molecular systems biology

Widespread translational control contributes to the regulation of Arabidopsis photomorphogenesis

Ming-Jung Liu, Szu-Hsien Wu, Ho-Ming Chen, Shu-Hsing Wu

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 June 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will the reviewers recognized that this work provided some interesting results demonstrating widespread translational control during photomorphogenesis. They, however, raised a series of important concerns, which will need to be convincingly addressed before this work can considered for publication. Two points seem particularly important, and appear to require additional controls and experiments:

1. Two reviewers had clear issues with the luciferase-based reporter experiments presented in Figure 8. In particular, they felt that it would be important to rigorously control for luciferase mRNA in the protoplast light-response experiments.

2. The first reviewer had a clear concern that proteolytic regulation of HY5 and BBX22 could confound results. Additional controls ruling out light-induced changes in proteolytic control, or similar experiments on other proteins that are not under proteolytic control, appear needed.

In addition, the reviewers noted several cases where additional details are needed to support the Figures. For Figure 3 it will be important to clarify whether the qRT-PCRs were conducted on independent biological samples or on one of the RNA isolations conducted for the microarray experiments -- if they are from one of the microarray RNA isolates then new tests from independent biological samples should be conducted. Similarly, for Figure 4 please state the number of independent biological samples that were used to estimate the values in panels B and C. Figure legends should state what error bars represent (s.e.m., standard deviation, etc).

Please note, that in addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data'

associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for Figures 3, 4, 8, and 9. Please see our Instructions to Authors for more details (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office msb@embo.org.

Yours sincerely,

Editor - Molecular Systems Biology

Referee reports

Reviewer #1 (Remarks to the Author):

The manuscript by Liu et al reports the genome-wide study of translational control during Arabidopsis photomorphogenesis. The authors used ribosome profiling to separate polysome portion from ribosme monomers and dimers, and further isolated mRNAs associated with polysomes (PL) and monomer-ribosomes (NP). Enrichment of transcripts in the PL portion was found for a significant portion of the transcriptome after light treatment. A carful comparison at half an hour and at four hours after white light treatment for PL and for NP revealed that enhanced translation is as important as enhanced transcription. The authors further reported stronger translation for stable transcripts and short transcripts. Two cis-elements were identified in the 5' UTR regions of transcripts with enhanced translation. One of them was experimentally tested for translation efficiency using an in vitro system and Arabidopsis protoplasts. Overall, this manuscript is well-written and the main point that translational control during photomorphogenesis is novel. Nevertheless, the new findings described in this paper were not so much different from previous genome-wide studies of translational changes during several other environmental responses, reported over the past 7 years or so (e.g. Kawaguchi et al., TPJ 2004; Nicolai et al., PP 2006; Branco-Prince et al., TPJ 2008; Matsuura et al., PCP 2010; Sormani et al., PCP 2011). Also, the manuscript could be more concise.

Other points

1) p. 13: The finding that short transcripts are highly translated is not first-time reported, not even in Arabidopsis.

2) Fig. 9: The two proteins, HY5 and BBX22, were selected for Western blotting, in combination with transcripts RNA blotting, to confirm enhanced translation. Both HY5 and BBX22 were previously reported under proteolysis control, making the Western results difficult to interpret.

3) The reported cis-element, TAGGGTTT, appeared to be a general enhancer of translation, instead of photomorphogenesis-specific.

Reviewer #2 (Remarks to the Author):

This manuscript presents evidence for large scale upregulation of protein synthesis in response to light. Translational regulation by light has been reported before in plants for specific transcripts, and here the authors should cite work by Petracek et al, Plant cell (1997) 9: 2291-2300; Kim et al, EMBO J (2003) 22, 935 - 944. However this has to my knowledge never been analysed in plants on a genome-wide basis. The upregulation but is remarkably widespread and the number of genes affected exceeds that for transcriptional regulation by at least one order of magnitude. The upregulation is relatively modest (3-4 fold at the most) but consistently observed using different experimental techniques. The authors suggest that this represents an effective mechanism to rapidly increase protein expression levels in response to environmental signals.

The manuscript is written very clearly (with the exception of a few specific sections, see below) and very well presented. The experimental data are of very high quality.

The only real issue that i had with the experimental data was in the functional analysis of a putative regulatory element using luciferase reporter construct. It is first shown in Figure 8D that this element enhances translation of the luciferase protein in an in vitro system, but this is unlikely to be relevant to light-regulated translation. In panel 8E the authors test for upregulation of their constructs in vivo in response to light. However upregulation of luciferase activity may be due to either transcriptional or translational upregulation. The authors need to analyse luciferase mRNA levels in parallel with luciferase activity levels to test whether regulation truly takes place at the translational level. In fact the Methods section mentions that this has been done but this is not shown.
Vertical axes in Figure 8 D-E are also misleading as they grossly exaggerate differences between the constructs which are actually pretty small. It would be better to have them ranging from zero to the maximum value in the experiment.
The legend to panel 8E should state that this was a transient protoplast transfection experiment.

Minor comments:

Figure 2B: it would be helpful to highlight on this figure (possibly in a different panel or by showing cut-off lines) the genes that were upregulated either at the mRNAss or at the mRNApl level. This would support the argument at the top of p. 9. The authors may also want to present this argument first as it is relatively simple. They should then explain their strategy to identify genes that are regulated at the translational level, i.e genes that showed more than 3 fold differences between mRNApl and mRNAss fold changes relative to D, and support that with the current panel shown in figure 2B.

Figure 3: do the error bars represent variation between technical replicates or between biological replicates? Were the biological samples independent from those analysed in the microarray experiment? It would be important here to have at least two biological replicates.

page 11, line 8. Supplementary figure 3, not 2.

page 19, line 20. The following statement is unclear: "the identities of those genes could not be revealed via transcriptome study of steady state mRNAs"

Methods section, page 25, line 18. The logic of the normalisation method should be described rather than the command lines used.

