Disrupted Genome Methylation in Response to High Temperature Has Distinct Affects on Microspore Abortion and Anther Indehiscence

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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.)

TPC2017-00656-RA 1st Editorial decision – *declined* **Sept. 24, 2017**

All reviewers appreciated your efforts to generate DNA methylome data and to try to understand the impact of high temperature stress on male sterility in cotton, which can be potentially useful in certain aspects of cotton breeding. Although the initial question is very interesting, the present manuscript did not address the most important question. The reviewers noted the lack of experimental replication and the need for better statistical treatment of the data. Some editors found it very difficult to sort through the data, and we wondered whether your work may have better impact when you hire a professional editor -- it is not a question of language, but rather a question of organization and presentation; planteditors.com or scienceeditorsnetwork.com are two companies that other authors have used. Finally, one editor noted issues of scholarship as well (i.e., inadequate citation of prior work).

Therefore, we cannot recommend resubmission of this work. The reviewers point out a number of areas in which the work could be strengthened, which may be helpful to you as you continue your project or revise your manuscript for submission elsewhere. If you decide to complete the story and resubmit to The Plant Cell, it will be evaluated as a new submission subject to full assessment by the editorial board, including pre-review and editor selection, and if sent for external review, a new set of reviewers is likely to be chosen.

-- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00074-RA Submission received **Jan. 29, 2018**

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

Reviewer #1:

The study by Ma and colleagues addresses the question of how exposure to high temperature causes male sterility in cotton. In a previous study, the authors had observed a global change in DNA methylation in pollen upon heat treatment. In the present study, they investigate whether changes in DNA methylation could be associated with male sterility, for which they perform whole-genome bisulfite sequencing of different pollen developmental stages in a heat-sensitive cotton cultivar.

The authors address an interesting phenomenon and start out from an interesting hypothesis. However, the present manuscript suffers from a series of conceptual and technical flaws that make it very difficult to trust the results, and hence the conclusions.

Point 1. My main question regarding the concept of the study is this: In their 2014 paper, the authors describe DNA methylation changes in both heat-insensitive (84021) and heat-sensitive (H05) cultivars. Although overall methylation seems to be lower in H05 compared to 84021, both showed a global change in DNA methylation upon high temperature (see Figure 3B in Min et al. 2014). Based on that observation, it would have been necessary to conduct the WGBS in both cultivars, and not exclusively in the sensitive one. In the current form of the manuscript, one cannot tell whether the observed differences are merely background noise that is unrelated to the observed phenotype or whether there is an actual link.

RESPONSE: Thanks for your suggestions. Actually, we had performed bisulfite sequencing (BS-seq) for the high temperature (HT)-tolerant line 84021 at the same time. In order not to confuse the reader (too much data), we just presented the results from H05 in the first version of the manuscript. For 84021, we found it showed hyper-CHH methylation under HT. The positive effect of RdDM on CHH methylation was also detected in 84021. Per your request and suggestions, we now added the results of BS-seq and small RNA seq of 84021 to the revised version of the manuscript. Thanks for the suggestions again!

Point 2. The second main criticism, which I will elaborate on in more detail below, regards the technical execution. I understand that cotton has a large genome and that WGBS analyses are therefore costly and computationally intense, but this does not justify the lack of replicates and the use of very reductionist statistical tools.

RESPONSE: Thanks for your comments. Actually, we had performed replications of BS-seq of both cultivars (see supplemental Table 1 in the new version). We chose the statistical tools by referring to (Calarco et al., 2012; Zhang et al., 2015; Groth et al., 2016; Wang et al., 2016)'s papers. Perhaps we did not present this very clearly in the old version of the manuscript. We will answer your following questions one by one and made modifications in the related text lines. Thanks for the suggestions again!

Point 3. What is the false methylation rate (fmr) in the WGBS data that is due to incomplete BS conversion? Variation in fmr might explain the big variation in the number of seemingly methylated CHH sites (Suppl. Table 2).

RESPONSE: Thanks for the suggestions. Actually, we had presented BS conversion in Lambda DNA to evaluate the fmr, which has been stated in the Methods section (DNA Extraction, Bisulfite Treatment and Library Construction) by referring to (Yong-Villalobos et al., 2015; Zhang et al., 2015; Wang et al., 2016), but we found that we may not have described this very clearly. We resequenced the BS-treated lambda DNA and uploaded the sequence reads under NCBI Sequence Read Archive (SRA) accession number: PRJNA393079.

We evaluated the non-conversion rate in three methylation contexts (0.003 in CG, 0.003 in CHG and 0.003 in CHH); the detailed sequencing information is presented in Supplemental Table 2. We believe that fmr could be eliminated according to the conversion rate.

