Oviedo, ….. 2022

Dear Editor,

We thank the opportunity to submit a new submission of the revised version of the manuscript “**Integrative analysis in Pinus revealed long-term heat stress splicing memory (MS ID#: TPJ-00081-2022)”.**

In this revised version we have addressed all suggestions and questions raised during review process, which have contributed to increase the overall quality of this work. According to referees, sanger sequencing data was provided, method section extended in relation to RT-PCR procedure and, omics data have been deposited to public databases (……, proteomics in ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD032754 and metabolomics in…..). Also, results and discussion sections have been rewritten according to all the issues addres from the reviewers, and additional figures and supplementary materiales were added. Detailed responses to reviewers are included below.

We believe our manuscript is now well suited for publication in “The Plant Journal” and we hope that you and the reviewer and editorial committee share our opinion.

Sincerely yours,



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**MS ID#: TPJ-00081-2022**

**MS TITLE: Integrative analysis in Pinus revealed long-term heat stress splicing memory**

***Editor's comments to for the Author:***

Dear Dr Meijón,

Thank you for submitting your manuscript to The Plant Journal. It has now been assessed by expert reviewers and their comments are copied below. They can also be viewed, along with the editorial correspondence, in your Author Centre on our online site https://mc.manuscriptcentral.com/tpj.

It has been now reviewed by two experts in the field, and both consider that the ms is not acceptable for publication as it stands but it should be provided you can address the concerns raised. While many of these might be easy to follow, the major concerns by reviewer 2 may require both additional measurements and better explanation of the methods used.

I therefore have decided to reject this version of the manuscript, but to offer you the option of resubmitting a revised version in case you were able to improve your ms by fulfilling all the reviewers’ demands. Thus, in revising the manuscript you should bear in mind all the reviewers' criticisms, with particular attention to those highlighted above.

I hope that the reviewers' comments are of help for improving your research, and I thank you again for considering TPJ for submitting your research.

# We wish to thank the Editor to give us this opportunity to resubmit our work and anonymous reviewers for their feedback which helped us to improve our manuscript. We really believe that our work provide new interesting data and a novel integrative approach about heat stress splicing memory in conifers. We have modified the manuscript according to all the reviewers’ criticisms.

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***Referee(s)' Comments to Author:***

*Reviewer: 2*

*Reviewer Report for the Authors*

The manuscript describes the modification in the pattern of alternative splicing (AS) induced by heat stress in the conifer “Pinus radiata”. The authors have found that the changes in AS induced by heat stress mainly alters the protein amount more than protein sequences affecting to genes/proteins that are known to be involved in the response to this kind of stress. Additionally, the authors have made an experiment to prove if there is a memory in the changes of AS caused by heat stress analyzing the isoforms from 8 different genes.

The work is meritorious in that it deals with a gymnosperm. This group of species is of great importance from the evolutionary point of view but has been little studied. However, work on gymnosperms can help us to better understand the molecular mechanisms and physiology of plants including evolutionary aspects.

# First, we want to thank the reviewer for the time taken in reviewing our manuscript. Their valuable comments will no doubt help improve our work. We appreciate and share the reviewers’ opinion on the subject: non-model organisms, like gymnosperms, knowledge can help plant science community to elucidate the core molecular mechanisms and physiology from an evolutionary perspective.

*Major concerns and Methods explanation:*

The work is well planning and developed, however, sometimes is too general without specific mention of the most relevant isoforms in the context of heat stress response.

# We completely agree with the reviewer. Because the paper could look dense, we adopted a more general approach to describe the main results. We have rewritten results and discussion sections expanding information about the possible role of the most relevant isoforms mentioned in the heat stress context. (Page ¿?, lines; Page ¿?, lines….). Also, new bibliography was added (Huang et al, 2010; Spitzer et al., 2015)

Some methods are described vaguely through cites that do not end up describing the method. The RT-PCR products should have been sequenced and their sequences presented in the article.

# Additional information about RT-PCR was added in methods (Page ¿?, lines¿?) and data of Sanger sequencing was provided in additional supplementary Table (Table S3). Due to our genome-free splicing analysis we always sequenced the longest isoform (designated as Upper) because the lower isoform did not differ too much from primers hybridization and we wanted to validate the sequence which variates according to the alternative splicing event. No tengo muy claro que quieres decir aqui… y tampoco si es conveniente decirlo, tengo miedo que nos mander secuenciar todas las isoformas.

There are different omics datasets that should have been submitted to public databases.

# Omics datasets have been submitted to public databases and included this information in Methods section (Page ¿?, line¿?).

