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Tyr-Asp inhibition of glyceraldehyde 3-phosphate dehydrogenase affects plant redox metabolism.

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 6th Nov 2020

Thank you for submitting your manuscript entitled "Tyr-Asp inhibits glyceraldehyde-3-phosphate-dehydrogenase affecting metabolism and stress tolerance" (EMBOJ-2020-106880) to The EMBO Journal. Please accept my sincerest apologies for the delay in getting back to you with our decision due to the difficulties in finding good referees for this study. We have now received comments from two trusted experts in the field, which are enclosed below for your information.

As you can see, the referees find your work potentially interesting. However, they also raise several major issues, in particular related to the experimental approach, that need to be addressed before they can support publication in The EMBO Journal.

We agree with the referees that these are important points and addressing them will be essential to pursue publication of this study in The EMBO Journal. Please note that we will need strong support from the referees for publication here. Given the overall interest of your study, I would like to invite you to submit a revised manuscript according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in the revised version.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

Before submitting your revised manuscript, deposit any primary datasets and computer code produced in this study in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password, in case such datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

Feel free to contact me if you have any questions about the submission of the revised manuscript to The EMBO Journal. I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Referee #1:

General comment.
Previous works by the same group have identified more than hundred dipeptides intearacting with proteins in Arabidopsis, among them Tyr-Asp interacts with cytosolic GAPDH (and other dipeptides interact with GAPDH as well as other proteins appeared to interact with Tyr-Asp). In this work it

shown that Tyr-Asp inhibits animal GAPDH activity in vitro and different plant GAPDH forms in vivo (GAPC/GAPCp/GAPA-B). Different binding sites for Tyr-Asp on both cytosolic and chloroplastic GAPDH are proposed. In Tyr-Asp treated At seedlings, total NADPH/NADP+ ratio increased as a result of stimulated pentose phosphate pathway. PEPCK was found to be inhibited by several dipeptides but not by Tyr-Asp. Plants treated with Tyr-Asp grow better under oxidative stress, but Arabidopsis mutants with no cytosolic GAPDH (gapc1/gapc2) do not. Proteins and metabolites affected by Tyr-Asp feeding are mostly related to proteostasis and redox control. Based on these data, Tyr-Asp is proposed to boost plant stress resistance in Arabidopsis and in more generale terms, dipeptides are proposed as important regulators of metabolism.
The manuscript deals with a novel and interesting topic for plant physiology, it is well written and contains a lots of interesting data. Most of the experiments are well designed and many results are convincing. Nevertheless, I found some critical points that I believe should be addressed.

Major concerns
1. The use of recombinant human or rabbit GAPDH to test the effect of Tyr-Asp on activity in vitro is possibly informative but not the best choice. As shown in Fig. 1 the sensitivity of these GAPDHs for Tyr-Asp is low (about 30% inhibition with 25 uM with rabbit GAPDH and 100 uM with human GAPDH) and it would be interesting to know whether the sensitivity of plant GAPDHs is also so low. By the way, if animal GAPDH have similar specific activity to plant GAPDH, than the units in Fig. 1 A and B are wrong by a factor of 100.
2. Enzymatic assays for GAPC/GAPCP and GAPA-B are not described in Materials and Methods (although references are provided). This is critical because GAPA-B has substantial activity with NAD(H) and therefore interferes with the assay of GAPC/GAPCP. GAPA-B (whose NADPH-dependent activity is 4-fold higher than GAPC/GAPCP in At rosette leaves; Fig. 1 and Fig. S1) appears to be also inhibited by Tyr-Asp. Based on these data, the NADH-dependent activity of GAPA-B may well be in the same order of the apparent activity of GAPC/GAPCP. Is it possible that GAPA-B does also contribute to the phenotype observed in Tyr-Asp fed plants?
3. Binding experiments. Same problem with activities: experiments with nanoDSF and MST were made with rabbit GAPDH. In silico docking experiments were made with Arabidopsis GAPDHs. Since thermal stability and dissociation constants may depend on single amino acid sustitutions, it is questionable whether the results obtained with rabbit GAPDH can be transferred to Arabidopsis GAPC. The Kd 5 uM calculated by docking Tyr-Asp to apo-GAPC is in fact very different from the Kd of 365 uM determined by MST on apo-GAPDH from rabbit. In general, all calculated or predicted Kd values are very high in comparison with the in vivo concentration of Tyr-Asp, this should be commented. Finally, it is not clear whether Tyr-Asp is predicted to interfere with NAD binding in GAPC as it does with CP12 in GAPA-B.