Point 4. The indications with regard to methylation rates (text lines 149 ff, and Suppl. Table 3) are ambiguous. For example: "CHH sites were only slightly methylated (less than 20%)": does this mean that 20% of all CHH sites were methylated, or does it mean that the average methylation rate of all methylated CHH sites was 20%? Please clarify for all contexts. First, indicate which fraction of cytosines was methylated in the genome; second, present a histogram of methylation rates for each context (instead of average).

RESPONSE: Thanks for the suggestions. Here we want to show that the numbers of mCs in the CHH context occupied a small proportion of the number of total Cs in the CHH context. After identifying methylation sites (mCs) through a classic binomial test, we calculated the percentage of mCs in total cytosines (mCs+unmethylatedCs)

covered by the sequencing reads (mCs/mCs+unmethylatedCs). We have modified the manuscripts in the related text line (line152-154). To avoid any misunderstanding, the methylation rate of mCs in three contexts are presented in Supplemental Figure 2, and the fractions of mCs in the genome are shown in Supplemental Figure 3.

Point 5. The WGBS data is not replicated.

RESPONSE: Thanks for the suggestions. Actually, we had presented replication and we also uploaded two biological replicates of BS-sequencing reads to NCBI Sequence Read Archive (SRA) accession number: PRJNA393079.

We performed reads mapping separately when we analyzing the two sets of BS-seq data. After reads mapping, we selected cytosines that were detected in two sets of BS-data to perform further analysis to avoid uncertain mCs calling. We have modified Supplemental Table accordingly. Now, Raw paired reads, number of reads covered cytosines are displayed in Supplemental Table 1.

Point 6. Figure 1D: The authors confuse relative and absolute enrichment. From their data, it is not clear whether short TEs are more abundant on chromosome arms (i.e., there are simply more of them than in other places), or if at chromosome arms they make up a larger fraction of the TEs that are present there. A better indicator would be number of TEs in 100 kb windows, for example.

RESPONSE: Thanks for the suggestions. We aimed to show that short TEs are much more abundant than medium and long TEs. Perhaps presenting relative enrichment would result in misunderstanding. Here, we presented a histogram of the numbers of three types of TEs on each chromosome in 1 Mb sections in Figure 1D. Meanwhile, we present the numbers of three types of TEs on each chromosome in 1 Mb sections in Supplemental Dataset 1. According to the figure and dataset, short TEs are more abundant than medium and long TEs on chromosome arms.

Point 7. Line 186 ff: Are the authors suggesting that Suppl. Table 2 and Suppl. Fig. 2 provide proof for increasing DNA methylation during pollen development? One cannot extrapolate the overall methylation from the sheer number of methylated sites, as this number also incorporates very lowly methylated sites and is affected by many factors such as coverage, fmr, etc. To prove my point: there are almost 1 mio more methylated CGs in HN-TDS than there are in HN-ADS (despite the authors' claim that CG methylated positions are similar in these two stages). Still, Figure 1F shows that overall, CG methylation levels are almost the same and are actually slightly DECREASED in HN-ADS.

RESPONSE: Thanks for the suggestions. Under normal temperature (NT), an increasing level of CHH methylation could be found during three developmental stages, as shown in Supplementary Table 3 (Supplementary Table 2 in previous version). This is a very interesting result; we thank you for your nice suggestion, and we would like to investigate this in our further work. However, in this manuscript, the key point we focused on is the changes in DNA methylation under high temperature and how DNA methylation regulates fertility under high temperature.

On the chromosomal scale, the number of CHH methylation sites was found to change significantly under HT. For example, at the tetrad stage, in H05 under HT, the number of CHH methylation sites decreased from 9,167,155 under NT to 4,301,205 under HT; meanwhile, the number of CG and CHG methylation sites decreased by 385,476 and 345,554, respectively. To avoid misunderstanding of the comparison of conditions, we removed the description of developmental stages from the manuscript

As for the issue that the "number incorporates also very lowly methylated sites", we referred to the methods published by (Calarco et al., 2012; Song et al., 2015; Zhang et al., 2015; Groth et al., 2016; Wang et al., 2016), which using classic binomial test to identify DNA methylation sites. Truly methylated Cs were determined based on p-value under the binomial distribution P = binomial (mCs, totalCs, error_rate), where mCs = number of mCs; totalCs =mCs+unmethylatedCs; error_rate was the error rate for nonconversion rate of the Lambda DNA.

We determined TRUE mCs with a p-value cutoff of 1e-4 using the above method; low methylated sites were filtered, true mCs were reserved. Meanwhile, we presented the methylation rates of mCs in three contexts, as in Supplemental Figure 2: the average methylation rate in mCs was more than 60%, which helps confirm the finding that these cytosines were truly methylated.

Point 8. The DMR calling presented in the paper is highly questionable. The authors use a simple 100 bp window approach with non-overlapping windows and perform a Fisher's Exact test on the C and T counts in each window to identify DMRs in pairwise comparisons. This has several flaws: This approach is suitable for DMP detection, but not for DMRs: because of the high read counts that result from summing up reads over a 100 bp sequence, Fisher's Exact test becomes very sensitive and reports very low p-values even when count differences are minor.

