nature portfolio

Peer Review File

AtPRMT3-RPS2B promotes ribosome biogenesis and coordinates growth and cold adaptation trade-off

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The Cao group previously discovered that AtPRMT3 and RPS2B play a role in pre-ribosome biogenesis in Arabidopsis. In this study, they conducted a genetic screen and identified a dominant repressor that partially restored the pleiotropic developmental defects observed in atprmt3 mutants. This repressor mutation was found to be a point mutation in PDCD2 (E350K). Through a co-immunoprecipitation assay, the researchers also discovered that PDCD2 interacts with RPS2B and other proteins that are part of the U3 snoRNP complex or the nucleus pore complex (NPC). Their findings suggest that PDCD2 is involved in the transport of pre-ribosomes from the nucleus to the cytoplasm, and the PDCD2E350K mutation enhances this process. Furthermore, the authors concluded that disruption of PRMT3-RPS2B enhances the translation of mRNAs related to cold stress response genes while impairing the translation of growth-related genes, leading to enhanced cold tolerance.

These results provide solid evidence for the involvement of AtPRMT3-RPS2-PDCD2 in pre-ribosome biogenesis, transport, and translation. The data presented in the study appear to be of high quality, and the report is of broad interest to the science community. Here are my suggestions for the authors to address my concerns and improve their work by providing additional data and/or offering more clarity in their interpretation and explanation of the data to make their conclusions more convincing.

1. The authors should clarify whether the pdcd2-D1 mutation is a dominant or semidominant mutation. Additionally, it would be helpful to provide information on the phenotype of its knockout mutation.

2. The authors have shown that PDCD2 normally functions in pre-ribosomal assembly/processing through its association with U3 snoRNP core proteins such as FIB1 and NOP56 (Fig 2C), and that the pdcd2-D1 mutation impairs this association (Fig 3B). If so, should the pdcd2-D1 mutation be expected to further impair ribosome biogenesis caused by the atprmt3 mutation? How to explain that the pdcd2-D1 mutation rescues, instead of exacerbates, the defect in ribosome biogenesis caused by atprmt3?

3. While the authors have shown that mutations in AtPRMT3, RPS2, and PDCD2 affect pre-ribosomal assembly and transport, as well as translation, the exact molecular functions of these components were not well defined. It would be beneficial if the authors could provide further elaboration on the specific roles and mechanisms of action of AtPRMT3, RPS2, and PDCD2 in these processes.

4. The authors concluded, based on RNA-seq, polysome-seq, and ribo-seq data, that the atprmt3 and rps2b mutations enhance the translation of growth-related genes while repressing the translation of stress-responsive genes. However, the exact role of these components in stress

responses is not sufficiently supported by the data presented. An alternative interpretation of the data is that these mutations induce general stress, resulting in the upregulation of stress-responsive genes. This, in turn, could indirectly influence the ribo-seq profiles. For instance, the increased levels of stress-related genes observed in the ribo-seq profiling could be attributed to the accumulation of stress-related transcripts (as shown in the RNA-seq data in Fig S8) rather than enhanced translation of these mRNAs.

5. I could not find the dataset files associated with the submission, such as RNA-seq, ribo-seq, and MS data. I only found the document in the source files that is the same as the main manuscript file.

Other comments:

1. In Figure 1D, it would be helpful if the authors could provide an explanation of what the probes S7 and S9 specifically detect. Do these probes only detect pre-rRNAs and not mature rRNAs?

2. The conclusion that PDCD2 functions in 'dynamic pre-ribosome assembly' may be premature. The authors base this conclusion on the finding (Fig 3A and 3B) that PDCD2 has a higher level in atprmt3 compared to the wild type, while PDCD2E350K displays a normal level in atprmt3. However, this difference could simply indicate that PDCD2E350K is less stable than PDCD2.

3. The sentence "The Nups identified in mass spectrometry were mainly located in the core scaffold of NPC 176 (Fig.3C)..." is misleading since Figure 3C does not provide any data showing that these proteins are located in the core scaffold of NPC.

4. The sentence "Here, we found that AtPRMT3-RPS2B-PDCD2 complex co-regulates the pre-ribosome assembly through NPCs" sounds confusing. There is a lack of evidence to support the claim that NPCs function in pre-ribosome assembly. NPCs are known to be involved in the transport of pre-ribosomes, but not in the assembly process itself.

5. The sentence "we created atprmt3cbfs quadruple mutants" requires clarification. What exactly does this quadruple mutant refer to?

Reviewer #2 (Remarks to the Author):

The paper by Wang et al is a potentially interesting study on the role of PRMT3 and RPS2B in ribosome biogenesis and translation regulation upon cold stress. This is a very important topic because little is known about ribosome biogenesis and translation regulation upon stress. The goal of the study is interesting but, however, the way the authors go about achieving it is not fully addressed.

The first part of the manuscript (Figures 1 to 3) is dedicated to the role of PDCD2 (identified using a forward genetic screen) in pre-ribosome assembly through its interaction with RSP2B. And then, the second part of the manuscript (Figures 4 to 6) is focused on the role of PRMT3 and RPS2B in translation regulation upon cold stress. My main concern is the lack of connections between the two parts. To complete the story, I think it is important to demonstrate the role of PDCD2 in pre-ribosome assembly and translation regulation upon cold stress. For example, a Ribo-seq approach on pdcd2 mutant in comparison to data presented on Fig5 could help. A phenotypic approach upon cold stress could be also helpful. Many experiments can be done to provide connections between the two parts. This is the clear weakness of the paper.

In addition to this main concern, I have additional comments.

Many phenotypes are presented in the manuscript (Figures 1A, 2B, 2F, 3E) without clear quantification. Additionally, the different genotypes are not presented on the same picture.

Fig2B. what is the level of the transgene PDCD2E350K&ΔZF-HA ? It is important to demonstrate that the transgene has a similar level than PDCD2 in WT background.

Fig3A. The authors suggest that PDCD2 is more accumulated in the nucleus in prmt3-3 mutant. The signal is very weak. A quantification with biological replicates is necessary.

Fig5C. Polysomes quantification with biological replicates is necessary.

FigS3. The data presented on this figure is not convincing. Please provide a better IP.

FigS4. The NA condition was only tested. I suggest testing also CA condition. Additionally, what is the phenotype of pdcd2 mutant ?

Reviewer #3 (Remarks to the Author):

The manuscript entitled "AtPRMT3-RPS2B promotes ribosome biogenesis and coordinates growth and cold adaptation trade-off at translational level" focuses on the role of three proteins PRMT3, RPS2B, and PDCD2 in ribosome biogenesis and translational control. These authors have previously published a different article describing the role of PRMT3-RPS2B in ribosome biogenesis, by fine-tuning the assembly/disassembling of the 90S/SSU processome. In this new manuscript, they identify PDCD2-D1 as a suppressor gene that reverts the phenotypes of the prmt3 and rps2b mutants. After this finding, authors explore the role of PDCD2 in this process and, finally, they provide evidence about the role of PRMT3 and RPS2A and 2B proteins in freezing tolerance. In my opinion, the experiments provide different interesting results regarding different aspects of each protein or pair of proteins, but sometimes they do not cover the three proteins and jump among pairs; In addition, authors do not reach a deep characterization of PDCD2 and lose the opportunity to analyze its relationship with freezing tolerance.

General and main concerns:

In some cases, I find that the description of the experiments is poor, which avoids revising/judging the results. Some examples are:

• Authors provide different lists of proteins that interact by co-IP with PDCD2; however, they do not give information about the controls that they have carried out and that support that the interaction is specific.

• They do not describe the polysome analyses and the Riboseq/RNAseq analyses have been done under control or after the freezing conditions. This makes it extremely difficult to judge if the results, regarding this part of the analyses, are coherent.

• They do not make reference to the criteria they have used to consider that a gene is changed in the mutants compared to wt at the transcriptional, translational, or regarding the TE levels. Which is the threshold of fold change and padj value they used to consider that a gene is regulated?

2. Authors show that PDCD2 interacts with RPS2B, but does PDCD2 also interact with PRMT3?

3. In their previous article, authors showed that RPS2B interacts with components of the U3snoRNP and that the mutant shows defects in ribosome ribogenesis (including defects in pre-RNA processing). In this case, they show that PDCD2 interacts with RPS2B and components of U3snoRNP, which seems to suggest that these two proteins form part of the same complex. If so, it is expected that the pdcd2 mutant shows a similar phenotype in terms of pre-RNA processing and ribosome ribogenesis as the rps2b mutant. Have authors tried to generate a pdcd2 mutant and analyze the molecular phenotypes? I suppose that they can use CRISPR-CAS techniques or RNAi (in case the full knockout mutant is lethal)? I am convinced that showing experiments with the PDCD2 mutant will allow them to further support their hypothesis and more clearly show the roles of PDCD2 in transport.

4. Authors clearly demonstrate that the suppressor PDCD2-D1 reverts the phenotype of the rps2b mutant, but does PDCD2-D1 also revert the molecular phenotypes of rps2b, especially those related to pre-RNA processing as they show for atprmt3 mutant?

5. It seems to me that the PDCD2 accumulation is reduced in the atprmt3 mutant in figure Fig S3 and Figure 3A; however, this reduction is not observed in Figure 3B. Could you comment on that? In addition, why are there higher levels of PDCD1-D1 in atpmr3-2? It seems to me that there are two bands of PDCD2 in the nucleus? Do you think that these two bands could suggest that the PDCD2 is post-translationally modified when located in the nucleus?

6. In Figure 3B, I can observe that PDCD2 is involved in high molecular complexes and that these complexes are highly accumulated in the atprmt3 mutant. This is an effect that is reverted in the PDCD2-D1 background. This is not striking, since other molecular phenotypes of atprmt3 are reverted in the PDCD2-D1 background. I have the same feeling with Fig. 3F and 3G. For me, it would have been more informative to carry out the gel filtration experiments separating the cytosolic and the nuclear fractions. This could provide more information on why the PDCD2-D1 protein acts as a suppressor of the atpmr3-2 phenotype?

7. What are the identities of the proteins identified in Figure 3F? Are those related to ribosome proteins? I suppose that this information, along with accumulation of ribosomal proteins in the nucleus in the PDCD2 mutant, is important if they want to establish that PDCD2 facilitates the export of pre-ribosomes to the cytoplasm.

Regarding the translation analyses, I have different important concerns.

8. In the protocols for Riboseq analyses, it is quite usual to generate fragmented fragments to analyze transcription. These fragments are small and this allows the comparison of the Riboseq and total RNA libraries, since they are done in a similar way (for example, using small fragments without enriching for polyA transcript). In this case, this has not been done, and authors have obtained small fragments for Riboseq and full transcripts polyA mRNAs to generate the libraries. I suppose that although not fully conventional, it is possible to compare Riboseq (small fragments) vs RNAseq. However, the different way to create the libraries could generate some bias and this probably affects the correlations when comparing Riboseqvs RNAseq.

9. Another issue is that authors do not specify if the polysome profiles and Riboseq/RNAseq analyses have been done in seedlings grown under control conditions or subjected to freezing and left to recover. This is important to judge the results. For example, since there are no control and freezing conditions, I suppose that the translation experiments are done under control conditions; However, if this is the case, how mutants with problems in ribogenesis (with problems in pre-RNA processing, and nucleolus stress) could display higher translation? If the translation analyses are done under freezing conditions, it would be also important to know the translational status under control conditions and explain why mutants in ribosome biogenesis have increased translation.

10. I am especially curious with the results they obtained for the rps2c-1/Col-0 mutant in Figure 5A. It seems that this mutant has no big changes in transcription compared to Col-0; however, the changes in translation are really high. However, this mutant does not show differences with the wt in the polysome profiles. This seems odd to me and suggests that probably this is a mutant highly (almost specifically) affected at the translational level.