Minor concerns
- Fig. 2C is not clear
- Provide a reference for the oxidative stress induced by catechin (l. 220)
- L.97 Bassham
- Fig. 4C, legend: the following sentence is misleading "Surface representation of the GAPC1 tetramer (dimer of O-R-dimer) with colors indicating the different chains and respective sequence identity (O=O´and R=R´)" because in GAPC all subunit has identical sequence.

Referee #2:

This is paper is part of a continuing story by the senior author's lab that is attempting to

characterize a definitive role for dipeptides as regulatory signals within plant metabolism. This area of research is highly novel, within the field of plant metabolism. This paper follows up previous reports of an interaction between Tyr-Asp and the cytosolic GAPDH isoform GAPC. It is satisfying that additional interaction studies presented in this paper, as well as some molecular modelling, have confirmed and solidified the presence of this interaction. The paper aims to provide a biological/regulatory reason for the interaction focused on post-translational control of GAPC activity. If some of the results and methods are strengthened then I feel this this study make an important contribution to the developing novel story about dipeptides within metabolic regulation.

Major Points

Line 161: You claim to test the in vivo effect of Try-Asp on GAPDH activity but the next two examples are clearly in vitro experiments, ie the crude extract activity assats and the Arabidopsis 4 week plants supplemented with 50 uM Tyr-Asp followed by crude extract activity assays. The fact that you fed the Tyr-Asp ahead of time is irrelevant given the time scales involved in extraction. The inhibition you see is likely attributable to the diluted Tyr-Asp in the extract, the ezyme will be in equiblibrium with that concentration not the concentration it experiences in the cell. I think your flux assay is a true in vivo assessment, though indirect. Nevertheless, establishing in in vitro effects of Tyr-Asp is still crucial. The key comparison that was left out was how does the inhibitor power of Tyr-Asp (e.g I50) compare to its estimated in vivo concentration (also refer to lines 483- 488 and lines 291 where you state a concentration of ~0.62 uM)?

Line 197 and methods: The description and interpretation of the 14CO_2 flux data is problematic and incomplete. No CO_2 is release by glycolysis, as stated. What about CO_2 released by PDC and the TCA cycle? Your interpretation of this data is very unclear. I think you need to show a diagram to explain what are you talking about. This would greatly benefit the reader who would not know about appositionally dependent release of CO_2 from glucose.

Also the use of the C6/C1 ratio is not clear to me. I can see how you would expect a difference in C1 release via OPPP. But if there is a difference in C6 release how do you explain this? C1 and C6 glucose are not fed to the same samples so presumably C6 cannot be used to normalize overall metabolic rate. Why not show the data for just C1 release also?

Line 236: The whole experiment (Fig 2A and sup 5) seems very batch dependent. Mock treatment levels show high variability between batches, which are actually larger then many of the treatment effects. The difference between the same treatments (ie mock) is greater than the difference between paired treatments (ie mock vs Tyr-Asp), so it seems like a dodgy effect of be quantifying. To what extent was the whole experiment reproduced?

Line 417: Your PEPCK assay is highly questionable for several reasons. First of all you are doing a fluorometric assay in crude leaf extract, so there may be very high background. Secondly, you add the coupling enzyme MDH, so you are measuring OAA conversion to malate and NADH consumption by fluoromitry. Crucially, this measurement is in the carboxylation direction, so you have measured PEPC activity, which is highly abundant in plant tissues, not PEPCK activity. Why did you add $KHCO_3$ (which is a PEPC substrate) and why don't you add any carbon substrate like PEP? Therefore, I do not think this assay is substantiated.

The whole PEPCK section does not fit with the rest of the paper. These results are comparatively undeveloped and the only link is that they are both glycolytic enzymes, but really PEPCK is only a gluconeogenic enzyme.

Line 525: It's not clear that PEPCK has any major involvement in plant glycolysis. Its role in seedling establishment cleary pertains to gluconeogenesis.
Supplemental Methods, Feeding experiments with 14C-labelled glucose Was this assay conducted in the dark? If not will it be impacted by photorespiration following any triosephosphate equilibration with the plastidial pool? Also mention for all enzyme assays whether tissue harvested in the light or dark as this can have a big effect on redox status.