RESPONSE: Thanks for the suggestions! Here, we referred to the methods that used Fisher's exact test from (Calarco et al., 2012; Zhang et al., 2015; Wang et al., 2016) to divide the genome into 100 bp bins and followed with Fisher's exact test with cutoffs of p-value<0.05 or p-value<0.01 to identify DMRs.

After identifying TRUE mCs, we mapped the mCs to 100 bp bins; we didn't map sequence reads to 100 bp bins, as mentioned in the comment. In 100 bp bins, there will be 100 mCs at most. Through this method, we believe that the DMRs identified by Fisher's exact test truly existed.

Point 9. The authors seem not to have applied any multiple testing correction. In a genome of this size (>2Gb), testing 100 bp windows results in 21 mio tests for any given pairwise comparison.

RESPONSE: Thanks for the suggestions. Exactly, simply using 100 bp bin with non-overlapping windows would result in a higher failure rate. Therefore, we divided the genome into 100 bp bins with 25 bp sliding windows to identify DMRs. Next, the putative DMRs were pooled via Fisher's exact test and chi-square test, and DMRs that were identified by both fisher's exact test and chi-square test were regarded as true DMRs. We calculated DMRs numbers in three types of methylation contexts and found that there were many more CHH DMRs than CG and CHG DMRs (see Supplementary Table 4 in the new version).

We mapped hyper-DMRs and hypo-DMRs to the genome and found that CG and CHG DMRs were randomly distributed in the genome, while CHH DMRs were uniformly distributed on each chromosome (see Supplementary Figure 6). To further confirm this result, we used Metilene (Juhling et al., 2016) software to perform DMR identification under cutoff with p-value<0.05. There were also fewer CG and CHG DMRs than CHH DMRs (see Supplementary Table 4 in the new version).

We next mapped Metilene CG, CHG and CHH DMRs to the genome and found the same distribution pattern as the fisher's exact DMRs (see Supplementary Figure 6). Therefore, we think our results are reasonable. The DMRs information calculated by two methods has been deployed in Supplemental Dataset 2-7 to have applied any multiple testing correction. In a genome of this size (>2Gb), testing 100 bp windows results in 21 mio tests for any given pairwise comparison.

Point 10. Although there is a clear hyper-methylation in CHH (Figure 1F), the number of hyper- and hypo-methylated DMRs is quite similar. This also indicates an unspecific DMR calling.

RESPONSE: Thanks for the suggestions. An increasing CHH methylation level is a very interesting phenotype during anther development. This question gave us an excellent opportunity to think about why CHH methylation is increasing during development. We are truly thankful for the suggestion for our future work! In this manuscript, we presented DMR calling between the normal temperature against high temperature in three developmental stages separately, not between the different developmental stages. For example, at the tetrad stage, H05 presented hypo-CHH methylation under HT, while 84021 showed hyper-CHH methylation, and the results of DMR calling also provided proof that CHH methylation indeed changed.

Point 11. The fact that CHH DMRs are uniformly distributed, which - as the authors state themselves - is in strong contrast to studies in other plant systems, is another indicator for an indiscriminate DMR calling and typical for uncorrected window-based approaches.

RESPONSE: Thanks for the suggestions. We used Metilene software (Juhling et al., 2016) to identify the DMRs (with p-value cutoff of 0.05) again. We mapped Metilene DMRs to the genome and found that the distribution of CHH DMRs was quite similar to that of the fisher's exact DMRs, so that we believe that our results are reasonable (see Supplementary Figure 7).

Point 12. There are by now much more elaborate tools for statistically robust DMR calling, some specifically designed for plant-specific DNA methylation data; I strongly advise the authors to refer to the recent literature.

RESPONSE: Thanks for the suggestions. Here, we used Metilene software (Juhling et al., 2016) to perform DMRs

identification based on two replicates which confirmed our results.

Point 13. The authors should provide information on DMRs (coordinates, methylation rate differences, etc.) in a supplemental dataset.

RESPONSE: Thanks for the suggestions. We have uploaded all of the DMRs information, including fisher's exact DMRs and Metilene DMRs, in Supplemental Dataset 2-7.

Point 14. I do not understand the author's logic in their conclusions from Figure 2B: siRNA+ regions have higher CHH methylation independent of the temperature treatment. The difference between siRNA+ and siRNA- regions is similar under HN and HH conditions, and therefore, the conclusion that siRNAs are related to CHH hypermethylation upon high temperature does not hold.