Reviewer #3 (Remarks to the Author):

In this study the authors show that the translation of a large number of mRNAs dramatically increases when etiolated seedlings are exposed to light, concomitant with photomorphogenesis. This response is shown to be due to rapid recruitment of mRNAs into polysomal complexes in illuminated seedlings. Using microarray technology they demonstrate that the translation status of individual mRNAs can be light regulated. The light regulated mRNAs include those encoding the photosynthetic apparatus and translational machinery. Their findings add to earlier studies that illustrating that environmental stimuli modulate translation of individual mRNAs, which in some cases is linked to mRNA stability. A nice addition to manuscript is the validation that light-induced increases in the association of BBX22 and HY5 mRNAs with polysomes correlates with increases in

these proteins.

There is one segment of the manuscript that is weak. This is their attempt to demonstrate that specific cis-acting motifs are responsible for the light-induced selective mRNA translation. The authors use a wheat germ in vitro translation system to test mRNAs that possess or lack a specific 5'UTR motif. The mRNAs put into this system are neither 5' capped nor 3' polyadenylated. It is extremely unlikely that a wheat germ provides information on mRNA translatability that can be extrapolated to dynamics in translation associated with photomorphogenesis. Most likely the mutations in the motif targeted can generally affect the translatability of an mRNA, either via initiation or some other mechanism, but this is not sufficient evidence to show an effect associated with light. In the second part of this experiment the same three constructs were tested in a protoplast system. It is probably an oversight in the methods, but there is no information given on the promoter used for these constructs although it is presented in the figure. Although the results look promising, they are not robust. This type of analysis is best performed by evaluating the test construct against a control construct, using two different LUC genes. Both mRNA abundance and protein product would need to be monitored in the protoplast system. Possibly the use of mutants could be used to bolster the validity of these results (i.e. a genotype that does not show the light-enhanced expression). Although experiments in this direction are needed, this analysis may be left out of the manuscript without compromising the study.

Specific comments:

(1) The authors have been thoughtful in their use of terms to describe the level of gene expression monitored and the level of gene regulation in evidence from their results. Howe
The English in the manuscript could benefit from editing by an individual familiar with the research.

(2) The authors use terms such as ribosome occupancy, translation rate and ribosome density. They need to be very careful to state only what was observed. For example, by measuring the ratio of mRNA in the polysomal versus total (steady-state) pools, they have assessed what is referred to as translation(al) status. By measuring mRNAs in sucrose gradient fractions of different density, they have an estimate for ribosome number per mRNA, which is okay to call ribosome or polysome occupancy. It would have been preferable for them to show this fractionating the entire gradient into more than four fractions and performing q-RT-PCR or a northern. The actual ribosome density would need to be assessed using a ribosome footprinting/mapping method. Finally, rate of translation was not assessed in this study, so this cannot be asserted.

Figure 3. The authors have measured "translation status" not ribosome occupancy. The legend of this figure is not sufficiently detailed. Some changes that are needed include, definition of all abbreviations, inclusion of Arabidopsis Gene Identifiers in the legend or figure for the genes with names, definition of the use of red font.

Figure 4. (a) The authors have used the term "ribosome density" but this analysis is better described as "ribosome occupancy". The authors have used their arrays to monitor the amount of individual gene transcripts in the steady-state and polysomal RNA preparations. This allows them to calculate the percentage of an mRNA in the polysomal complexes. This translation state information might be provided for these mRNAs in Table S3.
(b) Did the authors take into consideration length of transcripts in this analysis?
(c) Does this data represent the average from multiple experimental samples?
(d) The authors must also take into consideration that elongation of translation may be regulated. For example on p 10, line 20-24; they need to consider that increased ribosome occupancy could also reflect pausing. Ideally, they should have performed this experiment for HY5 and BBX22 as well.

Figure 5. (a) In both this figure and Supplemental figure 4 the authors have included a column to describe the level of gene regulation observed. They use the terms "mRNA" and "protein". Appreciating their aim, there may be less ambiguous terminology, such as "transcript accumulation" and "polysome association" to refer to "RNA (or steady-state)" and "Protein (polysome bound)", respectively.
(b) The figure should indicate the criteria for selection of differentially expressed genes. This is in the supplement, but belongs in the main body of the manuscript, especially since different criteria

were used.
(c) It is not clear why the authors combined clusters 3 and 4 in the figure presented in the main text. It would be fine to keep them separate, since their cluster 4 shows that some mRNAs are possibly destabilized even when translated. Turnover can be coupled to translation and their data may be showing this occurs.
(d) It is suggested that the authors include a Supplemental table giving the mean and error values for the four clusters for the four comparisons.

Figure 6. I had a very difficult time interpreting this figure from the legend and the text. It is a concern that the authors have used mRNA stability data derived from a cell culture analysis performed by another group. I feel this portion of the manuscript does not add significantly because it is not know if the stabilities of the mRNAs evaluated are similar in the seedling system evaluated.

Figure 8: Comments on this analysis were made in the opening statement. If included, the authors should provide a GO analysis on the mRNAs with these motifs. It seems that a table of these genes is not even presented or an alignment to show the consensus.

Supplemental Tables:
(a) The supplemental tables are prepared as PDF files. This made evaluation of this manuscript very difficult. In addition, providing this nice dataset in this manner to readers will diminish the value of this study. The authors should publish these as Excel files.
(b) Also, there are a few typos in the supplemental table headers. Here are some: Table S4: half-lifves were retrived ha; Table S7: expression

Discussion. The authors need to consider the work showing an intersection between COP9/eIF3/uORFs/translation.