In Figure 6A-B authors show the number of TE downregulated and upregulated genes in each mutant compared to Col-0. However, they do not define the criteria to ascribe the genes. In these

cases, it is important to describe the criteria of fold change and padj value, to allow to see if the differences are high enough.

10. I think that there is a mistake in Figures 6C or it is not clear enough, since they establish that the figure corresponds to TE-down regulated genes but in the scale bar TE varies from 6 to -6 (I suppose that it refers to log2 TE??)

11. Regarding the analyses of translation of the CHS and genes of the flavonoid metabolic pathway, I have serious doubts that some of them are regulated at the level of translation in both mutants atprmt3 and rps2a2b. For example, I am not sure that CHI is regulated at translational level in the mutants in fig S8. Indeed, you already show that CHI is upregulated to near 3x fold in the atprmt3-2 and 2.5 x fold in the rps2a2b-1 mutant already at the transcriptional level and these levels are highly similar to the ones you observed at the translational level. This seems clearer to me in the case of DFR in the rps2a2b-1 mutant (which shows small changes of induction at the level of transcription and translation). I have also doubts about other genes that are shown, since the changes between transcription and translation seem subtle and the criteria are not shown.

12. In the text, it is established that some genes have up-regulated expression patterns. Since you are looking at transcription and translation, it would be better to establish if the changes are at the transcriptional level, translation level or both.

13. Riboseq analyses allow to obtain a map of the positions of the ribosomes and to acquire more information such as if the ribosomes are stalled at uORFs etc. HAve you checked for specific issues of translational regulator? With this information it is possible that you can go further than to establish that a few genes are changed at the translational level.

We thank the reviewers for their effort in helping us to improve the quality of our manuscript. We have addressed the reviewers' comments by adding additional new experiments and analysis to further strengthen our conclusions. Please find the detailed responses as follows.

Reviewer #1 (Remarks to the Author)

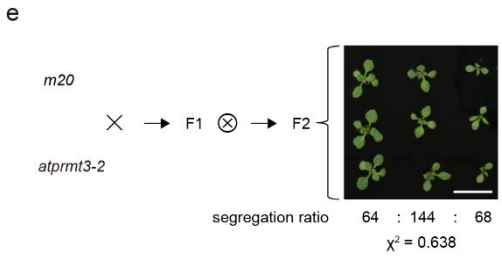
The Cao group previously discovered that AtPRMT3 and RPS2B play a role in pre-ribosome biogenesis in Arabidopsis. In this study, they conducted a genetic screen and identified a dominant repressor that partially restored the pleiotropic developmental defects observed in atprmt3 mutants. This repressor mutation was found to be a point mutation in PDCD2 (E350K). Through a co-immunoprecipitation assay, the researchers also discovered that PDCD2 interacts with RPS2B and other proteins that are part of the U3 snoRNP complex or the nucleus pore complex (NPC). Their findings suggest that PDCD2 is involved in the transport of pre-ribosomes from the nucleus to the cytoplasm, and the PDCD2E350K mutation enhances this process. Furthermore, the authors concluded that disruption of PRMT3-RPS2B enhances the translation of mRNAs related to cold stress response genes while impairing the translation of growth-related genes, leading to enhanced cold tolerance. These results provide solid evidence for the involvement of AtPRMT3-RPS2-PDCD2 in pre-ribosome biogenesis, transport, and translation. The data presented in the study appear to be of high quality, and the report is of broad interest to the science community. Here are my suggestions for the authors to address my concerns and improve their work by providing additional data and/or offering more clarity in their interpretation and explanation of the data to make their conclusions more convincing.

1. The authors should clarify whether the pdcd2-D1 mutation is a dominant or semidominant mutation. Additionally, it would be helpful to provide information on the phenotype of its knockout mutation.

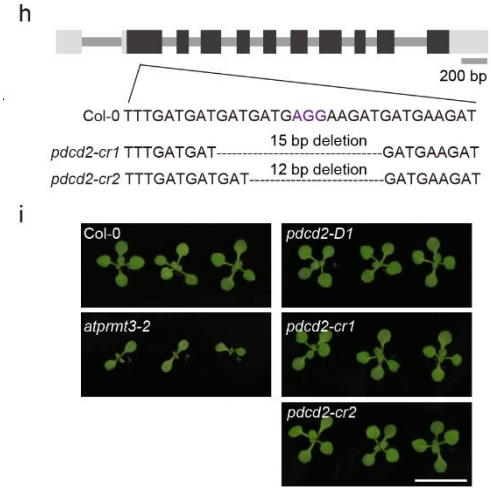
Response: We thank the reviewer for the comments.

1）We have backcrossed the suppressor *m20* with *atprmt3-2*, and determined the

separation ratio in F2 generation. Finding that the segregating ratio of well-rescued: partially-rescue: not-rescued seedlings is around 1:2:1 (**new Supplementary Fig. 1e**). It proves that the suppressor was a semidominant mutation.



2）We have designed many different targets on *PDCD2* for CRISPR-Cas9. And we got two lines named *pdcd2-cr1* and *pdcd2-cr2* mutants, with a 15-bp and 12-bp deletion, respectively **(new Supplementary Fig. 1h)**. Phenotype analysis shows that *pdcd2-cr1* and *pdcd2-cr2* mutants behave similar to Col-0 **(new Supplementary Fig. 1i)**.



2. The authors have shown that PDCD2 normally functions in pre-ribosomal assembly/processing through its association with U3 snoRNP core proteins such as FIB1 and NOP56 (Fig 2C), and that the pdcd2-D1 mutation impairs this association (Fig 3B). If so, should the pdcd2-D1 mutation be expected to further impair ribosome biogenesis caused by the atprmt3 mutation? How to explain that the pdcd2-D1 mutation rescues, instead of exacerbates, the defect in ribosome biogenesis caused by atprmt3?

Response: We apologize for this confusion. In our study, we found that there is an enhanced association between PDCD2 and U3 snoRNP in *atprmt3* mutants, and this

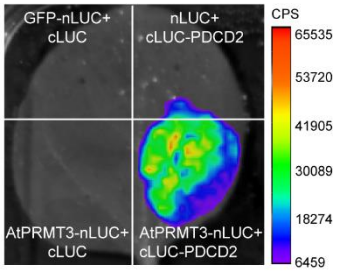
association is reduced with the *PDCD2-D1* mutation in the suppressor, so the suppressor can partially rescue the defective ribosome biogenesis in *atprmt3* mutants. The conclusion was based on the following results: In *atprmt3-2* mutants, we observed an enhanced associations between PDCD2 with U3 snoRNP in *atprmt3-2* mutants, and these associations were partially restored in suppressor *atprmt3pdcd2-D1* (**Supplementary Fig. 3b**). Additionally, we used gel-filtration assay to show that PDCD2 tends to form in a larger complex in *atprmt3-2* mutants, and the *PDCD2-D1* mutation in suppressor could restore the retarded complex to normal conditions **(Fig 3b)**. The above results suggest that PDCD2 could affect pre-ribosome biogenesis process in nucleus. Furthermore, we prove that *PDCD2-D1* mutation alleviate these defects mainly by promoting the export of retarded pre-ribosome to cytoplasm through NPC **(Fig. 3)**.

3. While the authors have shown that mutations in AtPRMT3, RPS2, and PDCD2 affect pre-ribosomal assembly and transport, as well as translation, the exact molecular functions of these components were not well defined. It would be beneficial if the authors could provide further elaboration on the specific roles and mechanisms of action of AtPRMT3, RPS2, and PDCD2 in these processes.

Response: We thank the reviewer for the comments. In general, we proved that AtPRMT3-RPS2 functions to facilitate ribosome biogenesis from the nucleus to the cytoplasm through PDCD2, thereby ensuring the accurate translation step in the cytoplasm. We have provided some extra experiments to reveal the mechanisms among AtPRMT3, RPS2, and PDCD2 in these processes.

1) In our previous manuscript we identified the interaction between RPS2 and PDCD2, here we also proved that AtPRMT3 could interact with PDCD2 (**new Fig. 2f**), which further proves the functional complex among AtPRMT3-PDCD2-RPS2.

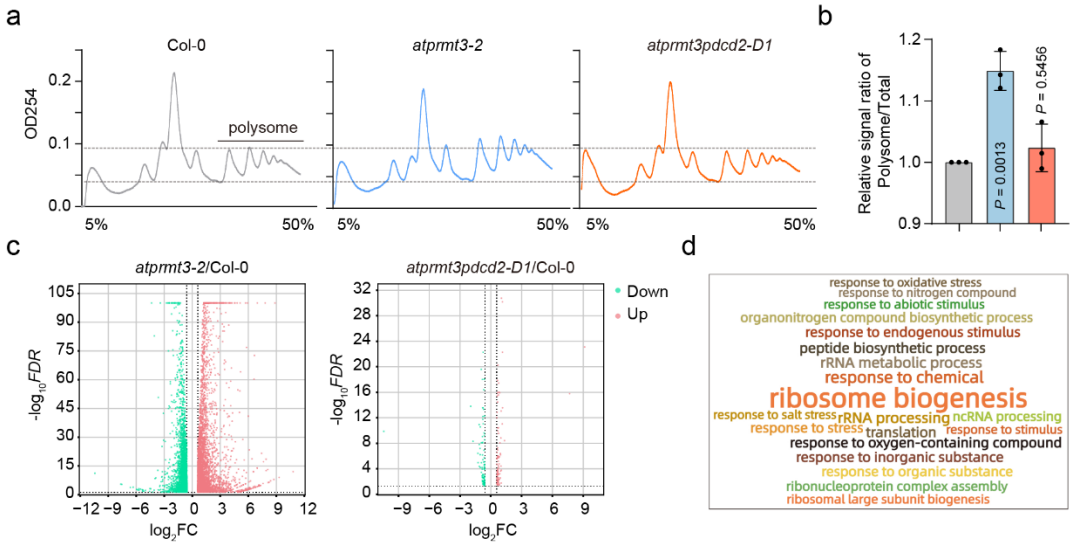
f



2) In our previous manuscript, we described the ribosome status and cold stress phenotypes related to AtPRMT3-RPS2. Here, we have included polysome profile data and gene expression analysis of the suppressors to further support the roles for AtPRMT3-RPS2-PDCD2 in ribosome biogenesis and cold stress response.

Firstly, we presented the polysome profile data, and demonstrated that the suppressor *atprmt3pdcd2-D1* is capable of partially restoring the abnormal polysome state in *atprmt3-2* mutants **(new Fig. 4a, b)**. This indicates that the suppressor not only rescues the faulty pre-ribosome biogenesis in the nucleus, but also rectifies the ribosome state in the cytoplasm.

Additionally, we conducted an analysis of gene expression of the suppressors. The results revealed that there are very few differentially expressed genes (DEGs) in the suppressor, indicating that the suppressor effectively rescues most of the DEGs in *atprmt3-2* mutants **(new Fig. 4c)**. Further analysis of these rescued DEGs showed that the GO analysis (Biological Process) is primarily associated with ribosome biogenesis, aligning with its ribosomal function **(new Fig. 4d)**. Moreover, we also observed enrichment of stress-responsive genes in this process **(new Fig.4d)**, implying their potential roles in stress response. Together with Fig. 5, it links AtPRMT3-PDCD2-RPS2 complex in the cold stress response process.



4. The authors concluded, based on RNA-seq, polysome-seq, and ribo-seq data, that the atprmt3 and rps2b mutations enhance the translation of growth-related genes while repressing the translation of stress-responsive genes. However, the exact role of these components in stress responses is not sufficiently supported by the data presented. An alternative interpretation of the data is that these mutations induce general stress, resulting in the upregulation of stress-responsive genes. This, in turn, could indirectly influence the ribo-seq profiles. For instance, the increased levels of stress-related genes observed in the ribo-seq profiling could be attributed to the accumulation of stress-related transcripts (as shown in the RNA-seq data in Fig S8) rather than enhanced translation of these mRNAs.