RESPONSE: Thanks for the suggestions. In Figure 2A, we aim to show that in each growth condition and developmental stage, the siRNA+ regions showed higher CHH methylation levels compared to siRNA- regions. This result showed that the effect of RdDM did exist, which suggested to us that the changes in siRNA may contribute to the alteration of CHH methylation under high temperature; therefore, we identified differentially siRNA mapped regions (DSRs) and checked DSR CHH methylation levels, as shown in Figure 2C.

Point 15. I am not sure I have fully followed the authors' argumentation on the anther analysis in Figure 4. If I understood correctly, high temperature but not zebularine treatment led to changes in secondary wall thickening, which would indicate that DNA methylation does not play a role in this process. Next, zebularine treatment results in barren pollen grains, but these are not observed at high temperature. Therefore, to me, the zebularine effect cannot be linked in any way to the DNA methylation change observed in HT, and does not support any claim that HT-induced methylation changes are responsible for male sterility.

RESPONSE: Thanks for the suggestions. Under high temperature, we found that both microspore abortion (Figure 4D and D') and secondary wall thickening (Figure 4F') occurred in H05. Zebularine treatment led to microspore sterility under normal temperature (Figure 4c) but didn't result in secondary wall thickening (Figure 4e'). Zebularine treatment is used to mimic the hypo-DNA methylation conditions under high temperature, so that we hypothesize that disrupted DNA methylation under high temperature may result in microspore sterility. We then performed transcriptome analysis and found that the differentially expressed genes may influence microspore development.

Reviewer #2:

In this article, Ma et al. performed whole genome bisulfite sequencing to investigate the regulatory roles of DNA methylation in male fertility under high temperature. They observed global disruption of DNA methylation, especially CHH methylation, which seems to result in male sterility in an HT-sensitive line. Changes in 24-nucleotide small interference RNAs were significantly associated with DNA methylation levels. Experimental suppression of DNA methylation led to pollen sterility in the HT-sensitive line under normal temperature, but did not affect the normal dehiscence of anther wall. The expression of genes in sugar and reactive oxygen species (ROS) metabolic pathways were modulated significantly, while auxin biosynthesis and signaling pathways remained unchanged, indicating that HT disorders sugar and ROS metabolism via disrupting DNA methylation, leading to microspore sterility.

I do acknowledge that this work generates big data on DNA methylation with regard to high temperature-induced male sterility in cotton, and believe that the data set may be useful in certain aspects of cotton breeding. I have several concerns that prevent me from recommending its publication in Plant Cell, at least not the current version.

Point 1. After reading of their Figure 1 "Phenotype of male fertility and DNA methylation pattern during another development under high temperature (HT) stress" and Figure 2 "RdDM involved in regulating CHH methylation under HT", my immediate question is what causes this significant CHH methylation disruption? Which gene in the RdDM pathway is responsible for controlling this molecular phenotype? Much to my dismay, they did not dig into these important and also imperative areas to decipher the mechanism. Instead, they continued in following aspects: Figure 3, "RdDM contributed to altering CHH methylation in promoter and down-stream regions of protein-coding genes (PC genes)." Figure 4, "DNA methylation suppression induces microspore sterility but does not affect anther dehiscence." Figure 5, "HT induced DNA methylation associated with altered sugar metabolism." and Figure 6, "Suppression of DNA methylation induced excessive ROS generation in anthers." All these are somewhat interesting, but certainly

not necessary and sufficient for their manuscript. These data does not answer the original question: How (or by what molecular pathway) CHH DNA methylation is disrupted in this high-temperature sensitive male sterile cotton line.

RESPONSE: Thanks for your nice questions! Your questions give us new insights to understand how HT disrupts DNA methylation. As you said, our paper aims to find out how DNA methylation controls male sterility under high temperature (HT). Actually, based on the big data analysis, we concluded that disrupted DNA methylation may induce microspore sterility; meanwhile miRNAs involved auxin may participate in altering anther wall dehiscence. We have also considered your question about why the DNA methylation showed totally different trends in two different materials, and why the DNA methylation changed under HT. All of your concerns are illustrated in Figure 8.

According to our results, we hypothesized that sugar may play an important role in altering the expression of genes that participate in RdDM. We may supply, in the future, soluble sugar to the bud to find whether DNA methylation would change, and we will create CRISPR vector to edit certain RdDM genes that changed significantly in H05 to understand their functions in high temperature-disrupt DNA methylation in anthers in the future. Thanks again for the suggestions for our future research.

Point 2. In Figure 4. "DNA methylation suppression induces microspore sterility but does not affect anther dehiscence." From the middle of line 4 after the title, they wrote "At TDS, HT induced microspore sterility in H05 following application of water (D)". I do not see significant differences between "C", "D" and "c", especially on the condition that "A", "B", "a", "b" is regarded as "no difference" by the authors. Also, one cannot conclude from a simple micrograph whether the microspore is sterile or not, one can only observe a phenotype, either tetrads or tapetum degrading, etc.

