PAPST2 Plays Critical Roles in Removing the Stress Signaling Molecule PAP from the Cytosol and its Subsequent Degradation in Plastids and Mitochondria

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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.)

TPC2017-00979-RA 1st Editorial decision – *declined* Feb. 13, 2018

The manuscript is potentially an important step forward in sulfur metabolism and retrograde signalling. The reviewers and the editors agree that this is a substantive study that provides new insight into PAP and PAPS transport and action. However, there were a series of critical comments by the three reviewers, including (i) the quality of localisation data, (ii) some overinterpretation where underlying mechanism were speculated but not demonstrated, and (iii) whether the advances were substantive enough for The Plant Cell. Also of note are the comments of reviewers #2 and #3 about the differential effects on growth (LD and SD). That is, the observation that the plants were bigger in the PAPST2 mutant due to changes in cytosolic PAP and smaller in the *sal1papst2* double mutant due to changes in cytosolic PAP. These two conclusions seem in juxtaposition. Also, it was not demonstrated whether the observed changes in PAP pools could be linked to the physiological changes you reported. In this respect, the recent publication by Phua et al. 2017 may be of benefit. A shared concern by the reviewers was that the data were at times over-interpreted and the experiments demonstrating causality were not undertaken, such as nuclear gene expression, changes in mitochondrial respiration, effect of PAP concentrations on size, etc. Another complication for the reviewers in interpreting your data was the challenge of the somewhat contrary observations that PAPST2 is proposed to be critical for PAP-mediate signalling, yet PAPST1 can mostly complement it. It will require some thought and experimentation to resolve these issues. The reviewers have provided a lot of advice and given considered reviews, and we hope you find this of use as you revise the manuscript for another journal.

Of course, this decision requires judgment and we recognize that we may sometimes mis-judge important work or misinterpret its scope. We hope that this is not the case here, but as working scientists who ourselves are subject to peer-review, we are willing to hear thoughtful rebuttals after you have had some time to digest these comments.

---------- Reviewer comments:

[Reviewer comments shown below, followed by the author response letter]

TPC2018-00512-RA Submission received July 6, 2018

Reviewer comments on previously declined manuscript; **author responses are shown below:**

Reviewer #1:

This study identifies/characterizes a functional homolog of the previously characterized PAPS1 plastid transporter. In contrast to PAPST1, PAPST2 is dually localized to mitochondria and plastids. However, as stated in the last sentence of the Introduction (page 6 - lines 149-151), the dual localized transporter is of minor importance in sulfur metabolism but is a key component for mitochondrial PAP degradation (since it seems to be the only PAP membrane transporter in mitochondria). This is a nice and high-quality study and also quite well written. However, it does feel somewhat incremental, as the major pathways and significance of PAP and PAPS have now been well established. But this new study does establish PAPST2 as critical for PAP degradation in mitochondria.

Point 1. Figure 1 & text - page. Why do the vesicles import ATP when preloaded with ATP?

Point 2. Page 9 - Describe the T-DNA lines that are not affected in mRNA level in the Methods section rather than the Results. I suggest showing the gel image with RT-PCR products including a control e.g. *ACTIN2*; add this to Supplemental Figure 1 as well as Figure 5C.

Point 3. Discussion - can the authors speculate on substrate affinities and fluxes when PAPST2 is located in envelopes versus mitochondrial membranes? Can one exclude that these PAPST2 properties are otherwise differentially regulated in these two different membrane systems? I think it would be good to elaborate more on the differential roles for mitochondria and plastid in the legend of Figure 11 (the schematic figure). I think it is important for the reader to appreciate the different metabolic roles and ATP/ADP concentrations in these organelles.

Reviewer #2:

The manuscript by Gigolashvilli et al. identified and characterized a PAPS/PAP transporter, PAPST2, which is dual-localized to both chloroplasts and mitochondria. The authors found that surprisingly, PAPST2 appears to be more important for removal of PAP into plastids and mitochondria for degradation and it plays a relatively minor role in PAPS supply to the cytosol compared to its homologue PAPST1. Based on biochemical and extensive genetic data, the authors suggest that PAPST2 may play an important role in organelle-to-nucleus PAP retrograde signalling. Overall, the authors' findings are solid and can make an important contribution to the field. However, in my view, there are also several aspects that need to be address

Point 1. Discussion and interpretation of the biochemical data need to be improved.
i. In Table 1, Coenzyme A (CoA) decreased PAPST2 activity by nearly half, yet this is not discussed at all. There is high utilization of CoA and its derivatives in plastids and mitochondria. Interestingly, PAPST1 is relatively impervious to CoA inhibition. These factors could also contribute to the contrasting growth phenotypes of *papst1* versus *papst2* mutants and need to be considered.
ii. There is inadequate discussion on why PAPST1 and PAPST2 show different substrate affinities and velocities in vitro - the sequence alignments in Gigolashvili 2012 show very high sequence similarity. Do the differences between these two proteins correspond to known binding site residues, etc.?

