**Reviewers’ comments:**

Below, we address all of the issues raised by the reviewers in a point-by-point manner. These include the clarification and correction of mistakes, other inaccuracies pointed out and the generation of new figures and supplemental data in the manuscript. Taking together the responses to the requirements of both reviewers, the new material in the manuscript includes:

* Text formatting and grammatical corrections.
* Minor text changes across the main text: see comments below. These changes are also included in the comments which are answered.
* Major text changes across the main text: see comments below. These changes mainly pertain to expand methods description and specific isoform relevance in the context of heat stress.
* Modified (mod) - new (new) figures and tables: mod Figure 2d; new supplemental table with cDNA Sanger sequencing data; new supplemental table with Figure S3 data; mod Figure 4; new supplemental table with all isoforms sequences, event type, annotations, expression values and stats; mod Figure 5; new supplemental table with Figure 5 data; mod Table S2 including the oligos used to verify the DNA contamination; new Figure 8 and mod Figure 1 following Reviewer 1 suggestions.
* Omics data deposited to public databases and the accesion codes included in the ‘Data Statement’.

**Reviewer: 1**

Comments to the Author

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.

We thank the reviewer for the time taken into reviewing the manuscript. We appreciate that the reviewer recognized the value of the experimental design and data visualization.

**1. 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-PCR 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.**

We agree with the observation of the reviewer. In the resubmitted manuscript we have incorporated a new main figure (Figure 8) with the memory experimental set up (previous Fig. 1B) and RT-PCR gels (previous Figure 7).

**2. It would help to introduce a quantification of the isoform based on the intensity of the bands in the gel. 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.

In a splicing context, despite the loss of quantitative information, we think that RT-PCR seems to be more straight forward than RT-qPCR when non-genic information is available.

**3. 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.**

The reviewer is absolutely right. We changed heat stress temperature from 40 ºC in the RNA-seq assay to 45 ºC in the memory-assay because we wanted to further characterize our already validated at 40 ºC 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 ºC because of the promising results obtained in Lamelas *et al*, 2020 and Lamelas *et al*, 2022. The analysis of the nuclear and chloroplast proteomes highlighted RNA metabolism and splicing relevance in response to 45 ºC.

In the original submitted manuscript at the beginning of ‘Plant material and growth conditions’ section in ‘Experimental Procedures’:

*Plant material was generated for (1) validating RNA-Seq based results and also for (2) testing the potential implication of AS in stress memory and checking AS patterns coherency at a more lethal temperature (****Figure 1****).*

We have expanded this comment (highlighted in red; see the full manuscript):

*Plant material was generated for (1) validating RNA-Seq based results in a 40 ºC assay and also for (2) further characterizing our validated AS events in a 45 ºC assay in order to check AS patterns coherency at a more lethal temperature, test the potential implication of AS in stress memory, and elucidate high temperature specific expression (****Figure 1; Figure 8a****).*

\*Lamelas L, Valledor L, Escandón M, Pinto G, Cañal MJ, Meijón M. (2020) Integrative analysis of the nuclear proteome in Pinus radiata reveals thermopriming coupled to epigenetic regulation. *Journal of Experimental Botany* 71(6): 2040–2057.

\*Lamelas, L., Valledor, L., López-Hidalgo, C., Cañal, M.J. & Meijón, M. (2022). Nucleus and chloroplast: a necessary understanding to overcome heat stress in *Pinus radiata*. *Plant, Cell & Environment*, 45, 446– 458.

**4. 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.**

**PENDING MM LV MJ**

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

We have added this information to the manuscript (highlighted in red), at the indicated place:

*Splicing and expression changes induced by high temperature were analyzed from RNA-seq data of P. radiata needles subjected to 40 ºC stress pulses. Briefly, heat stress was applied for 3 consecutive days during the central hours of the day, employing a temperature ramp from 15 ºC to 40 ºC over 5 h, holding high temperature for 6 h and then ramped down again to night values (****Figure 1****; see Material and Methods). De novo […]*

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

We thank the reviewer for the comment. The ovals are graphical representations of distinct molecular levels or regulatory layers. To avoid confusion, we modified Figure 5 legend (highlighted in red; see the full manuscript):

*(a) Several layers of coordinated molecular levels (represented as ovals) collectively and differentially participate in heat response definition. (b) Percentage […]*

**Reviewer: 2**

Comments to the Author

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.