RESPONSE: Thanks for your questions. Maybe the phenotypes presented in Figure 4C, D and c were not very clear. Many researchers have identified male sterility through the phenotype showing abnormal tetrads, wizened pollens/microspores, empty inclusion of pollens/microspores and indehiscent anther walls (Rhee et al., 2003; Mitsuda et al., 2005; Cecchetti et al., 2008; Hu et al., 2011; Xin et al., 2016; Cecchetti et al., 2017). In the figure, the microspores under HT ("D" and "d") showed abnormal shapes and not fully filled inclusion (showed with a red arrow with "MSP"), so we think that HT could result in microspore abortion no matter whether water or Zeb treatment is applied.

With the application of Zeb, we found that the microspores ("c") showed a similar phenotype to the microspores under HT ("D" and "d"). Due to this phenotype, we thought Zeb treatment could mimic HT stress in causing male sterility. The related micrographs ("C" and "c") have been changed to show a clear phenotype.

As for the question "A", "B", "a", "b" is regarded as "no difference", in the manuscript, normal tetrads showed wrapped microspores and abundant callose. In Figure "A", "B", "a" and "b", tetrads contained normal microspores, and the Aniline blue staining also showed callose around the microspores. Now the micrographs ("a" and "b") have been changed to show much clearer phenotypes. The related figure legend (line 1171-1173) has been modified.

Point 3. Figure 7, "Auxin biosynthesis and signaling pathway regulated indehiscence of endothecium is independent of HT-induced DNA methylation." Auxin biosynthesis and signaling has nothing to do with HT-induced DNA methylation, why a figure here. It obviously belongs to supplementary.

RESPONSE: Thanks for the suggestions. In our paper from 2014, we analyzed DNA methylation levels of H05 and found significant changes in DNA methylation under HT. Meanwhile, we displayed the results of gene expression profile analysis and found that sugar and auxin metabolism pathways changed significantly under HT.

In this paper, we aimed to investigate whether HT-disrupted DNA methylation is related to auxin and sugar metabolism pathways. We found there was a relationship between sugar metabolism pathway and DNA methylation. Next, we wanted to know whether the auxin metabolism pathway is related to DNA methylation, so we presented the results of auxin qualification and auxin immunohistochemical analysis. Maybe our statement caused misunderstanding.

In the heatmap in Figure7 A, many auxin biosynthesis and signaling genes were up-regulated under Zeb treatment (in red box), which means these genes may be regulated by DNA methylation. However, the auxin quantification and auxin immunohistochemical analysis showed no significant changes. Meanwhile, our recent study pointed out that the auxin metabolism pathway may be regulated by auxin-involved microRNAs (Ding et al., 2017), so we propose that the auxin metabolism pathway may be slightly influenced by DNA methylation but predominantly controlled by miRNAs under HT. The related text title (line 1223-1224) has been changed to "Auxin biosynthesis and signaling

pathway regulated indehiscence of endothecium is slightly influenced by HT-induced DNA methylation"

Actually, we could move Figure 7 to the supplementary file. In order to provide the reader with a complete understanding of the hypothesis that HT may drive DNA methylation, which may affect microspore sterility or anther indehiscence in two different ways as in Figure 8, we retained Fig 7 in this version of the manuscript.

Point 4. It is almost impossible to read the manuscript. The authors use abbreviations freely. One needs to refer back to the text several times in order to understand a particular figure legend.

RESPONSE: Thanks for your criticism. We realized there were too many abbreviations, which made the manuscript not easy to read. In the revised manuscript, we omitted many abbreviations by using full names, including materials, growth conditions, and developmental stages, in the figure legend and text lines (i.e. H05/HT).

Reviewer #3:

This study reports the impact of high temperature stress on the DNA methylomes of three different tissues in cotton. A genome-wide increase in CHH methylation was observed under high temperatures. Small RNA sequencing revealed that a substantial amount of the CHH intersected with regions that contained 24th siRNAs. Treatment with a DNA methylation analog inhibitor led to pollen sterility in normal treatments, but did not affect normal development of the anther wall. Lastly, RNA-seq data was generated and used to identify differentially expressed genes during the different stages of development under high temperature stress. These results are consistent with a genome-wide increase in CHH methylation in other species that have been heat stressed. However, the biological function of this increase, if any, is still unclear.

Overall, the data generated for this study is comprehensive and will serve as a useful resource to the field. However, most of the results are over interpreted, as there was no demonstration of a causal role for a change in DNA methylation causing a change in gene expression. Essentially distinct high-quality datasets were generated and intersecting lists were made without convincing the reviewer that these is a causal relationship. This is a major limitation of this work.

Point 1. No conversion rates for the BSseq data was presented. This is required, especially given how sensitive CHH methylation is to poor conversion reactions.

