REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a well-written concise and nice story on how the ER ligase NOT4b in the cytosol of Arabidopsis thaliana is important for intra-plastid gene expression through the chloroplast protein PGR3. The direct and functional link between NOT4b and PGR3 is not quite established, but nevertheless the functional connection between NOT4B and PGR3 is clearly established through this very comprehensive analysis. I was wondering if the authors did try to determine the poly-ubiquitination of cytosolic PGR3 under different conditions (eg after blocking proteasomal activity) and genotypic backgrounds (e.g. in wt and in the not4a line or various plastid mutants)? This could further eliminate regulation through PGR3 protein stability as opposed to its cytosolic translation.

I have a a number of specific comments that should be addressed:
1. Please provide the gene identification numbers for the three Arabidopsis NOT4 homologs in the text (I suggest at the start of the Results section). Providing these numbers is really important for the readers and also for text mining to improve gene annotation. Also provide the At id for PGR3. This is also very important because the text and suppl fig 1 do not match where describing the TDNA lines and the genes not4b and not4c.

Not4b - in suppl Fig 2 this is At3G45630 and SAIL_274_D03
Not4c - in Suppl Fig 2 this is At2G28540 and SALK_093123

However in the methods it states (page 13):
- not4b TDNA line is listed as SALK_079194 - this line has no TDNA insertion according to SALK.
- not4c is listed as SAIL-274_D03

So which gene is 4b and which is 4c? and which tDNA lines were actually used?

Also, in suppl fig 2, please indicate the positions of the primers used for genotyping

- figure 1 - explain RuBL (if rubisco large subunit - more common is RBCL. For the starch staining, when were the leaves harvested?
- figure 2a. This volcano would be more informative if the gene ids would be provided for those 8-10 genes that are highly significant and/or have extreme ratios. These ~8-10 data points could be numbered and the gene ids provided in the legend. Is PGR3 one of those data points?

- figure 3 and text (page 6 - line 204). What was the proteome that was extracted and analyzed? Total leaf rosette including membranes? Or soluble proteomes? Perhaps this is the same as in the citation (Helm) but it be very useful to mention this here.

- please submit the proteome/mass spec data sets to ProteomeXchange /PRIDE

Page 7 line 208. Why is it so remarkable? The plants are yellow - hence one expects to see loss of thylakoid proteins. And what is meant by central enzymes? The Calvin cycle proteins collectively seem not much reduced (fig 3C). (There is another 'remarkably a few lines lower - line 216 on the same page)

- fig 4d - it would be great to include standard deviations for panel d (D1 protein)

Figure 6a. same comment as for the volcano plot in figure 2a.

Reviewer #2 (Remarks to the Author):

The experiments in this manuscript explore the function of an Arabidopsis homolog of a protein called NOT4 that had been studied previously in animals and yeast. NOT4 has both a RING Finger and RRM domain- the former involved in ubiquitin-mediated proteolysis and the latter in RNA
binding. Quality-control functions have been attributed to NOT4 homologs in animals and yeast, and these involve cotranslational mRNA and protein degradation.

Analysis of a mutant lacking one of the NOT4 paralogs in Arabidopsis, NOT4A, revealed a defect in chloroplast biogenesis. This effect was then attributed to the absence of the mRNA encoding a PPR protein called PGR3. PGR3 activates the expression of several chloroplast genes by stabilizing specific RNAs and/or activating translation. The loss of PGR3 mRNA in the mutant was shown by RNA-seq and by qRT-PCR, so this seems to be convincing. This is intriguing for many reasons, including that, to my knowledge, NOT4 homologs in other organisms have been shown only to decrease gene expression (by RNA or protein degradation), not increase expression as is suggested by these results for NOT4A.

However, the data are too preliminary to instill confidence in the key conclusion that the lack of PGR3 expression underlies the phenotype of the not4a mutant (examples below). Furthermore, even if that were convincingly shown, I feel that some bit of insight into mechanism would be needed to make this study suitable for publication in this journal. This is especially important given that NOT4 in other systems stimulates RNA decay, whereas NOT4A here is proposed to stabilize a specific RNA.

Major points.

1. The authors conclude that the absence of PGR3 causes the chloroplast defects in the not4a mutant. However, the chloroplast defects were analyzed only superficially, and the defects detected (reduced translation, reduction in several photosynthetic complexes) are extremely common.

To support this key conclusion, it is essential to show that the not4a mutant phenocopies pgr3 in assays that are truly diagnostic for pgr3. The most convincing assay would be northern blot analysis of petL transcripts, because PGR3 is the only protein known to be required to stabilize these RNAs. The pgr3 mutant should be included on the same gel, for comparison.

Similarly, the analysis of chloroplast rRNAs in Fig 3e and Supp Fig 5 (not supp fig 4 as stated in fig 3) is inadequate because the assay (Agilent tapestation analysis of total RNA) did not resolve the incompletely processed isoforms that have been reported for pgr3. Northern blots should be probed for 16S and 23S rRNA, again with the pgr3 mutant included for comparison.

2. 2D immunoblots were used to examine the abundance of photosynthetic complexes. Although the results do show the loss of the cytochrome b6f complex, they are less informative with regard to the abundance of other complexes. It would be much more informative to examine the degree to which various complexes are reduced by using standard SDS-PAGE western blots probed for subunits of each complex. This is a standard, simple and more quantitative approach. The pgr3 mutant should be analyzed in an adjacent lane so that the two can be directly compared.

The proteome data in Figure 3 were used to conclude a specific loss of 30S ribosomal proteins. First, this needs to be validated by quantitative western blots. Second, this needs to be compared directly to a pgr3 mutant to determine whether they behave similarly.

3. The authors claim that a PGR3-YFP fusion restores the not4 phenotype to the wild-type. However, this is only relevant to the hypothesis if the same construct complements the pgr3 phenotype, and this does not go without saying: it would not be surprising if the large C-terminal extension interferes, in particular, with PGR3’s translation activation functions. It is essential to show that the same construct complements a pgr3 mutant to support these claims (or cite a paper that shows that a similar construct complements).

4. Line 389-391: Because mRNA from the pgr3-yfp transgene accumulated to high levels in the
not4A background, the authors conclude that NOT4A must act on sequences that were not present in the transgene—"such as the 3’ UTR or distal elements". Given the highly surprising nature of these data, this should be tested. This would be straightforward by using reporter transgenes fused to the PGR3 3’ UTR and “distal elements”.

Minor points.

The title refers to regulating chloroplast translation. But PGR3’s effects are not simply at the translational level.

Much of the data shown in the body of the paper would be more appropriate for supplemental figures or could be eliminated entirely. E.g. The transcriptome overview in Fig 3 is not informative because mutants that look the way this one looks always have changes in the nuclear transcriptome due to retrograde signaling effects. The summaries of proteome data in Figure 3 are suggestive only and should be supplemental data, with validating western blots shown in the body of the paper.