**1. 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 added new comments and improved this sections to expand the heat stress context of the most relevant isoforms mentioned (highlighted in red; see the full manuscript):

***Results***

*[…]*

***Biological functions regulated at splicing and transcription levels***

*[…]*

*These insights were supported by some of the most relevant isoforms for each comparison, such as protein homeostasis HEAT SHOCK PROTEIN 21, CHLOROPLASTIC (HSP21) required for chloroplast development under heat stress, probably maintaining plastid-encoded RNA polymerase-dependent transcription; phytohormone action ABCISIC STRESS-RIPENING PROTEIN 5 (ASR5) involved in the common cross heat-drought responses playing a positive role by regulating abcisic acid signaling, stomatal closure and possibly preventing stress-related proteins from inactivation; cell wall organisation GLYCINE-RICH CELL WALL PROTEIN (EMB31); and secondary metabolism FLAVONOID 3’,5’-HYDROXYLASE (CYP75A2) which is part of anthocyanins biosynthesis that are known to be associated to stress damage photoprotection as antioxidants (****Figure 4b; Supplemental******Figure S4a****) (Bateman et al., 2021).*

*[…]*

*Thus, while general RNA-processing isoform expression changes were not highlighted at T1 (****Figure 4a****), on DD level we saw a significative enrichment in this term ratified by MULTIPLE ORGANELLAR RNA EDITING FACTOR 3, MITOCHONDRIAL (MORF3) relevance which is coordinating mitochondrial expression via epi- and post-transcriptional regulation (****Figure 4b, c****). General photosynthesis-associated expression increased at T3 (****Figure 4a****) supported by INNER MEMBRANE PROTEIN ALBINO 3, CHLOROPLASTIC (ALB3) importance in stress vs control volcano analysis (****Figure 4b****). ALB3 plays a key role in the insertion of some light harvesting chlorophyll-binding proteins into the thylakoid membrane and, therefore, recovering thylakoid integrity associated to potential stress acclimation (Bateman et al., 2021).*

*[…]*

*Similarly, solute transport also appeared as inconsistent between general dynamics and DD regulation in stress-specific contrast (****Supplemental******Figure S4a, b****). The emergence of several isoforms such as PROBABLE AQUAPORIN PIP1-4 (PIP1.4) and REF/SRPP-LIKE PROTEIN At3G05500 (At3G05500) pointed to a possible water deprivation during the heat assay possibly connecting high temperature to drought stress signalling (****Figure 4b; Supplemental Figure S4a****).*

*[…]*

***Relative contribution of multiple regulatory layers to heat stress response***

*[…]*

*Additionally, we detected an enrichment in secondary metabolism functions which confirmed the prior role of metabolism layer in this latent factor (****Figure 5e****). For instance, this is illustrated by the relevance of splicing in PHENYLALANINE AMMONIA-LYASE (PAL) which catalyzes the production of an important variety of well-known stress-responsive secondary metabolites like the aforementioned flavonoids and anthocyanins (Huang et al., 2010).*

*[…]*

***Meta-network analysis reveals isoform co-function modules***

*[…]*

*Interestingly, we found well-known regulators such as SPLICING FACTOR U2AF SMALL SUBUNIT B (U2AF35B) (module 2) (Laloum et al., 2018); isoforms earlier proposed in volcano and integrative analysis (****Figure 4b and******Figure 5e****) like ALB3 (module 1), METALLOTHIONEIN-LIKE PROTEIN (EMB30) (module 1 and 5), SERINE/ARGININE-RICH SPLICING FACTOR RSZ22 (RSZ22) (module 2), CYP75A2 (module 4) and ASR5 (module 3); and new ones for instance 50S RIBOSOMAL PROTEIN 5, CHLOROPLASTIC (PSRP5) (module 1). In agreement with previous highlighted isoforms, PSRP5 plays a critical role in the translation of chloroplast encoded genes and it is specially related to thylakoid membrane proteins (Bateman et al., 2021).*

*[…]*

***Discussion***

*[…]*

*; by the same token coordinated control of RNA biosynthesis (****Figure 5e and Figure 6b; Supplemental Figure S4b****) between nucleus and chloroplast is also emphasized including probably retrograde signaling acting as mediator of nuclear photosynthetic gene expression and straight-forward regulation of nuclear transcription through control of RNA polymerase II dynamics and bivalent epigenetic signals (both active and repressive) represented by CHLM, TFB5 and HDT2- UBC36 events, respectively (Bateman et al., 2021).*

*[…]*

*We validated the acquisition of heat stress-linked splicing-memory in a diverse subset of genes associated to aforementioned pathways and others previously described (Lamelas et al., 2020), such as proteins degradation illustrated by CHMP1B event which is particularly required for autophagic degradation of plastid proteins to the vacuole; again, denoting the importance of chloroplast proteome turnover during the acclimation as one of the main targets of heat stress damage (Spitzer et al., 2015).*

