Responses to Comments

Dear the Editor,

We resubmitted the revised manuscript entitled “Genome-wide identification and expression profiling of *DREB* Genes in *Saccharum spontaneum*” by Li *et al.* for publication in BMC Genomics. In this revised version, we have addressed all of the reviewers’ comments and included new results from additional analyses suggested by the reviewers.

In order to highlight the changes, all of these rewritten sentences and words are in red. And the detailed point-to-point responses are listed as following, besides the summary of the major points in this revision.

1. We have performed qRT-PCR by using two reference genes to verify the gene expression results.

2. We have provided the detailed methods as the reviewer pointed out.

3. We have added some contents about the novelty of our present study compared to the work by Huang *et al.* into the background section of the manuscript.

4. We have added the phylogenetic tree to Figure 5, 6, and 7, and merged Figure 8 and 9 to Figure 8 in the revised manuscript.

Thank you very much for your consideration.

Best regards!

Jisen Zhang

To the editor,
Editorial Board Member comments
Please, consider the manuscript for revise under the reviewers points. I also found a published paper with a similar evolutionary history which need to me consider by your group. I suggest you to consider the previous publication from Huang et al., 2020 entitled "Genome-Wide Analysis of the DREB Subfamily in Saccharum spontaneum Reveals Their Functional Divergence During Cold and Drought Stresses" published in Frontiers in Genetics. They conducted a large genome-wide analysis with DREB subfamily in S. spontaneous and your work contain related information. So please, specify the novelty of your work compared to Huang and collaborators and introduce

thoseinformation into the manuscript. Please, take care about gene name as he published it first.

Response: Thank you very much for your positive and valuable comments for improving our present manuscript!

S. spontaneum is an autopolyploid, and the homologous genes at the same locus on homologous chromosomes are defined as alleles in autopolyploid genomes (Zhang *et al.*, 2018). In "Genome-Wide Analysis of the DREB Subfamily in *Saccharum spontaneum* Reveals Their Functional Divergence During Cold and Drought Stresses", however, the authors didn't analyze the allelic levels for *DREB* genes and mixed the gene and gene allele, which is inappropriate for the gene identification in the autopolyploid genome. In our present study, we have discriminated the gene and their alleles, so we re-named these *DREB1s* and *DREB2s* according to Liu *et al.* (Liu *et al.*, 2013).

In this study, we analyzed the allele-defined genes for *DREB1s* and *DREB2s*, and explored the gene function based on the large scales of expression profiles from RNA-seq data sets including leaf developmental gradient, diurnal cycle, development stage, drought stress and cold stress. Thus, the present study provided insights into the polyploid characterizes for the *DREBs* and functions relative to photosynthesis and plant development besides the drought stress.

Reference:

Zhang J, Zhang X, Tang H, Zhang Q, Hua X, Ma X, Zhu F, Jones T, Zhu X, Bowers J *et al*: **Allele-defined genome of the autopolyploid sugarcane *Saccharum spontaneum* L.** *Nat Genet* 2018, 50(11):1565-1573.

Liu S, Wang X, Wang H, Xin H, Yang X, Yan J, Li J, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K *et al*: **Genome-wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L.** *PLoS genetics* 2013, 9(9):e1003790.

To Reviewer 1

Saccharum spontaneum is the founding species which contributes the genetic background of stress tolerance including the drought resistance for the sugarcane hybrids. And drought is considered as significant abiotic stresses for sugarcane production particular in China as over 70% were cultivated in the hilly area. The dehydration-responsive element-binding proteins (DREBs) are important transcription factors that involve in response to multiple abiotic stresses in plants. Identification and potential function analysis of DREB genes in S. spontaneum may provide foundation for the improvement of drought tolerance in sugarcane. In the study, Li and colleagues have performed genome-wide study for the DREB genes, explored the gene evolution. Noteworthy, they further estimated the potential function based on large set of RNA-seq from the different development stage, developing leave segments, different time

periods, drought stress and cold stress. They also verify RNA-seq based expressions of SsDREB genes by RT-PCR. Thus, the study provided new insights into the DREB genes in sugarcane. The manuscript is globally well-written. But minor revision is essential for the manuscript before publication.

Response: Thank you very much for your positive and valuable comments! Indeed, the large set of transcriptome datasets are very helpful resource for this study. *DREB* genes were considered to the drought response genes, in our present study, with the large set of RNA-seq analysis, these genes were indicated to relative to photosynthesis and plant development beside the drought stress.

Some specific comments on the analyses conducted and the writing:

1. For Key words: Genome-wide should be used as key words since it is too indistinct.

Response: We had changed "Genome-wide" to "Phylogenetic analysis" (L39). Thank you!

2. S. spontaneum are polyploidy with variant ploidy level. In result section, the author should clarify what is the ploidy level of the genome they used in this study.

Response: We have clarified what is the ploidy level of the *S. spontaneum* used in this study (L93). Thank you!