1st Revision - authors' response 24 October 2011

Reviewer #1 (Remarks to the Author):

The manuscript by Liu et al reports the genome-wide study of translational control during Arabidopsis photomorphogenesis. The authors used ribosome profiling to separate polysome portion from ribosome monomers and dimers, and further isolated mRNAs associated with polysomes (PL) and monomer-ribosomes (NP). Enrichment of transcripts in the PL portion was found for a significant portion of the transcriptome after light treatment. A carful comparison at half an hour and at four hours after white light treatment for PL and for NP revealed that enhanced translation is as important as enhanced transcription. The authors further reported stronger translation for stable transcripts and short transcripts. Two cis-elements were identified in the 5' UTR regions of transcripts with enhanced translation. One of them was experimentally tested for translation efficiency using an in vitro system and Arabidopsis protoplasts. Overall, this manuscript is well-written and the main point that translational control during photomorphogenesis is novel. Nevertheless, the new findings described in this paper were not so much different from previous genome-wide studies of translational changes during several other environmental responses, reported over the past 7 years or so (e.g. Kawaguchi et al., TPJ 2004; Nicolai et al., PP 2006; Branco-Prince et al., TPJ 2008; Matsuura et al., PCP 2010; Sormani et al., PCP 2011). Also, the manuscript could be more concise.

Response: We thank the reviewers for recognizing the novelty of the translational control during Arabidopsis photomorphogenesis. This is a previously neglected aspect of gene expression regulation in this important developmental stage. The massive polysomal shift in response to environmental changes has been discussed in previous reports, which reflects that the shift is a commonly adopted mechanism for plants to respond to environmental challenges. These previous reports served as solid foundation for us to pursue this research. Our conclusions were based on vigorous statistical evaluations and the integration of multiple dimensions of experimental data, which were not included or may not be accessible in previous reports. In addition, our report includes the validation of both the experimental data and the hypothesis driven by our data. For example, we validated the light-regulated translational control of selected genes and the cis-element contributing to the translational control. Also, we reported and experimentally validated for the first time that HY5, the key transcriptional regulator in the light signaling pathway, is regulated at the translational level. We thus consider that our report expands the scope and further emphasizes the importance of translation control in regulation of Arabidopsis gene expression.

Other points

1) p. 13: The finding that short transcripts are highly translated is not first-time reported, not even in Arabidopsis.

Response: Indeed, shorter transcripts have been found highly translated from genome-wide studies in yeast and human, as we acknowledged in the manuscript (p.13). Whether shorter transcripts are also highly translated in Arabidopsis was primarily discussed in 2 research articles with different observations. First, no clear

preference for shorter transcripts in polysome loading was observed in Arabidopsis seedlings under normal conditions, and the polysome loading for shorter transcripts clearly decreased under dehydration conditions (Kawaguchi & Bailey-Serres, 2005). Second, an anti-correlation between translation efficiency and the ORF length was reported for Arabidopsis under hypoxia stress (Branco-Price et al, 2005). We have confirmed that, in addition to environmental stress (hypoxia), translation control also favors shorter transcripts in Arabidopsis responding to the light-regulated developmental cue. This point was added in the revised manuscript (p.21).

2) Fig. 9: The two proteins, HY5 and BBX22, were selected for Western blotting, in combination with transcripts RNA blotting, to confirm enhanced translation. Both HY5 and BBX22 were previously reported under proteolysis control, making the Western results difficult to interpret.

Response: As pointed out by the reviewer, the post-translational protein degradation might complicate the interpretation of the western blot analyses of HY5 and BBX22 shown in Figure 9. To circumvent this limitation, we have compared the protein abundance of HY5 and BBX22 with and without the proteasome inhibitors MG115/MG132. The original conclusion remains the same after eliminating the impact of post-translational regulation. The result obtained was included in the revised manuscript (p.17-18 and Supplementary Figure S6). Briefly, the addition of proteasome inhibitors stabilized BBX22 at L4h, which is consistent with previous results (Chang et al, 2011). For HY5, although the transcript level is high at L0.5h, no HY5 protein could be detected, even in the presence of MG115/132. At L4h, the *HY5* transcript level decreased, but the protein could be readily accumulated. That the HY5 protein abundance is not further increased in the presence of proteasome inhibitors indicates that translational regulation is primarily responsible for the HY5 protein detected. Thus, the HY5 protein accumulation at L4h, when the transcript level is low, represents the result of selective protein translation of *HY5* transcripts.

3) The reported cis-element, TAGGGTTT, appeared to be a general enhancer of translation, instead of photomorphogenesis-specific.

Response: We agree with the reviewer that from results in Figure 8B and 8C, the cis-element TAGGGTTT is more likely a general enhancer of translation. We discuss this on p.16.

Reviewer #2 (Remarks to the Author):

This manuscript presents evidence for large scale upregulation of protein synthesis in response to light. Translational regulation by light has been reported before in plants for specific transcripts, and here the authors should cite work by Petracek et al, Plant cell (1997) 9: 2291-2300; Kim et al, EMBO J (2003) 22, 935 - 944. However this has to my knowledge never been analysed in plants on a genome-wide basis. The upregulation but is remarkably widespread and the number of genes affected exceeds that for transcriptional regulation by at least one order of magnitude. The upregulation is

relatively modest (3-4 fold at the most) but consistently observed using different experimental techniques. The authors suggest that this represents an effective mechanism to rapidly increase protein expression levels in response to environmental signals.

The manuscript is written very clearly (with the exception of a few specific sections, see below) and very well presented. The experimental data are of very high quality.
Response: We thank the reviewer for recognizing our efforts. We have added the citations suggested by the reviewers (p.5 of the revised manuscript).