Point 2. Localization data are not high quality and require improvement.
i. The images are all too dark and cannot be easily interpreted. While this is understandable for the native promoter GFP fusions, I do not see why signal intensity was a problem for the 35S constructs.
ii. It is surprising that mitochondrial localization was only observed in root cells (3C), but not in the mesophyll protoplasts in Figs 3A and B.
iii. In Figs 3A and B, it was not clear whether PAPST2 was similarly targeted to both the chloroplast membrane and also thylakoid membranes like PAPST1 is.

Point 3. Some aspects of the genetic data are confusing/contradictory, in particular the relationship between PAPS/PAP levels, SOT regulation, and growth phenotypes.
i. The enhanced growth of *papst2* (Fig 5) and the up-regulation of several SOTs in this mutant (Fig S3) were attributed to the decreased PAP abundance in organelles and more PAP in the cytosol for papst2. Yet in Fig 9, the authors now propose that enhanced cytosolic PAP concentration (and less PAP in organelles) had an opposite effect and contributed to the retarded growth in *fry1papst2*. This is contradictory.
ii. Also, other groups have shown that even intermediate PAP levels retard growth e.g., *apk1apk2sal1* in Estavillo 2011 TPC, so the authors need to show convincing data that higher PAP levels in the cytosol can indeed lead to enhanced growth in *papst2*.
iii. Other alternative explanations for the enhanced growth in *papst2* were not considered e.g., altered energy metabolism, which would be very likely since *papst2* shows different growth under short vs. long days. This could be due to the altered transport of ATP, ADP and CoA or an inhibitory effect of PAP on mitochondrial and plastid metabolism. A simple test for mitochondrial respiration in *papst2* under long vs. short days would address this hypothesis.
iv. I was surprised by the enhanced growth of the *fry1apk1apk2apk3* and *fry1apk2apk4* quadruple mutants relative to the parental *apk1apk2apk3*, *apk2apk2apk4* and *fry1*. The quadruple mutants would have the same PAPS limitation as the triple mutants, yet now the PAPS limitation is affecting growth to a lesser extent. Why is this the case?

Point 4. The connection to mitochondrial retrograde signalling involving SAL1 in mitochondria is an interesting aspect. I think most of the follow-up work would be beyond the scope of this present manuscript. However, one aspect in particular should be tested now. If there is indeed a higher concentration of cytosolic PAP in *papst2*, then there is presumably also a higher PAP concentration in the nucleus. The authors should test whether PAP-responsive genes in *fry1* like *APX2* are up-regulated in *papst2*, and whether then there is also altered stress tolerance in *papst2* as would be expected for a mutant with altered PAP distribution/plastidic access to PAP for degradation.

Reviewer #3:

Importance of findings: The manuscript characterizes the function of the mitochondrial membrane transporter PAPST2, which exchanges several adenosine compounds. PAPST1 is dual localized in chloroplasts and mitochondria and is doing largely the same. The relevance of the manuscript comes from the assumed function of PAP as a retrograde signal from chloroplast to nucleus. If PAP cannot be removed from the cytosol into the organelles for degradation by the enzyme SAL1/FRY1, stress signaling is disturbed. Using crosses of *sal1/fry1* with *papst* mutants, the authors show that the mitochondria-localized PAPST2 transporter is relevant for PAP removal from the cytosol and that PAPST1 is more relevant for PAPS export from plastids. These are significant findings for the metabolic part of PAP retrograde signaling, but it is hard to judge from the way the results are presented (see manuscript title!) how interesting this aspect is for a broader readership.

Quality of experiments: In general, the experiments are well prepared, but there are exceptions (particularly the localization data are actually insufficient). There are lots of minor shortcomings, and numerous cases of over-interpretation of results, leading the reader to conclusions that are not fully evidenced and justified. A large part dealing with sulfate reduction and glucosinolates is circumstantial and adds little to the main findings.

Point 1. Fig. 1: Clear uptake and liposome experiment, but the shown rates are very much in the same range of Vmax and also initial slopes differ only marginally. What is known about intracellular concentrations of ATP, ADP and PAPS?