3. It is interesting see to gene structure of allelic variation. I would suggest the authors to present a graphic for the gene with four alleles in Figure 2.

Response: We have presented a graphic for the gene with four alleles (Figure 3). Thank you very much for your suggestion!

4. mya should be Mya

Response: Changed (L154-L157). Thank you!

5. The phylogenetic relationship for the genes could be added Figure 5, 6 and 7. And Figure 8 and 9 could be merged into one figure

Response: We have added the phylogenetic tree to Figure 5, 6, and 7, and merged Figure 8 and 9 to Figure 8 in the revised manuscript. Thank you very much for your suggestion!

6. In discussion section, "And the gradually increased expression pattern from the near-terrestrial end to the distal of steam at the stage of sugar accumulating suggested that SsDREB1L may participate in sugar metabolism." In my opinion, the authors only can conclude that SsDREB1L may paly role relative to sugar metabolism.

Response: It is a good suggestion! We have rephrased it (L290). Thank you!

7. There are still few spell issues, Line 147: related diploid species sorghum had been estimated by zhang et al should Zhang et a.; Line: 277: Given, gene expression patterns are highly corrected with their functions in plants, "," should be deleted.

Response: Changed into "Zhang *et al.*" (L153), and deleted "," (L283). Thank you very

much!

To Reviewer 2

General Comment:

Li and colleagues performed the genome-wide analysis of the DREB gene family in Saccharum spontaneum. The research topic is of interest, but the methods are not novel for the community, and the work is not based on an original hypothesis. On the other hand, it serves its purpose, justifying this genome-wide study. For that reason, I suggest the following minor revisions:

Response: Thank you very much for your comments! Our present study indicated *DREB* genes were relative to photosynthesis and plant development, except the drought stress.

Specific remarks:

-Clarify the purpose of this genome-wide study in the Abstract and not only in the main text.

Response: We have clarified the purpose of genome-wide study in the Abstract (L23-L26). Thank you!

-All the sections, including Abstract, Introduction, Materials and Methods, Results and Discussions, are presented in the correct order. However, the Materials and Methods and legends need to be revised to include all the details to be reproducible. Important information has not been given.

Response: We have revised the Materials and Methods and legends (L330-L405, L765-L800). Thank you!

- The "Plant materials" section needs to be more informative. I understand that the description of sample material was done previously by Li and colleagues, 2020, but a brief description or an additional table describing the material is desirable. It is also necessary to describe in details the varieties and treatments in "They collected the primary meristem of heartleaf (2-3 cm) in three sugarcane varieties under different degrees of drought treatment for RNA-seq library construction" (line 330).

Response: We have added the description of sample material as your suggestion (L332-L351). Thank you!

-The "Expression profiling analysis of DREBs in S. spontaneum based on RNA-seq" section needs to be re-written in details to include the version of the software that was used for each case, and also the parameters that were employed or state that standard ones were used". It is also not clear the source of the "reference gene models" for the alignment with the reads.

Response: We have re-written the section in details. The version of TRINITY is 2.8.5 and the reference gene models for the alignment with the reads is *S. spontaneum* AP85-441 (v20180123) (Zhang et al., 2018, L388-L395). Thank you very much for your suggestion!

Reference: Zhang J, Zhang X, Tang H, Zhang Q, Hua X, Ma X, Zhu F, Jones T, Zhu X, Bowers J *et al*: **Allele-defined genome of the autopolyploid sugarcane *Saccharum spontaneum* L.** *Nat Genet* 2018, 50(11):1565-1573.

- In the "Experimental validation of DREB gene expression level by qRT-PCR" section, the authors don't justify using a single reference gene, failing to follow the MIQE guidelines (https://doi.org/10.1373/clinchem.2008.112797). And also, don't describe the quantification method used. Justification is needed.
Response: We have performed qRT-PCR with two reference genes (*GAPDH* and *eEF-1a*) and re-written the quantification method (L398-L405). Thank you!

-Apart from the "Phylogenetic analysis" section, the authors fail to address the softwares' versions (or day of access) and used parameters in all the sections. This needs to be revised.
Response: We have added the version of the software 'MEGA' (version 7.0). The parameters were described in the "Phylogenetic analysis": the bootstrap test replicated 1000 times, the Poisson model, and Pairwise deletion (L374-378). Thank you!

-What is the justification for the tree scale in Figure 1? And for the choice of the labels DREB1s and DREB2s instead of DREB1 and DREB2?
Response: Thank you very much for your suggestion!

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

In Figure 1, there are more than one DREB1 and DREB2, so we named these two branches as DREB1s and DREB2s, respectively, followed Liu *et al.* (*Liu et al.*, 2013).

Reference: Liu S, Wang X, Wang H, Xin H, Yang X, Yan J, Li J, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K et al: **Genome-wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L.** PLoS genetics 2013, 9(9):e1003790.