-Transcriptomics data have been submitted to…..(Reviewer account details:…..; Password: ….)

-Proteomics data have been submitted to ProteomeXchange (Reviewer account details:…..; Password: ….)

-Metabolomics have been submitted to…..(Reviewer account details:…..; Password: ….)

*Detailed comments and Minor concerns:*

1. Page 2, line 20: Modify the sentence to match the following one. For example, “…from heat stress in conifers, a group wich…”

# It has been corrected (Page ¿?; Line ¿?).

2. Page 2, line 29: “seems” instead of “seem”

# It has been corrected (Page ¿?; Line ¿?).

3. Page 2, line 31: “These discoveries” instead of “This discoveries”

# It has been corrected (Page ¿?; Line ¿?).

4. Page 3, line 42: Be careful with the term evolution, many authors consider that the processes involved in biological evolution are discontinuous. Support this sentence with bibliographic references and nuance the sentence.

# We recognize the issue pointed out by the reviewer and the sentence have been rectified deleting the term continuous (Page ¿?, line¿?).

5. Page 3, line 56: There are more appropriate bibliographic references (e.g., One Thousand Plant Transcriptomes Initiative. One thousand plant transcriptomes and the phylogenomics of green plants. Nature 574, 679–685 (2019). https://doi.org/10.1038/s41586-019-1693-2). In general, evolutionary studies suggested that gymnosperms are composed only by two evolutionary lineages or clades, Gnetophytes-Conifers and Cycads-Ginkgo, including the four classical taxonomic groups.

# The reviewer is right. The sentence was reformulated to pinpoint only two lineages of gymnosperms and added the recommended reference (Page ¿?, line¿?).

6. Page 5, lines 39-45 and Figure 2d: Please, include symbols (as asterisks) into the figure to highlight significant changes of the proportions.

# Figure 2d was modified including asterisks to highlight significant changes in the proportions as requested by the reviewer (Page ¿?, line¿?).

7. Page 5, line 47: Please, include the employed method to generate these clusters.

# The method to generate the cluters was added (Page ¿?, line¿?).

8. Page 5, lines 51. Figure S3: Better than this graphical representation in this case it would be better to include this data in a supplemental table highlighting significant changes.

# A new Supplemental Table S4 including the requested information was added. La Figura S3 ahora sobra??? No entiendo muy bien la tabla nueva… mejorar las leyendas en todo caso…

9. Page 6, line 15: Please, include a table with your results for each AS types. In fact, you must include supplemental datasets with all your isoforms (sequences), event type, expression values and stats. Without this information, it is very difficult to follow the manuscript or even to review it.

# We apologize for the inconvenience. Initially all the isoforms data was deposited in the github repository specified in ‘Data Statement’ https://github.com/RocesV/AS\_heat\_Pra/ at tree/main/Data/ in 1.KisSplice, 2.KissDE and 3.DESeq2. Nevertheless, we added a new Supplemental Table S5 with all isoforms information in different sheets and reference added across the text.

10. Page 6, line 39: If it is OK for Figure S6 position here, please, change the number to Figure S4. There is no mention to Figures S4 and S5 before. Figure numbers should correspond to their order of appearance in the text.

# The reviewer is completely right and we changed Supplemental Figures order according to this comment.

11. Page 6, line 46. Figure 4a: Highlight the significant terms in the heatmap with different font colours.

# Significant terms has been highlightedin the heatmap following the reviewer’s suggestion. Hay que indicar en la leyenda de la figura porque se destacan esas categorías.

12. Page 7, lines 24-29: I insist, you must include supplemental datasets with individual values for the different isoforms. It is impossible to verify the results (e.g., AS isoforms) for the different genes mentioned in the text. In this sense, nobody could reproduce your results in another laboratory.

# Table S5 was included. Please, see response above in relation to this point.

13. Page 8, line 11. Figure 5a. The panel “a” of the figure 5 is not relevant, it must be eliminated.

# Panel “a” of Figure 5 has been removed. Victor, no te compliques la vida… yo creo que lo podemos eliminar del todo sin mayor problema. Al revisor se le da la razón en todo, o casi todo… y más si son cosas menores como estas. Poner en la leyenda que indican los colores en el panel d.

14. Page 8, lines 26-29: I don’t know if it is because my limitations in statistics, but I’m amazed of your variance results because the sum of the variance percentages explained by each component is higher than 100%.

# MOFA can be viewed as a versatile and statistically rigorous generalization of principal component analysis to multi-omics data, however each analysis have several key features. So that, while principal components (PCs) derived from PCA are orthogonal and they explain independent sources of variation summing up to 100 % with enough number of PCs, each Latent Factors (LF) of MOFA can explain until 100 % variance because they do not explain explicitly independent sources of variation (are not orthogonal) being 400 % the total of variation in this case. The data shown in the manuscript refers to the sum of each omics layer in all the LF.