Minor

Intro, Line 64, and discussion line 453: "....redirect the glycolytic flux..." Has this been shown in plants? In animals and yeast the regulation of the glycolytic pathway shows substrantial differences to plants. So the intro should not jump back and forth between different kindgoms without justification

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Line 511: Why would you expect the activities of different dipeptides to be the same? They are after all different chemicals. This is a straw man argument. Also, the discussion of Tyr-Ala possibly linking to Tyr-Asp at a mechanistic level seems overly speculative.

Line 532: "Dipeptide inhibition of PEPCK activity could serve 530 to promote the PPDK route. Dipeptide levels would then provide direct information 531 about the rate of proteolysis." This is overly speculative. Are not PEPCK and PPDK both gluconeogenic, what effect would choosing one route over another have?

EMBOJ-2020-106800

Rebuttal Letter

Referee #1:

- *Previous works by the same group have identified more than hundred dipeptides interacting with proteins in Arabidopsis, among them Tyr-Asp interacts with cytosolic GAPDH (and other dipeptides interact with GAPDH as well as other proteins appeared to interact with Tyr-Asp). In this work it shown that Tyr-Asp inhibits animal GAPDH activity in vitro and different plant GAPDH forms in vivo (GAPC/GAPCp/GAPA-B). Different binding sites for Tyr-Asp on both cytosolic and chloroplastic GAPDH are proposed. In Tyr-Asp treated At seedlings, total NADPH/NADP+ ratio increased as a result of stimulated pentose phosphate pathway. PEPCK was found to be inhibited by several dipeptides but not by Tyr-Asp. Plants treated with Tyr-Asp grow better under oxidative stress, but Arabidopsis mutants with no cytosolic GAPDH (gapc1/gapc2) do not. Proteins and metabolites affected by Tyr-Asp feeding are mostly related to proteostasis and redox control. Based on these data, Tyr-Asp is proposed to boost plant stress resistance in Arabidopsis and in more generale terms, dipeptides are proposed as important regulators of metabolism. The manuscript deals with a novel and interesting topic for plant physiology, it is well written and contains a lot of interesting data. Most of the experiments are well designed and many results are convincing. Nevertheless, I found some critical points that I believe should be addressed.*

Thank you. We are grateful for the reviewers' comments we addressed them as follows:

Major concerns

- *Enzymatic assays for GAPC/GAPCP and GAPA-B are not described in Materials and Methods (although references are provided). This is critical because GAPA-B has substantial activity with NAD(H) and therefore interferes with the assay of GAPC/GAPCP. GAPA-B (whose NADPH-dependent activity is 4-fold higher than GAPC/GAPCP in At rosette leaves; Fig. 1 and Fig. S1) appears to be also inhibited by Tyr-Asp. Based on these data, the NADH-dependent activity of GAPA-B may well be in the same order of the apparent activity of GAPC/GAPCP. Is it possible that GAPA-B does also contribute to the phenotype observed in Tyr-Asp fed plants?*

In the revised version of the manuscript, we included a new experiment designed to disentangle which GAPDHs are Tyr-Asp targets. We measured GAPC, GAPA-B, and GAPN activities in protein extracts from WT plants and the *gapc1gapc2* double mutant. We resorted to a different enzymatic assay successfully used in the past to differentiate between GAPC and GAPA/B activities (Rius et al, 2006, Plant Mol Biol 61, 945–95). Our results show that GAPC activity is reduced by Tyr-Asp in the WT but not in

the *gapc1gapc2* double mutant. Conversely, no significant differences were observed for GAPA-B or GAPN activities in the presence of Tyr-Asp, either in the WT or the *gapc1gapc2* double mutant. Considering that plastidic GAPC (GAPCp) activity in protein extracts from leaves harvested in the light is negligible (Muñoz-Bertomeu et al, 2009, Plant Physiology 151, 541–558), we conclude that cytosolic GAPC is the main target of Tyr-Asp. We could also show that the GAPC inhibition is specific for the Tyr-Asp dipeptide, with no effect measured for tyrosine and aspartic acid. Not to mix results obtained using two different enzymatic assays we replaced all the GAPDH enzymatic results with the new data (new Figure 1). The results, discussion, and methods sections were modified accordingly.