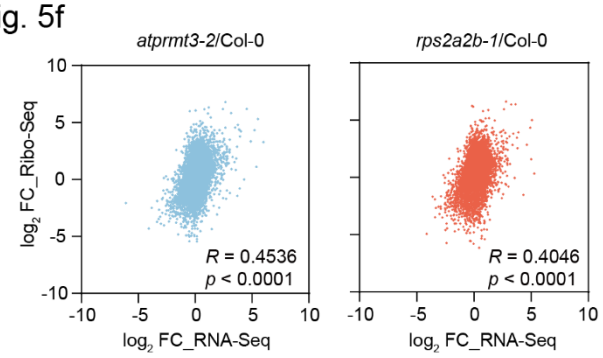
Response: We thank the reviewer for the comments. We conducted RNA-seq and Ribo-seq analyses to investigate the changes in the transcriptome and translatome, translational efficiency of mRNA was then determined by counting Ribo-seq versus RNA-seq [1,2]. We observed a moderate correlation (R≈0.4) between translational (Ribo-seq) and transcriptional changes (RNA-seq) in *atprmt3* and *rps2a2b* mutants **(Fig. 5f)**, indicating the presence of a regulatory mechanism at the translational level in these mutants. Furthermore, the translational efficiency also showed poor correlation with transcriptional levels **(Supplementary Fig. 6)**, providing further evidence for regulatory processes occurring at the translational level. The above findings demonstrate that both transcriptional and translational levels are altered in

atprmt3 and *rps2a2b* mutants, with the translational changes being partially dependent on the transcriptional changes.

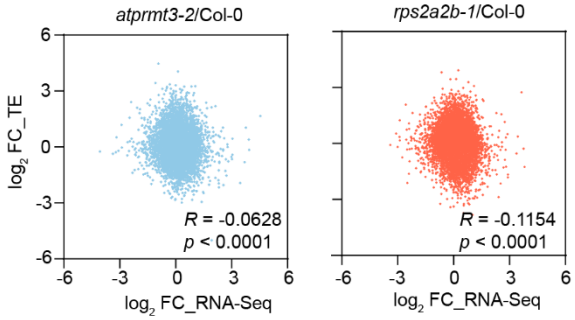
Thus, our results suggest the existence of regulatory mechanisms at the translational level in these mutants. So, these results could answer the reviewer's concern about the ribo-seq results were attributed to the transcriptional changes.

But as the reviewer suggested, the transcription was also changed, so the transcription and translation level are both affected in these mutants, we have also updated this information in the revised manuscript.

Fig. 5f

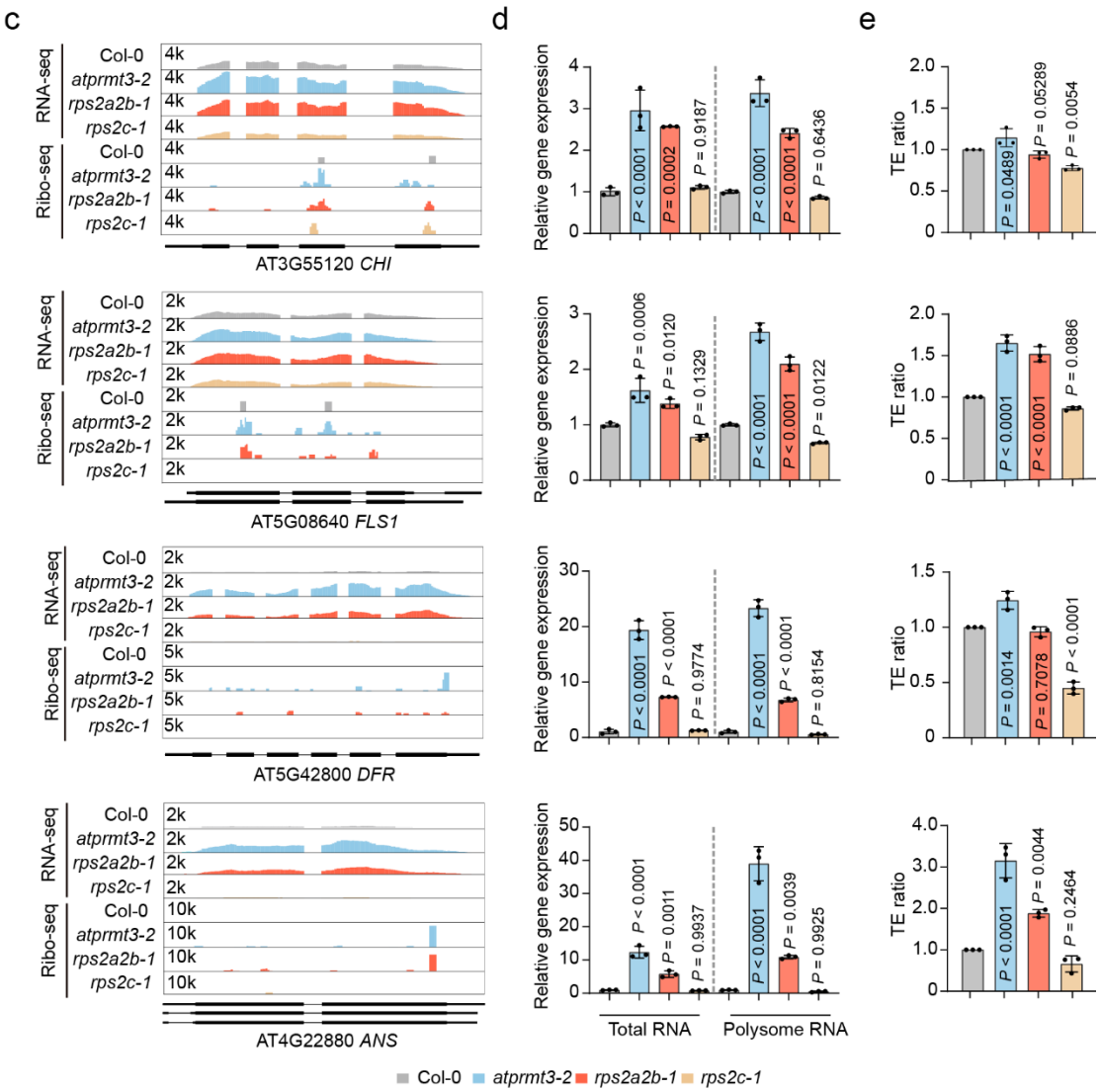


Supplementary Fig. 6



Regarding previous Fig S8 **(now Supplementary Fig. 8c-e)**. In order to better exhibit the translational changes, we calculated TE for each gene **(new Supplementary Fig. 8e)**. The results show that almost all of the genes have up-regulated level for RNA-seq and Ribo-seq in *atprmt3* and *rps2a2b* mutants. Additionally, *FLS1* and *ANS* also show up-regulated TE in both *atprmt3* and *rps2a2b* mutants.

Above all, we can conclude that both the transcriptional and translational level are affected in *atprmt3* and *rps2a2b* mutants, with that translational regulation is not solely dependent on transcription.



5. I could not find the dataset files associated with the submission, such as RNA-seq, ribo-seq, and MS data. I only found the document in the source files that is the same as the main manuscript file.

Response: We thank the reviewer for pointing out this issue. We have provided this information in supplementary dataset.

Other comments:

1. In Figure 1D, it would be helpful if the authors could provide an explanation of what the probes S7 and S9 specifically detect. Do these probes only detect pre-rRNAs and not mature rRNAs?

Response: We thank the reviewer for the suggestions. Yes, these probes can only detect pre-rRNAs instead of the mature rRNAs, because these probes are located in

ITS (Internal Transcribed Spacer) regions, probe S7 in ITS1, and probe S9 in ITS2, it is reported in previous publication [3], so they could detect the pre-rRNA processing intermediates.

And we have added the description of what kind of pre-rRNA intermediates could be detected by different probes in the revised manuscript. “For example, probe S7 detected that the suppressor rescued the accumulated level of 33S(P’)/32S and 18S-A2/A3 intermediates, and decreased level of P-A2/S3 intermediates in *atprmt3-2* mutants; probe S7 proved that the accumulated level of 33S(P’)/32S, 27SA/27SB and pre-5.8S intermediates were also rescued in the suppressor.”

2. The conclusion that PDCD2 functions in 'dynamic pre-ribosome assembly' may be premature. The authors base this conclusion on the finding (Fig 3A and 3B) that PDCD2 has a higher level in atprmt3 compared to the wild type, while PDCD2E350K displays a normal level in atprmt3. However, this difference could simply indicate that PDCD2E350K is less stable than PDCD2.

Response: We thank the reviewer for pointing out this issue. We have modified this conclusion to “Mutation in *PDCD2* rescues the retarded pre-ribosome assembly in *atprmt3* mutants”. The conclusion was based on the following results. Firstly, we observed that PDCD2 could interact with pre-ribosome proteins such as U3 snoRNP, and this interaction was enhanced in *atprmt3* mutants **(Supplementary Fig.3b and Supplementary Table 1)**. The suppressor was able to rescue this defect, demonstrating it could affect pre-ribosome assembly **(Supplementary Fig.3b)**. Subsequently, we found that the protein density of PDCD2 decreased in the cytoplasm and increased in the nucleus in *atprmt3* mutants **(Fig.3a)**, indicating its transport between cytoplasm and nucleus may be disturbed. Gel filtration proved that PDCD2 is accumulated in a larger complex in *atprmt3-2* mutants **(Fig.3b)**, and these larger fractions are mainly in nucleus **(Supplementary Fig.3c)**. These findings suggest that both PDCD2 and pre-ribosomes are delayed in the nucleus in *atprmt3* mutants, and PDCD2 mutation rescued this defect. Further experiments demonstrated that PDCD2 primarily functions by mediating nucleocytoplasmic transport of pre-ribosomes, leading to the rescue of the delayed dynamic pre-ribosome process in the suppressor

(Fig.3).

Therefore, we agree with the reviewer that PDCD2 is not directly involved in dynamic pre-ribosome assembly, but rather plays a role in facilitating pre-ribosome transport, so we changed this conclusion to “Mutation in *PDCD2* rescues the retarded pre-ribosome assembly in *atprmt3-2* mutants”.

In response to the reviewer's concern regarding the protein stability of PDCD2/ PDCD2^{E350K}, we have found that there are no obvious changes in the total protein levels of PDCD2 and PDCD2^{E350K} **(Fig.3a)**. As a result, we suppose that the stability of PDCD2 and PDCD2^{E350K} may be similar.

3. The sentence "The Nups identified in mass spectrometry were mainly located in the core scaffold of NPC 176 (Fig.3C)..." is misleading since Figure 3C does not provide any data showing that these proteins are located in the core scaffold of NPC.

Response: We thank the reviewer for pointing out this issue. We have changed this sentence to “The Nups identified in mass spectrometry were important components of NPC…” in the revised manuscript.

4. The sentence "Here, we found that AtPRMT3-RPS2B-PDCD2 complex co-regulates the pre-ribosome assembly through NPCs" sounds confusing. There is a lack of evidence to support the claim that NPCs function in pre-ribosome assembly. NPCs are known to be involved in the transport of pre-ribosomes, but not in the assembly process itself.

Response: We thank the reviewer for pointing out this issue. We have removed this confusing sentence.

5. The sentence "we created atprmt3cbfs quadruple mutants" requires clarification. What exactly does this quadruple mutant refer to?

Response: We thank the reviewer for pointing out this issue. *cbfs* means *cbf1cbf2cbf3* triple mutants, and we have added the description in the revised manuscript.