The only real issue that i had with the experimental data was in the functional analysis of a putative regulatory element using luciferase reporter construct. It is first shown in Figure 8D that this element enhances translation of the luciferase protein in an in vitro system, but this is unlikely to be relevant to light-regulated translation. In panel 8E the authors test for upregulation of their constructs in vivo in response to light. However upregulation of luciferase activity may be due to either transcriptional or translational upregulation. The authors need to analyse luciferase mRNA levels in parallel with luciferase activity levels to test whether regulation truly takes place at the translational level. In fact the Methods section mentions that this has been done but this is not shown.
Vertical axes in Figure 8 D-E are also misleading as they grossly exaggerate differences between the constructs which are actually pretty small. It would be better to have them ranging from zero to the maximum value in the experiment.
The legend to panel 8E should state that this was a transient protoplast transfection experiment.
Response: As the reviewer noticed, we have quantified both mRNA and enzyme activity for the reporter gene *LUC2*. The results shown in Figure 8E of the previous submission have taken consideration of both mRNA and luciferase activity. However, we completely agree that the light-regulated translational enhancement by this cis-element is less than prominent. Since this element is also over-represented in 5' UTRs of transcripts with altered translatability in response to hypoxia stress (Figure 8B), it is likely a common translational enhancer and not specific to the light signal. The *in vitro* transcription and translation assay supports this notion (Figure 8C). We have modified the conclusion and discussion accordingly in the revised manuscript. Also as suggested, we have re-plotted Figure 8 and improved the legend and methodology for better clarity.

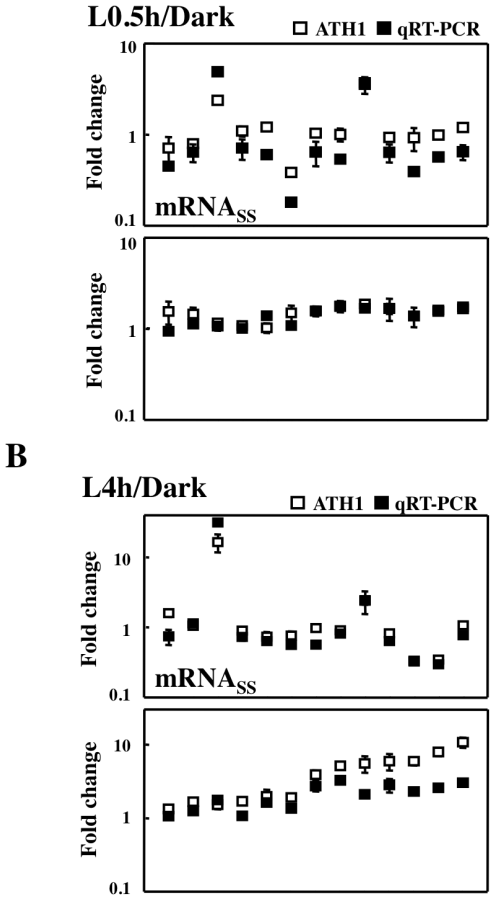
Minor comments:

Figure 2B: it would be helpful to highlight on this figure (possibly in a different panel or by showing cut-off lines) the genes that were upregulated either at the mRNAss or at the mRNApl level. This would support the argument at the top of p. 9. The authors may also want to present this argument first as it is relatively simple. They should then explain their strategy to identify genes that are regulated at the translational level, i.e genes that showed more than 3 fold differences between mRNApl and mRNAss fold changes relative to D, and support that with the current panel shown in figure 2B.
Response: We thank the reviewer for this suggestion. In the revised manuscript, we have swapped the original Figure 2B and 2C. As suggested, we first present the bar

graph to highlight the clear changes of genes at the $mRNA_{PL}$ level, then describe genes with 3-fold or higher expression of $mRNA_{PL}$ than $mRNA_{SS}$ (Figure 2C in the revised manuscript). The concept of more genes upregulated at the $mRNA_{PL}$ level than at the $mRNA_{SS}$ level is clearly presented in Figure 2B of the revised manuscript; thus, we do not highlight the genes or show the cutoff lines in Figure 2C.

Figure 3: do the error bars represent variation between technical replicates or between biological replicates? Were the biological samples independent from those analysed in the microarray experiment? It would be important here to have at least two biological replicates.

Response: The original Figure 3 was generated from samples used for transcriptome analyses. As suggested, we have analyzed two additional biological replicates in preparing the re-submission. Both replicates showed similar results. One representative result is shown in Figure 3 of the revised manuscript, with error bars indicating variation of 3 technical replicates. Results for the other biological replicate are included in Supplementary Figure S1 for reviewer.



Supplementary Figure S1 for reviewer An independent biological replicate for results in Figure 3.

page 11, line 8. Supplementary figure 3, not 2.
Response: It has been corrected in the revised manuscript. Thank you.

page 19, line 20. The following statement is unclear: "the identities of those genes could not be revealed via transcriptome study of steady state mRNAs"
Response: As advised, we have re-written this sentence on p.19 of the revised manuscript.

Methods section, page 25, line 18. The logic of the normalisation method should be described rather than the command lines used.
Response: We have re-drafted the methodology as suggested (p.25 of the revised manuscript).

Reviewer #3 (Remarks to the Author):

In this study the authors show that the translation of a large number of mRNAs dramatically increases when etiolated seedlings are exposed to light, concomitant with photomorphogenesis. This response is shown to be due to rapid recruitment of mRNAs into polysomal complexes in illuminated seedlings. Using microarray technology they demonstrate that the translation status of individual mRNAs can be light regulated. The light regulated mRNAs include those encoding the photosynthetic apparatus and translational machinery. Their findings add to earlier studies that illustrating that environmental stimuli modulate translation of individual mRNAs, which in some cases is linked to mRNA stability. A nice addition to manuscript is the validation that light-induced increases in the association of BBX22 and HY5 mRNAs with polysomes correlates with increases in these proteins.

