Plastidial NAD-Dependent Malate Dehydrogenase: A Moonlighting Protein Involved in Early Chloroplast Development Through its Interaction with an FtsH12-FtsHi Protease Complex

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00121-RA 1st Editorial decision – revision requested Feb. 9, 2018

(i) Results and discussion concerning NAD+/NADH supply to plastids. As explained in comment 1 by Reviewer #3, the presented data and their interpretation are inconclusive. A malate valve can operate in both directions (supply of plastids with redox power or export of redox power), in particular if MDH activity is not under thioredoxin control, as assumed for NAD-MDH. Since this part is not essential to the main conclusions of the manuscript, it could be either toned down or removed.

(ii) Activity gels, protein gel blots: Both R#1 and R#3 are concerned that the data might be confusing to the readers and criticize that the data are not of high quality. In the post-review consultation, both reviewers acknowledged the difficulties with detecting NAD-ME in gel assays, and we are aware that it might not be possible to produce higher quality gels. However, as indicated by Reviewer #1, it would certainly be possible to include lanes loaded with (a dilution series of) recombinant proteins as positive controls. Further, a better description of the band patterns observed, highlighting which ones are important and which ones are non-specific, would certainly be helpful. This point requires some additional experimentation. It may be possible to put some of the dilutions or other validations into the supplementary information if it meets the criteria for supplementary information.

Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00121-RAR1 1st Revision received May 21, 2018

Reviewer comments and author responses:

Reviewer #1:

The manuscript by Schreier et al. describes the further characterization of the plastidial NAD-MDH loss-of-function mutation that the authors initiated some time ago (Beeler et al., 2014). The previous study indicated a lethal
phenotype of the mutant. In this report, using very nicely designed complementary approaches, the authors provide a functional characterization of this mutation and demonstrate a novel function of the pNAD-MDH as a stabilizer of FtsH12 during plastid biogenesis. On the whole, the manuscript is very well written and the results are of high quality and support the conclusions drawn. I do, however, have a few concerns/issues that I believe should be addressed before the manuscript would be suitable to publication.

Point 1. Page 6 lines 155-158: Genotyping is not presented and I don’t think this is imperative, but it would be informative to mention the number of independent lines that were selected for further analysis.

RESPONSE: These details have been added to the text (line 144-145).

Point 2. Page 9 paragraph 187-199: The analysis presented is not well introduced and was difficult to follow. In line 188, the authors mention "homozygous pdnad-mdh seedlings"; is this correct? These seeds are actually not viable. I think they refer to PABI3:pdNAD-MDH-YFP. The same is true in line 207; here it surely referring to the rescued pdnad-mdh.

RESPONSE: We have added details of seedlings used for this analysis (line 184) and have improved the clarity of the entire paragraph.

Point 3. Page 11 lines 212-220: I am very disappointed by the presentation/resolution/quality of the western blot and in gel activity assays presented (actually throughout the whole manuscript).

1- The antibodies, although used in the previous publication, is of very low specificity and this should be mentioned in the description of the results to help the reader understand the analysis presented (Figure 3A). The only protein band of interest is the one in Ler at 35 kDa, but a control with the isolated protein showing reactivity at this molecular weight is lacking. What about all other reactive bands observed? Are they recognising other NAD-MDHs? And why do the antibodies recognise proteins of different molecular weight in the WT (55 kDa) and miR-mdh-2 and PABI3:pdNAD-MDH-YFP (much higher than 55 kDa)? This should be mentioned in the Results.

RESPONSE: In Figure 3A, the pdNAD-MDH antibody, which was raised against a 15 amino-acid peptide, unfortunately recognises several unspecific bands in leaf extracts of wild type, miR-mdh-1 and pdnad-mdh PABI3:pdNAD-MDH-YFP plants. This is despite having purified it against the antigen. We have now clarified in the figure legend that all bands except for those indicated as pdNAD-MDH are unspecific. We added to the Results section: “We observed several unspecific bands on the blot, but our previous cross-reactivity test of this antibody suggests that they are unlikely to be other MDH isoforms (Beeler et al., 2014).” (line 196-198). We prefer to not just to show the part of the blot with our protein of interest.