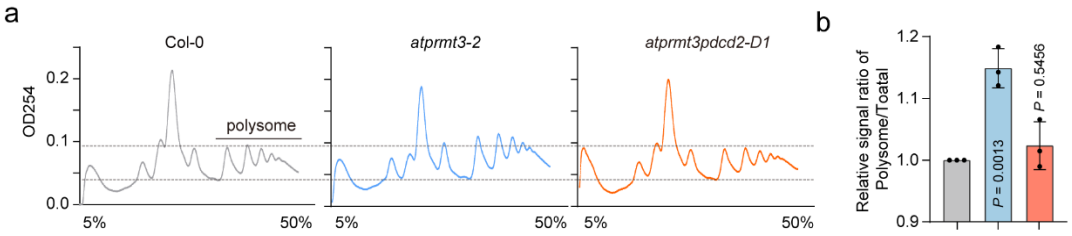
Reviewer #2 (Remarks to the Author)

The paper by Wang et al is a potentially interesting study on the role of PRMT3 and RPS2B in ribosome biogenesis and translation regulation upon cold stress. This is a very important topic because little is known about ribosome biogenesis and translation regulation upon stress. The goal of the study is interesting but, however, the way the authors go about achieving it is not fully addressed. The first part of the manuscript (Figures 1 to 3) is dedicated to the role of PDCD2 (identified using a forward genetic screen) in pre-ribosome assembly through its interaction with RSP2B. And then, the second part of the manuscript (Figures 4 to 6) is focused on the role of PRMT3 and RPS2B in translation regulation upon cold stress. My main concern is the lack of connections between the two parts. To complete the story, I think it is important to demonstrate the role of PDCD2 in pre-ribosome assembly and translation regulation upon cold stress. For example, a Ribo-seq approach on pdcd2 mutant in comparison to data presented on Fig5 could help. A phenotypic approach upon cold stress could be also helpful. Many experiments can be done to provide connections between the two parts. This is the clear weakness of the paper.

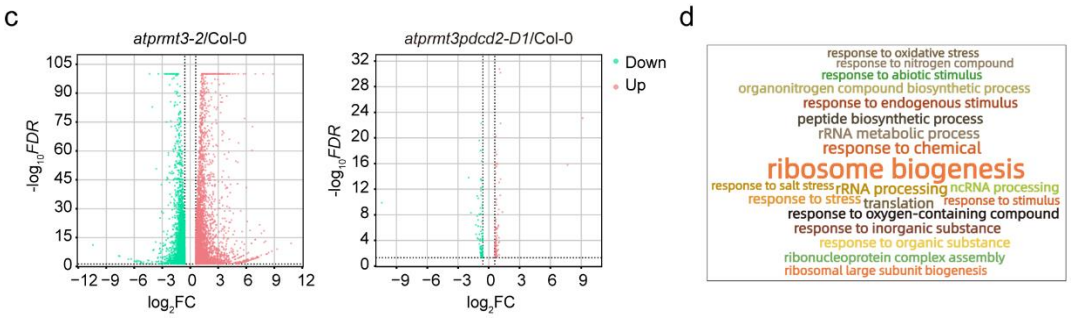
Response: We really appreciate your comments and suggestions on improving the quality of our manuscript. In this manuscript, we revealed the mechanisms of AtPRMT3-RPS2 in orchestrating ribosome assembly and managing translational regulation during stress-growth trade-off. But as the reviewer pointed out, these two parts seems lack of connection. As suggested by the reviewer, PDCD2 is an intermediate connecting these two parts, so we have reorganized the figure and conducted additional experiments to make the manuscript more fluent and coherent.

1) We have incorporated the polysome data of the suppressors to bridge the gap in the manuscript. The first part of the manuscript primarily discusses pre-ribosome biogenesis in the nucleus, and the suppressor *atprmt3pdcd2-D1* could rescue this abnormal ribosome biogenesis process. To further understand the final ribosome state in the cytoplasm, we performed polysome profile in the suppressors. Our

findings demonstrate that the suppressors effectively rescue the abnormal polysome state in *atprmt3-2* mutants **(new Fig. 4a, b)**. These results indicate that the aberrant polysome state restores to normal in the suppressors.

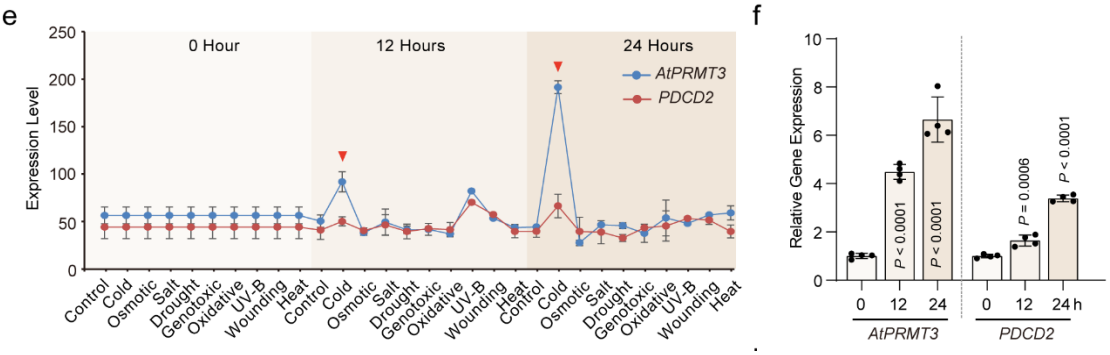


2) Additionally, we conducted an analysis of gene expression of the suppressors. The results revealed that there are very few differentially expressed genes (DEGs) in the suppressor, indicating that the suppressor partially rescues most of the DEGs in *atprmt3-2* mutants (**new Fig. 4c)**. Further analysis of these rescued DEGs showed that the GO analysis (Biological Process) is primarily associated with ribosome biogenesis, aligning with its ribosomal function **(new Fig. 4d)**. Moreover, we also observed enrichment of stress-responsive genes in this process **(new Fig.4d)**, implying the involvement of AtPRMT3-PDCD2 in stress response, which connects to the next part of the manuscript fluently.

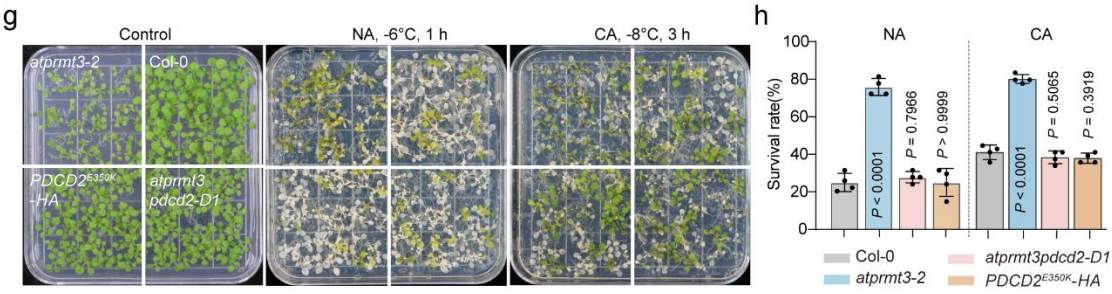


3) The above results indicates that AtPRMT3-PDCD2-mediated ribosome biogenesis process may be involved in stress response process, so it connects to the second part of the manuscript. In our previous manuscript, we only presented the expression of *AtPRMT3* upon cold stress. As suggested by the reviewer, in this revised version, we have included the molecular phenotype of *PDCD2* under stress conditions **(new Fig. 4e, f)**. Our analysis revealed that the expression of PDCD2 was also induced under cold stress, similar to AtPRMT3. The above results indicate that *PDCD2* and *AtPRMT3* has potential roles in cold stress

response.



4) Next, we determined whether they have biological functions in cold stress process. We have added the freezing phenotype of the suppressors under CA conditions and reorganized the figure **(new Fig. 4g, h)**. We performed freezing assays under both cold acclimation (CA) or non-acclimation (NA) conditions. The results showed that *atprmt3-2* mutants displayed enhanced freezing tolerance with higher survival rates compared with wild-type Col-0, and the suppressor *atprmt3pdcd2-D1* rescued the freezing tolerant phenotype of *atprmt3-2* mutants under both NA and CA conditions. These results suggest that AtPRMT3-PDCD2 has biological functions in freezing stress response.



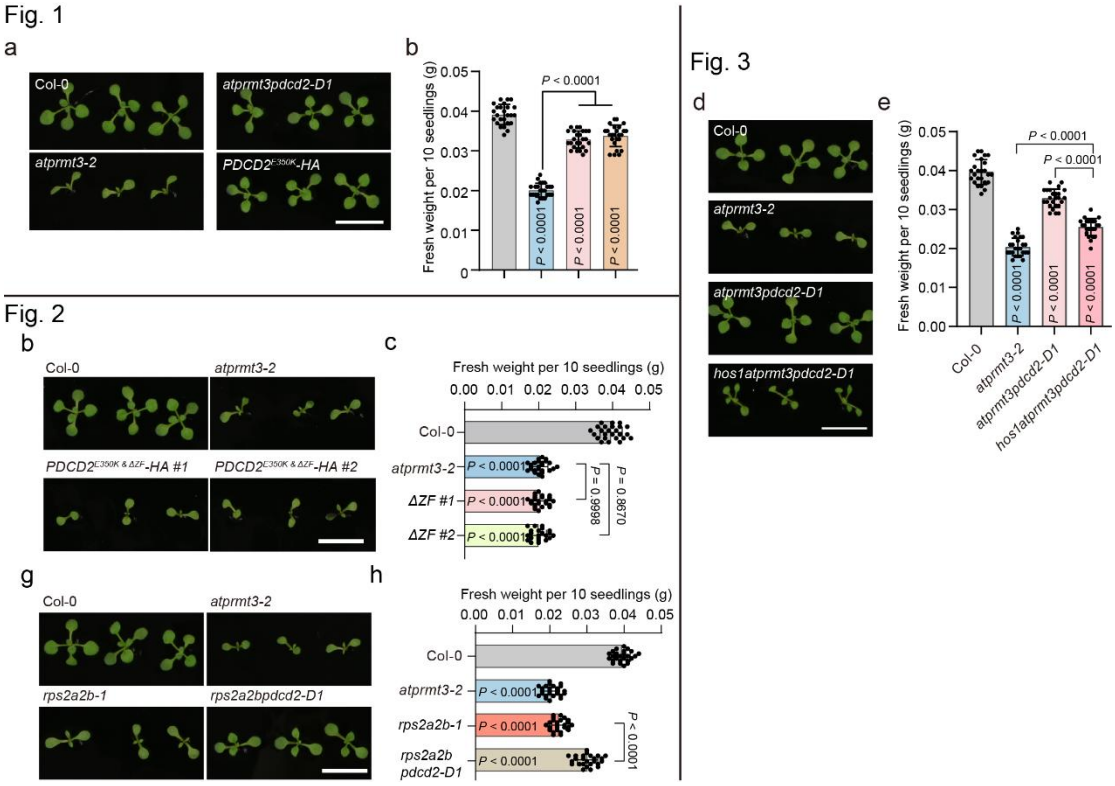
In addition to this main concern, I have additional comments.

(1) Many phenotypes are presented in the manuscript (Figures 1A, 2B, 2F, 3E) without clear quantification. Additionally, the different genotypes are not presented on the same picture.

Response: We thank the reviewer for pointing out these issues. We have calculated the fresh weight of the seedlings to make a clear quantification for these phenotypes (**new Fig. 1a-b, 2b-c, 2g-h, 3d-e**).