Unusual methods were used to assess effects on chloroplast translation, when standard approaches (e.g. pulse labeling, polysome assays) would be more informative. The puromycin data in Fig 4c are mysterious. What are the bands? This needs more explanation and the pgr3 mutant should be included for comparison.

The authors model would seem to require that NOT4A acts quite specifically on the PGR3 mRNA. What could the basis for this specificity be? It seems unlikely that NOT4A is a sequence-specific RNA binding protein, given that it has just one RRM domain. Do NOT4 homologs show sequence-specific binding to particular mRNAs? This point should be discussed much more thoroughly.

Line 424: this important sentence is confusing: “Not4A may inhibit the 3’ UTR recruitment of PGR3 to mRNA decay in plants.” I think this is saying that Not4A prevents decay of PGR3 mRNA. Is there any precedent for NOT4 proteins acting in this way? I believe that the characterized members STIMULATE RNA decay. This needs to be explained and discussed more clearly/fully in the discussion.
Response to reviewers

We thank the reviewers for their useful and detailed comments on this manuscript. Below we outline our responses to each point in blue.

Reviewer #1 (Remarks to the Author):

I was wondering if the authors did try to determine the poly-ubiquitination of cytosolic PGR3 under different conditions (eg after blocking proteasomal activity) and genotypic backgrounds (e.g. in wt and in the not4a line or various plastid mutants)? This could further eliminate regulation through PGR3 protein stability as opposed to its cytosolic translation.

We have now included new data (new Figure S8) where we analyzed the poly-ubiquitination of PGR3-YFP by performing anti-GFP immunoprecipitation from Col-0 and not4a transgenic lines treated with bortezomib and deubiquitinating enzyme inhibitor, followed by anti-Ub Western blotting (line 378). This shows that polyubiquitination of PGR3 could not be detected.

Please provide the gene identification numbers for the three Arabidopsis NOT4 homologs and PGR3 in the text (I suggest at the start of the Results section)

We have included gene IDs when genes are first mentioned in the results section, as well as in the methods section.

Correct inconsistency error of not4b and no4c SALK and SAIL mutant lines

We apologise for this inconsistency and have now corrected this error in the methods section.

Also, in suppl fig 2, please indicate the positions of the primers used for genotyping

We have now added primer positions to Figure S2A.

Figure 1. explain RuBL (if rubisco large subunit - more common is RBCL.)

We have corrected to the conventional RBCL gene-name in Figure 1H, legend and main text.

For the starch staining, when were the leaves harvested?

We have added harvest age to the main text (line 166), in addition to the methods section.

Figure 2a. This volcano would be more informative if the gene ids would be provided for those 8-10 genes that are highly significant and/or have extreme ratios. These ~8-10 data points could be numbered and the gene ids provided in the legend. Is PGR3 one of those data?

The top 8 DEGs have now been labelled in Figure 2A with the gene IDs provided in the legend. This has also been undertaken for the proteomics volcano (Figure 3A). PGR3 was undetected in these analyses and only featured in WT plants, not the mutant, as highlighted in Figure 6A and the main text (line 308).

Figure 3 What was the proteome that was extracted and analyzed? Total leaf rosette including membranes? Or soluble proteomes? Perhaps this is the same as in the citation (Helm) but it be very useful to mention this here.
We have added additional information describing the tissue used and procedure undertaken, in the main text (line 210) and methods.

Please submit the proteome/mass spec data sets to ProteomeXchange /PRIDE

This will be done prior to publication.

Page 7 line 208. Why is it so remarkable? The plants are yellow - hence one expects to see loss of thylakoid proteins. And what is meant by central enzymes? The Calvin cycle proteins collectively seem not much reduced (fig 3C). (There is another ‘remarkably a few lines lower - line 216 on the same page)

We have now removed ‘remarkably’ and ‘central enzymes’ and improved the narrative accuracy in this section.

Figure 4d. It would be great to include standard deviations for panel d (D1 protein)

We have now included a graph (including standard error bars) that is averaged across three biological replicates in Figure 4D (this was previously one representative replicate).

Figure 6a. Same comment as for the volcano plot in figure 2a.

The top 8 DEGs have now been labelled in Figure 6A with the gene IDs provided in the figure legend.

Reviewer #2 (Remarks to the Author):

Major points.

1. The authors conclude that the absence of PGR3 causes the chloroplast defects in the not4a mutant. However, the chloroplast defects were analyzed only superficially, and the defects detected (reduced translation, reduction in several photosynthetic complexes) are extremely common.

We present data indicating that not4a lacks PGR3 expression (Figure 6B and S6B), which is restored in NOT4A complementation lines, and we were able to complement much of the dysfunction of the not4a mutant with heterologous expression of PGR3 (Figures 7D, E). Further, we demonstrate extensive parallels between a null pgr3 mutant and the not4a mutant (Figures 6C,D,E,F,G and H), including new western blotting data as requested (and outlined further below). As such we believe we have provided robust evidence that the defects we observe in not4a are largely consequence of a loss of PGR3 expression.

Whilst many of the phenotypes observed may be common amongst mutants in chloroplast function, petL and petG, as you highlight, are direct targets of PGR3 required for their stabilization, and we show levels of these to be specifically reduced in the not4a and pgr3 mutants (Figure 6F). Additionally, we have included new Western blot data (Figure 6E) showing the 30S subunits RPS1 and RPS7 are similarly reduced in abundance in pgr3 as they are in the not4a mutant, which complements existing data demonstrating reduced 30S-specific plastid ribosomal RNA abundance in both mutants (Figure 6E and G).

The depletion of the 30S subunit of the chloroplast ribosome, from a proteomics perspective, is truly unique, suggesting specificity in the underlying mechanisms. We reached this conclusion by comparing the proteome data acquired here with a set of quantitative
proteome variations identified for different mutant lines in S. Baginsky’s group. In all proteomics data the abundance of 30S and 50S subunits is co-regulated. For example, we generated a reference proteome for albino/pale-green mutants (APG) in order to map the effects of proteome adaptation to a defect in photosynthesis (Motohashi et al., 2012). This analysis identified many characteristics of proteomes of PS-depleted plants, among them elevated abundance of plastid ribosomes that encompass both subunits. While this is in part due to normalization effects in the absence of photosynthetic proteins, it indicates that both subunits are co-regulated in the different APG lines used for our analysis. This notion was also substantiated by the proteome of two mutants (ppi2 and tic56) with a defect in ribosome assembly. Here, both subunits were depleted, confirming the joint regulation and accumulation of both chloroplast ribosomal subunits (Köhler et al., 2016). We have highlighted the uniqueness of this molecular phenotype in the main text (line 226).