- *The use of recombinant human or rabbit GAPDH to test the effect of Tyr-Asp on activity in vitro is possibly informative but not the best choice. As shown in Fig. 1 the sensitivity of these GAPDHs for Tyr-Asp is low (about 30% inhibition with 25 uM with rabbit GAPDH and 100 uM with human GAPDH) and it would be interesting to know whether the sensitivity of plant GAPDHs is also so low. By the way, if animal GAPDH have similar specific activity to plant GAPDH, then the units in Fig. 1 A and B are wrong by a factor of 100.*
- *Binding experiments. Same problem with activities: experiments with nanoDSF and MST were made with rabbit GAPDH. In silico docking experiments were made with Arabidopsis GAPDHs. Since thermal stability and dissociation constants may depend on single amino acid sustitutions, it is questionable whether the results obtained with rabbit GAPDH can be transferred to Arabidopsis GAPC. The Kd 5 uM calculated by docking Tyr-Asp to apo-GAPC is in fact very different from the Kd of 365 uM determined by MST on apo-GAPDH from rabbit. In general, all calculated or predicted Kd values are very high in comparison with the in vivo concentration of Tyr-Asp, this should be commented. Finally, it is not clear whether Tyr-Asp is predicted to interfere with NAD binding in GAPC as it does with CP12 in GAPA-B.*

We agree that *in vitro* binding and enzymatic analysis should be performed with the Arabidopsis rather than rabbit protein. We attempted to purify the AtGAPC1 protein, and we also ordered it from a commercial provider. Alas, we failed in obtaining an active enzyme. Not to confuse the readers by "jumping between" mammalian and plant GAPDH, in the revised version of the manuscript, we removed all the results obtained using rabbit GAPDH. However, we understand the added value of having the K_d and IC50 information; we would argue that the lack of it does not compromise our study's main conclusions, especially considering the new enzymatic results. Most importantly, by measuring GAPC activity in protein extracts from WT plants and the *gapc1gapc2* double mutant, we now show that 100µM Tyr-Asp leads to almost complete inhibition of the GAPC activity. Moreover, by recalculating Tyr-Asp concentration, considering subcellular compartmentalization, we demonstrate that 100µM Tyr-Asp lies within low to mid µM cellular concentrations of the endogenous Tyr-Asp.

From the manuscript, p10 “We subsequently removed the NAD+ molecule from the crystal structure (PDB-ID 4z0h), resulting in Tyr-Asp binding at the position of the removed adenosine of NAD+, binding to the amino acid residues “I”, “SAP”, “ASC”, “T”, “R”, “NE”, “Y” (11, 119-121, 147-149, 179, 231, 313-314, 317, and with a Kd of 5.1 µM; Figure 4B).” So, this could be interpreted as interference/competitive binding, though we cannot answer whether NAD^+ would expel Tyr-Asp from the pocket.

Minor concerns

- *Fig. 2C is not clear*
- *Provide a reference for the oxidative stress induced by catechin (l. 220)*
- *L.97 Bassham*
- Fig. 4C, legend: the following sentence is misleading "Surface representation of the GAPC1 tetramer (dimer of O-R-dimer) with colors indicating the different chains and respective sequence identity (O=O´and R=R´)" because in GAPC all subunit has identical sequence.

We addressed all the minor concerns. Regarding Fig. 2C we have now explained that is a black and white image used to measure leaf surface in the software ImageJ.

Referee #2:

This is paper is part of a continuing story by the senior author's lab that is attempting to characterize a definitive role for dipeptides as regulatory signals within plant metabolism. This area of research is highly novel, within the field of plant metabolism. This paper follows up previous reports of an interaction between Tyr-Asp and the cytosolic GAPDH isoform GAPC. It is satisfying that additional interaction studies presented in this paper, as well as some molecular modelling, have confirmed and solidified the presence of this interaction. The paper aims to provide a biological/regulatory reason for the interaction focused on post-translational control of GAPC activity. If some of the results and methods are strengthened then I feel this this study make an important contribution to the developing novel story about dipeptides within metabolic regulation.

Thank you. We are grateful for the reviewers' comments we addressed them as follows:

Major Points

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plastidic GAPC (GAPCp) activity in protein extracts from leaves harvested in the light is negligible (Muñoz-Bertomeu et al, 2009, Plant Physiology 151, 541–558), we conclude that cytosolic GAPC is the main target of Tyr-Asp. Most importantly, by measuring GAPC activity in protein extracts from WT plants and the *gapc1gapc2* double mutant, we now show that 100µM Tyr-Asp leads to almost complete inhibition of the GAPC activity. Moreover, by recalculating Tyr-Asp concentration, taking into account subcellular compartmentalization, we demonstrate that 100µM Tyr-Asp lies within low to mid µM cellular concentrations of the endogenous Tyr-Asp. Not to mix results obtained using two different enzymatic assays, we replaced all the previous GAPDH enzymatic results with the new data (new Figure 1). The results, discussion, and methods sections were modified accordingly. Following the reviewer's suggestion, we don't refer to the lysate experiments as *in vivo*.

