The authors thank the reviewers and the editor for their comments and greatly appreciate the detailed feedback regarding our manuscript. We have made the required changes and are happy to submit a revised version of the manuscript for further review. Much of the manuscript has been revised based on recommendations by the reviewers including substantial revision to the methods section to shorten this section and make it less cumbersome. Response to the reviewers' comments are provided in detail below.

The editor and reviewers noted six major concerns. We would like to start with addressing these major concerns and then move on to the each reviewer's individual comments.

Major concern 1: Statements regarding MatK affinity for each of the different substrates is speculative.

- **We agree that this was highly speculative and, without further investigation, reasons for MatK's activity on one versus the other substrate cannot be determined. We have, therefore, removed this section from the manuscript as suggested.**

Major concern 2: Un-spliced product of *rps12* was not assayed.

- **We have assayed un-spliced product levels of *rps12-2* as well as managed to determined un-spliced levels of *rpl2* using a more extensive dilution series of standards. Relative quantity of un-spliced product for both substrates is reported and discussed.**

Major concern 3: Total amount of spliced and un-spliced product had not been reported.

- **As the authors understand it, this concern was based on the possible impact of RNA degradation on observed levels of spliced and un-spliced product. We have addressed possible RNA degradation as stated in Major concern 4. Percentage of total RNA spliced vs. un-spliced is more difficult to ascertain. This is because different standard curves were utilized (based on size differences of substrate needed for amplification – spliced product being much smaller than un-spliced) for qPCR. As such, total RNA would need to be assessed with each standard curve and with the same primer efficiency to be reliable. Though we were able to do this to a limited extant for *rpl2*, we were not able to assess total RNA of *rps12-2* due to primer and funding issues. We hope that the added information regarding un-spliced substrate levels and total *rpl2* RNA levels is sufficient to address the concerns of the reviewer.**

Major concern 4: It was unclear whether RNA degradation impacted the study.

- **We thank the reviewers for pointing this caveat of the study out. We attempted to assay total RNA levels using exon only primers to determine if total RNA levels decreased from the beginning to the end of the reaction. This worked well for *rpl2* but not for *rps12*. The qPCR amplification for *rps12* using exon only primers resulted in a low efficiency of amplification and highly questionable results. However, levels of un-spliced *rps12* RNA substrate when incubated in buffer alone or with the MatK maturase appear to not be altered in treatments based on relative quantity at 0 and 60 min. These results support that RNA has little to any degradation over the incubation time and changes in spliced product levels may be due to another factor. We maintain our hypothesis that reverse-splicing is occurring since un-spliced substrate levels had an increase from 30 to 60 minutes for *rps12-2* that corresponded well to the decrease in spliced product in the presence of MatK but also include the possibility that RNA degradation may contribute to the decreased levels of spliced product.**

Major concern 5: Absence of the negative control in Figure 4a.

- **The negative control has been added. We also have re-evaluated these data with new software and repeated the experiment to validate data.**

Major concern 6: The results (i.e. p values) of student's t-test are not presented in Figures 4a, 4c, 5a, 6a, and 6b.

- **We have re-assessed statistical significance of products from our assays using the Bio-Rad CFX Maestro™ software package. At the time the manuscript was originally submitted we only had Bio-Rad CFX Manager software for qPCR analysis. This software does not allow for statistical analysis of samples which resulted in great difficulty to run data through appropriate statistical tests. We now have run all data through Bio-Rad CFX Maestro™ software which provides robust statistical analysis of data as well as export of algorithms to R to further extend statistical analyses if desired. We were able to better determine significance among the various treatments we analyzed in the study using the Maestro™ software package and have provided corresponding p values both in the results section and figures where appropriate.**

Reviewer #1:

This manuscript describes the establishment of an in vitro assay to analyze splicing of chloroplast RNAs in the presence or absence of the chloroplast encoded maturase MatK. The authors use highly sensitive qRT-PCR to identify spliced products of the reaction. They

can demonstrate self-splicing in water, which is in fact working better than in the presence of 5 mM Mg, an unsuspected finding in the light of data from bacterial group II introns. Splicing of one of the two introns tested is improved in the presence of MatK, while the other intron does not show any reaction to MatK, but surprisingly to a mock protein extract from *E. coli*. Although there is only one assay showing a MatK effect on splicing of a single intron, this is still interesting information for the (small) community of researchers on self-splicing introns. I do however disagree with the strong conclusion that the different effect of MatK on the two tested introns is due to affinity differences. Affinity is not directly tested here and numerous other factors might explain this behavior just as well.

Major points

1. Please delete all speculations on affinity differences of MatK to the two target introns (e.g. line 614). This would require a completely different set of experiments. Affinity is not measured in such an in vitro splicing assay. The difference in splicing might be due to folding differences of the intron or missing additional factors required in vitro. Affinity measurements would require gel shifts or filter binding assays, which are not presented here.

- **We have removed this section as suggested.**

Minor points

1. The discussion is excessively long when compared to the results presented her. First, there are repetitions of information already presented in the intro (or that should belong into the introduction). Please delete or shorten massively: Lines 508-518; 526-551; 628-636.