To support this key conclusion, it is essential to show that the not4a mutant phenocopies pgr3 in assays that are truly diagnostic for pgr3. The most convincing assay would be northern blot analysis of petL transcripts, because PGR3 is the only protein known to be required to stabilize these RNAs. The pgr3 mutant should be included on the same gel, for comparison. We previously presented qPCR analysis, a highly specific and quantitative approach, to demonstrate that petL and petG transcripts are significantly less abundant in both the not4a and pgr3 mutants than WT controls (Figure 6F), indicating reduced stabilization. In addition to this, we have included new Western blot data (new Figure 6E) confirming that petC and NdhH subunits are reduced in both the not4a and pgr3 mutants, as well as a specific reduction in 30S subunits and rRNAs (Figure 6E and G); a unique molecular phenotype that both mutants share (as discussed above).

Similarly, the analysis of chloroplast rRNAs in Fig 3e and Supp Fig 5 (not supp fig 4 as stated in fig 3) is inadequate because the assay (Agilent tapestation analysis of total RNA) did not resolve the incompletely processed isoforms that have been reported for pgr3. Northern blots should be probed for 16S and 23S rRNA, again with the pgr3 mutant included for comparison.

This approach semi-quantitatively demonstrates reduction in total abundance of the 16S rRNA, specific to the 30S ribosomal subunit. Although it does not resolve incompletely processed rRNAs, these would not be incorporated into assembled functional ribosomes. We use this data to support our conclusion that the 30S ribosome subunit (and not the 50S) is reduced in both the pgr3 and not4a mutants. Both of these mutants have reduced abundance of 16S rRNAs, which correlates with a proportional reduction in assembled 30S subunits, irrespective of rRNA processing. To substantiate this claim our new comparative data (Figure 6E) indeed confirms the finding from our quantitative proteome analysis that the 30S subunits RPS1 and RPS7 are reduced in abundance in both the pgr3 and not4a mutants. We have also corrected the Supp figure cross referencing error.

2. 2D immunoblots were used to examine the abundance of photosynthetic complexes. Although the results do show the loss of the cytochrome b6f complex, they are less informative with regard to the abundance of other complexes. It would be much more informative to examine the degree to which various complexes are reduced by using standard SDS-PAGE western blots probed for subunits of each complex. This is a standard, simple and more quantitative approach. The pgr3 mutant should be analyzed in an adjacent lane so that the two can be directly compared.

In addition to our quantitative proteomics analysis (Figure S4D) which supports the 2D results, we have included new Western blot data (new Figures 2E, 3D and 6E) showing
that the NDH subunit ndhH and the Cyt b6f subunit PetC, are reduced in abundance in both the not4a and pgr3 mutants when compared in adjacent lanes from plants grown under the same conditions.

The proteome data in Figure 3 were used to conclude a specific loss of 30S ribosomal proteins. First, this needs to be validated by quantitative western blots. Second, this needs to be compared directly to a pgr3 mutant to determine whether they behave similarly.

In addition to the comparative analysis indicating a 30S-specific reduction of the 16S rRNA (Figure 6G) we have included new data (new Figure 3D and 6E) using available commercial antibodies (Agrisera) to show that in the pgr3 and not4a mutants, the 30S subunits RPS1 and RPS7 are reduced in abundance, unlike the 50S subunits RPL2 and RPL4. The observed abundances are similar in not4a and pgr3, and reflect the patterns observed in the quantitative proteomics (Figure 3F, red arrows).

3. The authors claim that a PGR3-YFP fusion restores the not4 phenotype to the wild-type. However, this is only relevant to the hypothesis if the same construct complements the pgr3 phenotype, and this does not go without saying: it would not be surprising if the large C-terminal extension interferes, in particular, with PGR3’s translation activation functions. It is essential to show that the same construct complements a pgr3 mutant to support these claims (or cite a paper that shows that a similar construct complements).

We have now included this important control as new data (new Figure S7a), showing that the PGR3-YFP construct complements the pgr3-4 mutant as it does the not4a mutant. Further, we have included new data (new Figure S7c) confirming that the not4a mutant complemented with PGR-YFP still lacks NOT4A expression. These new data support the conclusion that the functional PGR3-YFP construct is responsible for complementing the not4a phenotype, providing additional support to our conclusion that not4a mutant defects are largely due to a lack of PGR3 expression.

4. Line 389-391: Because mRNA from the pgr3-yfp transgene accumulated to high levels in the not4A background, the authors conclude that NOT4A must act on sequences that were not present in the transgene- “such as the 3’ UTR or distal elements” Given the highly surprising nature of these data, this should be tested. This would be straightforward by using reporter transgenes fused to the PGR3 3’ UTR and “distal elements”.

NOT4 proteins in mammals and yeast have varied functions related to protein ubiquitination and turnover, ribosome function, and mRNA processing via deadenylation. As such, there are a range of potential mechanisms (either direct or indirect) through which NOT4A could be regulating production of PGR3. Full exploration of these potential scenarios will be the focus of future studies, but this will take quite some time and is beyond the scope of this initial report that connects NOT4A to chloroplast function. We have expanded and improved the discussion, and in particular have highlighted that there are several ways in which NOT4A might be acting to control PGR3 production, based on known functions of these enzymes in other species.

Minor points.

The title refers to regulating chloroplast translation. But PGR3’s effects are not simply at the translational level.

The title has been adjusted to encompass these wider functions in “chloroplast function”.

Much of the data shown in the body of the paper would be more appropriate for supplemental figures or could be eliminated entirely. E.g. The transcriptome overview in Fig
3 is not informative because mutants that look the way this one looks always have changes in the nuclear transcriptome due to retrograde signaling effects. The summaries of proteome data in Figure 3 are suggestive only and should be supplemental data, with validating western blots shown in the body of the paper.

To make transcriptomic and proteomic volcano plots more informative the top 8 DEGs have been labelled (Figure 2A, 3A, 6A, as requested by reviewer 1) with the gene IDs provided in the figure legend. As requested, we have also included the new western blots in the main body of the paper (Figures 2E, 3D and 6E) showing that the NDH subunit ndhH and the Cyt b6f complex subunit petC and the 30S ribosomal subunits RPS1 and RPS7 are reduced in abundance in both the not4a and pgr3 mutants.

Unusual methods were used to assess effects on chloroplast translation, when standard approaches (e.g. pulse labeling, polysome assays) would be more informative. The puromycin data in Fig 4c are mysterious. What are the bands? This needs more explanation and the pgr3 mutant should be included for comparison.

This is an analogous approach to radio pulse labelling but removes the need to handle radioactive material, hence is a safer alternative. It has been used to measure total translation in plants previously (Van Hoewyk, 2016) and is widely used in other model organisms, including in studies of mitochondrial translation (Wu et al 2018). We have included further explanation of the puromycin incorporation assay in the main text (line 247-252).