New bibliography added:

\*Junli Huang, Min Gu, Zhibing Lai, Baofang Fan, Kai Shi, Yan-Hong Zhou, Jing-Quan Yu, Zhixiang Chen, Functional Analysis of the Arabidopsis PAL Gene Family in Plant Growth, Development, and Response to Environmental Stress. (2010). Plant Physiology, Volume 153, Issue 4, Pages 1526–1538,

\*Spitzer C, Li F, Buono R, Roschzttardtz H, Chung T, Zhang M, Osteryoung KW, Vierstra RD, Otegui MS. The endosomal protein CHARGED MULTIVESICULAR BODY PROTEIN1 regulates the autophagic turnover of plastids in Arabidopsis. (2015). *Plant Cell*. 27(2), 391-402.

**2. Some methods are described vaguely through cites that do not end up describing the method; 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. Page 18, line 49: Provide the sequences of the oligos used to verify the DNA contamination.**

We grouped reviewer comments that could be answered together. We have added this information to the manuscript (highlighted in red; see the full manuscript):

***Experimental Procedures***

***In silico analysis of AS events***

*[…]*

***Annotation and functional enrichment analysis***

*[…]*

*Gene Ontology (GO) (Carbon et al., 2021) annotations were obtained using the RSQLite package (v.2.2.0) with custom R scripts (see Data Statement). Gene Ontology tree maps were generated using REVIGO (Supek et al., 2011).*

*[…]*

***Experimental validation of AS events***

*[…]*

***RT-PCR analysis***

*RNA of the samples was extracted as described by Valledor et al. (2014) and then quantified in a Nabi UV/Vis Nano Spectrophotomer. RNA integrity was checked by agarose gel electrophoresis, and potential DNA contamination by PCR employing GLYCERALDEHYDE-3-212 PHOSPHATE DEHYDROGENASE (GADPH)* *primer pair (****Supplemental******Table S2****). cDNA was obtained from 500 ng of RNA using the RevertAid kit (ThermoFisher Scientific) and random hexamers as primers following the manufacturer’s instructions. RT-PCR was performed with DreamTaq DNA Polymerase under the following conditions: 95 ºC for 4 min, continuing with 40 cycles of: 95 ºC for 30 sec, 60 or 61 ºC for 30 sec and 72 ºC for 1 min; ending with 72 ºC for 10 min and 8ºC hold until the use of the product. Primers for each AS event were designed to amplify both two splice variants in a single reaction. Sequence of different splice variants were validated by Sanger sequencing (Stab Vida, Lda; Portugal).*

**3. The RT-PCR products should have been sequenced and their sequences presented in the article; Page 10, line 52. Figure 7: These results are very interesting, but you must sequence the amplified cDNA fragments to validate your RT-PCR results, especially because you have obtained unexpected bands in some cases.**

We thank the reviewer for their comment. Initially all the sanger sequencing data validating our 8 candidate splicing events was deposited in the github repository specified in ‘Data Statement’ [https://github.com/RocesV/AS\_heat\_Pra/ at tree/main/Data/5.Validation/Bio](https://github.com/RocesV/AS_heat_Pra/%20at%20tree/main/Data/5.Validation/Bio). Nonetheless, we agree with the reviewer and to avoid confusion we added a new supplementary table to present this data in the article. See ‘RT-PCR analysis’ in ‘Experimental Procedures’ (highlighted in red):

***RT-PCR analysis***

*RNA of the samples was extracted as described by Valledor et al. (2014) and then quantified in a Nabi UV/Vis Nano Spectrophotomer. RNA integrity was checked by agarose gel electrophoresis, and potential DNA contamination by PCR employing GLYCERALDEHYDE-3-212 PHOSPHATE DEHYDROGENASE (GADPH)* *primer pair (****Supplemental******Table S2****). cDNA was obtained from 500 ng of RNA using the RevertAid kit (ThermoFisher Scientific) and random hexamers as primers following the manufacturer’s instructions. RT-PCR was performed with DreamTaq DNA Polymerase under the following conditions: 95 ºC for 4 min, continuing with 40 cycles of: 95 ºC for 30 sec, 60 ºC for 30 sec and 72 ºC for 1 min; ending with 72 ºC for 10 min and 8ºC hold until the use of the product. Primers for each AS event were designed to amplify both two splice variants in a single reaction. Sequence of different splice variants were validated by Sanger sequencing (Stab Vida, Lda; Portugal) (****Supplemental Table S3****).*

*[…]*

***Supporting Information***

*[…]*

***Supplemental Table S3*** *Sanger sequencing data validating RT-PCR results.*

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.