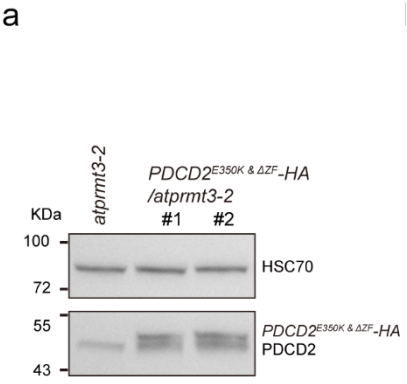
Additionally, we have renewed all the phenotypes, which are grown for the same day,

and took a picture from the same view (**new Fig. 1a-b, 2b-c, 2g-h, 3d-e**).



(2) Fig2B. what is the level of the transgene PDCD2E350K&ΔZF-HA? It is important to demonstrate that the transgene has a similar level than PDCD2 in WT background.

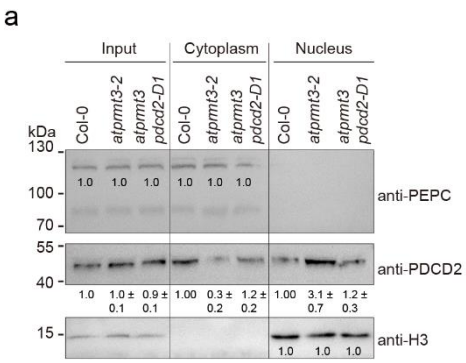
Response: We thank the reviewer for pointing out these issues. We used western blot to detect that the transgenic PDCD2$^{E350K\&\Delta ZF}$-HA has a similar level with endogenous wild-type PDCD2 **(new Supplementary Fig. 3a)**.



(3) Fig3A. The authors suggest that PDCD2 is more accumulated in the nucleus in prmt3-2 mutant. The signal is very weak. A quantification with biological replicates is necessary.

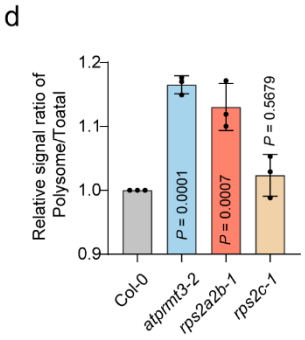
Response: We thank the reviewer for pointing out this issue. We have replaced the

previous figure with a new one, and the quantification of the band density from three biological replicates are also indicated.



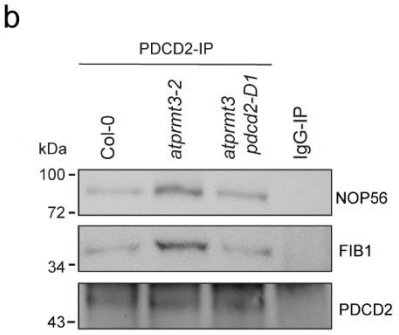
(4) Fig5C. Polysomes quantification with biological replicates is necessary.

Response: We thank the reviewer for pointing out this issue. We have analyzed the relative ratios of polysome/total from three independent biological replicates (**new Fig. 5d**). The polysome quantification shows clearly that *atprmt3-2* and *rps2a2b* mutants have obvious increased polysome ratios than Col-0 and *rps2c* mutants.



(5) FigS3. The data presented on this figure is not convincing. Please provide a better IP.

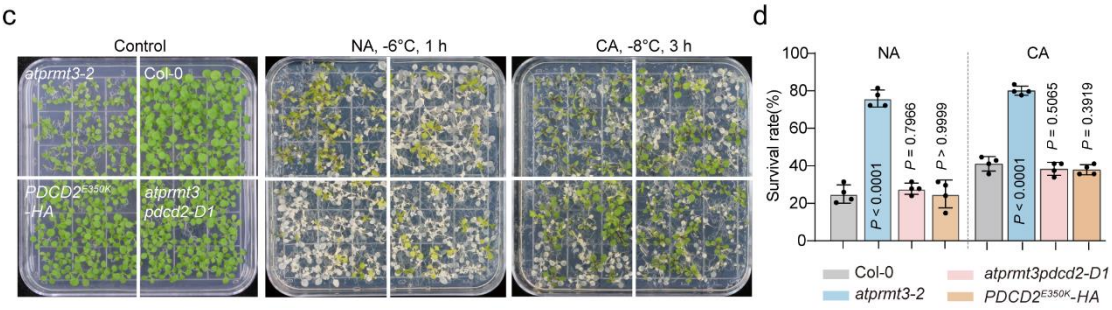
Response: We thank the reviewer for pointing out this issue. We have provided a better IP figure in the revised manuscript **(new Supplementary Fig. 3b)**.



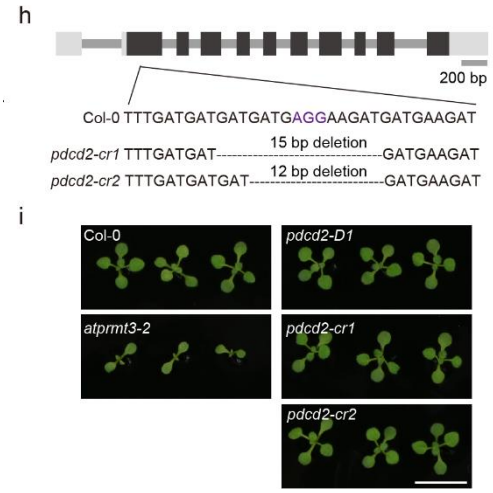
(6) FigS4. The NA condition was only tested. I suggest testing also CA condition.

Additionally, what is the phenotype of pdcd2 mutant?

Response: We thank the reviewer for the suggestions. We have tested the freezing phenotypes of the suppressors under CA conditions, finding that the suppressor also rescued the tolerant freezing phenotype of *atprmt3-2* mutants similar to NA conditions, and we moved this supplementary figure to main figure as new **Fig. 4c and 4d**.



We have designed many different targets on *PDCD2* for CRISPR-Cas9. And we got two lines named *pdcd2-cr1* and *pdcd2-cr2* mutants, with a 15-bp and 12-bp deletion, respectively **(new Supplementary Fig. 1h)**. Phenotype analysis shows that *pdcd2-cr1* and *pdcd2-cr2* mutants behave similar to Col-0 **(new Supplementary Fig. 1i)**.



Reviewer #3 (Remarks to the Author):

The manuscript entitled "AtPRMT3-RPS2B promotes ribosome biogenesis and coordinates growth and cold adaptation trade-off at translational level" focuses on the role of three proteins PRMT3, RPS2B, and PDCD2 in ribosome biogenesis and translational control. These authors have previously published a different article describing the role of PRMT3-RPS2B in ribosome biogenesis, by fine-tuning the assembly/disassembling of the 90S/SSU processome. In this new manuscript, they identify PDCD2-D1 as a suppressor gene that reverts the phenotypes of the prmt3 and rps2b mutants. After this finding, authors explore the role of PDCD2 in this process and, finally, they provide evidence about the role of PRMT3 and RPS2A and 2B proteins in freezing tolerance. In my opinion, the experiments provide different interesting results regarding different aspects of each protein or pair of proteins, but sometimes they do not cover the three proteins and jump among pairs; In addition, authors do not reach a deep characterization of PDCD2 and lose the opportunity to analyze its relationship with freezing tolerance.

General and main concerns:

1. In some cases, I find that the description of the experiments is poor, which avoids revising/judging the results. Some examples are:

• Authors provide different lists of proteins that interact by co-IP with PDCD2; however, they do not give information about the controls that they have carried out and that support that the interaction is specific.

Response: We thank the reviewer for pointing out these issues. We used IgG as controls to perform IP-MS, and the new IP-MS results were provided as **Supplementary Table 1** in the revised manuscript.

• They do not describe the polysome analyses and the Riboseq/RNAseq analyses have been done under control or after the freezing conditions. This makes it extremely difficult to judge if the results, regarding this part of the analyses, are coherent.

Response: We thank the reviewer for pointing out these issues. We have done Riboseq/RNA-seq analyses under normal conditions. The reason is that *atprmt3-2*

mutants are freezing tolerant under non-acclimation (NA) conditions, indicating that the mutants have already accumulated some anti-freezing substances under normal conditions. So, we focused on the gene expression on control conditions. In order to make it clearer, we have introduced these descriptions in the revised manuscript.

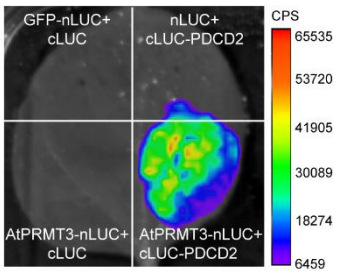
• They do not make reference to the criteria they have used to consider that a gene is changed in the mutants compared to wt at the transcriptional, translational, or regarding the TE levels. Which is the threshold of fold change and padj value they used to consider that a gene is regulated?

Response: We thank the reviewer for pointing out these issues. In this manuscript we use the threshold of fold change>=1.5 and padj value <0.05 to consider a gene is regulated, and we have specified them in the revised method part to make it clearer.

2. Authors show that PDCD2 interacts with RPS2B, but does PDCD2 also interact with PRMT3?

Response: We thank the reviewer for the suggestions. Yes, we used Luciferase complementation assay to prove that PDCD2 also interacts with AtPRMT3 (**new Fig. 2f**), which further proves the potential functional complex among AtPRMT3-PDCD2-RPS2B.

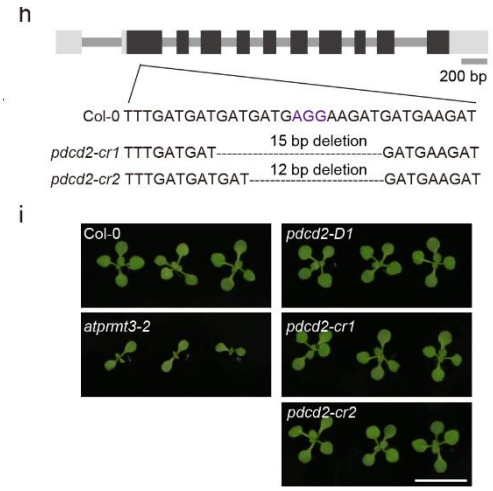
f



3. In their previous article, authors showed that RPS2B interacts with components of the U3snoRNP and that the mutant shows defects in ribosome ribogenesis (including defects in pre-RNA processing). In this case, they show that PDCD2 interacts with RPS2B and components of U3snoRNP, which seems to suggest that these two proteins form part of the same complex. If so, it is expected that the pdcd2 mutant shows a similar phenotype in terms of pre-RNA processing and ribosome ribogenesis as the rps2b mutant. Have authors tried to generate a pdcd2 mutant and analyze the

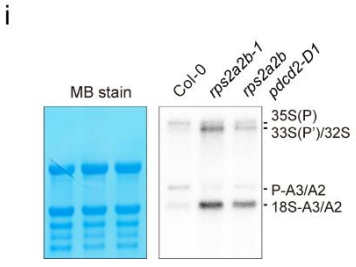
molecular phenotypes? I suppose that they can use CRISPR-CAS techniques or RNAi (in case the full knockout mutant is lethal)? I am convinced that showing experiments with the PDCD2 mutant will allow them to further support their hypothesis and more clearly show the roles of PDCD2 in transport.

Response: We thank the reviewer for the comment. We have designed many different targets on *PDCD2* for CRISPR-Cas9. But we only got two lines named *pdcd2-cr1* and *pdcd2-cr2* mutants, which have a 15-bp and 12-bp deletion, respectively (**new Supplementary Fig. 1h**). Phenotype analysis shows that these two weak alleles *pdcd2-cr1* and *pdcd2-cr2* mutants behave similar to Col-0 (**new Supplementary Fig. 1i**).