We have also included additional data supporting the plastid translation defects of not4a in our analysis of the chloroplast encoded D1 protein re-synthesis (Figure 4D), which now presents an average of three biological replicates with standard error bars (previously one representative replicate). The translational defects are further supported by the severe sensitivity of the not4a mutant to lincomycin, a plastid ribosome inhibitor (Figure 4A and B).

Although some of these analyses have not been performed on the pgr3 mutant we have demonstrated extensive parallels between a null pgr3 mutant and the not4a mutant using many other assays (Figures 6C,D,E,F,G and H), including lincomycin sensitivity, chloroplast rRNA analysis and the new Western blot data showing reduced protein abundance of 30S ribosomal subunits in the pgr3 mutant compared to not4a. Moreover, we have shown that ectopic expression of PGR3 restores a WT-like phenotype to not4a.

The authors model would seem to require that NOT4A acts quite specifically on the PGR3 mRNA. What could the basis for this specificity be? It seems unlikely that NOT4A is a sequence-specific RNA binding protein, given that it has just one RRM domain. Do NOT4 homologs show sequence-specific binding to particular mRNAs? This point should be discussed much more thoroughly.

We have expanded and improved our discussion to address your points, including discussion of NOT4 RNA binding and clarification of possible activities of NOT4 that may regulate PGR3 expression which are not limited to direct binding of the PGR3 mRNA. We have been careful to not include a model, per se, but rather to present different scenarios that will be the subject of future investigation.

Line 424: this important sentence is confusing: “Not4A may inhibit the 3’ UTR recruitment of PGR3 to mRNA decay in plants.” I think this is saying that Not4A prevents decay of PGR3 mRNA. Is there any precedent for NOT4 proteins acting in this way? I believe that the characterized members STIMULATE RNA decay. This needs to be explained and discussed more clearly/fully in the discussion.
We have reworded and elaborated upon the sentence causing confusion, as part of a wider rewrite of the discussion. We further discuss the expansion of NOT4 genes in plants and the possible functional divergence of the CCR4-NOT complex through which NOT4 stimulates mRNA decay in other organisms (which may target negative regulators of PGR3 expression in plants).

We have also included several further references in the paper to support new information and concepts that are presented.

Köhler, D., Helm, S., Agne, B., and Baginsky, S. (2016). Importance of Translocon Subunit Tic56 for rRNA Processing and Chloroplast Ribosome Assembly. Plant Physiology 172, 2429–2444.

Motohashi, R., Rödiger, A., Agne, B., Baerenfaller, K., and Baginsky, S. (2012). Common and specific protein accumulation patterns in different albino/pale-green mutants reveals regulon organization at the proteome level. Plant Physiology 160, 2189–2201.

Van Hoewyk, D. (2016). Use of the non-radioactive SUnSET method to detect decreased protein synthesis in proteasome inhibited Arabidopsis roots. Plant Methods 12, 20.

Wu, Z., Wang, Y., Lim, J., Liu, B., Li, Y., Vartak, R., Stankiewicz, T., Montgomery, S., and Lu, B. (2018). Ubiquitination of ABCE1 by NOT4 in Response to Mitochondrial Damage Links Co-translational Quality Control to PINK1-Directed Mitophagy. Cell Metabolism 28, 130–144.e137.
REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

the authors responded satisfactory to both reviews.

Reviewer #2 (Remarks to the Author):

This is a revised version of a manuscript that comes to the intriguing conclusion that the NOT4A ubiquitin ligase increases abundance of the mRNA encoding the chloroplast-localized PPR protein PGR3, and acts specifically on this mRNA. This is an intriguing finding for many reasons, including the specificity for PGR3 (other NOT4 homologs do not show this degree of specificity, to my knowledge) and that (to my knowledge) other characterized NOT4 homologs decrease gene expression (by RNA or protein degradation), rather than increase expression.

A key prediction of the authors’ model is that not4a mutants should phenocopy pgr3 mutants. My primary concern previously was lack of confidence that this had been rigorously demonstrated. This is straightforward to test conclusively, and I had suggested specific ways to do so. Yet this revised manuscript side-steps the experiments that would have provided the most compelling evidence.

Major points.

1. The authors added a Western blot (Fig 2e) showing data for subunits of the two complexes that are known to be reduced in pgr3 mutants (PetC for cytochrome b6f and NdhH for the NDH complex). However, a general chloroplast translation defect (as likely occurs in not4a mutants) would lead to the loss of these proteins, as well as to loss of all other complexes that include subunits encoded in the chloroplast genome. The relevant question is whether there is a SPECIFIC loss of these two complexes (as reported for pgr3 mutants). In fact, other new data (S4C, S4D) show that PSI and ATP synthase are reduce in not4A mutants. These complexes are not known to be reduced in pgr3 mutants. So, it remains unclear whether the protein phenotypes of the two mutants are similar.

Therefore, it is essential to show the analogous western blot data for subunits of those complexes that are not strongly affected in pgr3 mutants: Rubisco, PSI, PSII, ATP synthase. To be convincing, pgr3 and not4a mutants should be grown in parallel and analyzed on the same gel, and the gels should include dilutions of Col-0 samples to aid in quantitative comparisons. The authors model predicts that these complexes would be relatively unaffected in not4a mutants, as is known to be the case in pgr3 mutants.

(ii) The single most diagnostic feature of pgr3 mutants is the specific loss of petL-containing transcripts. This is readily detected by Northern blotting. I requested this experiment, but the authors instead present other, less compelling pieces of evidence that pgr3 and not4a phenocopy one another.

- they argue that the specific loss of 30S subunits is a diagnostic feature of pgr3 mutants. However, this is not the case. For example, ppr4 mutants, which are defective in splicing a chloroplast mRNA encoding a protein in the 30S subunit, shows a stronger defect in 30S than 50S accumulation.

- qRT-PCR data are presented to show the loss of petL and petG RNA. However, these transcripts (together with many others) are reduced in all mutants with defects in chloroplast translation. This is believed to be a consequence of reduced transcription by the plastid-encoded polymerase. I suspect that analogous qRT-PCR assays of many other chloroplast genes encoding photosystem subunits who should show an analogous decrease in not4a mutants (but not in pgr3 mutants). The authors claim in the response to reviews that new data in Figure 6F show that petL and expression
is "specifically reduced in the not4a and pgr3 mutants." However, this is not true: only petL and petG RNAs were examined, so specificity for these two genes cannot be assessed.

It is essential to show Northern blot data that illustrate the unique features of RNA accumulation from the petL-petG region in pgr3, side-by-side with not4a. If they cleanly show a similar transcript pattern, I would be convinced!