Reviewer#1 strongly argued that *in vitro* binding and enzymatic analysis should be performed with the Arabidopsis rather than rabbit protein. We attempted to purify the AtGAPC1 protein, and we also ordered it from a commercial provider. Alas, we failed in obtaining an active enzyme. Not to confuse the readers by "jumping between" mammalian and plant GAPDH, in the revised version of the manuscript, we removed all the results obtained using rabbit GAPDH. However, we understand the added value of having the K_d and IC50 information; we would argue that the lack of it does not compromise our study's main conclusions, especially considering the new enzymatic results.

- *Line 197 and methods: The description and interpretation of the 14CO2 flux data is problematic and incomplete. No CO2 is release by glycolysis, as stated. What about CO2 released by PDC and the TCA cycle? Your interpretation of this data is very unclear. I think you need to show a diagram to explain what are you talking about. This would greatly benefit the reader who would not know about appositionally dependent release of CO2 from glucose. Also the use of the C6/C1 ratio is not clear to me. I can see how you would expect a difference in C1 release via OPPP. But if there is a difference in C6 release how do you explain this? C1 and C6 glucose are not fed to the same samples so presumably C6 cannot be used to normalize overall metabolic rate. Why not show the data for just C1 release also?*

Thank you for pointing this out. We now included the requested diagram (New Figure S2A), which shows that C_1 release is preferentially from the OPPP while C_6 can come from glycolysis and the OPPP. Previously feeding with $C_{3\text{-}4}$ labeled glucose was also performed, but this is no longer available. The samples were fed individually as labeled CO_2 released from the C_1 and C_6 position is identical. These experiments are the classical route for determining the relative activities as presented in the manuscript (detailed in ap Rees, T. (1978), Assessment of the contribution of metabolic pathways to plant respiration. In the Biochemistry of Plants, D. D. Davies (ed.). Academic Press Incorporated, San Diego. 2, pp 1-27.) While revising the text, we realized that we made an error that is now corrected. Was: $^{14}CO_2$ release from C_1 is related to the activity of both PPP and glycolysis, whilst from C_6 only of glycolysis. Should be: $^{14}CO_2$ release from C_6 is related to the activity of both PPP and glycolysis, whilst from C_1 only of glycolysis.

- *Line 236: The whole experiment (Fig 2A and sup 5) seems very batch dependent. Mock treatment levels show high variability between batches, which are actually larger than many of the treatment effects. The difference between the same treatments (ie mock) is greater than the difference between paired treatments (ie mock vs Tyr-Asp), so it seems like a dodgy effect of be quantifying. To what extent was the whole experiment reproduced?*

The reviewer rightly pointed the variability in the presented stress experiments, explaining why we restricted our comparisons to plants treated and grown in the same 24-well plate. Each treatment was replicated with multiple plants and independent experiments. To address the reviewer's point, in the revised version of the manuscript, we introduced New Supplemental Dataset S1, which contains results for all the Tyr-Asp treatments under control and oxidative stress conditions. The key result, demonstrating higher biomass in plants subjected to the various stress treatments (salt, catechin, or H2O2) in the presence of Tyr-Asp could be reproduced in a total of 8 experiments, 4 for Arabidopsis and 4 for tobacco plants, either in liquid and/or solid artificial medium.

- *Line 417: Your PEPCK assay is highly questionable for several reasons. First of all you are doing a fluorometric assay in crude leaf extract, so there may be very high background.*

In general, fluorometric assays have higher sensitivity and lower background than spectrophotometric assays. As mentioned in the Supplemental Information file, PEPCK activity is very low in Arabidopsis rosettes. Thus, we adapted the fluorometric method described by Rojas et al. (2018) Biochem J 476: 2939-2952 to assess PEPCK activity in such samples. Indeed, the fluorometric PEPCK assay has very low background (see Supplemental Figure 11). Based on these results, we conclude that the fluorometric method used in this work is very sensitive and reliable.