There is one segment of the manuscript that is weak. This is their attempt to demonstrate that specific cis-acting motifs are responsible for the light-induced selective mRNA translation. The authors use a wheat germ in vitro translation system to test mRNAs that possess or lack a specific 5'UTR motif. The mRNAs put into this system are neither 5' capped nor 3' polyadenylated. It is extremely unlikely that a wheat germ provides information on mRNA translatability that can be extrapolated to dynamics in translation associated with photomorphogenesis. Most likely the mutations in the motif targeted can generally affect the translatability of an mRNA, either via initiation or some other mechanism, but this is not sufficient evidence to show an effect associated with light. In the second part of this experiment the same three constructs were tested in a protoplast system. It is probably an oversight in the methods, but there is no information given on the promoter used for these constructs although it is presented in the figure. Although the results look promising, they are not robust. This type of analysis is best performed by evaluating the test construct against a control construct, using two different LUC genes. Both mRNA abundance and protein product would need to be monitored in the protoplast system. Possibly the use of mutants could be used to bolster the validity of these results (i.e. a genotype that does not show the light-enhanced expression). Although experiments in this direction are needed, this analysis may be left out of the manuscript without compromising the study.

Response: We agree with the reviewer that *in vitro* translation assay supports only the general enhancement of translatability by the cis-element we analyzed. As suggested, we have described the *T7* promoter used for *in vitro* transcription in the legend of Figure 8 in the revised manuscript for better clarity.
As for the assay performed in protoplasts, we indeed measured both the mRNA and protein products. Nevertheless, we also agree that the experimental outcome was not robust. For dual luciferase assay, the inclusion of an additional luciferase gene, such as Renilla luciferase gene, in the transient assay relies on the assumption that the mRNA and protein activity of this control gene are not regulated by light. Since such information is currently unavailable for Renilla luciferase gene, this gene is unsuitable as an internal control. Since the element we analyzed is also over-represented in 5' UTRs of transcripts with altered translatability in response to hypoxia stress (Figure 8B), it is likely a common translational enhancer and not specific to the light signal. We have thus chosen to focus on its ability in enhancing *in vitro* translation in the revised manuscript. We have modified the conclusion and discussion accordingly.

Specific comments:

(1) The authors have been thoughtful in their use of terms to describe the level of gene expression monitored and the level of gene regulation in evidence from their results. Howe The English in the manuscript could benefit from editing by an individual familiar with the research.
Response: We thank the reviewer for recognizing our efforts in choosing appropriate terminology. Both the original and the revised manuscript have been carefully edited by a professional English editor and also by our colleagues sharing similar research interests.

(2) The authors use terms such as ribosome occupancy, translation rate and ribosome density. They need to be very careful to state only what was observed. For example, by measuring the ratio of mRNA in the polysomal versus total (steady-state) pools, they have assessed what is referred to as translation(al) status. By measuring mRNAs in sucrose gradient fractions of different density, they have an estimate for ribosome number per mRNA, which is okay to call ribosome or polysome occupancy. It would have been preferable for them to show this fractionating the entire gradient into more than four fractions and performing q-RT-PCR or a northern. The actual ribosome density would need to be assessed using a ribosome footprinting/mapping method. Finally, rate of translation was not assessed in this study, so this cannot be asserted.
Response: To avoid introducing additional jargon from related research fields, we adopted the common terminology of "ribosome occupancy" and "ribosome density" from previous literature (Arava et al, 2003; Lackner et al, 2007; Piques et al, 2009). Ribosome occupancy refers to the proportion of the transcript abundance in PL fractions. However, ribosome density, infers the number of ribosomes on transcripts. The association with more ribosomes on a transcript, i.e. higher ribosome density, infers a higher translation rate of this mRNA.
We agree that the actual translation rate is only inferred but not directly

measured. We have modified the "translation rate" in the revised manuscript. We also agree that the combination of ribosome footprinting/mapping and northern blot analysis would provide a more precise measurement of ribosome density for a given mRNA. However, the results we show in Figure 4 have provided clear evidence that light also regulates the translatability of mRNAs by altering their ribosome densities.

Figure 3. The authors have measured "translation status" not ribosome occupancy. The legend of this figure is not sufficiently detailed. Some changes that are needed include, definition of all abbreviations, inclusion of Arabidopsis Gene Identifiers in the legend or figure for the genes with names, definition of the use of red font.

Response: As mentioned above, we chose the terminology to meet general interest. We have elaborated the meaning of "ribosome occupancy" in the revised manuscript for clarity. Arabidopsis Gene Identifiers (AGIs) were used for all genes for better consistency in Figure 3B. In the figure legend, we have added full names and AGIs for all gene abbreviations. The use of red font was also defined in the figure legend.

Figure 4. (a) The authors have used the term "ribosome density" but this analysis is better described as "ribosome occupancy". The authors have used their arrays to monitor the amount of individual gene transcripts in the steady-state and polysomal RNA preparations. This allows them to calculate the percentage of an mRNA in the polysomal complexes. This translation state information might be provided for these mRNAs in Table S3.

(b) Did the authors take into consideration length of transcripts in this analysis?

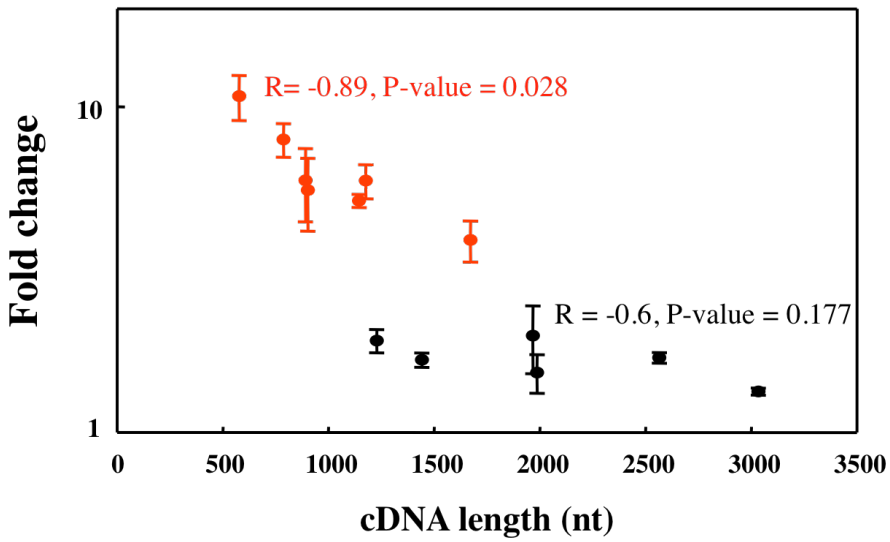
(c) Does this data represent the average from multiple experimental samples?