Minor points

The Abstract states that PGR3 is required for "translating 30S ribosome subunits and several thylakoid-localized photosynthetic components." I feel that this distorts what is known about PGR3 by over-emphasizing its role in chloroplast translation. I mention this because the foundational work on PGR3 (Shikanai lab) did not detect effects on chloroplast translation. This work involved alleles that may not have been null. Still, those results made clear that the major function of PGR3 is to stabilize petL mRNA and stimulate petL translation. The more recent analysis of a null allele by ribosome profiling (Rojas et al, NAR) confirmed that the major effect is on petL-petG expression, and that there is also a small defect in rps8-rpl14 expression. The rRNA analysis in the latter paper showed a minor decrease in mature 16S and 23S rRNAs and an increased abundance of precursor forms of both rRNAs. Those Northern blot results did not show a strong bias toward loss of 16S accumulation/processing (compared to 23S), differing from claims here. I am puzzled by this difference.
Response to reviewers Reviewer #1 (Remarks to the Author):

We are pleased that reviewer 1 was satisfied with the resubmitted version of this work. Below we outline our responses (in blue) to the remaining queries raised by reviewer 2.

Reviewer #2 (Remarks to the Author):

This is a revised version of a manuscript that comes to the intriguing conclusion that the NOT4A ubiquitin ligase increases abundance of the mRNA encoding the chloroplast-localized PPR protein PGR3, and acts specifically on this mRNA. This is an intriguing finding for many reasons, including the specificity for PGR3 (other NOT4 homologs do not show this degree of specificity, to my knowledge) and that (to my knowledge) other characterized NOT4 homologs decrease gene expression (by RNA or protein degradation), rather than increase expression.

A key prediction of the authors’ model is that not4a mutants should phenocopy pgr3 mutants. My primary concern previously was lack of confidence that this had been rigorously demonstrated. This is straightforward to test conclusively, and I had suggested specific ways to do so. Yet this revised manuscript side-steps the experiments that would have provided the most compelling evidence.

We do not believe that NOT4A exclusively regulates the $PGR3$ mRNA, but rather that the major chloroplast defects we observe in not4a can be ascribed to the fact that $PGR3$ expression does not occur in this mutant. In addition to extra experimental data added in the previous and current revisions, we believe we have already provided compelling evidence for this:

Firstly, we show that $PGR3$ is not expressed in not4a (it is not detected in RNA seq [Fig 6A], and not detected by qPCR [Fig 6B]), and that its expression is fully restored in the $pNOT4A$-NOT4A complementation line (Fig 6B). This means that (i) NOT4A is required to express $PGR3$ and (ii) the not4a mutant will by definition share all the same defects as pgr3, because it does not make this transcript.

Secondly, we show that ectopic expression of $PGR3$-YFP in the not4a mutant restores WT-like phenotypes (e.g. Fig 7E and D), despite NOT4A expression still being absent (Supp Fig 7C, new data added in the previous revision). The fact that transgenic $PGR3$ (which we showed could complement pgr3-4 as per the reviewer’s previous request; Supplemental Fig 7a) can compensate for a lack of NOT4A in the not4a mutant is further compelling evidence that a majority of the defects observed in not4a are a consequence of loss $PGR3$ of expression.

We fully expect that NOT4A (either alone, or in redundancy with NOT4B and C) will have other targets, and some of these may also partly contribute to the chloroplast defects reported here. But our work clearly shows a connection between NOT4A and PGR3 within the context of the phenotypes we discuss. We have now modified the title of the manuscript to acknowledge this, which now reads:

“The Arabidopsis NOT4A E3 ligase promotes PGR3 expression and regulates chloroplast function”

We have now also added further data to the manuscript in response to the reviewer’s specific requests below.

Major points.

1. The authors added a Western blot (Fig 2e) showing data for subunits of the two complexes that are known to be reduced in pgr3 mutants (PetC for cytochrome b6f and NdhH for the NDH complex). However, a general chloroplast translation defect (as likely occurs in not4a mutants)
would lead to the loss of these proteins, as well as to loss of all other complexes that include subunits encoded in the chloroplast genome. The relevant question is whether there is a SPECIFIC loss of these two complexes (as reported for pgr3 mutants). In fact, other new data (S4C, S4D) show that PSI and ATP synthase are reduce in not4A mutants. These complexes are not known to be reduced in pgr3 mutants. So, it remains unclear whether the protein phenotypes of the two mutants are similar.

Therefore, it is essential to show the analogous western blot data for subunits of those complexes that are not strongly affected in pgr3 mutants: Rubisco, PSI, PSII, ATP synthase. To be convincing, pgr3 and not4a mutants should be grown in parallel and analyzed on the same gel, and the gels should include dilutions of Col-0 samples to aid in quantitative comparisons. The authors model predicts that these complexes would be relatively unaffected in not4a mutants, as is known to be the case in pgr3 mutants.

To our knowledge there has not been an exhaustive analysis (i.e. proteome assessment) of photosynthetic protein abundance in pgr3 mutants, and many of the previous studies have been conducted on point mutation alleles (pgr3-1, -2 and -3) that are incomplete knockouts (i.e. hypermorphs), as reviewer 2 also notes below. As such we do not know the full spectrum of photosynthetic proteins that would be mis-regulated in a full knockout of PGR3. However, Yamazaki et al (2004) and Fujii et al (2013) did conduct western blots for several photosynthetic components, showing specific depletion of Pet and NDH subunits in some of the pgr3 alleles, but no obvious reduction in the steady state levels of some subunits from other complexes.

We showed that not4a, similarly to pgr3-4, has highly reduced levels of PetC and NdhH (Fig), but we do agree with the reviewer that western blot analysis of other photosynthetic complex subunits would provide broader evidence for the similarities in not4a and pgr3. As such, we now provide new western blotting data (new Figure S4D) for a range of nuclear- and chloroplast-encoded proteins from different photosynthetic complexes (for which we have available antibodies), and as requested we have conducted dilutions of WT samples for comparison. This includes: (1) Chloroplast-encoded Rubisco subunit RBCL; (2) Chloroplast-encoded PSII subunit D1/PsBA; (3) nuclear encoded LHCA4, which associates with PSI; (4) nuclear encoded PSII subunit PsbP. For all four of these proteins we see the same pattern - no significant depletion in either not4a and pgr3 - which is in keeping with the conclusion/model that not4a and pgr3-4 share similar and specific defects linked to Pet, NDH and 30S ribosomes. The blot is shown here:

(ii) The single most diagnostic feature of pgr3 mutants is the specific loss of petL-containing transcripts. This is readily detected by Northern blotting. I requested this experiment, but the authors instead present other, less compelling pieces of evidence that pgr3 and not4a phenocopy one another.
- they argue that the specific loss of 30S subunits is a diagnostic feature of pgr3 mutants. However, this is not the case. For example, ppr4 mutants, which are defective in splicing a chloroplast mRNA encoding a protein in the 30S subunit, shows a stronger defect in 30S than 50S accumulation.