Point 2. Fig. 2: Nice loading experiment. Differences between exchange rates of ATP, ADP, PAPS and PAP are marginal. How were these three independent experiments normalized to each other? Significance analysis is missing. Only one time point is shown: Was the exchange linear during this period?
Line 175 pp.: A comparison with the PAPST1 uptake data from Gigolashvili et al. (2012) actually reveals relatively little differences with respect to preferences of PAP competition of ATP transport (18% for PAPST2, 22% for PAPST1) or PAPS (38% for PAPST2, 31% for PAPST1). A bit stronger are the differences for ATP uptake in loaded

liposomes: PAPST2 shows 70% for PAPS and 120% for PAP relative to ATP, and PAPST1 shows 80% for PAPS and 60% for PAP. Despite the two-fold difference in the PAP exchange rate, this appears to be in a similar physiological range. There may be in vitro preferences recognizable, but these need to be related to intracellular concentrations of the compounds. The conclusions should therefore be much more precise and cautious.
Line 190: Km/Ki ratios should be properly named here. A thorough comparison of kinetic data of PAPST2 and PAPST1 from Gigolashvili et al. (2012) would be really helpful. To this end, the same kinetic parameters should be calculated for all substrates; otherwise, it is impossible for the reader to find out similarities and differences between the transporters.

Point 3. Fig. 3: The approach of using 35S and native promoter constructs is good, but the quality of the confocal imaging for subcellular localization is really weak and not convincing at all. 3AB: Better adjustments of the confocal required, especially in B, barely acceptable. 3C: This could be anything, controls or markers are missing. 3D-H: Inacceptable. Nothing is clearly visible, overlays are missing.
Line 203 pp.: The PAPST2 knock-out has different consequences compared to the PAPST1 KO because it is not only localized in chloroplasts but also in mitochondria (assuming the confocal data are correct). It is therefore essential that this dataset is really convincing.

Point 4. Fig. 4: Two different promoter sizes were used for the GUS fusions. The legend fails to state which one is shown in A to H. In this context, it would be interesting to compare the expression levels of *PAPST1* and *2* in the wild type. Which one is stronger?

Point 5. Fig. 5A: A proper growth curve would tell if *papst2* plants grow faster or reach larger final rosette size. Why does this larger size appear only under short day/long night conditions? Why would PAP retrograde signaling be affected by day length? In this context, it is important to indicate for each experiment whether plant genotypes were grown under SD or LD conditions. The phenotypic difference between the two mutant lines is crucial, but the analyses shown are rather superficial.
Fig. 5B: Which generation are the amiRNA lines? Was *PAPST1* expression affected or was the amiRNA specific for *PAPST2*? The same would be good to know for the *papst* T-DNA mutants, together with the comparison of expression levels of *PAPST1* and *2* e.g. in leaves as a possible indication of their importance.

Point 6. Fig. 6A: Total glucosinolate levels are significantly reduced in *papst1* and *2*, but more so in *papst1* (and not marginally, as concluded in the paragraph title p. 10. Indole glucosinolate levels are not changed at all, only those of aliphatic glucosinolates. How can this be explained by PAP transport?
Fig. 6B: desulfo-glucosinolates indeed accumulate strongly in both mutants: about 10x in papst2 and 120x in *papst1*. Thus, PAPST2 appears to have a stronger influence (on desulfo-glucosinolate accumulation) than PAPST1. This is correlated with the expression patterns of the sulfotransferase gene family (Suppl. Fig. S3). The authors claimed that only the ST belonging to glucosinolate biosynthesis shows higher expression in *papsts1* than in *papst2*. I suppose it is *AtSt5a*, but please indicate this. More importantly, this gene is about 2-4-fold upregulated, but two other family members are up about 8- to 30-fold. How specific are these STs really, and how pleiotropic are the *papst1* or *2* effects? Where are these strongly upregulated STs localized in the cell? In general, these data are rather circumstantial and distract from the main story and could go into the Supplement. They add little to the significance of PAP removal by PAPST2.

Point 7. Fig. 7: The titles of the legend and corresponding paragraph (p. 11) should avoid the statement that the sulfate reduction pathway was affected or stimulated. Correct is that *papst1* and *2* inactivation results in the accumulation of thiols (possibly indicative of an enhanced flux in the pathway). It would be supportive to see at least the expression profile of the sulfate reduction-related genes. Gamma-EC levels in wild type appear to be higher than those of cysteine? Very unusual.