(d) The authors must also take into consideration that elongation of translation may be regulated. For example on p 10, line 20-24; they need to consider that increased ribosome occupancy could also reflect pausing. Ideally, they should have performed this experiment for HY5 and BBX22 as well.

Response:

(a) Also as mentioned above, we hope the reviewer can agree with the common terminology used. As suggested, we have included the percentage of polysome association for mRNA examined in Figure 4 in the source data for Figure 4.

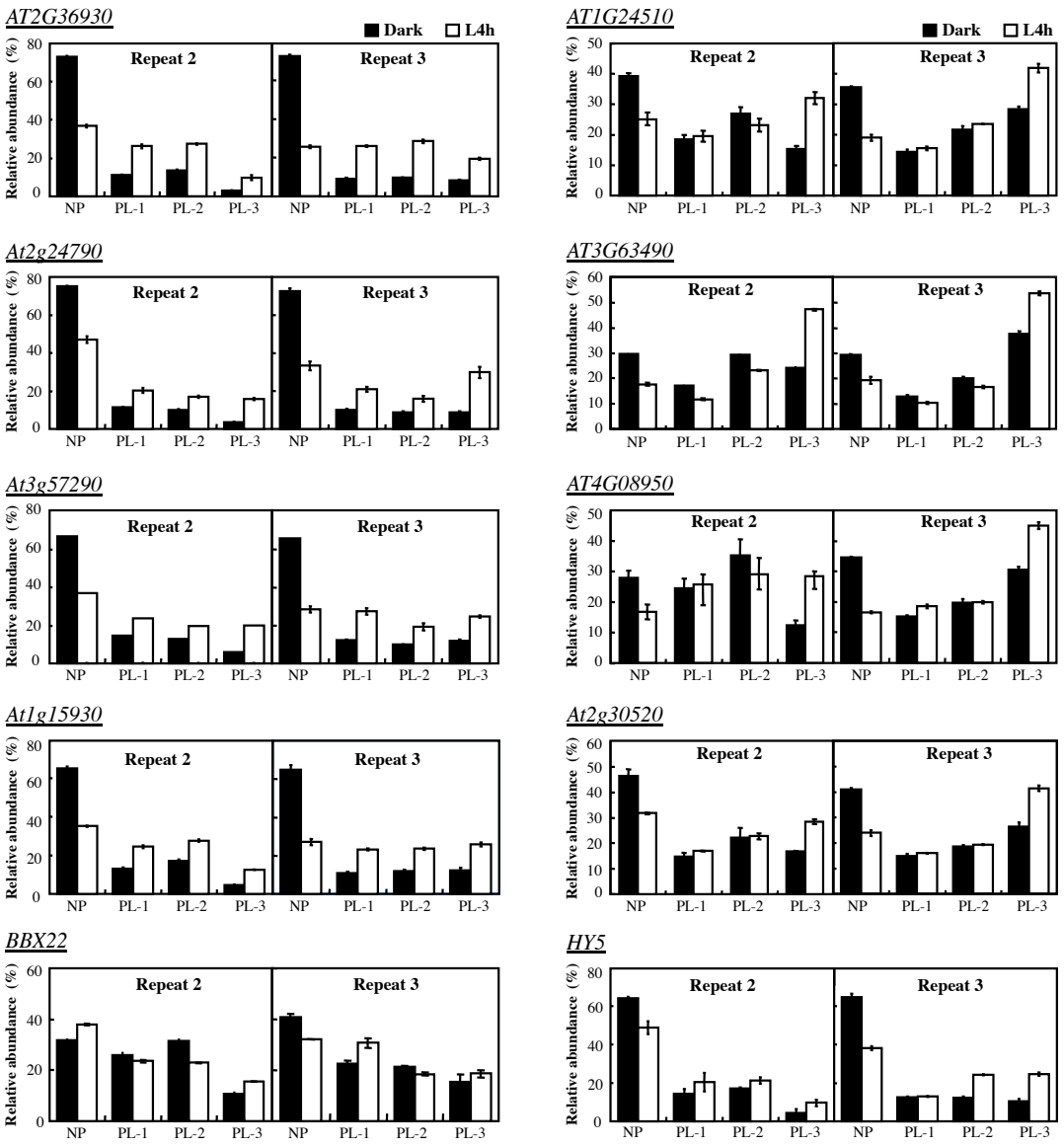
(b) As suggested by the reviewer, we have performed statistical analyses on the 13 genes examined in Figures 3 and 4. Results in "Supplementary Figure S2 for reviewer" indicated that the 7 genes categorized as regulated at the translational level have a shorter transcript length (Spearman rank correlation R=-0.89, P=0.028). The result is consistent with the conclusion that light-mediated translational control favors shorter transcripts.



Supplementary Figure S2 for reviewer The correlation of cDNA length and the degree of translational regulation (fold change) for genes in Figure 3 evaluated by Spearman rank correlation. Only genes regulated at the translational level (in red) show clear anti-correlation between cDNA length and the degree of translational regulation (R=–0.89, P=0.028).

(c) The data presented in Figure 4 are for one representative biological replicate. The error bars are variations of 3 technical replicates. Results for the other 2 independent biological replicates are included in "Supplementary Figure S3 for reviewer".