We thank the reviewer for highlighting this for us. This would make sense given that ppr4 specifically targets the 30S subunit rps12 plastid RNA for trans-splicing, and so could also be considered a specific case, like we are reporting here for not4a. We had previously identified PPR4 (At5g04810) as upregulated in not4a relative to WT (see heatmap figure S6B). We have modified the text at line 226 (and included two new PPR4 references), which now reads:

“This is reminiscent of maize and Arabidopsis ppr4 mutants, which have a specific depletion of the small ribosomal subunit due to the role of PPR4 plays in trans-splicing plastid rps12 transcripts (Schmitz-Linneweber et al. 2006; Tadini et al. 2018). Such an observation indicates a highly specific defect in not4a, since proteomes of most pale green mutants consistently display co-regulation of 50S and 30S abundance (Köhler et al., 2016; Motohashi et al., 2012).”

And also Line 307: “Amongst these genes were 34 chloroplast targeted PPR proteins, including PPR4 (an essential factor for ribosome small subunit biogenesis), which collectively may be upregulated to compensate for compromised translation in the mutant (Figure S6B, Data file 2)”

- qRT-PCR data are presented to show the loss of petL and petG RNA. However, these transcripts (together with many others) are reduced in all mutants with defects in chloroplast translation. This is believed to be a consequence of reduced transcription by the plastid-encoded polymerase. I suspect that analogous qRT-PCR assays of many other chloroplast genes encoding photosystem subunits would show an analogous decrease in not4a mutants (but not in pgr3 mutants). The authors claim in the response to reviews that new data in Figure 6F show that petL and expression is “specifically reduced in the not4a and pgr3 mutants.” However, this is not true: only petL and petG RNAs were examined, so specificity for these two genes cannot be assessed.

It was previously reported (although the data was not shown) in Yamazaki et al (2004) that PetA, B, and D transcripts are not reduced in the pgr3 hypermorph mutants. We have now conducted additional qPCRs to detect these transcripts in both not4a and pgr3-4. New Figure S6D shows none of these chloroplast-encoded Pet RNAs are depleted in either mutant (in fact, PetA is increased in both mutants relative to their WT), which does confirm specificity towards PetL and PetG. The fact these transcripts occur at a similar level to WT in both mutants also argues against ‘reduced transcription by the plastid encoded polymerase’. The data are shown here:
It is essential to show Northern blot data that illustrate the unique features of RNA accumulation from the petL-petG region in pgr3, side-by-side with not4a. If they cleanly show a similar transcript pattern, I would be convinced!

Instead of providing Northern blots, a technique we do not have established in the lab, we have provided the qPCR data outlined above. In Yamazaki et al (2004), it was shown by RNA-gel blot using probes specific to either PetL or PetG that both RNAs were depleted in abundance in two of the three pgr3 alleles investigated (Fig 7a in that paper). We believe that our qPCRs, which are also specific to each of these RNAs, are an appropriate alternative to the Northern blotting method for showing this diagnostic result, particularly now that we have provide the requested evidence that other chloroplast-encoded Pet RNAs are not affected.

Minor points

The Abstract states that PGR3 is required for “translating 30S ribosome subunits and several thylakoid-localized photosynthetic components.” I feel that this distorts what is known about PGR3 by over-emphasizing its role in chloroplast translation. I mention this because the foundational work on PGR3 (Shikanai lab) did not detect effects on chloroplast translation. This work involved alleles that may not have been null. Still, those results made clear that the major function of PGR3 is to stabilize petL mRNA and stimulate petL translation. The more recent analysis of a null allele by ribosome profiling (Rojas et al, NAR) confirmed that the major effect is on petL-petG expression, and that there is also a small defect in rps8-rpl14 expression. The rRNA analysis in the latter paper showed a minor decrease in mature 16S and 23S rRNAs and an increased abundance of precursor forms of both rRNAs. Those Northern blot results did not show a strong bias toward loss of 16S accumulation/processing (compared to 23S), differing from claims here. I am puzzled by this difference.

We have reordered the wording in the abstract and relevant points throughout the text so that the role for PGR3 in Cytochrome b₆/NDH regulation is listed first. Ribosome defects were not reported in the Shikanai lab papers, where hypermorphic alleles were analysed; the influence on ribosomal subunit RNA processing was reported more recently in pgr3-4, which has an insertion that disrupts the ORF (as opposed to single point mutations) and is therefore likely to be a null allele (Rojas et al 2018). It was also previously reported that the maize PGR3 homolog has reduced chloroplast ribosomes (Belcher et al 2015).

In our study, we further characterise pgr3-4, showing that that 30S ribosome subunits are depleted (westerns Fig 6e) and also that pgr3-4 possesses hypersensitivity to lincomycin, data that complements and corroborates the ribosomal defects described in Rojas et al. Thus, with this work we are also providing additional phenotypic information connecting PGR3 activity to ribosome production (in common with not4a), which does further indicate a core role for this PPR protein in ribosome regulation, in addition to its longer established functions related to Pet and NDH processing.

With regards to the rRNA analysis: In our work we assessed rRNAs using tapestation, which is different to the method used in Rojas et al (2018). We saw a modest but clear reduction in 16s rRNAs that was similar in both not4a and pgr3. In accordance with Nature Communications policy, and for clarity, we also provide the tapestation traces in supplemental Fig 5, and the corresponding raw data reads from which the graph in Fig 6g was generated are provided in the source data file.
Reviewer #2 (Remarks to the Author):

This is my third time reviewing this manuscript. To put my response in context, the two major concerns in my initial review are reiterated below:

(i) The authors conclusion predicted that the not4a and pgr3 mutants should phenocopy one another, but that was not shown rigorously. This seems particularly important due to the surprising nature of the conclusions (in light of what is known about other NOT4 homologs).

(ii) Even if that were convincingly shown, some bit of insight into mechanism seems like a reasonable expectation for publication in a journal like Nature Communications. This is especially important given that NOT4 in other systems stimulates RNA decay, whereas NOT4A here is proposed to stabilize a specific RNA.

With regard to the second point: the authors modified the paper to suggest a variety of mechanisms that are consistent with what is known about NOT4 in other systems. However, no evidence was presented to address these mechanisms. As such, I continue to feel that the observations, although intriguing, are not sufficiently developed for a journal like Nature Communications.

With regard to the first point, I requested the following in my prior review:

“... it is essential to show the analogous western blot data for subunits of those complexes that are not strongly affected in pgr3 mutants: Rubisco, PSI, PSII, ATP synthase. ... The authors model predicts that these complexes would be relatively unaffected in not4a mutants, as is known to be the case in pgr3 mutants.”

This is relevant because a chloroplast translation defect (as expected to occur from the ribosome defect highlighted by the authors) would lead to a reduction of all of the photosynthetic complexes that include chloroplast-encoded subunits.

To address this, the authors added a new figure, Supp Fig 4e, showing that RbcL and D1 (chloroplast-encoded subunits of Rubisco and PSII, respectively) accumulate normally in not4a and pgr3 mutants.