RESPONSE: Thanks for your suggestions. Actually we had presented BS conversion in the Lambda DNA to evaluate the non-conversion rate, which has been stated in the methods section (DNA Extraction, Bisulfite Treatment and Library Construction) by referring to (Yong-Villalobos et al., 2015; Zhang et al., 2015; Wang et al., 2016), but maybe we didn't make this very clear.

We resequenced the BS-treated lambda DNA and we also uploaded the sequence reads to the NCBI Sequence Read Archive (SRA) accession number: PRJNA393079. We evaluated the non-conversion rate in three methylation contexts (0.003 in CG, 0.003 in CHG and 0.003 in CHH); the detailed sequencing information is presented in the Supplemental Table 2. We believe that CHH methylation sites have been efficiently converted. Thanks for the suggestions again!

Point 2. The zeb treatments have no specificity, so they cannot be interpreted as presented. There are so many possibilities. Further, it was not shown if DNA methylation was actually reduced in the zeb-treated plants.

RESPONSE: Thanks for your suggestions. We have made note of this question and carried out additional bisulfite sequencing in H05 at the tapetum degradation stage (TDS) under normal temperature (NT) and high temperature (HT) under zeb treatment (see Supplemental Table 6 and Supplemental Figure 13). We found that the DNA methylation was reduced specifically under zeb treatment.

Point 3. Line 124 states that "HT directs DNA methylation to cause". There is no data to support causality. This is a major statement that would be worthwhile of publication if there was supporting evidence.

RESPONSE: Thanks for your suggestions! Maybe we didn't state this very clearly, but here we wanted to indicate that HT could disrupts DNA methylation, and aberrant DNA methylation could disrupt sugar and ROS metabolism pathways, which may result in microspore sterility. This question gives us a new insight into investigating how HT disrupts DNA methylation in anthers, which we would like to investigate in future work. The related text line (line

125) has been modified to "HT disrupts DNA methylation to cause sterility in the anther". Thanks for your suggestions again!

Point 4. It is stated repeated that the CHH methylation occurs specifically in the euchromatic arms, but it is clearly presented across the entire genome, including the pericentromere (Figure 1C).

RESPONSE: Thanks for your suggestions! In fact, on the chromosomal scales, there is relatively hyper CHH methylation (not specifically) in chromosomal arms compared to the pericentromere region, which was different from the CG and CHG contexts (Figure 1C). Perhaps our description was confusing. The related text lines (line 156-157, line 168-169, line 286 and line 513) have been modified to "CHH sites were relatively enriched in chromosomal arms".

Point 5. The affect of CHH on TEs is the same regardless of size. It is stated that the mid size TEs are not affected, but there is the same magnitude of an increase of CHH at TEs whether they are short, mid or long.

RESPONSE: Thanks for your suggestions! We believe that CHH methylation has effects on all kinds of TEs in the genome. Actually, we want to investigate the reasons why CHH methylation patterns were different from the CG and CHG contexts. I(Song et al., 2013; Wang et al., 2016) found that different CHH methylation patterns occurred on the different kinds of TEs. Also referring to (Wicker et al., 2007)'s paper, we classify TEs into short, medium and long TEs to investigate the CHH methylation pattern on these TEs. We found that short TEs and long TEs bodies showed relatively hyper CHH methylation compared to the up-stream and down-stream regions, but the mid TEs bodies didn't show very significant hyper-methylation. Meanwhile, short TEs occupied a large number of total TEs through the genome (Figure 1D and Supplemental Dataset 1). We believe CHH methylation may affect mid TEs, as is the case for short and long TEs, but we think hyper-CHH methylation on the short and long TEs in the genome may result in the hyper-CHH methylation levels on chromosomal arms.

Point 6. HT and NT are used throughout the manuscript but NH and HH are used in the figures. This is very confusing.

RESPONSE: Thanks for your suggestions! HN and HH referred to the H05 under NT and H05 under HT. To avoid this problem, we added the full names, including materials, growth conditions and developmental stages to the figures (i.e. H05/HT). Thanks for your suggestions again!

Point 7. The CHH changes in heat-treated anthers is the most important observation of this work, but there is no data to support why this is happening.

RESPONSE: Thanks for your comments. We are very glad to know this is an important observation. As you mentioned, CHH methylation was significantly altered under HT in anthers, prompting us to investigate how CHH methylation resulted in male sterility under HT. According to our results, we hypothesized that sugar may play an important role in altering the expression of genes that participate in RdDM. In the future, we may apply soluble sugar to buds to find out whether the DNA methylation would change, and we may create a CRISPR vector to edit certain RdDM genes that changed significantly in H05 to understand their functions.

Point 8. 25,000 promoter siRNA clusters were identified. This almost matches the number of genes in the genome. What is the significance of this? It is found in expected regions with expected molecular phenotypes? There is no data to support cause vs. effect.

