may be better to use reduced, but not "loss".

We have corrected the sentence. Please see line 257 of the revised manuscript.

11. In the line 346, the end of sentence is missing.

We have corrected the sentence. Please see line 345 of the revised manuscript.

12. Line 337-338, what interaction?

We have corrected the sentence. Please see lines 346-347 of the revised manuscript.

13. What is the circle in the Fig 1A? please indicate it in the figure legend. That is easier for readers to follow.

We have corrected the figure legend. Please see lines 714-715 of the revised manuscript.

14. The names of "*Spo11-1-1 wt-like 1*" and "*spo11-1-w1*" are confusing and may mislead. (in the table).

We have renamed to wild-type looking lines in Table S1.

15. In the Fig 4A, there is a yellow arrow on the up-right corner. What does it point at?

We have corrected Fig.4A.

16. In the Fig 4H, chromosomes are tangled? Is it just the cell or it is common in the line?

It was just a randomly selected cell. We have replaced it with a different one in the revised Fig.4H.

17. In the Line 227-228, Use brackets for sentence "Please see Materials and Methods for more information."

We have corrected the sentence. Please see lines 234 of the revised manuscript.

18. In the Fig 5B, Significant difference should be shown by drawing a line above relevant bar and putting asterisk above the line, instead of putting asterisk on two relevant bars.

We have revised Fig. 5B accordingly.

19. Please provide the source information of antibodies in the "Materials and Methods" section.

We have included the source information for antibodies in the revised manuscript. Please see lines 4449-446 and 539-542.

20. In Line 416-419, the internal control of real time PCR is *ACTIN2*. But in the Figure legend for Fig 1B, *ACTIN4* was used for the internal control. Please revise.

We have corrected the information in revised Fig.1B.

Reviewer #3:

I am satisfied that the authors have dealt with the issues I raised in reviewing the original manuscript, except for my request for a description of how numbers of foci were quantified. This has been dealt with by referring to "Image Tool 3.0", with a reference to a 2007 article summarising ImageJ (Collins et al), which doesn't clarify things. This work presents an analysis of the effects of reduced DSB numbers in hypomorphic *spo11* mutants and while I don't doubt that the authors have quantified them with care, stating how they did it seems important. This is a relatively minor issue and should be easy to fix.

We have added more information on quantifying immunoblot foci in the revised manuscript. Please refer lines 450-454.

Comments in the pdf file:

We have corrected the flaws indicated in the pdf file in the Figures and Tables.

TPC2018-00531-RAR1 2nd Editorial decision – *acceptance pending* **Sept. 17, 2018**

We are pleased to inform you that your paper entitled "The number of meiotic double-strand breaks influences crossover distribution in Arabidopsis" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **Sept. 30, 2018**