**4. There are different omics datasets that should have been submitted to public databases; 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). It is mandatory.**

**PENDING MM LV MJ**

**5. Page 2, line 20: Modify the sentence to match the following one. For example, “…from heat stress in conifers, a group wich…”. Page 2, line 29: “seems” instead of “seem”. Page 2, line 31: “These discoveries” instead of “This discoveries”.**

Again, we thank the reviewer for pointing out these errors which we have corrected.

**6. 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 we have rectified the sentence deleting the term continuous.

**7. 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**](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 and we reformulated the sentence to pinpoint only two lineages of gymnosperms and added the recommended reference.

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

We modified the Figure 2d including asterisks to highlight significant changes in the proportions as requested by the reviewer.

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

We added that the clusters were generated with k-means as the reviewer suggested.

**10. 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.**

We added a new Supplemental Table S4 including the requested information.

**11. 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; 16. 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.**

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.

**12. 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.

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

We highlighted the terms following the reviewer instructions.

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

As the reviewer pointed, we deleted panel “a” reference from Figure 5 because its low relevance in the results. We decided to maintain the infographic in the Figure because we considered that could be helpful for the future potential readers as this section starts an integrative systems-biology approach as contrary to the previous ones that were focused only in isoforms.

**15. 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%.**

We thank the reviewer for the comment. While Multi-omics Factor Analysis (MOFA) Latent Factors (LF) could be interpreted as a versatile and statistically rigorous generalization of principal component analysis (PCA), each analysis have several key features that are important to take into account. For example, LFs will never be able to explain 100% variance because they are not orthogonal (do not explain explicitly independent sources of variation). Principal components (PCs) derived from PCA are orthogonal so they explain independent sources of variation summing up to 100% with enough number of PCs.

We did not understand the term ‘component’ used by the reviewer because we did not perform PCA. We apologize for the inconvenience so we decided to give two possible explanations depending if we understand ‘component’ as latent factor (LF) or ‘component’ as regulatory level:

1) If we understand ‘component’ as LF. The sum of the variance percentages explained by all LF for each regulatory layer is specified in ‘Results’ at ‘Relative contribution of multiple regulatory layers to heat stress response’ section:

*MOFA inferred four latent factors (LF1, LF2, LF3 and LF4) with common and unique contributions from each molecular level (****Figure 5b****). Overall, isoforms represented the majority of total variance (R2=84 %), followed by metabolites (R2=62 %) and proteins (R2=43 %). We further inspected the top two factors (LF1 and LF2) sorted by variance explained, which defined control vs stress and stress intensities differences, respectively (****Figure 5c****).*

We did not observe that the sum of total variance explained for each regulatory level was higher than 100% in any case (84% for isoforms, 62% for metabolites and 43 % for proteins).

2) If we understand ‘component’ as regulatory level. It is not correct to sum the total variance explained by all regulatory layers because R2 computation is done for each regulatory level independently and LFs do not explain independent sources of variation for each one as mentioned above. Similar appreciations could be done in other bulk multi-omics articles that performed MOFA integration including the original article cited Argelaguet *et al*., 2018.

**16. 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.**

We agree with the reviewer. We corrected the mistake and added a new Supplemental Table S6 with the information requested (MOFA weights with functional bins enriched). Initially MOFA-associated data was deposited in the github repository specified in ‘Data Statement’:https://github.com/RocesV/AS\_heat\_Pra/tree/main/Data/4.Integration\_Metanetworks.

The reviewer is absolutely right. 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 (this can be easily checked at <https://www.plabipd.de/portal/mercator4>). In order to maintain a results uniform level of consistency we performed all functional analysis using term categories (not subterms).

**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: (highlighted in red; see the full manuscript):

*[…]*

*, we propose that simultaneous availability of weak and strong splice sites due to transcription loss of control (Luco et al., 2011) could result in favoured retention of introns flanked by weak splice sites.*

**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. Page 13, line 60: “…P. radiata? The…” instead of “…P.radiata? . The…” Page 14, line 13: There are only two groups of seed plants (gymnosperms and angiosperms). Modify the sentence.**

We grouped reviewer comments that could be answered together. The reviewer is right. We rectified the sentences, deleted the word strange and replaced “two biggest groups of seed plants” by “both groups of seed plants”.

**19. 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; Page 14, line 47: See the precedent comment.**

Again 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 (highlighted in red; see the full manuscript):

*2) All splicing events validated are intron complete retention-independent.  
Despite of its conservation, these results suggest that the processes that drive splicing-memory are substantially different between angiosperms and gymnosperms, probably because of distinct IR relevance due to genome architecture divergence (****Figure 3 and Figure 8b****).*