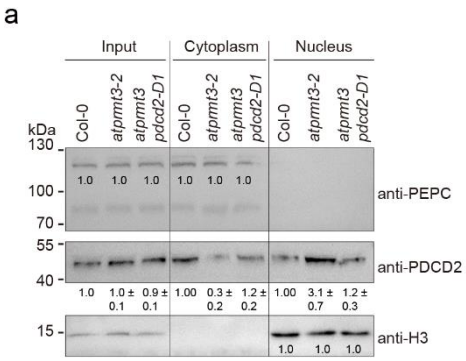
4. Authors clearly demonstrate that the suppressor PDCD2-D1 reverts the phenotype of the rps2b mutant, but does PDCD2-D1 also revert the molecular phenotypes of rps2b, especially those related to pre-RNA processing as they show for atprmt3 mutant?

Response: We thank the reviewer for the suggestions. In order to answer this question, we performed Northern blot to prove that *PDCD2-D1* could also partially rescue the pre-rRNA processing defects in *rps2a2b* mutants **(new Fig. 2i)**.

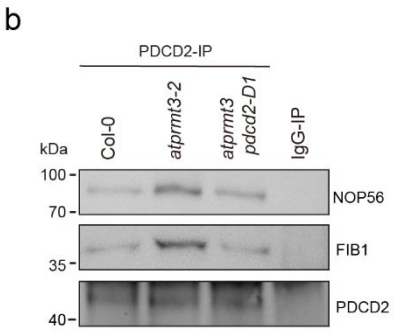


5. It seems to me that the PDCD2 accumulation is reduced in the atprmt3 mutant in figure Fig S3 and Figure 3A; however, this reduction is not observed in Figure 3B. Could you comment on that? In addition, why are there higher levels of PDCD1-D1 in atpmr3-2? It seems to me that there are two bands of PDCD2 in the nucleus? Do you think that these two bands could suggest that the PDCD2 is post-translationally modified when located in the nucleus?

Response: We apologize for the misleading information. The expression of PDCD2 is not reduced in *atprmt3-2* total proteins, because it is not repeatably reduced in other biological replicates, we have replaced with other replicates and added the quantification of band signal from three biological replicates **(new Fig. 3a)**.



As for previous Fig. S3 **(now new Supplementary Fig. 3b)**, this figure is used to show the association between PDCD2 and U3 snoRNP (FIB1 and NOP56) in different mutants. In *atprmt3-2* mutants, these associations are enhanced, but they were partially rescued in the suppressors. The reduced amount of PDCD2 is due to unequal loading. We have provided a better co-IP figure as also requested by reviewer #2.



Regarding Fig. 3b, these three samples (Col-0, *atprmt3-2*, and suppressor) are from different membranes and therefore not directly comparable; Fig. 3b is used to

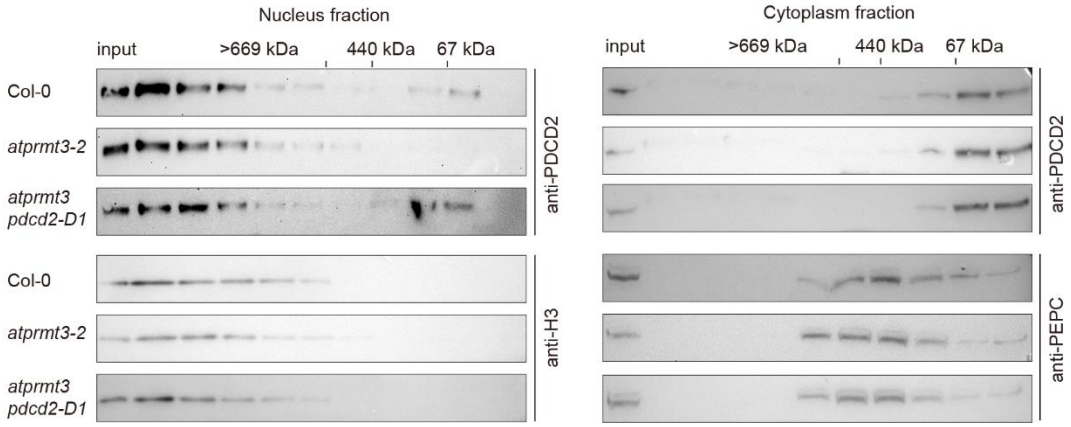
demonstrate the different distribution pattern of PDCD2 between high-molecular fractions (left) and low-molecular fractions (right) on the same membrane.

In response to the reviewer's concern of PDCD2 is post-translationally modified. We suspect that one of the two bands of PDCD2 in the nucleus is non-specific, as they are not consistently observed in other biological replicates or fractions. Therefore, the bottom band is likely non-specific.

6. In Figure 3B, I can observe that PDCD2 is involved in high molecular complexes and that these complexes are highly accumulated in the atprmt3 mutant. This is an effect that is reverted in the PDCD2-D1 background. This is not striking, since other molecular phenotypes of atprmt3 are reverted in the PDCD2-D1 background. I have the same feeling with Fig. 3F and 3G. For me, it would have been more informative to carry out the gel filtration experiments separating the cytosolic and the nuclear fractions. This could provide more information on why the PDCD2-D1 protein acts as a suppressor of the atprmt3-2 phenotype?

Response: We thank the reviewer for the constructive suggestions. We carried out gel filtration with cytosolic and the nuclear fractions, respectively. The results show that in nucleus PDCD2 is mainly distributed in larger molecular fractions, which correlates with its interaction with pre-ribosome complex (Supplementary Table 1); PDCD2 is mainly distributed in small molecular fractions in cytoplasm, suggesting that it is newly synthesized in this cellular compartment **(new Supplementary Fig. 3c)**. These findings imply that PDCD2 is synthesized in the cytoplasm and then transported into the nucleus, where it interacts with pre-ribosomes. Subsequently, it could facilitate the export of pre-ribosomes into the cytoplasm through the nuclear pore complex (NPC).

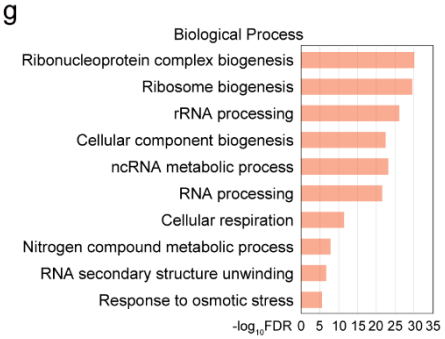
c



7. What are the identities of the proteins identified in Figure 3F? Are those related to ribosome proteins? I suppose that this information, along with accumulation of ribosomal proteins in the nucleus in the PDCD2 mutant, is important if they want to establish that PDCD2 facilitates the export of pre-ribosomes to the cytoplasm.

Response: We thank the reviewer for the important suggestions. We analyzed these accumulated proteins in *atprmt3-2* mutants, which were rescued in suppressor *atprmt3pdcd2-D1*, finding that these proteins were mainly associated with ribosome biogenesis process, indicating that PDCD2 facilitates the export of pre-ribosomes to the cytoplasm.

g



Regarding the translation analyses, I have different important concerns.

8. In the protocols for Riboseq analyses, it is quite usual to generate fragmented fragments to analyze transcription. These fragments are small and this allows the comparison of the Riboseq and total RNA libraries, since they are done in a similar way (for example, using small fragments without enriching for polyA transcript). In this case, this has not been done, and authors have obtained small fragments for Riboseq and full transcripts polyA mRNAs to generate the libraries. I suppose that

although not fully conventional, it is possible to compare Riboseq (small fragments) vs RNAseq. However, the different way to create the libraries could generate some bias and this probably affects the correlations when comparing Riboseqvs RNAseq.

Response: We thank the reviewer for the comment. In preparing RNA-seq library, after polyA mRNAs enrichment, fragmentation was also performed, then the fragmented mRNAs were subjected to library construction. We have added the fragmentation step for RNA-seq in the library preparing flowchart in **Fig.5e**. And this kind of RNA-seq strategies is widely used in many different Ribo-seq researches [4-6].

In response to the reviewer's concern of the comparing bias between Ribo-seq vs RNA-seq, we also agree with the reviewer. But in this manuscript, we don't compare the correlation between Ribo-seq vs RNA-seq directly, instead we compared their fold changes of Mutant vs WT, thus it could eliminate their bias between different libraries.

9. Another issue is that authors do not specify if the polysome profiles and Riboseq/RNAseq analyses have been done in seedlings grown under control conditions or subjected to freezing and left to recover. This is important to judge the results. For example, since there are no control and freezing conditions, I suppose that the translation experiments are done under control conditions; However, if this is the case, how mutants with problems in ribogenesis (with problems in pre-RNA processing, and nucleolus stress) could display higher translation? If the translation analyses are done under freezing conditions, it would be also important to know the translational status under control conditions and explain why mutants in ribosome biogenesis have increased translation.

Response: We thank the reviewer for the comment. The polysome profiles and Riboseq/RNAseq analyses have been done under control conditions, we have specified them in the revised manuscript.

Our investigation into the ribosome-defective *atprmt3* and *rps2a2b* mutants revealed a higher polysome ratio, indicating a defect in ribosome biogenesis. To further understand the impact on overall translation, we conducted ribo-seq analysis to assess their translational status, and used translational efficiency to quantify these changes. Using TE as a measure, we found that the balance between stress-responsive

and growth-related gene expression is disrupted in these mutants (Fig. 6). Despite polysome ratio was increased, it does not mean the overall translation is elevated. Instead, there is an increase in stress-responsive gene expression and a decrease in growth-related gene expression. Overall, it demonstrates that these mutants have defective ribosome biogenesis as well as aberrant translation status, leading to an unbalanced growth-stress trade-off.

10. I am especially curious with the results they obtained for the rps2c-1/Col-0 mutant in Figure 5A. It seems that this mutant has no big changes in transcription compared to Col-0; however, the changes in translation are really high. However, this mutant does not show differences with the wt in the polysome profiles. This seems odd to me and suggests that probably this is a mutant highly (almost specifically) affected at the translational level.

Response: We thank the reviewer for the comments. Yes, we also noticed that the transcription of *rps2c* mutant is similar to that in Col-0, however its translational level changes a lot. Our explanation is that in *atprmt3* and *rps2a2b* mutants, they have severe pre-rRNA processing and pre-ribosome assembly defects[3,7], which results in aberrant ribosome state evidenced by increased polysomes in cytoplasm. However, the existing results show that RPS2C do not obviously affect ribosome biogenesis, so the polysome state seems similar to Col-0 in cytoplasm.

Although RPS2C is not as important as RPS2A2B in pre-rRNA processing, but *RPS2C* is highly expressed (https://bar.utoronto.ca/eplant/), the knockout of RPS2C could results in the disappearance of ribosomes composed of RPS2C, leading to the production of heterogeneous ribosomes. Ribosome heterogeneity includes sequence variation of rRNAs, absence of specific ribosomal proteins (RP), modification of rRNAs/RPs, different auxiliary factors, etc [8,9]. In *Arabidopsis* RP gene has multiple paralogs, some of them display sequence variations and are differentially expressed during development. There is evidence to suggest that heterogeneous ribosomes preferentially translate different subpools of mRNAs [10]. Therefore, it is likely that the translation status is different among different *rps2* family mutants. Additionally, while the *rps2c* mutant may not display obvious seedling phenotypes under normal

conditions, it remains to be determined if it has other defects during development or under stress conditions. We also would like to thank the reviewer for giving us a hint for future heterogeneous ribosome translation research.

11. In Figure 6A-B authors show the number of TE downregulated and upregulated genes in each mutant compared to Col-0. However, they do not define the criteria to ascribe the genes. In these cases, it is important to describe the criteria of fold change and padj value, to allow to see if the differences are high enough.