The unspecific bands that run above pdNAD-MDH are seen in all samples (also previously; Beeler et al. 2014), and we have added the following to the Results section: “These bands were visible in all lines, but variable in abundance possibly because of variation in the amounts of chloroplastic proteins such as Rubisco between the lines (gels were loaded on an equal protein basis).” (line 198-201) The fact that such variation was not observed in the pale leaves analysed in Figure 4B (loaded by equal leaf area rather than equal protein) is consistent with this explanation.

Point 4. As far as I know, NAD-MDH would form active dimers. The authors present an in gel activity assay showing many bands giving reaction and indicate with arrows two bands that should be the ones corresponding to pdNAD-MDH. I see that these bands are not present in miR-mdh-2 and PABI3:pdNAD-MDH-YFP, but the authors should present a biochemical justification that those bands correspond to pdNAD-MDH. Later in the paper, they mentioned the heterologous production of pdNAD-MDH. A control using this protein in native gel should be shown to justify the assumption.

RESPONSE: The reviewer makes a valid criticism and we appreciate the editors understanding that routine in-gel separation and detection of NAD-MDH activity is difficult. We already optimised our method extensively (as documented in the supplementary data of Beeler et al., 2014), where in gel activity assays were performed with various NAD-MDH mutants to assign bands to specific isoforms. The pdNAD-MDH dimer by itself runs as a lower band that is usually masked by other NAD-MDH bands. However, the two upper bands (which correspond to pdNAD-MDH in complex with other proteins) are easily detected. We know the identity of these interactors (LSF1 and beta-amylases), which are amongst the proteins identified in our IP experiments, but as these interactions are not the focus of this paper, we do not elaborate on them here.
As requested, we have run the recombinant protein on the native gel next to the plant extracts (see Response Figure 1, below). We also added recombinant protein to one of the plant extracts and observed that activity is enhanced in the lower region of the gel containing unresolved NAD-MDH activity. Adding active recombinant protein to extracts containing inactive MDH complex bands did not make these bands visible, probably because the inactive MDH is already occupying in these complexes (even after a 12-hour incubation of the plant extract with the recombinant protein).

We added to the following text to the revision: “On native-PAGE gels, pdNAD-MDH runs as three distinct activity bands - where the lower band corresponds to the free dimer, while the two upper bands correspond to pdNAD-MDH in protein-protein interactions. The lower band is difficult to resolve from other NAD-MDH isoforms (Beeler et al. 2014). Thus, it should be noted that only part of the total pdNAD-MDH in the extract can be observed with this technique.” (line 201-206). We did not include these data because the major points were already demonstrated in the supplementary data of Beeler et al. (2014).

Point 5. Figure 4 B and C.
1- The actin control in not mentioned in the Figure description and is thus confusing.
2- The pattern of reactive band (with the exception of actin) is different from the one shown in Figure 3A. Give an explanation for this. Again, a control with the isolated protein is lacking.

RESPONSE: We added a description of how actin was detected simultaneously in the blot into the legend. The pattern of reactive bands is different to 3A because, as mentioned, these gels were loaded on equal leaf area basis, not equal protein.

Point 6. Figure 7 D. Again, I am very disappointed by the resolution and quality of the activity gel presented. The pattern of reactive bands is different from the ones shown in Figure 3. I highly recommend that the authors eliminate this figure from the manuscript. If the authors want to keep it, controls with recombinant proteins to characterize the bands observed should be presented.

RESPONSE: We realise that the different gels in the original submission were not uniformly presented (the low-mobility peroxisomal isoforms were not always shown), which we believe prompted the reviewers to be critical. Beside their resolution, the gels run quite consistently. However, we agree that the data in the original Figure 7D are not crucial for the story since none of these isoforms could rescue the embryo-lethal phenotype. Nevertheless, we feel it is important that we can detect the activities of at least some of these introduced isoforms. We have repeated these experiments several times with the same results. Thus, we have kept these results in but moved them to the Supplementary Material. We have also revised the presentation of all the native page gels to make them uniform (in Figures 3, S4 and 8).