However, I have three concerns with this figure.

i) I requested that the authors probe for subunits of each photosynthetic complex that includes chloroplast-encoded subunits, but they did not do this: There was no probing for an ATP synthase subunit, and PSI was represented only by LHCA4, which is well known to accumulate independently of the PSI core complex (the location of chloroplast-encoded subunits, which are the relevant ones). The failure to probe for a PSI core subunit is especially pertinent because Supp Fig 4d claims that PSI core proteins are reduced to the same degree as cyt b6f proteins in not4a mutants, and this is not a known feature of pgr3 mutants. Antibodies to PSI and ATP synthase core subunits are readily available from commercial sources (eg Agrisera).

ii) It was disappointing that the new Supp Fig 4e does not include probings with NdhH and PetC, to make it unambiguous that these proteins are missing in the same samples that accumulate normal levels of RbcL and D1. It is standard to show both affected and unaffected proteins from the same samples in the same figure in analyses of this type. This is especially important given the puzzling behavior described in my next point.

iii) A further mystery is that RbcL and D1 accumulate normally, yet the authors describe these mutants as being deficient for chloroplast ribosomes. Both RbcL and D1 require chloroplast ribosomes for their synthesis. The accumulation of D1, in particular, is very sensitive to small defects in chloroplast translation because the protein is unstable. The normal accumulation of D1
seems inconsistent with the emphasis on a ribosome defect. These results are not consistent with the following statement in the abstract: “Loss of NOT4A function leads to a strong depletion of cytochrome b6f and NDH complexes, as well as plastid 30S ribosomes”. A strong depletion for 30S ribosomes would lead to a strong decrease in all plastid-encoded proteins. Pgr3 mutants have only a subtle decrease in plastid ribosomes- as shown here and in the previous work on the same allele.

2. The strong loss of NdhH in total leaf extracts of not4a and pgr3 mutants (Figure 6E) is difficult to reconcile with previous information about pgr3. The Shikanai group reported that NdhH accumulates to normal levels in total leaf extracts of their strongest pgr3 allele, but is absent from thylakoid membranes (Cai et al, 2011; Yamazaki et al, 2004). NdhH is in the stromal subcomplex of NDH, so this behavior led to the inference that the NDH defect in pgr3 mutants results from failure to express a subunit in the membrane-bound subcomplex.

Why is NdhH missing from total leaf extract of pgr3 mutants in this study but not in the Shikanai work? One might argue that it results from use of different alleles, but this seems unlikely to me, because a stronger loss of the membrane subcomplex should not affect NdhH accumulation in the stroma.

3. Line 226- 230. Previously, the authors claimed that the specific depletion of 30S ribosomal subunits in not4A mutants provided strong evidence that they phenocopy pgr3. In response, I pointed out the existence of ppr4 mutants, to illustrate that this is not a unique feature of pgr3 mutants. In response to this, the authors now cite work on ppr4 (which was not my intent and isn’t necessary or relevant to this paper), but they continue to make the same basic conclusion as before: "Such an observation indicates a highly specific defect in not4a,"

To reinforce my original point, I would like to point out additional mutants that exhibit a stronger defect in 16S/30S accumulation than 23S/50S accumulation. The purpose of this list is to point out that this feature is not particularly uncommon. (This is not a request to cite the papers).
Kleinknecht et al, RAP, Plant Cell 2014.
Fristedt et al, RBF1, Plant Phys 2014
Janowski et al, AtRsgA, Plant J 2018.

Minor points:
The last line of the abstract does not make sense: PGR3 does not “coordinate chloroplast protein synthesis”.

Line 137- when PGR3 is first mentioned and described, it would be appropriate to cite the extensive work on pgr3 from the Shikanai lab that underlies the statement.

Supp Fig 4d should indicate the source of data underlying the displayed heat map. Was this based on proteomics? 2D gels?
RESPONSE TO REVIEWERS’ COMMENTS

Reviewer #2 (Remarks to the Author):

This is my third time reviewing this manuscript. To put my response in context, the two major concerns in my initial review are reiterated below:

(i) the authors conclusion predicted that the not4a and pgr3 mutants should phenocopy one another, but that was not shown rigorously. This seems particularly important due to the surprising nature of the conclusions (in light of what is known about other NOT4 homologs).

>> We believe that our diverse experimental approaches have shown the strikingly similar phenotypes of not4a and pgr3. In response to editorial requests, we have now modified the text throughout the manuscript to ensure that we do not explicitly state the not4a phenotypes are due to a lack of PGR3, but rather that the two mutants show very similar defects in Cytochrome B6, NDH, and 30S ribosomes/chloroplast translation.

(ii) Even if that were convincingly shown, some bit of insight into mechanism seems like a reasonable expectation for publication in a journal like Nature Communications. This is especially important given that NOT4 in other systems stimulates RNA decay, whereas NOT4A here is proposed to stabilize a specific RNA. With regard to the second point: the authors modified the paper to suggest a variety of mechanisms that are consistent with what is known about NOT4 in other systems. However, no evidence was presented to address these mechanisms. As such, I continue to feel that the observations, although intriguing, are not sufficiently developed for a journal like Nature Communications.

>> As the reviewer points out, we propose a number of potential mechanistic options in the discussion of this manuscript; deciphering these will be a major focus of our future research efforts but are beyond the scope of this current work. We believe that the exciting results published here (connecting a conserved cytosolic E3 ligase to the regulation of chloroplast function) are of interest in their own right and therefore require timely publication.

With regard to the first point, I requested the following in my prior review:

“… it is essential to show the analogous western blot data for subunits of those complexes that are not strongly affected in pgr3 mutants: Rubisco, PSI, PSII, ATP synthase. … The authors model predicts that these complexes would be relatively unaffected in not4a mutants, as is known to be the case in pgr3 mutants.”

>> Our new Figure S4E does confirm that the proteins we tested in response to this request are similarly unaffected in both not4a and pgr3.

This is relevant because a chloroplast translation defect (as expected to occur from the ribosome defect highlighted by the authors) would lead to a reduction of all of the photosynthetic complexes that include chloroplast-encoded subunits. To address this, the authors added a new figure, Supp Fig 4e, showing that RbcL and D1 (chloroplast-encoded subunits of Rubisco and PSII, respectively) accumulate normally in not4a and pgr3 mutants.

However, I have three concerns with this figure.

i) I requested that the authors probe for subunits of each photosynthetic complex that includes chloroplast-encoded subunits, but they did not do this: There was no probing for an ATP synthase subunit, and PSI was represented only by LHCA4, which is well known to accumulate independently of the PSI core complex (the location of chloroplast-encoded subunits, which are the relevant ones). The failure to probe for a PSI core subunit is especially pertinent because Supp Fig 4d claims that PSI core proteins are reduced to the
same degree as cyt b6f proteins in not4a mutants, and this is not a known feature of pgr3 mutants. Antibodies to PSI and ATP synthase core subunits are readily available from commercial sources (eg Agrisera).