Response: We thank the reviewer for pointing out these issues. We have specified them in the method part in the revised manuscript. Genes with RPKM ≥ 1 in ribo-seq and FPKM ≥ 1 in RNA-seq were kept for the calculation of translational efficiency. The translational efficiency was calculated as the ratio between RPKM in the Ribo-seq and that of FPKM in the RNA-seq, genes with fold change of TE ≥ 1.5 were identified as differentially expressed.

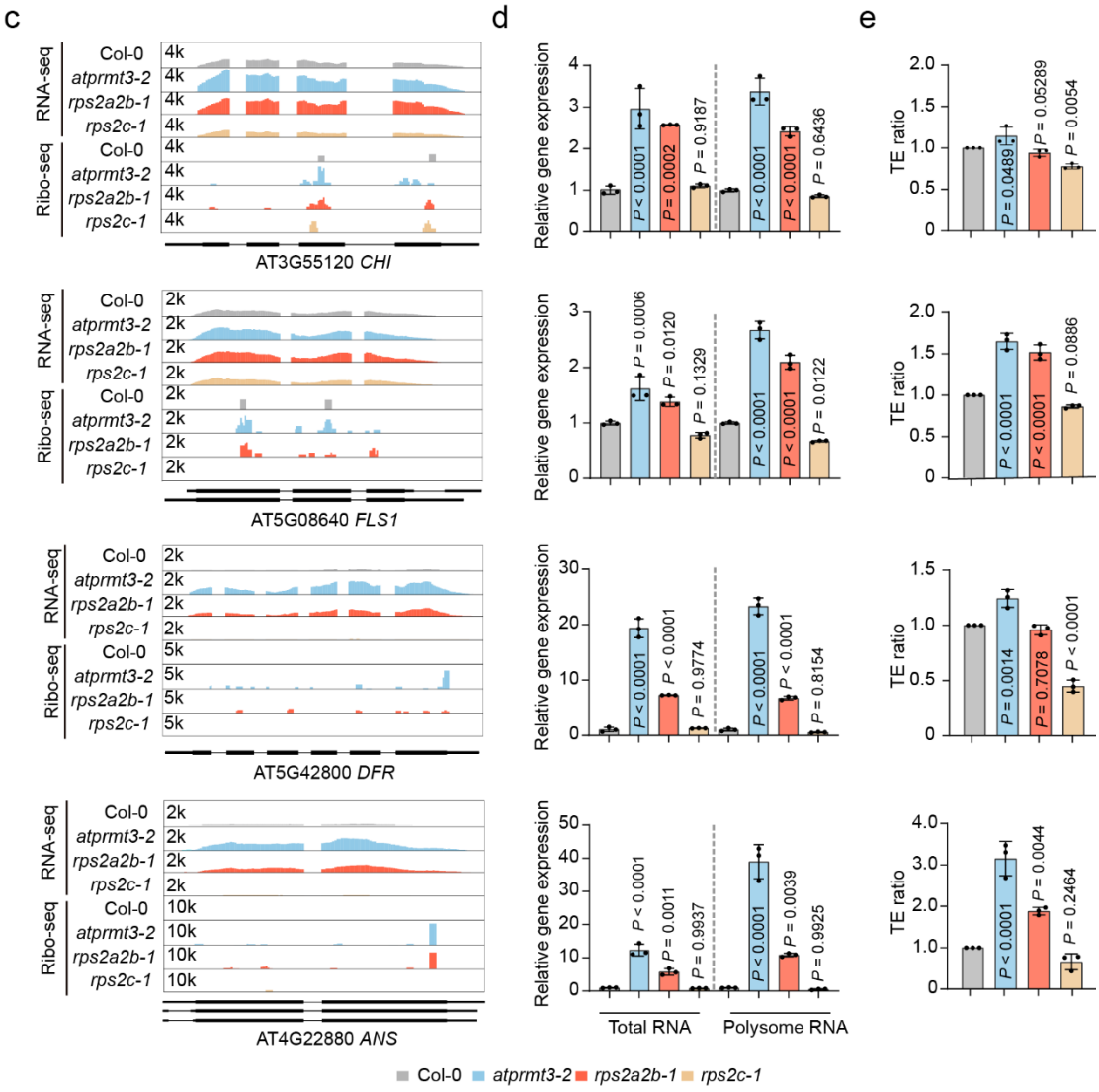
12. I think that there is a mistake in Figures 6C or it is not clear enough, since they establish that the figure corresponds to TE-down regulated genes but in the scale bar TE varies from 6 to -6 (I suppose that it refers to log2 TE??)

Response: We thank the reviewer for pointing out these issues. Yes, the scale bar refers to log_2TE, and we specified them in figure legend in the revised manuscript.

13. Regarding the analyses of translation of the CHS and genes of the flavonoid metabolic pathway, I have serious doubts that some of them are regulated at the level of translation in both mutants atprmt3 and rps2a2b. For example, I am not sure that CHI is regulated at translational level in the mutants in fig S8. Indeed, you already show that CHI is upregulated to near 3x fold in the atprmt3-2 and 2.5 x fold in the rps2a2b-1 mutant already at the transcriptional level and these levels are highly similar to the ones you observed at the translational level. This seems clearer to me in the case of DFR in the rps2a2b-1 mutant (which shows small changes of induction at the level of transcription and translation). I have also doubts about other genes that are shown, since the changes between transcription and translation seem subtle and the criteria are not shown.

Response: We thank the reviewer for the comments. In our previous manuscript, we

presented the expression patterns for a number of genes in the flavonoid pathway at both the transcriptional and translational levels. We observed up-regulated gene expression at the transcriptional level for all the genes, and for some, we also observed up-regulation at the translational level. To better illustrate the translational changes, we calculated the translational efficiency (TE) to quantify these changes **(new Supplementary Fig. 7e)**. Our analysis revealed that some of the genes such as *FLS1* and *ANS* were indeed have up-regulated TE in *atprmt3* and *rps2a2b* mutants, the significant differences are shown on the figures.



14. In the text, it is established that some genes have up-regulated expression patterns. Since you are looking at transcription and translation, it would be better to establish if the changes are at the transcriptional level, translation level or both.

Response: We thank the reviewer for the comments. From the Ribo-seq and RNA-seq

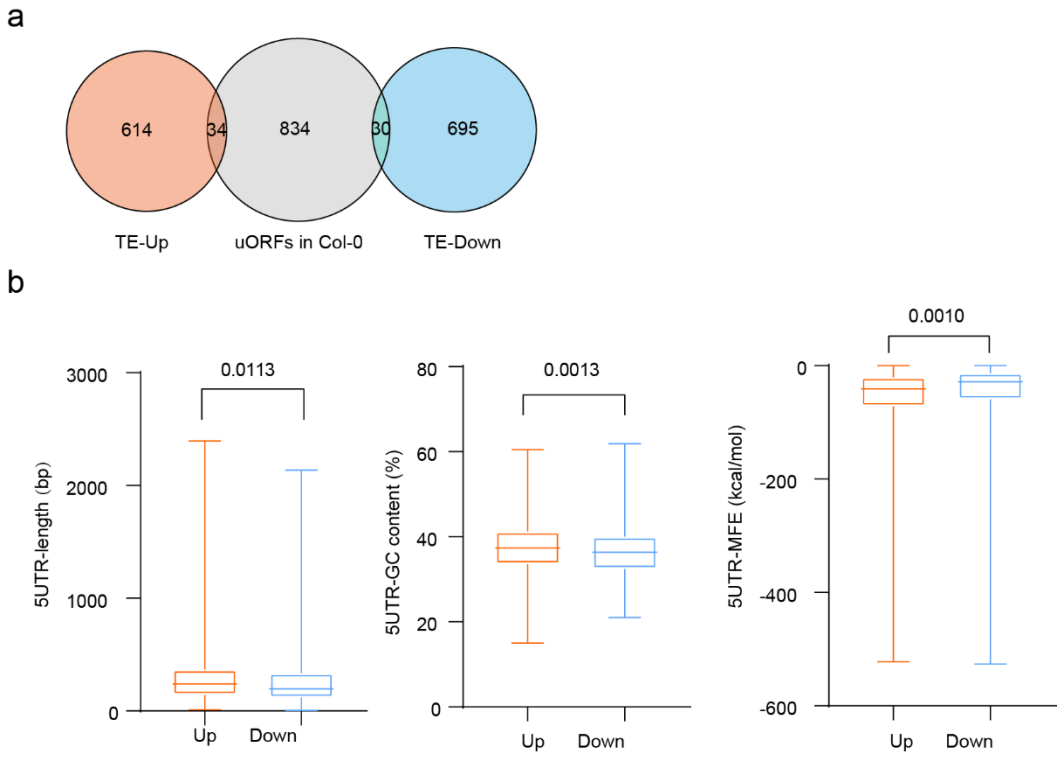
analysis, we observed a moderate correlation (R≈0.4) between translational (Ribo-seq) and transcriptional changes (RNA-seq) in *atprmt3* and *rps2a2b* mutants **(Fig. 5f)**, indicating the presence of a regulatory mechanism at the translational level in these mutants. It proves that the transcription and translation level are both affected in these mutants. To further distinguish the impact at the translational level, we utilized translation efficiency (TE) analysis and found that genes with up-regulated TE are primarily stress-responsive genes, down-regulated genes are mainly growth-related genes **(Fig. 6a-6d)**. In conclusion, the data suggests that both transcription and translation are affected in these mutants, and we have updated the manuscript accordingly.

15. Riboseq analyses allow to obtain a map of the positions of the ribosomes and to acquire more information such as if the ribosomes are stalled at uORFs etc. HAve you checked for specific issues of translational regulator? With this information it is possible that you can go further than to establish that a few genes are changed at the translational level.

Response: We thank the reviewer for the comments. The mRNA contains some specific features that could influence translation, such as 5′ Cap, 5′ UTR, uORF, polyA tail, and 3′ UTR and so on [11-13]. In response to the reviewer's suggestions, we conducted an analysis of the uORF, an important translation regulator in the 5′ UTR. Upon comparison of TE-Up/Down-regulated genes with uORFs identified in Col-0, we found that there is little overlap (rebuttal figure a). As a result, the function we studied here may have little link with uORFs.

To further expand our understanding of translational regulation, we also investigated additional sequence features such as sequence length, minimal free energy (MFE), and GC content in 5′ UTR regions, and estimated their potential effect on TE of corresponding genes. It is generally believed that genes with complex secondary structures in GC-rich 5′ UTRs are associated with lower translation [12]. Interestingly, our findings revealed that genes with up-regulated TE preferably have long 5′ UTR, high GC content and lower MFE in 5′ UTR in *atprmt3* and *rps2a2b* mutants (rebuttal figure b). These observations suggest that the AtPRMT3-RPS2-

mediated ribosome translation may play a regulatory role in this process. Additionally, it is possible that the RNA secondary structure also influences this process. It still needs to be explored in future. We have incorporated this into discussion and are grateful to the reviewer for providing constructive suggestions to further deepen the scope of this project.



References:

1. Xu, G. *et al.* Global translational reprogramming is a fundamental layer of immune regulation in plants. *Nature* **545**, 487-490 (2017).
2. Merchante, C. *et al.* Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. *Cell* **163**, 684-97 (2015).
3. Hang, R.L. *et al.* Arabidopsis protein arginine methyltransferase 3 is required for ribosome biogenesis by affecting precursor ribosomal RNA processing. *Proc Natl Acad Sci U S A* **111**, 16190-16195 (2014).
4. Hang, R.L. *et al.* HOT3/eIF5B1 constrains rRNA RNAi by facilitating 18S rRNA maturation. *Proc Natl Acad Sci U S A* **120**(2023).
5. Guo, Y. *et al.* The translational landscape of bread wheat during grain

development. *The Plant Cell* **35**, 1848-1867 (2023).