- *Secondly, you add the coupling enzyme MDH, so you are measuring OAA conversion to malate and NADH consumption by fluoromitry.*

It is important to note that our fluorometric method does not measure the intrinsic fluorescence of NADH, but that originating from fluorescent adducts obtained by alkaline treatment of the generated NAD^+. These adducts have higher fluorescence than NADH. This is clearly discussed in the work of Rojas et al. (2018) Biochem J 476: 2939-2952, cited in our manuscript.

- *Crucially, this measurement is in the carboxylation direction, so you have measured PEPC activity, which is highly abundant in plant tissues, not PEPCK activity. Why did you add KHCO3 (which is a PEPC substrate) and why don't you add any carbon substrate like PEP? Therefore, I do not think this assay is substantiated.*

We thank the reviewer for pointing this out. We did consider PEPC activity when performing PEPCK assays by including a blank without ADP, as it was previously done by Martin et al. (2007), Physiol. Plant. 130, 484-494. However, we did not include this information in the first version of the manuscript. Additionally, we forgot to mention that PEP was included in the reaction mixture. This information was corrected and moved to the Materials and Methods section in the new version of the manuscript.

- *The whole PEPCK section does not fit with the rest of the paper. These results are comparatively undeveloped and the only link is that they are both glycolytic enzymes, but really PEPCK is only a gluconeogenic enzyme. Line 525: It's not clear that PEPCK has any major involvement in plant glycolysis. Its role in seedling establishment cleary pertains to gluconeogenesis.*

We agree with the reviewer, PEPCK is a gluconeogenic enzyme. This is why we refer to glycolysis/gluconeogenesis in different parts of the manuscript. Our intention was to show that GAPC is not the sole target of dipeptides and, at the same time, to validate the results previously reported in the PROMIS database.

- *Supplemental Methods, Feeding experiments with 14C-labelled glucose Was this assay conducted in the dark? If not will it be impacted by photorespiration following any triosephosphate equilibration with the plastidial pool?*

Yes, the assay was performed in the absence of photosynthetically active radiation.

- *Also mention for all enzyme assays whether tissue harvested in the light or dark as this can have a big effect on redox status.*

Samples for measuring enzyme activities in crude extracts were harvested in the light. This information has been included in the revised version of the manuscript. It is worth mentioning that the extraction buffers used for preparing protein extracts contain either 0.5 mM DTT (for GAPDHs) or 5 mM β-mercaptoethanol (for PEPCK).

Minor

- *Intro, Line 64, and discussion line 453: "....redirect the glycolytic flux..." Has this been shown in plants? In animals and yeast the regulation of the glycolytic pathway shows substrantial differences to plants. So the intro should not jump back and forth between different kindgoms without justification.*

We used reviewers' comments to improve both introduction and discussion.

- *Line 71, consider rewording "belongs to the biochemistry curriculum", I think it means textbook example but had to stop and think about it.*

Thank you for pointing this out. We changed it accordingly.

- *Line 192: "distribution" You didn't really measure the distribution of the label. You only measured $^{14}CO2$*

Thank you for pointing this out. It has now been corrected.

- *Line 211: You mean to say that you did not find evidence that Tyr-Asp changes the subcellular localization of GAPDH.*

Yes, that is what we meant. We changed accordingly.

- *Line 221: what are in vitro plants?*

We meant plants grown on synthetic MS media. The sentence was changed.

- *Line 338: figure says 4 metabolites, here you say 3. What are they? dont make the reader go digging in the supplemental. Asp, Tyr, Try-Asp and what?*

The figure was revised accordingly.

- *Line 511: Why would you expect the activities of different dipeptides to be the same? They are after all different chemicals. This is a straw man argument. Also, the discussion of Tyr-Ala possibly linking to Tyr-Asp at a mechanistic level seems overly speculative.*

Following on the reviewers' point we rewrote the discussion by removing the specificity argument.