15. Page 8, line 47. Figure 5e: There is a mistake in the panel “e”. “Nutrient uptake” instead of “nutrien uptake”. The figure is interesting, but you must include the entire results in a supplemental dataset. Additionally, I don’t know if NiR and GS1 are included in the Nutrient uptake term of the Mapman ontology, but they are related to N assimilation and not with N uptake.

# Typo was corrected and added a new Supplemental Table S6 with the information requested (MOFA weights with functional bins enriched). Mercator 4 functional ontology is hierarchically organized so, despite NiR and GS1 are part of the 25.1 | nitrogen assimilation subterm these genes are englobed in 25 | Nutrient uptake term. In order to maintain a results uniform level of consistency we performed all functional analysis using term categories (not subterms). However, term of the category in Figure 5e was changed to “nutrient uptake and assimilation”. Yo creo que asi dejariamos content al revisor.

17. Page 13, lines 29-32: Please, explain who proposed this hypothesis the authors or Luco et al.? If Luco et al., you must say that your results support this previous hypothesis.

# The hypothesis is proposed by the author inspired by Luco et al., 2011 suggested splicing mechanisms. In order to avoid confusion, we moved the reference and reformulated the sentence (Page ¿?, Lines¿?).

18. Page 13, line 47: The case of Gnetum is not “strange”. With only three studies about AS in gymnosperms is excessive to describe this case as strange.

# We agree, sentence has been rewritten deleted the word “strange” (Page ¿?, line¿?).

19. Page 13, line 60: “…P. radiata? The…” instead of “…P.radiata? . The…”

# Sorry for the typo, it has been changed (Page ¿?, line¿?).

20. Page 14, line 13: There are only two groups of seed plants (gymnosperms and angiosperms). Modify the sentence.

# Sentecen has been modified and remplaced “two biggest groups of seed plants” by “both groups of seed plants” (Page ¿?, line¿?).

21. Page 14, lines 17-21: I don’t understand this sentence. If the splicing-memory seems to be conserved, what are the differences between angiosperms and gymnosperms? What results? What mechanism? Please support this sentence with mentions to specific results.

22. Page 14, line 47: See the precedent comment. La línea 47 tb habría que reformularla un poco. Cambiar lo de “a particular way of remembering” por algo más concreto.

# We thank the reviewer for these remarks. We wanted to communicate that despite the validation of splicing memory in two distant seed plants, the results obtained in the angiosperm Arabidopsis thaliana by Ling et al., 2018 placed intron retention (IR) as one of the major contributors to heat response and memory while the discoveries revealed in the present report with the gymnosperm *Pinus radiata* suggest a potential low relevance of IR in both processes. This is supported by our validation of 8 not complete intron retention AS events in both assays and the results in the intron morphology comparative analysis (Figure 3) section. In order to clarify the reviewer comment, we expanded the sentence and supported it with mention to specific results (Page ¿?, line¿? and line¿?).

23. Page 15, line 11: In the cited reference there is no transcriptomic data, only RT-qPCRs. In fact, you must submit your omics data to the appropriate public databases (e.g., transcriptomics data to the NCBI’s GEO).

# References has been modified (Page, lines¿?) and all omics datasets have been submitted to public databases (Please, see response above in relation to this point). Victor, en esa linea tenemos que añadir la referencia del paper de Monica E de transcriptoma y se indica que está en revisión.

24. Page 18, line 49: Provide the sequences of the oligos used to verify the DNA contamination.

# Information was added in the text and Supplemental Table where is provided the sequences referenced (Page ¿?, line ¿?).

25. Page 18, line 53: Please, make a complete description of the RT-PCR procedure including reaction mixes, incubation conditions and cycles. The bibliographic reference doesn’t describe the method and not even the work cited in Ling et al.

# Information was added (Please, see response above in relation to this point).

26. Page 19, lines 5-7: See my precedent comment. You must submit your omics data to public databases. This is mandatory.

# All omics datasets have been submitted to public databases (Please, see response above in relation to this point).

*Reviewer: 1*

*Reviewer Report for the Authors*

In the manuscript by Roces et al, the authors study transcriptomic response to heat stress in Pinus. The authors identify large impact on alternative splicing (AS) during heat stress response. Next, they integrate previous publish data on metabolites and proteomics to globally address heat stress response components. Finally, they explore heat stress AS memory.