Point 7. Figure 8E is of very poor quality: very high background and a control to justify that the bands indicated with arrows correspond to pdNAD-MDH is lacking.

RESPONSE: We repeated this experiment and replaced the figure with a new gel that has lower background activity that is consistent with our other gels.

Point 8. Page 21 lines 398-412: the authors do not comment on why they do not further analyse interaction with YCF2, as they do in the case of the proteins involved in starch degradation.

RESPONSE: Analysis of the pdNAD-MDH-Ycf2 interaction is difficult. Ycf2 is encoded in the chloroplast genome. No mutants are available in Arabidopsis, and RNAi approaches are not possible. This is why we chose to focus on FtsH12. However, YCF2 shares a common evolutionary origin to the FtsH subunits and was consistently identified in the IPs with both NAD-MDH and FtsH12. Furthermore, work in tobacco suggests that it is also an essential gene, like pdNAD-MDH and FtsH12. Thus, we feel it is reasonable to include it in our model in Figure 11.

Point 9. Page 23, Figure 9C upper gel:
1- Justify that the band higher than 100 kDa is FtsH12.
2- In the miR-mdh-1 and amiRNA FtsH12 lines, similar bands (that are not seen in the WT) are recognised by the antibodies. What is the explanation for this observation?

RESPONSE: FtsH12 is 115 kDa including the chloroplast transit peptide (49 amino acids), and the mature peptide is predicted to be 110 kDa. These molecular weights have now been added to the text (line 453-455). We observed a band at the correct molecular weight with the FtsH12 antibody, and its intensity is reduced/abolished in two
independent artificial microRNA silencing lines of FtsH12 (amiRNA FtsH12 A/B). This makes it very likely that the band is FtsH12.

As for Figure 3, gels were loaded on an equal protein basis. Differences in the abundance of chloroplast proteins like Rubisco in the leaf extracts of the different lines may explain why the detection of unspecific bands is enhanced in the pale mutants.

Reviewer #2:
Several issues, minor in my opinion, could be addressed in a final version of this manuscript:

Point 1. As FtsH12 and its proteolytically inactive variants are poorly studied thus far, mentioning their interaction with pdNAD-MDH in the title is justified. Maybe the authors could consider adding the following at the end of the title: "... through its interaction with a FtsH12-FtsHi complex".

RESPONSE: We have revised the title of our manuscript as suggested.

Point 2. As MDHs are essentially soluble proteins, the question of how much of pdNAD-MDH is associated with the inner envelope membrane / the FtsH12 complex could be discussed. Data on distribution of pdNAD-MDH within the chloroplast may be found in chloroplast proteomics data (e.g., in the At_Chloro database).

RESPONSE: In the At_Chloro database, pdNAD-MDH is reported to be completely stromal. We now discuss this on line 651-655. However, Cvetic et al. (2008) identified pdNAD-MDH both in stroma and chloroplast envelope fractions of spinach leaf chloroplasts, which we now cite in the revised manuscript.

Point 3. The absence of FtsH12 in the miR-mdh-1 line (that does not accumulate pdNAD-MDH) is intriguing. I wonder whether the authors have lines accumulating lower levels of pdNAD-MDH, where the level of FtsH12 can be determined. If a positive correlation between the levels of remaining pdNAD-MDH and FtsH12 can be established, this would strongly support the suggestion that the presence of the former stabilizes the latter.

RESPONSE: We thank the reviewer for this suggestion. For the current study, we have strengthened the observation that FtsH12 levels are reduced in miR-mdh-1 via quantitative western blots. We have added these data to the revised manuscript (Supplementary Figure 7). (line 459-460). We agree that further work will be necessary to determine exactly how pdNAD-MDH controls FtsH12 levels, but suggest that this goes beyond the scope of this manuscript.

Point 4. At the current stage, it is not clear whether the developmental role of FtsH12 is dependent on its proteolytic activity, or else, solely on its ATPase one. This point could be mentioned in the Discussion.