6. Kim, E.Y. *et al.* Ribosome stalling and SGS3 phase separation prime the epigenetic silencing of transposons. *Nature Plants* **7**, 303-309 (2021).
7. Hang, R. *et al.* Protein arginine methyltransferase 3 fine-tunes the assembly/disassembly of pre-ribosomes to repress nucleolar stress by interacting with RPS2B in arabidopsis. *Mol Plant* **14**, 223-236 (2021).
8. Martinez-Seidel, F., Beine-Golovchuk, O., Hsieh, Y.C. & Kopka, J. Systematic Review of Plant Ribosome Heterogeneity and Specialization. *Front Plant Sci* **11**, 948 (2020).
9. Shi, Z. & Barna, M. Translating the genome in time and space: specialized ribosomes, RNA regulons, and RNA-binding proteins. *Annu Rev Cell Dev Biol* **31**, 31-54 (2015).
10. Shi, Z. *et al.* Heterogeneous Ribosomes Preferentially Translate Distinct Subpools of mRNAs Genome-wide. *Mol Cell* **67**, 71-83.e7 (2017).
11. Merchante, C., Stepanova, A.N. & Alonso, J.M. Translation regulation in plants: an interesting past, an exciting present and a promising future. *Plant J* **90**, 628-653 (2017).
12. Leppek, K., Das, R. & Barna, M. Functional 5′ UTR mRNA structures in eukaryotic translation regulation and how to find them. *Nature Reviews Molecular Cell Biology* **19**, 158-174 (2018).
13. Wu, H.-Y.L., Jen, J. & Hsu, P.Y. What, where, and how: Regulation of translation and the translational landscape in plants. *The Plant Cell* **36**, 1540-1564 (2023).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript addressed some of my concerns; however, I still have some lingering issues:

1. While they demonstrated the direct interaction between PDCD2 and PRMT3 through a split luciferase assay, PRMT3 is not among the proteins co-immunoprecipitated by PDCD2 (Table S1). Further evidence is needed to confirm the PDCD2-PRMT3 interaction.

2. More evidence is needed to support their main conclusion on the role of these proteins in translational regulation. For example, the conclusion that the mutations of RPMT3-RPS enhance translation of stress-responsive genes, like flavonoid genes, is not sufficiently supported by the data. There appears a lack of a distinct phenotype in the mutants that are often observed with other mutants with significant accumulation of flavonoids. Could the higher abundance of Ribo-seq transcripts from the flavonoid genes be due to stalled/slow translation (therefore protected from RNAse digestion) due to the defect in ribosomal assembly by these mutations? It would help if they can determine the protein levels like CHS, CHI, etc., through Western blotting, as well as quantifying flavonoid levels.

Other points for consideration:

3. The authors suggest that PDCD2, PRMT3, and RPS are involved in various steps from pre-ribosome processing to translational control. The evidence supporting their roles in ribosome processing/assembly is stronger than for other functions. Could their primary function be in ribosomal biogenesis, indirectly affecting translation processes?

4. Each dataset should be titled to aid reader comprehension.

5. Consistency should be maintained in referring to the suppressor mutation. They used PDCD2-D1 and pdcd2-D1.

6. This sentence: ‘atprmt3cbfs mutants also show reduced survival rate compare to atprmt3-2 mutants, indicating that CBFs have partially functions in this process, but mainly through CBF-independent pathway’, needs to be revised as the sentence sounds like ‘CBFs mainly function through CBF-independent pathway’.

7. This statement is not well supported by data: ‘The freezing tolerant phenotype of atprmt3-2 mutants in NA conditions indicates that some cold-protective substances have already been accumulated in the mutants, thus our work mainly focus on the mechanism of how they are regulated in NA conditions’. The mutant does not have to have cold-protective substances

accumulated under the normal condition as the freezing tolerance phenotype could also simply be due to a stronger response to cold treatment than wt.

Reviewer #2 (Remarks to the Author):

I really appreciate the new results provided in the revised version. The additional data drastically strengthen the story. I have no more comments.

Reviewer #3 (Remarks to the Author):

In this new version, authors have successfully addressed all my questions and concerns. They have provided new experiments that strengthen the conclusions. The manuscript has been deeply improved.

I have no additional concerns.

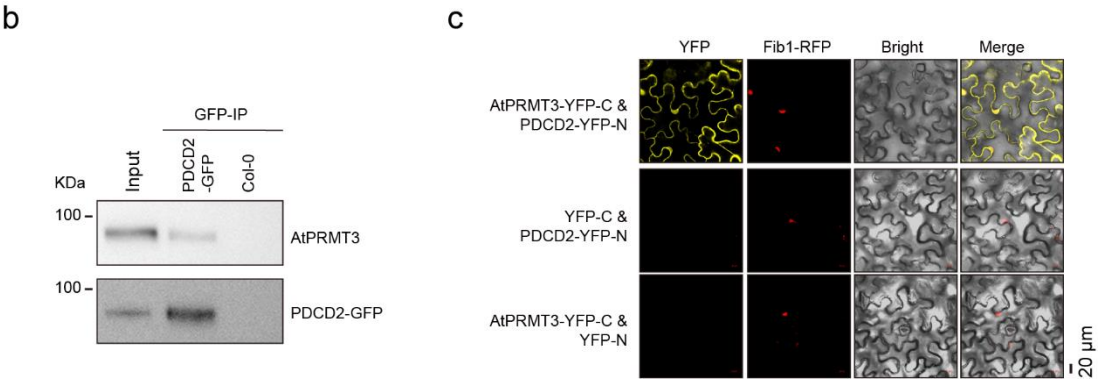
Reviewer #1 (Remarks to the Author):

The revised manuscript addressed some of my concerns; however, I still have some lingering issues:

We are grateful for your valuable comments and suggestions on improving the quality of our manuscript. We have revised and added new experiments to further enhance the support for our conclusions.

1. While they demonstrated the direct interaction between PDCD2 and PRMT3 through a split luciferase assay, PRMT3 is not among the proteins co-immunoprecipitated by PDCD2 (Table S1). Further evidence is needed to confirm the PDCD2-PRMT3 interaction.

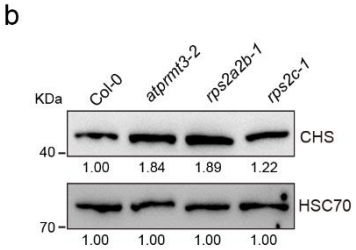
Response: We thank the reviewer for pointing this issue. In order to further prove the interaction between PRMT3 and PDCD2, we used co-IP and bimolecular fluorescence complementation (BiFC) assay to confirm their interaction (Supplementary Fig. 3b, c).



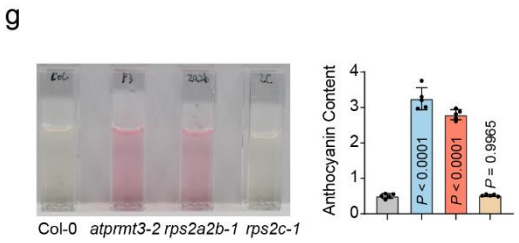
2. More evidence is needed to support their main conclusion on the role of these proteins in translational regulation. For example, the conclusion that the mutations of RPMT3-RPS enhance translation of stress-responsive genes, like flavonoid genes, is not sufficiently supported by the data. There appears a lack of a distinct phenotype in the mutants that are often observed with other mutants with significant accumulation of flavonoids. Could the higher abundance of Ribo-seq transcripts from the flavonoid genes be due to stalled/slow translation (therefore protected from RNAse digestion) due to the defect in ribosomal assembly by these mutations? It would help if they can determine the protein levels like CHS, CHI, etc., through Western blotting, as well as

quantifying flavonoid levels.

Response: We thank the reviewer for the constructive suggestions. As suggested by the reviewer, we have investigated the protein expression level of CHS, an important gene in the flavonoid pathway. Our results indicate that CHS has exhibited a higher expression level in *atprmt3* and *rps2a2b* mutants, which is consistent with the its increased translation (Supplementary Fig. 7b).



Anthocyanins, a type of flavonoid, are present in plants and play a role in cold acclimation [1-4]. We found that *atprmt3* and *rps2a2b* mutants have higher levels of anthocyanin compared with Col-0 and *rps2c* mutants, which corresponds to their freezing phenotypes (Supplementary Fig. 7g).



Other points for consideration:

3. The authors suggest that PDCD2, PRMT3, and RPS are involved in various steps from pre-ribosome processing to translational control. The evidence supporting their roles in ribosome processing/assembly is stronger than for other functions. Could their primary function be in ribosomal biogenesis, indirectly affecting translation processes?

Response: We thank the reviewer for the comments. Yes, we agree with the reviewer, these proteins have primary functions in ribosome biogenesis, their defects could ultimately disturb the ribosome state in the cytoplasm, which finally affect translation process.

4. Each dataset should be titled to aid reader comprehension.

Response: We thank the reviewer for pointing this issue. We have added the

description title in each dataset excel, and we also added a supplementary dataset description file in the revised manuscript.

5. Consistency should be maintained in referring to the suppressor mutation. They used PDCD2-D1 and pdcd2-D1.

Response: We thank the reviewer for pointing this issue. We have made the correction. We used *pdcd2-d1* when referring to mutants, and *PDCD2-D1* when referring to the gene.

6. This sentence: 'atprmt3cbfs mutants also show reduced survival rate compare to atprmt3-2 mutants, indicating that CBFs have partially functions in this process, but mainly through CBF-independent pathway', needs to be revised as the sentence sounds like 'CBFs mainly function through CBF-independent pathway'.

Response: We thank the reviewer for pointing this issue. We have revised this sentence to "…*atprmt3cbfs* mutants also show reduced survival rate compare to *atprmt3-2* mutants, suggesting the partial function of CBFs in this process…"

7. This statement is not well supported by data: 'The freezing tolerant phenotype of atprmt3-2 mutants in NA conditions indicates that some cold-protective substances have already been accumulated in the mutants, thus our work mainly focus on the mechanism of how they are regulated in NA conditions'. The mutant does not have to have cold-protective substances accumulated under the normal condition as the freezing tolerance phenotype could also simply be due to a stronger response to cold treatment than wt.

Response: We thank the reviewer for pointing this issue. We have revised this sentence to "The freezing tolerant phenotype of *atprmt3* mutants in NA conditions indicating their stronger response to cold stress, and our work focuses on understanding the mechanism through which they are regulated under these conditions.".

Reviewer #2 (Remarks to the Author):

I really appreciate the new results provided in the revised version. The additional data drastically strengthen the story. I have no more comments.

Response: We thank the reviewer for the positive comments.

Reviewer #3 (Remarks to the Author):

In this new version, authors have successfully addressed all my questions and concerns. They have provided new experiments that strengthen the conclusions. The manuscript has been deeply improved. I have no additional concerns.

Response: We thank the reviewer for the positive comments.

References:

1. Dong, N.Q. & Lin, H.X. Contribution of phenylpropanoid metabolism to plant development and plant-environment interactions. *J Integr Plant Biol* **63**, 180-209 (2021).
2. Li, P. *et al.* The Arabidopsis UDP-glycosyltransferases UGT79B2 and UGT79B3, contribute to cold, salt and drought stress tolerance via modulating anthocyanin accumulation. *Plant J* **89**, 85-103 (2017).
3. Schulz, E., Tohge, T., Zuther, E., Fernie, A.R. & Hincha, D.K. Flavonoids are determinants of freezing tolerance and cold acclimation in Arabidopsis thaliana. *Sci Rep* **6**, 34027 (2016).
4. Li, Y. *et al.* The CRY2–COP1–HY5–BBX7/8 module regulates blue light-dependent cold acclimation in Arabidopsis. *The Plant Cell* **33**, 3555-3573 (2021).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript has adequately addressed my main concerns.