Point 8. Fig. 8A/p. 12 - line 329: 'hypothesis that PAPSt2 is more important in organellar PAP translocation' What is this supposed to mean relative to all the finding in the previous figures where PApexport was attributed to both mutants and *papst1* had supposedly stronger metabolic defects?
Line 333: two *fry1/sal1* mutant lines are mentioned but only one is investigated. Why mention the other one?
Line 340: why could the *fry1papst2* double mutant partially be rescued in the *fry1/papst1* double mutant due to the

ADDITIONAL ABSENCE of PAPST1? Is this a typo?
Fig. 8B/p.12: The phenotype of *fry1papst2* of anthocyanins, failure of seed production and particularly chlorotic areas in cotyledons are not well documented. In addition to photos (not convincing, some of the cotyledons of other genotypes look similar), some sort of quantification would be appropriate.
Line 351: The different levels of PAP in *fry1papst1* and *fry1papst2* (12 vs. 6 pmol/mg fw) as alleviating effect is statistically significant but not clearly proof for physiological relevance. It remains a 6-fold accumulation in *fry1 papst1*!
Besides, between Figs. 9A and B, the mean level of PAP in *fry1* also differs by 25%. Likewise, the difference between *fry1* and *fry1papst2* levels (Fig. 9A) may be statistically significant but with 20-25% difference, this appears physiologically meaningless. It is an over-interpretation to link these differences as an 'accumulation-related phenotype' (line 360). Similar statement in line 351, but there is only correlation, whereas the authors insinuate a functional link that is simply not proven.
Line 366 How do we know about the cytosolic PAP concentration in *fry1papst2*? What is the evidence other than indirect interpretation?
Line 374: Indeed, all mutant combinations carrying inactive *apk1* show no PAP accumulation. But what is the explanation?

Point 9. Fig. 11: The figure is quite useful. However, the indicated substrates and transport directions of PAPST1 and 2 are very much over-simplified. Both transporters evidently can carry out the same transport processes and it is the localization that makes the important difference.

AUTHOR RESPONSE LETTER: Following your decision letter regarding the manuscript "*Identification of Organellar 3'-Phosphoadenosine 5'-Phosphate Transport Systems of Arabidopsis thaliana*", we have substantially revised the previous manuscript and would like to submit it again. We thank the Editor and reviewers for their very helpful and supportive comments. Taking their advice, we have significantly improved the previous manuscript, added new experiments substantiating our main findings, worked through the details in the comments of the reviewers, and revised the paper accordingly. The main points of the revision are as follows:

(i) **To reveal physiological differences between PAPST1 and PAPST2 *in planta* and to resolve their redundancy, we performed complementation experiments by expressing *PAPST2* and *mitPAPST1* in *papst2*. These experiments clearly demonstrated that opposite to *PAPST2,* which restores *papst2* performance, *mitPAPST1* is unable to complement *papst2* as it causes worsening of plant growth and bleaching of leaves (Figure 6). We believe that this strong phenotype results from the depletion of cytosolic PAPS (due to import of PAPS into mitochondria) and the lack of sulfated hormone-like peptides (like PSK, PSY, RGFs etc.), as we observed the upregulation of *PSKs* in *mitPAPST1ox* plants (Supplemental FigureS2). The inability of PAPST1 to rescue *papst2* provided additional evidence for PAPST1 serving as a PAPS exporter from plastids whereas PAPST2 functions as an importer of PAP into both mitochondria and plastids.**

(ii) **To address the role of PAPST2 in the modulation of cellular PAP levels, we measured PAP concentrations in chloroplasts and the cytosol in a set of selected mutants including *papst1, papst2, fry1papst2* and *fry1papst2* by non-aqueous fractionation (NAF). This method revealed a significant accumulation of PAP in the cytosol of *papst2*and *fry1papst2* (but not in *papst1 or fry1papst1*), substantiating the importance of PAPST2 in PAP transport. While moderately increased cytosolic levels of PAP in *papst2* induce growth promotion, the strong increase in PAP levels in *fry1papst2* cells has growth inhibiting effects, as it causes an activation of stress-responsive genes.**

(iii) **To assess the growth phenotype of *papst2* and *amiRNA* plants, we present an anatomical cross-section showing a significant increase in size of palisade and parenchyma cells (Figure 5B). We also made clear that the growth phenotype of *papst2* is present both at LD and SD conditions, although sometimes it might be better visible under SD.**