- *Line 532: "Dipeptide inhibition of PEPCK activity could serve 530 to promote the PPDK route. Dipeptide levels would then provide direct information 531 about the rate of proteolysis." This is overly speculative. Are not PEPCK and PPDK both gluconeogenic, what effect would choosing one route over another have?*

Reviewer rightly points out that both PEPCK and PPCK are gluconeogenic enzymes important for seedling establishment. However, Eastmont et al. (2015), Nat. Commun. 6, 6659, demonstrated that these enzymes channel carbon derived from different sources. PEPCK uses carbon released from lipid reserves, while PPDK channels carbon released from protein reserves. Further, circumstantial evidence linking protein degradation and so dipeptides with the PEPCK activity comes from the work of (Raineri et al., 2016, Plant Cell Rep. 35, 1875-1890). Transgenic Arabidopsis lines with impaired protein degradation show higher rates of lipid consumption, with a concomitant increase in PEPCK activity. Moreover autophagy, which we previously showed is a source of dipeptides, is also important for seedling establishment (Avin-Wittenberg et al., 2015, Plant Cell 27, 306-322). We revised the discussion accordingly.

1st Revision - Editorial Decision 29th Apr 2021

Thank you for submitting your revised study. The manuscript has now been sent back to the original referee #1, whose comments are appended below.

As you will see, this referee finds that most criticisms have been adequately addressed. However, s/he also stresses that the lack of in vitro data to validate the in vivo results would need to be further discussed in the discussion section.

In addition, there are few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

Referee #1:

In the revised version, the authors addressed most of the points raised by the reviewers and several problems have been satisfactorely solved. In vitro experiments with mammalian GAPDH which have been criticized in the first revision, were removed but could not be substituted with similar experiments performed with the plant enzyme because of technical problems. So, the current manuscripts shows no in vitro data to further circumstanciate the in vivo evidence. On the other hand, the results from the in vivo experiments showing the positive effect of Tyr-Asp on biomass yield under stress are solid and statistically supported. The problem tackled by this paper is in my opinion very interesting and potentially very relevant.
These are the points that I think still need to be considered:
1. In the rebuttal letter it is reported that the assay used in this paper can differentiate between GAPC and GAPA/B activities, but I think this not correct. Figure 1 clearly shows that the GAPC assay measures also the NAD-dependent activity of GAPA/B (Fig. 1B), which is the activity detected in gapc1gapc2 mutants. Indeed, the NAD-activity of GAPA/B (as detected with the GAPC assay, Fig. 1B gapc1gapc2) is much higher than the activity of GAPC itself (Fig. 1B wt-gapc1gapc2). The reason why GAPA/B appears to function with NADP (Fig. 1C) at similar rates as with NAD (Fig. 1B gapc1gapc2) is possibly because GAPA/B is not fully activated. Nevertheless the data of Fig. 1, thanks to the comparison between wt and gapc1gapc2 plants, also show that GAPC and not GAPA/B nor GAPN are the targets of Tyr-Asp inhibition. So, I would not consider the unspecificty of the assay as a major limitation for the interpretation of the results. Moreover, Fig. 1 also shows that GAPC activity is almost completely inhibited by the Tyr-Asp treatment and raises the question whether lower concentraions of Tyr-Asp might also be effective. That GAPC appers to be fully inhibited in 100 uM Tyr-Asp experiments is commented in the letter but I think should be underlined also in the paper.
2. Line 224-232. Tyr-Asp is estimated to be ca. 1 uM in seedlings cells, that would correspond to 26 uM if it was concentrated in the cytosol. However, there's no evidence that Tyr-Asp is confined in the cytosol, and using this value to suggest that 100 uM Tyr-Asp treatments are in line with physiological concentrations is questionable. All treatmentes in the current paper were performed with 100 uM Tyr-Asp and, at the present state, there's unfortunatley no indication that this treatment generates a cytosolic concentration of Tyr-Asp that is in the range of physiological or pathological concentrations.
3. As written above, the previous version of the paper included in vitro experiments with mammalian GAPDH. Now these have been deleted without being substituted with same experiments with plant GAPDH due to technical problems. I agree with the choice of avoiding the confusion between animal and plant enzymes, but I have to say that the absence of in vitro experiments on the Tyr-Asp/GAPC interaction, which may say which are the relevant concentrations involved, reduces somehow the overall strength of the paper.
4. Line 247. The authors revised this part on the C1/C6 ratioes, but the corrected version was the first one: C1 is released by both PPP and glycolysis/TCA while C6 is released by glycolysis/TCA but not PPP, as also shown by the new supplementary Fig. 2.
5. Line 360 and Fig. 4. If I understood well Fig. 4, the position 1 and 2 of Tyr-Asp binding to GAPC are close to the nicotinamide and adenine moiety of NAD+, respectively, and not vice versa as reported in the text.

The authors performed the requested editorial changes.