(d) As pointed out by the reviewer, we have included the possibility of "translational pausing" in p.10 of the revised manuscript. We have also performed ribosome density assays for *HY5* and *BBX22* as suggested. The results shown in Supplementary Figure S6 confirmed that *HY5* is regulated at the translational level. As well, the increase in ribosome occupancy of *HY5* transcripts is evenly distributed among the three PL fractions analyzed, which indicates that the increased ribosome density is not a primary cause of the translational control of *HY5* transcript.



Supplementary Figure S3 for reviewer Two independent biological replicates for results in Figure 4 and Supplementary Figure S6.

Figure 5. (a) In both this figure and Supplemental figure 4 the authors have included a column to describe the level of gene regulation observed. They use the terms "mRNA" and "protein". Appreciating their aim, there may be less ambiguous terminology, such as "transcript accumulation" and "polysome association" to refer to "RNA (or steady-state)" and "Protein (polysome bound)", respectively.
(b) The figure should indicate the criteria for selection of differentially expressed genes. This is in the supplement, but belongs in the main body of the manuscript, especially since different criteria were used.
(c) It is not clear why the authors combined clusters 3 and 4 in the figure presented in the main text. It would be fine to keep them separate, since their cluster 4 shows that some

mRNAs are possibly destabilized even when translated. Turnover can be coupled to translation and their data may be showing this occurs.
(d) It is suggested that the authors include a Supplemental table giving the mean and error values for the four clusters for the four comparisons.

Response:

(a) If possible, we prefer to use the terminology of "RNA" and "Protein" in the figure so it is easier for readers to cross-reference between the manuscript text and the figure. However, we appreciate the suggestions on using a more precise terminology. Thus, in addition to the description in the legend of Figure 5, we have added the description to clarify that "RNA" and "Protein" represent gene expression regulation at the "transcript accumulation" and "polysome association" levels, respectively, in the text when these terms were first mentioned.

(b) The figure legend has been improved as suggested. Criteria used for clustering analysis were also indicated in the "Materials and methods" section of the revised manuscript.

(c) This point was well taken and was the reason we included a Supplementary Figure S3 and Table S2 to show all 4 clusters. The identities of genes in cluster 4 are also listed in Supplementary Table S2. In the revised manuscript, we have added discussion on the possibility of coupled translation and mRNA degradation on p.20-21. Because both cluster 3 and 4 are subjected to regulation at the "Protein" level, we feel it is most beneficial to combine them for the analyses presented in Figures 6 and 7.

(d) As suggested, the mean and standard deviations are listed in Supplementary Figure S3.

Figure 6. I had a very difficult time interpreting this figure from the legend and the text. It is a concern that the authors have used mRNA stability data derived from a cell culture analysis performed by another group. I feel this portion of the manuscript does not add significantly because it is not know if the stabilities of the mRNAs evaluated are similar in the seedling system evaluated.

Response: We agree that the stability for any given mRNA species could vary between a cell culture and young seedlings under the experimental condition we applied. However, the genome-wide mRNA stability data for our experimental condition is currently unavailable. To avoid misleading readers, we have emphasized this point on p.13 of the revised manuscript.

Figure 8: Comments on this analysis were made in the opening statement. If included, the authors should provide a GO analysis on the mRNAs with these motifs. It seems that a table of these genes is not even presented or an alignment to show the consensus.

Response: We apologize for not including a gene list in support of Figure 8. Source data for Figure 8B are included to show genes with two elements reported. We have also performed alignment of the sequences harboring the core element and the flanking sequences extracted from this list of genes (Supplementary Figure S5). The GO analysis of these genes is also included in Supplementary Figure S5.

Supplemental Tables:
(a) The supplemental tables are prepared as PDF files. This made evaluation of this manuscript very difficult. In addition, providing this nice dataset in this manner to readers will diminish the value of this study. The authors should publish these as Excel files.
(b) Also, there are a few typos in the supplemental table headers. Here are some: Table S4: half-lifves were retrived ha; Table S7: expression
Response:

(a) As suggested and also to follow the journal guidelines, the Supplementary tables are resubmitted as Excel files and presented as source files whenever they are associated with specific figures.

(b) We thank the reviewer for reading the draft thoroughly; we have carefully edited the headings for all tables.

Discussion. The authors need to consider the work showing an intersection between COP9/eIF3/uORFs/translation.
Response: We thank the reviewer for this suggestion. We have elaborated on COP9/eIF3/uORFs/translation in the introduction of the revised manuscript (p.4-5).

Literature cited

Arava Y, Wang Y, Storey JD, Liu CL, Brown PO, Herschlag D (2003) Genome-wide analysis of mRNA translation profiles in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **100:** 3889-3894

Branco-Price C, Kawaguchi R, Ferreira RB, Bailey-Serres J (2005) Genome-wide analysis of transcript abundance and translation in Arabidopsis seedlings subjected to oxygen deprivation. *Ann Bot* **96:** 647-660

Chang CS, Maloof JN, Wu SH (2011) COP1-Mediated Degradation of BBX22/LZF1 Optimizes Seedling Development in Arabidopsis. *Plant Physiol* **156:** 228-239

Kawaguchi R, Bailey-Serres J (2005) mRNA sequence features that contribute to translational regulation in Arabidopsis. *Nucleic Acids Res* **33:** 955-965

Lackner DH, Beilharz TH, Marguerat S, Mata J, Watt S, Schubert F, Preiss T, Bahler J (2007) A network of multiple regulatory layers shapes gene expression in fission yeast. *Mol Cell* **26:** 145-155

Piques M, Schulze WX, Hohne M, Usadel B, Gibon Y, Rohwer J, Stitt M (2009) Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in Arabidopsis. *Mol Syst Biol* **5:** 314

2nd Editorial Decision 21 November 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from referee who agreed to evaluate your revised study. As you will see, the referee felt that the revisions made had satisfied his/her main concerns, and is now largely supportive. S/he has some final suggestions for modifications and clarifications, and the Editor has some minor format and content issues, which we would ask you to carefully address in a revision of the present work.