The authors use a really nice experimental set up, they employ ramping temperatures that mimic day and night cycles. This represent a more natural situation that a constant high temperature often used in heat-stress experiments.

The paper is well structured and the data are well presented, also the interactive figures helps to explore the dataset.

# Thank you for your comments. We appreciate that the reviewer recognized the value of the experimental design and data visualization.

*Major Concerns:*

I suggest to modify Figure 7, and divide the data over two separate figures: figure 7 with the confirmation of the RNA-seq data by RT-qPCR and a new figure “Figure 8” with the RT-PCR gels of the memory experiment. Also, the experimental set up (Fig. 1B) would fit best in the new Figure 8. It would help to introduce a quantification of the isoform based on the intensity of the bands in the gel.

# According to reviewer’s suggestion a new main figure (Figure 8) with the memory experimental set up (previous Fig. 1B) and RT-PCR gels (previous Figure 7) has been incorporated. Figure 1 have been redesigned. La figura 1 tal como está no me gusta, el muestreo en T5 habria que quitarlo o pensar como reahacerla, y tal como quedan citada la figura 1 en los resultados tampoco lo veo. Quizas el panel 8a también habría que dividirlo.

Why the authors use RT-PCR instead of qPCR to monitor AS memory?

# We appreciate the comment made by the reviewer. Although quantitative information it is indeed interesting to explore isoform-specific trends, the main focus of our experimental validation was the qualitative association between isoforms from the same splicing event (event-specific trends) to memory or different stress phases. This was driven by two main reasons: 1) we considered that qualitative changes are stronger signals than quantitative ones in order to detect stress and/or memory biomarkers. 2) To perform RT-qPCR analysis we should design isoform-specific primers so we could not directly compare both isoforms from the same splicing event. On the other hand, performing RT-PCR with primers designed to amplificate both isoforms allowed us to discuss the results at the event-level in all sampling points taking into account both isoforms usage and switch. This trade-off decision between quantitative information and comparison clarity was also influenced by the fact that in our de-novo genome-free (no gene-level information) splicing analysis, each alternative splicing event is constituted only by two isoforms (aqui no entiendo muy bien que quiere decir). En este punto corremos el riesgo que nos diga que una vez se detectan las dos isoformas porque no hicimos también qPCR con primer específicos para cada una… algo que creo que seria bastante complicado de hacer en términos de diseño de primers, por otro lado.

It is not clear why in the RNA-seq experiment the authors used a heat stress temperature of 40ºC while in the memory experiments they used 45ºC. It should be explained in the text the reason of changing the heat stress temperature.

# Heat stress temperature from 40 °C in the RNA-seq assay was increased to 45 °C in the memory-assay because we wanted to further characterize our already validated at 40 °C candidates splicing events in a more exploratory fashion. Increasing temperature and changing our experimental design allowed not only to test the existence of acquired long-term splicing memory but also: 1) To check alternative splicing patterns coherency at a more lethal temperature. 2) To elucidate high temperature specific expression. For example, despite the clear splicing pattern of *UBIQUITIN-CONJUGATING ENZYME E2 36 (UBC36)* at 40 ºC, this event was not expressed at 45 ºC. 3) To get more robust conclusions as we used different plants/populations for each assay so the results remained consistent taking into account the natural variation in the experimental set up. Moreover, we were particularly interested in splicing patterns at 45 °C because of the promising results obtained in Lamelas *et al*, (2020) (doi: [10.1093/jxb/erz524](https://doi.org/10.1093/jxb/erz524)) and Lamelas *et al*, (2022) (doi: [10.1111/pce.14238](https://doi.org/10.1111/pce.14238)). The analysis of the nuclear and chloroplast proteomes highlighted RNA metabolism and splicing relevance in response to 45 °C. Aditional information in relation this change of temperature was provided in Methods section (Page¿?, line¿?).

Also, in the experimental procedure the authors mention that they used previously generated transcriptomic data “In this work we studied *P. radiata* response to heat (40ºC) employing already generated transcriptomics, proteomics, ….” while from the main text it seems the RNA-seq was generate in this study. Authors must clarify this point and revise the manuscript accordingly.

# Any possible confusion generated with this issue in the main text has been corrected. Porfa, revisalo Victor.

*Minor Concerns:*

Page 4 line 41. Add brief description of the experimental set up in the results section.

# According to reviewer’s suggestion a brief description of the experimental system has been added (Page ¿?, line¿?).

Figure 5a. Is not clear what the ovals represent.

# The ovals are graphical representations of distinct molecular levels or regulatory layers. To clarify this issue, legend of Figure 5 has been rewritten.