EMBOJ-2020-106800 Tyr-Asp inhibition of glyceraldehyde 3-phosphate dehydrogenase affects plant redox metabolism.

Rebuttal Letter

Referee #1:

We are grateful for the reviewers' comments we addressed them as follows:

1. *In the rebuttal letter it is reported that the assay used in this paper can differentiate between GAPC and GAPA/B activities, but I think this not correct. Figure 1 clearly shows that the GAPC assay measures also the NAD-dependent activity of GAPA/B (Fig. 1B), which is the activity detected in gapc1gapc2 mutants. Indeed, the NAD-activity of GAPA/B (as detected with the GAPC assay, Fig. 1B gapc1gapc2) is much higher than the activity of GAPC itself (Fig. 1B wt-gapc1gapc2). The reason why GAPA/B appears to function with NADP (Fig. 1C) at similar rates as with NAD (Fig. 1B gapc1gapc2) is possibly because GAPA/B is not fully activated. Nevertheless the data of Fig. 1, thanks to the comparison between wt and gapc1gapc2 plants, also show that GAPC and not GAPA/B nor GAPN are the targets of Tyr-Asp inhibition. So, I would not consider the unspecificty of the assay as a major limitation for the interpretation of the results. Moreover, Fig. 1 also shows that GAPC activity is almost completely inhibited by the Tyr-Asp treatment and raises the question whether lower concentraions of Tyr-Asp might also be effective. That GAPC appers to be fully inhibited in 100 uM Tyr-Asp experiments is commented in the letter but I think should be underlined also in the paper*

We thank you, reviewer, for bringing this point up. We rewrote the result section accordingly.

2. *Line 224-232. Tyr-Asp is estimated to be ca. 1 uM in seedlings cells, that would correspond to 26 uM if it was concentrated in the cytosol. However, there's no evidence that Tyr-Asp is confined in the cytosol, and using this value to suggest that 100 uM Tyr-Asp treatments are in line with physiological concentrations is questionable. All treatmentes in the current paper were performed with 100 uM Tyr-Asp and, at the present state, there's unfortunatley no indication that this treatment generates a cytosolic concentration of Tyr-Asp that is in the range of physiological or pathological concentrations.*

Reviewer is correct, hence we removed the statement about 100 µM concentration of Tyr-Asp being close to physiological.

3. *3. As written above, the previous version of the paper included in vitro experiments with mammalian GAPDH. Now these have been deleted without being substituted with same experiments with plant GAPDH due to technical problems. I agree with the choice of avoiding the confusion between animal and plant enzymes, but I have to say that the absence of in vitro experiments on the Tyr-Asp/GAPC interaction, which may say which are the relevant concentrations involved, reduces somehow the overall strength of the paper.*

We agree with the reviewer; therefore, we introduced a paragraph in the discussion touching on the importance of in vitro experiments to understand the physiological significance of Tyr-Asp inhibition of the GAPC activity.

4. *4. Line 247. The authors revised this part on the C1/C6 ratioes, but the corrected version was the first one: C1 is released by both PPP and glycolysis/TCA while C6 is released by glycolysis/TCA but not PPP, as also shown by the new supplementary Fig. 2.*

Is now corrected.

5. *5. Line 360 and Fig. 4. If I understood well Fig. 4, the position 1 and 2 of Tyr-Asp binding to GAPC are close to the nicotinamide and adenine moiety of NAD+, respectively, and not vice versa as reported in the text.*

Is now corrected.

2nd Revision - Editorial Decision 13th May 2021

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Aleksandra Skirycz
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-106800

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was based on analogous experiments in the past, following field standards.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were analyzed assuming normal distribution based on the character of the measured traits and small-sample size (n=3-12).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
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http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Variance was estimated using F-test statistics embedded in the Graphpad Prism software.
Is the variance similar between the groups that are being statistically compared?	Yes (see above) and hence data were analysed assuming similar variance between the groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Figure S11: Membranes were incubated with rabbit polyclonal antisera raised against recombinant AthPEPCK1 (Rojas et al, 2020). The load control was performed by stripping the membrane and incubating with rabbit polyclonal antisera raised against recombinant Triticum aestivum GAPDH (TaeGAPDH) (Piattoni et al, 2017).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Proteomics data were submitted to PRIDE; submission number PXD019332. https://www.ebi.ac.uk/pride/markdownpage/submitdatapage
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A