RESPONSE: From our results, we cannot deduce whether the proteolytic activity or only the ATPase activity is important for the role of FtsH12 in chloroplast development and embryogenesis. We added to the discussion “However, further investigations are needed to determine whether the function of FtsH12 in chloroplast development is dependent on its proteolytic activity, or solely on its ATPase activity.” (line 712-714)

Point 5. The MS analysis of co-IPed proteins presented in Figures 1-4 is qualitative, or maybe semi-quantitative. The similar patterns of association in the different tissues and lines do compensate for the absence of a quantitative approach, in my opinion, and do justify the conclusion proposed. Nevertheless, showing the raw MS data, including the negative controls that were performed (on WT), in a Supplemental Table could increase the confidence of readers.

RESPONSE: We have noted in the text that peptide counts are not strictly correlated with protein abundance (line 390-392). We included a Supplementary Dataset with the raw data for each individual IP experiment, including control samples.

Reviewer #3:
This manuscript addresses the function of plastidial NAD-dependent malate dehydrogenase (pdNAD-MDH) in Arabidopsis. A knock-out mutation has previously been shown to be lethal at the embryo stage, and miRNA silencing of the pdNAD-MDH gene produced pale plants with defective plastids. In contrast, knock-out mutants of plastidial NADP-dependent MDH have previously been shown to grow relatively normally. This enzyme is redox sensitive and was proposed to function in the light, such as in the operation of a ‘malate valve’ as a means to export reducing
equivalents from the plastid. These observations collectively suggest that pdNAD-MDH could function in the malate valve, consistent with its lack of redox regulation, or that it could serve other essential roles in plastid development or function, even in the presence of pdNADP-MDH.

Schrier et al. conducted a series of detailed experiments employing a range of different genetic and biochemical approaches to determine the function of pdNAD-MDH. They discovered that pdNAD-MDH is essential for plastid development, not as a functional MDH enzyme, but as an essential protein constituent of the FtsH1 (Filamentous temperature sensitive) complex located at the plastid envelope inner membrane. This complex is essential for protein processing during plastid biogenesis. Remarkably, pdNAD-MDH with mutations that abolish its enzyme activity are still able to function in the FtsH complex and to complement the pdnad-mdh knock-out mutant.

These results reveal an unexpected role for this protein, in addition to its function as an MDH enzyme. Evidence for yet further functions is suggested by data indicating that pdNAD-MDH can interact with enzymes of starch metabolism. These studies open up a new and exciting area of plastid biology.

Approaches included embryo-specific gene expression (ABI3 promoter) to rescue the pdnad-mdh mutant, estradiol-inducible silencing in leaves, measurement of galactolipids, carotenoids and protochlorophyllide, and TEM analysis of plastids. The work shows that other NAD-MDH enzymes (from peroxisome, mitochondrion, cytosol and yeast) could not complement when targeted to the plastid. Site-directed mutagenesis of pdNAD-MDH was conducted and loss of enzyme activity confirmed by expressing recombinant proteins in E. coli. Isothermal Calorimetry was used to confirm that NADH binding was abolished. The ability of such proteins to complement the pdnad-mdh phenotype was established in transgenic plants. Immunoprecipitations (pull downs) were conducted, and peptides associated with pdNAD-MDH identified by LC/MS-MS. Functional interaction of pdNAD-MDH with FtsH was established with both enzymatically active and inactive MDH. No other NAD-MDH enzymes interacted with FtsH. A knock-out mutant of FtsH was shown to be embryo lethal, and miRNA silencing of FtsH produced plants resembling those in which pdNAD-MDH is silenced. pdNAD-MDH was shown to be required to stabilise FtsH, but not the converse.

The approaches adopted are very impressive in their range and detail. This is a very rigorous study.