In addition, to the comments raised by Reviewer #3, please address these minor issues before submitting your revised work:

1. Unfortunately, we cannot accept figures in PPT format. We generally prefer EPS files, but PDF files may also be acceptable. Please submit a single image file for each figure, without the figure legend. The resolution of the heatmap shown in Figure 5 remains rather low, please make sure that it is presented in higher-resolution when resubmitting. You will get the best results if the figures are made directly in a high-quality vector graphics program like Illustrator or the free, opensource alternative Inkscape, and saved as EPS.

2. In general, we prefer article titles without colons (":"), and we think that this work could potentially benefit from a more declarative title. For example, "Widespread translational control contributes to the regulation of Arabidopsis photomorphogenesis."

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology

Referee reports

Reviewer #3 (Remarks to the Author):

In this revised manuscript the authors have presented convicing evidence that mRNAs present but poorly translated in seedlings grown in the dark are shifted into polysomal complexes in response to illumination. Their evaluation identifies the regulated mRNAs and goes as far as finding a 5'UTR motif that functions to enhance translation in an in vitro system. This is the first genomic level demonstration that translational control contributes to photomorphogenesis. This solid work should be of interest to researchers in the photomorphogenesis and translation fields.

I evaluated the manuscript as well as the responses to the reviewers. My specific comments have been appropriately addressed. I do have a number of minor comments:

(1) Page 10 line 5. I still find the authors' use of "ribosome occupancy" to be undesirable. At the very least a citation for the terminology is required. Perhaps the authors will reconsider using terms/phrases to describe mRNA association with polysomes that have already be put forth in the literature. A quick survey of similar plant indicates that most have used the more descriptive phrases including, "mRNA association with ribosomes or polysomes"; "polysome-bound mRNAs" or "polysome loading". There is one plant paper that uses "polysome occupancy". None have used the term "ribosome occupancy".
(2) Page 11, line 17, Figures 5 and S3: The authors' should be encouraged to use Figure S3 instead of Figure 5. These two figures overlap in content. Figure S3 provides a more complete analysis,

including one additional cluster. However, use of S3 will require some modification to the accompanying text because it seems to suggest that the "RNA" regulated genes are also regulated at the level of translation in response to the light treatment.
(3) Page 14, lines 12-13. Please check if this statement only refers to in vitro translation systems. If so, it is misleading.
(4) Page 15, line 10. Here the authors take advantage of a dataset that evaluated translational regulation in response to hypoxia in Arabidpsis. It is important to point out whether or not the genes in the "hypoxia" group were those downregulated in response to the stress and upregulated in response to reoxygenation, at the level of polysome loading. I cannot deduce this from the supplemental table because it does not include gene annotation information. (I recommend that the supplemental data be more accessible to future readers by including gene annotation information). I believe the observation is that these motifs are in the genes that are putatively stored in dark as well as during hypoxia. This point can be made.

2nd Revision - authors' response 25 November 2011

Responses to Reviewer #3

In this revised manuscript the authors have presented convincing evidence that mRNAs present but poorly translated in seedlings grown in the dark are shifted into polysomal complexes in response to illumination. Their evaluation identifies the regulated mRNAs and goes as far as finding a 5'UTR motif that functions to enhance translation in an in vitro system. This is the first genomic level demonstration that translational control contributes to photomorphogenesis. This solid work should be of interest to researchers in the photomorphogenesis and translation fields.

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Response: As suggested, we have included citations for the terminology of "ribosome occupancy" used in the studies performed in yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and *Arabidopsis thaliana* (p.10, lines 5-6).

(2) Page 11, line 17, Figures 5 and S3: The authors' should be encouraged to use Figure S3 instead of Figure 5. These two figures overlap in content. Figure S3 provides a more complete analysis, including one additional cluster. However, use of S3 will require some modification to the accompanying text because it seems to suggest that the "RNA" regulated genes are also regulated at the level of translation in response to the light treatment.
Response: In the revised manuscript, both Figure 5 and Supplementary Figure S3 are cited to support the conclusion summarized in p. 11, lines 16-18. We agree with the reviewer that Supplementary Figure S3 has additional details in the clustering analyses. However, the difference in the mRNAss fold changes for clusters 3 and 4 is marginal (1.2 ± 0.2 and 0.9 ± 0.3, respectively). We thus combined these 2 clusters and defined both of them are regulated at the "Protein" level as shown in Figure 5. Genes in the "Protein" group were then used for the analyses shown in Figures 6-8.

(3) Page 14, lines 12-13. Please check if this statement only refers to in vitro translation systems. If so, it is misleading.
Response: We have modified the text into "….. whether the 5' UTRs of transcripts have cis-elements that can benefit the translation." in the revised manuscript (p. 14, lines 17-18).

(4) Page 15, line 10. Here the authors take advantage of a dataset that evaluated

translational regulation in response to hypoxia in Arabidpsis. It is important to point out whether or not the genes in the "hypoxia" group were those downregulated in response to the stress and upregulated in response to reoxygenation, at the level of polysome loading. I cannot deduce this from the supplemental table because it does not include gene annotation information. (I recommend that the supplemental data be more accessible to future readers by including gene annotation information). I believe the observation is that these motifs are in the genes that are putatively stored in dark as well as during hypoxia. This point can be made.

Response: As suggested by the reviewer, we have added the expression data of these genes upon oxygenation in Supplementary Table S6. The description of these genes was also listed in source data for Figure 8B. As the reviewer pointed out, most genes repressed at the translational level under hypoxia will be engaged in translation upon reoxygenation. It is possible that transcripts of these genes are “transiently” stored under unfavorable conditions (dark or hypoxia) and quickly engaged in translation when conditions permit (4 hr of light treatment or 1 hr of reoxygenation). This point was further elaborated in the revised manuscript (p.20, lines 15-26). Because only a small fraction of these genes possess the 2 cis-elements we identified (discussed in p.23), additional sequence features may contribute to the “temporary storage” of these transcripts.