>> We carried out western blotting on a range of different subunits. Although the reviewer indicated which complexes they wished to see, they did not state specifically which proteins. As such we used antibodies that we had available and, with the exception of ATP synthase, we were able to show that results for RbcL, PSII components and the PSI-affiliated LHCA4 abundance fit with our predictions and support our conclusion that not4a and pgr3 have similar phenotypes. We do not think that further analysis of even more photosynthetic proteins is necessary to support the conclusions we make. We also note that since the original submission we have performed western analysis on 10 new proteins at the request of reviewer 2 - 4 ribosomal proteins, PetC, NdhH, RbcL, LHCA4, PsbP and D1. In each case we observed the predicted result in both not4a and pgr3-4.

ii) It was disappointing that the new Supp Fig 4e does not include probings with NdhH and PetC, to make it unambiguous that these proteins are missing in the same samples that accumulate normal levels of RbcL and D1. It is standard to show both affected and unaffected proteins from the same samples in the same figure in analyses of this type. This is especially important given the puzzling behavior described in my next point.

>> The reviewer did not specifically ask for us to redo the PetC and NdhH (which we did in response to their first review) in their second review. The depletion of PetC and NdhH in both not4a and pgr3 (Figure 6E) was very strong and therefore did not require dilutions to show the differences. We can confirm that the samples used for Figure S4E were the exact same samples as those for Figure 6E. We have now added a comment in the legend of Figure S4E that highlights this, to avoid ambiguity.

iii) A further mystery is that RbcL and D1 accumulate normally, yet the authors describe these mutants as being deficient for chloroplast ribosomes. Both RbcL and D1 require chloroplast ribosomes for their synthesis. The accumulation of D1, in particular, is very sensitive to small defects in chloroplast translation because the protein is unstable. The normal accumulation of D1 seems inconsistent with the emphasis on a ribosome defect. These results are not consistent with the following statement in the abstract: “Loss of NOT4A function leads to a strong depletion of cytochrome b6f and NDH complexes, as well as plastid 30S ribosomes”. A strong depletion for 30 S ribosomes would lead to a strong decrease in all plastid-encoded proteins. Pgr3 mutants have only a subtle decrease in plastid ribosomes- as shown here and in the previous work on the same allele.

>> Our diverse data clearly show 30S ribosome deficiency in not4a. However, we do indeed show that under non-stressed conditions, D1 and RbcL accumulate normally. This is observed in Figure S4E (which we included in our second revision), but for D1 can also be seen in Figure 4D, which was in our original submission (note that Figure 4D is presented as relative data; the raw data are in the source data sheet). In Figure 4D, we monitored D1 levels following high-light stress, which requires effective D1 translation for recovery. Here, we clearly show defective D1 translation in not4a relative to WT. This suggests that the depletion of 30S ribosomes in not4a still permits basal translation under non-stressed conditions, but stress-situations that require rapid and effective translation for recovery are severely affected. This is a plausible explanation for the results presented in e.g. Figure S4E.

We have now included brief new statements in the main text that refer to this, and thank the reviewer for raising this as we think it is important to note in the paper:
Comparable levels of D1 in *not4a* and Col-0 before HL stress suggests sufficient basal translation under non-stressed conditions, despite reduced ribosome abundance (Figure 4D and Source Data)

Thus, reduced abundance of plastid ribosomes in *not4a* leads to severely reduced translational capacity in response to stress."

2. The strong loss of NdhH in total leaf extracts of *not4a* and *pgr3* mutants (Figure 6E) is difficult to reconcile with previous information about *pgr3*. The Shikanai group reported that NdhH accumulates to normal levels in total leaf extracts of their strongest *pgr3* allele, but is absent from thylakoid membranes (Cai et al, 2011; Yamazaki et al, 2004). NdhH is in the stromal subcomplex of NDH, so this behavior led to the inference that the NDH defect in *pgr3* mutants results from failure to express a subunit in the membrane-bound subcomplex. Why is NdhH missing from total leaf extract of *pgr3* mutants in this study but not in the Shikanai work? One might argue that it results from use of different alleles, but this seems unlikely to me, because a stronger loss of the membrane subcomplex should not affect NdhH accumulation in the stroma.

>> As the reviewer points out, Shikani *et al.* used different (weaker) alleles. Without a direct comparison of protein extracts from the alleles side by side, we cannot fully comment on this apparent disparity. We note that the mechanistic connection between PGR3 and NdhH is still to be resolved, as it was reported in previous Shikanai papers that PGR3 does not seem to directly affect metabolism/translation of any Ndh RNAs (in contrast to its effects on Pet RNAs). Therefore, we cannot draw any conclusions about the mechanistic basis for this loss, and here we are simply reporting our observations and show the similarities in *not4a* and *pgr3*.

3. Line 226-230. Previously, the authors claimed that the specific depletion of 30S ribosomal subunits in *not4A* mutants provided strong evidence that they phenocopy *pgr3*. In response, I pointed out the existence of *ppr4* mutants, to illustrate that this is not a unique feature of *pgr3* mutants. In response to this, the authors now cite work on *ppr4* (which was not my intent and isn’t necessary or relevant to this paper), but they continue to make the same basic conclusion as before: “Such an observation indicates a highly specific defect in *not4a,*” To reinforce my original point, I would like to point out additional mutants that exhibit a stronger defect in 16S/30S accumulation than 23S/50S accumulation. The purpose of this list is to point out that this feature is not particularly uncommon. (This is not a request to cite the papers).

Kleinknecht et al, RAP, Plant Cell 2014.
Fristedt et al, RBF1, Plant Phys 2014
Janowski et al, AtRsgA, Plant J 2018.

>> In response to this and the editorial requests, we have reworded the text in the main document so that we no longer refer to this observation as being specific. As such we have also removed references to *PPR4*, which are no longer relevant to our narrative.

Minor points:
The last line of the abstract does not make sense: PGR3 does not “coordinate chloroplast protein synthesis”.

Line 267: “Comparable levels of D1 in *not4a* and Col-0 before HL stress suggests sufficient basal translation under non-stressed conditions, despite reduced ribosome abundance (Figure 4D and Source Data)”

and

Line 276: “Thus, reduced abundance of plastid ribosomes in *not4a* leads to severely reduced translational capacity in response to stress.”
Our work identifies NOT4A as crucial for ensuring robust photosynthetic function during development and stress-response, through promoting PGR3 production and regulating chloroplast protein synthesis.

Line 137- when PGR3 is first mentioned and described, it would be appropriate to cite the extensive work on pgr3 from the Shikanai lab that underlies the statement.

Supp Fig 4d should indicate the source of data underlying the displayed heat map. Was this based on proteomics? 2D gels?

This is based on proteomics data and we have now cross referenced to the relevant Datafile where the source data is found.