Point 1. PAGE 9 (187-199) and Supp Fig 1. What is the argument that assumes that the mdh mutant will have deficiency in NAD+ in plastid? Does this assume that if it serves as a ‘malate valve’, MDH would be expected to oxidise NADH to NAD and then export malate? But theoretically MDH could oxidise incoming malate and reduce NAD to NADH. The use of 2-oxoglutarate (2.5 mM) plus glutamine (10 mM) seems to be an imprecise way to address this question. 2OG would potentially be used by 2OGDH to reduce NAD to NADH. The text is also unclear because it refers to ‘deficiency of NAD+’ and ‘increasing cellular NAD+ levels’, which can be misunderstood as NAD metabolism rather than oxidation and reduction. I think this is the weakest part of the Results, and needs better explanation or could be omitted without detracting from the manuscript.

RESPONSE: We agree that the malate valve is reversible. We also agree that the experiments with 2OG and glutamine are unnecessary for supporting our major conclusions. Thus, Supplementary Figure 1 D/E and the corresponding text have been omitted from the revised manuscript, as recommended.

Point 2. PAGE 18. MDH activity gels are inconclusive because there are so many bands of activity (Fig 7D). The appearance of a new band is only ‘consistent’ with the proposal that it is derived from an introduced gene. As it stands, there is such evidence for only two of the MDH transgenes. Did the authors attempt to isolate chloroplasts for native PAGE assay to obtain clearer results?

RESPONSE: We have moved this figure to the Supplementary Material (Figure S4), as discussed above.

Point 3. PAGE 25. The authors tested binding of NADH to MDH proteins using ITC. Why were malate, OAA and NAD not tested for binding? This would seem to be relatively straightforward and informative.

RESPONSE: We tested the interaction of NAD+ with recombinant pdnAD-MDH protein. However, no binding was observed using 400 µM of NAD+ (the same concentration used for NADH). Only weak binding was observed when we increased the concentration of NAD+. OAA and malate titrations were not tested and are not straightforward with
this method; these substrates only bind to the protein when the cofactor (NAD+/NADH) is already bound and a conformational change has occurred. ITC experiments with multiple substrates (and those involving conformational changes) are challenging to interpret, as observed heat exchanges cannot be attributed to the direct interaction between one substrate and the protein.

Point 4. PAGE 25. Do SEX4 and BAM peptides appear in the IP using non-catalytic pdNAD-MDH as 'bait'? It is an obvious question that the authors can answer with current data, and it would be a pity if such information is being withheld. However it may be understandable if this information provides the basis for a future paper on the interactions between NAD-MDH and enzymes of starch metabolism.

RESPONSE: We indeed found that the non-catalytic pdNAD-MDH mutant forms still interact with the LSF1 and BAMs. These data are now available in Supplemental Dataset 1.

Point 5. PAGE 30 637-640. Authors should not claim priority or ownership. Perhaps this experiment can be proposed as a worthwhile approach.

RESPONSE: We have reworded this sentence to: “As the loss of NAD-MDH activity alone also has a relatively mild impact on plant growth (Hebbelmann et al., 2012), generating lines expressing the inactive pdNAD-MDH constructs in the pdnad-mdh nadp-mdh double mutant background would be highly valuable for re-assessing the importance of MDH activity and the malate valve in the chloroplast.” (line 641-643)

Point 6. 121-133. Most of this is summary and interpretation of results rather than introduction. Traditionally this paragraph should describe the aims and approach, but does not describe the results. Moonlighting proteins are not part of the background, but are a product of the experiments.

RESPONSE: This has been corrected in the revised manuscript. Most of this paragraph has been moved to expand the section on moonlighting enzymes in the Discussion. In the Introduction, the paragraph has been replaced with a short sentence describing the aims of the work.

“Here, we aimed to investigate the role of pdNAD-MDH in both embryo development and post-embryonic growth, focussing on chloroplast development, and to test the importance of NAD-MDH activity in these processes.” (line 120-123).

TPC2018-00121-RAR1 2nd Editorial decision – acceptance pending June 11, 2018

We are pleased to inform you that your paper entitled "Plastidal NAD-Dependent Malate Dehydrogenase: A Moonlighting Protein Involved in Early Chloroplast Development Through its Interaction with an FtsH12-FtsHi Complex" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor June 19, 2018