- **The authors thank the reviewer for pointing out the redundancy in this section. We have removed or shorten these passages as suggested by the reviewer.**

2. Discussion, lines 662-680: the speculation that there is a reverse splicing reaction responsible for the loss of signal after 60 minutes compared to 30 minutes is not based on any evidence. Much more likely, residual nuclease activities in the protein preparation lead to slow loss of nucleic acid template in the reaction. Please delete this paragraph.

- **We agree that it is very possible that RNA degradation is to blame for the observed decline in spliced product levels for *rps12*-2. However, examination of total RNA from the 15 minutes to the 60 minutes time points for *rpl2* and un-spliced substrate levels for *rps12-2* both support that RNA only had limited, if any, degradation during the reaction. Further, the corresponding increase in un-spliced substrate to decrease in spliced product observed from qPCR data of *rps12-2* support our hypothesis that reverse-splicing is occurring. Based on this evidence the authors kept the paragraph concerning reverse splicing in the**

discussion section but have modified it to include the likelihood that RNA degradation may also contribute to the observed changes in product levels.

3. Discussion, chapter 4.2: I do not see that these minor details and speculative thoughts on the expression of MatK could be of interest to a broader readership. I suggest deleting this chapter.

- **This section was included to address questions that may arise regarding discrepancy in mass of expressed MatK protein used in our study and native MatK protein of rice. As stated in this section, MatK protein identified through Western blot analysis of rice protein extracts is typically ~55 kDa in size. This is smaller than the protein product we produced by about 10 kDa. The difference, however, is due to the initiation codon used for expression. We have included a brief paragraph explaining this discrepancy in mass in the results section to alleviate any concerns from readers knowledgeable about MatK expression and deleted the longer explanation in the discussion section.**

4. Figure 3: please explain in the legend, what "Fraction 2" is. The reader should not be forced to check this in the methods section.

- **There was a statement in the Figure 3 legend about what "Fraction 2" is but we have moved that statement up to make it more visible in the figure legend.**

5. Figure 4: please explain in the legend, how the absolute quantification was done (copies per µl), i.e. present a short version of what is explained in the methods.

- **We have included a better explanation of how qPCR data was obtained and evaluated. We have included these statements in the legends for Figures 4, 5, and 6.**

6. Figures 5a, 6a: the line "MatK alone" is not visible. Is there a way to display this differently, i.e with thinner lines or a more stretched Y-axis?

- **We have used a different color and size of line to aid visualization of "MatK alone" in Figures 5 and 6.**

Reviewer #2:

Please consider the suggestions below:

p.2 39/40 Abbrevs: inconsistent

- **We have added the missing parenthesis to keep formatting consistent with abbreviations. We also have gone through the manuscript to ensure that all abbreviations were used in a consistent manner.**

p.3 66 citation?

- **More appropriate citations for this information have been added to the manuscript.**

p.5 126 hour

- **The term 'hour' has been added to provide units for the photoperiod.**

p.5 126 is lux an accepted term?

- **The amount of light has been converted from lux to PPFD based on the type of bulb used for lighting.**

p.5 133-142 cDNA synthesis protocol incomplete: RT units and definition?

- **We have added units for the reverse transcriptase but we are unclear what is meant by 'definition' here**.

p.6 153-160 reaction details?

- **The specifics of the reaction including final concentration of primer and cycling conditions has been added.**

p.7 193 sonicator model?

- **We have added this information to the methods**.

p.10 290 published (accessible) review?

- **Reference to a recent paper that has utilized qPCR to quantitatively assess splicing events was added to this section of the manuscript.**

p.13 360: A sizing step might provide additional clues.

- **The authors appreciate this suggestion. However, many of the contaminating proteins have similar molecular mass as MatK prohibiting size exclusion for further purification.**

p.14 393ff (chapter 3.1 and Fig.4): Interesting technical details, but how relevant to subsequent maturase (buffered) assays (Figs.4/5)? Consider Fig.4 --> Fig.S3

- **Although the authors agree that in some ways the self-splicing detracts from the main point of the paper regarding MatK activity, we also believe this information is necessary to address questions regarding possible self-splicing of these substrates. The group IIA introns of *rps12-2* or *rpl2* were not known to self-splice prior to this assessment. Therefore, we do believe that this is an important figure in the manuscript.**

p.16 460: Chapter 3.2 (?)

- **We have fixed the numbering system in this section.**

p.18 518: add cited reference here: "introns (Liere and Link 1995; Zoschke et al. 2010)"

- **This paragraph has been altered for brevity but the citation to Liere and Link's 1995 work has been added in the appropriate place to cite MatK RNA binding studies. We greatly thank the reviewers for catching this missing citation.**

p.34 979 (Fig.4): consider "RNA + buffer - heat", "RNA + buffer + heat", "RNA + water"

- **We have altered our nomenclature to more clearly explain each treatment.**

p.34 983 (Fig.4) control sequence data: please add "not shown"

- **"not shown" has been added.**

p.34 985 (Figure 4) Controls (Mock, Neg., NoRT etc.) should be more clarified

- **We have tried to make these descriptions more clear for the reader.**

p.34 998 (Fig.5) add control reaction containing heat-denatured maturase?