RESPONSE: Thanks for the suggestions! In our results, we found that RdDM changed significantly and that the changes in 24nt siRNAs contributed to altering CHH methylation in anthers under HT. Meanwhile, the siRNAs mapped to promoters showed hyper-methylation levels compared with those to which no siRNAs mapped, which means that the effect of siRNAs on CHH methylation indeed exists.

Upland cotton contains almost 70,000 genes in the genome. Here, about 25,000 genes were identified in both 84021 and H05. We wanted to find the potential RdDM-related genes, especially in 84012 or H05, to determine whether there is a difference in the enrichment of metabolism pathway in these two cultivars. Therefore, we performed gene ontology (GO) analysis for the selected specific RdDM-related genes and found there were some different GO terms enriched in these two cultivars (i.e. in H05, carbohydrate derivative binding and oxidation-reduction; in 84021, fatty acid metabolic process and signal transducer activity).

We have received reviews of your manuscript entitled "High temperature stress disrupts genome methylation distinctively affects microspore abortion and anther indehiscence." These were forwarded to you previously and you submitted a comment on the criticism of the statistics provided by Reviewer 1. The reviewer has now read your comment and provided a recommendation, which I discuss below. Based on this, the editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

The comment of reviewer 1 is pasted below verbatim:

"According to their statements, the authors have applied more stringent statistics to their methylation analysis and come up with what I would consider reasonable numbers. Their analysis still relies on sliding windows, which is not ideal, but at least they now apply the necessary stringency (and they are by far not the only ones still applying this method). To what extent this will affect the conclusions of the manuscript I presently cannot say; this would become apparent only in a revised manuscript.

The second unresolved issue is that of the false methylation rate, i.e., the efficiency of bisulfite-mediated conversion of unmethylated cytosines. To do it the correct way, the authors would need to repeat everything, which is a bit much to ask at this point. I don't know the quality of the cotton genome, nor do I know whether the genomes of chloroplast and/or mitochondria exist as (partial) copies in the nuclear genome, but one option to get an idea of the fmr in each sample would be analyzing the methylation state of the organellar genomes. This would at least add some certainty to their independent lambda sequencing. So, in summary: I think another revision might be justified, but the validity of the study will likely need to be checked again based on the new results. "

We invite you therefore to submit a revised version of your manuscript that makes the changes to the statistical analysis proposed in your rebuttal and which takes account of the comments above from reviewer 1. The revised version will be sent again to this reviewer, who has offered to rapidly assess the second revision.

-- Reviewer comments:

[Provided below along with author responses]

TPC2018-00074-RAR1 1st Revision received May 21, 2018

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

Reviewer #1

The authors formulated responses to all points raised by the reviewers, but I was disappointed to see that they answered only a fraction of them. I have found of my criticisms (and those of the other reviewers) not addressed properly or addressed in an evasive or unclear manner. The manuscript, albeit containing a serious load of data, still leaves the reader with many open questions on some of the major conclusions. I can therefore not recommend publication of the manuscript.

Point 1. Their analysis still relies on sliding windows, which is not ideal, but at least they now apply the necessary stringency (and they are by far not the only ones still applying this method). To what extent this will affect the conclusions of the manuscript I presently cannot say; this would become apparent only in a revised manuscript.

> **RESPONSE: Thanks for the suggestions! Based on your nice suggestions, we fully understood that DMRs identified from sliding window analysis may contain higher false positive rates, so we removed the sliding windows while we performed the DMRs calling.**
>
> **The table (Supplemental Table 5) listed the number of DMRs in the last response letter, which in this version of the manuscript was based on the results using no sliding window. We analyzed the distribution of DMRs in the genome. The results showed that CG and CHG methylation changes slightly (Supplemental Figure 6) and that the CHH methylation changed across the whole genome (Figure 2B). The gene bodies contained more CG and CHG DMRs**

than those in the promoter and downstream regions. Meanwhile, the CHH DMRs were relatively enriched in the promoters and downstream regions (Supplemental Figure 10).Thanks for the nice suggestion again!

Point 2. The second unresolved issue is that of the false methylation rate, i.e., the efficiency of bisulfite-mediated conversion of unmethylated cytosines. To do it the correct way, the authors would need to repeat everything, which is a bit much to ask at this point. I don't know the quality of the cotton genome, nor do I know whether the genomes of chloroplast and/or mitochondria exist as (partial) copies in the nuclear genome, but one option to get an idea of the fmr in each sample would be analyzing the methylation state of the organellar genomes. This would at least add some certainty to their independent lambda sequencing. So, in summary: I think another revision might be justified, but the validity of the study will likely need to be checked again based on the new results. "

RESPONSE: Thanks for the suggestions! According to the results of (Zhang et al., 2015b), the quality of the cotton genome was relatively good for the methylation analysis. The papers by (Liu et al., 2013) and (Chen et al., 2017) showed that the mitochondrion genome contains a few segments (not whole copies) in the nuclear genome. Because there are few chloroplasts in cotton anthers, we just mapped the BS-seq reads of each replicate of each sample to the mitochondrion genome and performed methylated cytosines calling to evaluate the methylation status. The results showed that the methylated cytosines occupied a low proportion of total cytosines in each sample (less than 0.004). The methylation status of the mitochondria genome is presented in Supplemental Table 3. We referred to the paper by (Zhang et al., 2015a), in which the BS-seq of leaves revealed 0.0048–0.0053 methylation levels of the chloroplast genome. Our results showed that fmr was low in each replicate of each sample. We truly thank you for the nice suggestion!