- **Although this would be a nice control, we have addressed the question of RNA degradation by analyzing un-spliced and total RNA levels. The authors feel that we already have an abundance of controls for these assays. Both the MatK alone (just the MatK protein in buffer without any additional components) and mock-induction alone (the background protein eluted from Ni-NTA columns in buffer without additional components) are controls for contamination that could come from the protein isolation itself. Mock-induction + RNA is a control for any change in RNA levels due to background protein isolated from Ni-NTA columns; and RNA alone is a control for RNA self-splicing activity. The authors feel that these controls along with checking levels of un-spliced substrate and total RNA are sufficient to demonstrate that alterations in levels of spliced products observed in the activity assays are the result of RNA excision events and not any external factors. Further, we do not have the funding to perform the additional reverse-transcription reactions or qPCR required to test a set of denatured maturase controls in triplicate, with all four time points, for both substrates.**

  p.35 999 (Fig.6) see comment regarding controls in Fig.5
- **Please see the comment above.**

  Reviewer #3:

  Barthet et al., examined the function of MatK maturase in chloroplast group IIA intron excision in an in vitro system. It is an interesting topic; however, the method in the study could be improved. Meanwhile, the methods are extremely long and hard to follow. Some of the contents are repeated in the results.

  First, I think the main problem in this study is that they only **examined the spliced products**. Even though the authors mentioned that they measured the unspliced product of rpl2, and it turns out the concentration is too low to be detected. **How about rps12**? In addition, they could still examine the amount of all products (within the exon) after incubation so that degradation would not affect the result.

- **The authors thank the reviewer for the suggestion regarding examining total products by using exon only primers. The idea presented by reviewer #3 that even if RNA is degraded, by looking at the proportion of product spliced verses un-spliced, from remaining un-degraded RNA, we may be able to determine any significant difference in splicing activity by MatK is much appreciated.**

However, since un-spliced and spliced product assessment requires two different standard curves, trying to determine the percentage of each from the total also requires two separate assessments. Unfortunately, we have run out of funding for this project and supplies needed to perform the additional qPCR assays. We were able to determined total RNA using the spliced standard curve for *rpl2* (Figure S3) but not for *rps12-2* nor using the un-spliced product standard curve for each assay. The total RNA assay for *rpl2* shows fairly consistent levels of RNA present in the assay from 15 to 60 minutes of incubation (Figure S3). Assessment of un-spliced substrate for *rps12-2* also remained fairly unchanged from 0 to 60 minutes of incubation for RNA alone and RNA + MatK (Figure 5b). Based on these assessments, we believe that we had little, if any, RNA degradation throughout the assay for these treatments.

Second, the authors mentioned they performed **t-tests for significance**. However, it seems that from their article (at least from their description), **nothing turns out to be statistically significant,** which is hard to believe. They need to revisit their statistical tests. There should be a p-value for each data point (Figure 4,5,6).

- **Since the original submission of this manuscript, we have acquired the Bio-rad Maestro software which enabled us to combine assays from multiple plates using internal calibrators. This increased technical replicates for most of the assays to three or more technical replicates, and in the case of self-splicing, we also increased some of the experimental replicates. Overall, the larger assessment reduced plate variability and increased statistical power of our analyses. Statically significant findings are presented in the results section and *p*-values are presented in figures and/ or figure legends.**

Figure 1: I would suggest not using S for stop codon. Although they are highlighted, they could cause misunderstanding as S also means serine. They should also label the deletion in 1a.

- **The stop codon has been noted with an asterisk instead and highlighted.**

Figure 4: Figure 4a does not have a **negative control**. The authors should specify whether neg. in 4d equals to water in 4c. They should replace 'no heat','plus heat' to 'no heat + buffer' and 'plus heat' + buffer for clarification. How long were those treatments? In the results, the authors may have mentioned 0 min, but they need to specify in the figure legend. And if the treatment lasts 0 min, why is the number different in 4c and 4e?

- **The nomenclature as well as other issues with this figure have been addressed. The relative quantity was different in the original Fig. 4e due to not having "RNA + water" on the same figure. The Bio-Rad Manager software calibrates relative quantity based on the highest expressed value of each set of compared samples. When "RNA + water" was present (original Fig. 4c), this treatment had the highest level of expression and, therefore, was set to 1.00 with all other quantities shown relative to this. Without the "RNA + water" treatment, the 15 min. *rpl2* spliced product levels were set to 1.00 and '0' time increased due the different calibrator. We have removed Fig. 4e to avoid confusion and left reporting of change in *rpl2* spliced product levels to when reporting results of the activity assays in Fig. 6.**

  Minor concerns:
  Table 1 could go to supplemental.
- **This table has been moved to supplemental data as well as primers added that were used for determining *rps12* un-spliced product and total RNA of both *rps12* and *rpl2*.**