Reviewer #2:

In the current version, the authors performed whole genome bisulfite sequencing to investigate the possible roles of DNA methylation in male fertility under HT. Global disruption of DNA methylation, especially CHH methylation, was found in an HT-sensitive line. Also, they found that changes in 24-nucleotide small interference RNAs were significantly associated with DNA methylation levels. Suppression of DNA methylation led to pollen sterility in the HT-sensitive line under NT. Transcriptome analysis of the anther showed that genes in sugar and reactive oxygen species (ROS) metabolic pathways, but not those in auxin biosynthesis and signaling, were under the regulation of DNA methylation. This study links DNA methylation to microspore sterility and possibly high temperature tolerance in cotton for the first time. I think that this revised form can be accepted for publication in the Plant Cell.

Point 1. I suggest that they delete their Figure 8, the schematic chart. It does not provide anything new except the stuff that we already know from the title of their paper. Also, they may need to shorten their Discussion part. Anything they say here are guesses, not really science.

RESPONSE: Thanks for supporting our results! We have now removed Figure 8 from the manuscript, and we also shortened the Discussion part by deleting some hypothetical statements to make the manuscript much more scientific. Thanks again for the comments!

Reviewer #4:

The study by Ma et al. identified the associations/connections among heat stress, CHH hypo-methylation, sugar contents, ROS metabolism and male sterility in cotton, which is very interesting. After careful reading the previous comments by all three reviewers and the authors' response letter, I think the authors have addressed all comments appropriately and revised the manuscript accordingly. In particular, the authors have done additional experiments and added large amounts of data that were not included previously. More technical details were also added. As such, the work is more complete, and the conclusions are much stronger. I think this is a solid study with sufficient novelty and significance. The manuscript in general is well-written. I only have the following moderate and minor comments.

Point 1. In Figure 1. D, it's hard to prove "medium TEs were concentrated near heterochromatic regions" by using the current chromosomal scale in the x-axis.

RESPONSE: Thanks for the suggestion! We removed the related content in the figure legend (Figure 1D) and modified the related text lines (lines 182) to avoid potential misunderstanding.

Point 2. Authors proposed a working model showing how HT induces male sterility in Figure 8. I do not fully understand why the sugar content signal is upstream of CHH hypo-methylation after heat stress. The CHH methylation decreased substantially in the tetrad stage (TS) and to a much lesser extent in the tetrad degradation stage (TDS) when compared between the HT and NT conditions (Figure 1F and Figure 3A), but it increased more significantly in TDS than in TS compared HT with NT. Could this imply that CHH hypo-methylation probably responds earlier than the sugar content after heat stress? And the accumulation of sugar across anther development might be due to the suppression of starch formation by some CHH methylation-regulated key starch synthesis genes, which finally causes pollen abortion. I think it would help if the authors could further elaborate the model.

RESPONSE: Thanks for the suggestion! The increase in CHH methylation during the development stage is a very interesting observation. It is a very good idea that CHH methylation responds earlier than the sugar; we really appreciate the suggestions for our further work.

According to the results of (Yu et al., 2013; Thalmann et al., 2016), glucose could repress miRNA transcription and post-transcription. Thalmann et al 2016 found that starch was degraded during the stress response. In our previous work, glucose content was reduced under high temperature, however, the total soluble sugar content was increased in both 84021 and H05 (Min et al., 2014). Meanwhile, (Min et al., 2013) found that casein kinase I (GhCKI), a key protein involved in sugar signaling, exhibits starch synthase kinase activity, which was disrupted under high temperature stress, leading to disordered starch synthesis.

We therefore think that hexose (like glucose and sucrose, shown as small circles in the model) is the first signaling molecule that functions during high temperature stress defense. Considering that there was too much hypothetical information in the model, we removed the model (Figure 8), as suggested by Reviewer 2. Thanks for the nice suggestion again!

TPC2018-00074-RAR1 2nd Editorial decision – *acceptance pending*	**May 22, 2018**

Thank you for the revised version of your manuscript, which takes account of the requests of Reviewer 1 for additional statistical analysis of the data. We appreciate your patience in the delayed handling of this manuscript. We are pleased now to inform you that your paper entitled "High temperature stress disrupts genome methylation distinctively affects microspore abortion and anther indehiscence" 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	**May 30, 2018**