(iv) **Concerning the juxtaposed effects of two different PAP concentrations on plant growth, we made clear that two different concentration ranges of PAP activate two different plant responses and two different sets of genes. While a low-to-moderate increase in PAP levels (mainly regulated by PAPST2) activates genes related to growth promotion, high PAP levels induce the *fry1* phenotype and activation of e.g. stress-responsive genes. The concentration range of about 0.5-1.0 pmol/mg PAP in the cell is growth**

promoting, whereas 10 pmol/mg and higher (as observed in *fry1* and *fry1papst2)* is growth inhibiting. Upregulation of stress-responsive genes in *fry1,* as previously documented {Estavillo, 2011 #235}, has been attributed to high cytosolic concentrations. In a follow up paper (which will be also submitted this summer), we will be addressing in more detail the growth-promoting effect of moderately increased PAP level concentrations in improved plant growth.

(v) **To address the possible role of PAPST1 and PAPST2 in the accumulation of adenylates (ATP, ADP and AMP) and to address the possible correlation of their concentrations with the growth of *papst2*, we measured ATP, ADP and AMP levels in chloroplasts and cytosol using NAF (Supplemental Figure S6). Interestingly, the accumulation of ATP was increased in chloroplasts of *papst2, fry1* and *fry1papst2* mutants – i.e. in all mutants accumulating PAP. This changes in ATP accumulation in chloroplasts did not correlate with improved (in *papst2*) or worsened (in *fry1* and *fry1papst2*) growth of mutants. We believe that ATP accumulates in chloroplasts because it is an excellent counter-exchange substrate against PAPS in response to increased concentrations of cellular PAP. This is e.g. the case in *fry1, papst2* and *fry1papst2* mutants. As PAPS export from the chloroplasts is absolutely essential for the survival of the plant and PAP cannot move against its concentration gradient, PAPS seems to be counter-exchanged against ATP.**

(vi) **We revised Figure 3 showing the subcellular localization of PAPST2-GFP and exchanged faint photographs by better ones. Supplemental Table S2 additionally supports the localization statement by summarizing the results of nine localization prediction algorithms for both chloroplasts and mitochondria, as well as an empiric proteomics study showing the presence of PAPST2 in these organelles.**

(vii) **We also reshaped the biochemical studies showing the activities of PAPST2 and PAPST1 by placing kinetic parameters of PAPST1 (from Gigolashvili et al., 2012) next to PAPST2. Nevertheless, biochemical characterization revealed that PAPST2 shows approximately nine times lower (~340 µM, Supplemental Table S1) than that of PAPST1 (~40 µM, Gigolashvili et al., 2012). In line with this, analysis of plants revealed that PAPST2 is critical for PAP transport, whereas PAPST1 is mostly important for PAPS transport *in vivo*. Both transporters can only partially complement each other.**

TPC2018-00512-RA 1st Editorial decision – *revision requested* **Aug. 15, 2018**

The reviewers and editors agree that the manuscript has substantially improved and, with further revision, would be of interest to the plant biology community. However, it is critical that you address the concerns of the reviewers and provide a "response to reviewers" letter detailing what you have done, highlighting them in a version of the manuscript showing changes. If a request is not directly addressed in the manuscript, explain why not.

The major concerns relate to
- Targeting. Please provide additional controls, scale bars etc. as requested and better answer the concerns raised. Beyond that, I think only limited additional work is required.
- Growth. Please address the concerns for better analysis of the growth experiments - photos of plants are not sufficient. I think the suggestion for biomass, both fresh weight and dry weight, can be done for one set of key comparisons and quantification of plant area of the existing photos using image analyses for the rest.
- Stress. I do not think you need to do more work on the stress tolerance or lack thereof, but I do agree with the arguments made by the reviewer and request you modify the text accordingly.
- GUS. It is essential to show the number of lines in a promoter:GUS experiment (and ideally this should be 10-20 due to position effect). Please correct as requested and move this to suppl. data. As long as the number of lines is greater than five and the similarity of them is described in the suppl. Figure, this will be sufficient. Also, fix the Methods on this section too, as it doesn't describe the number of lines etc.
- Statistical analyses, Methods, figure legends. Each reviewer raised different concerns about this. Please check very carefully, correct and add requested information both from this round of review and the prior round.
- "Page 7 and Figure 2. I don't understand this section - if PAP is so efficient at driving ATP uptake, why is ATP uptake (%) so low? And I don't follow the rest of this figure/text either." Please address this query by the reviewer.
- Bleaching - please address the concerns raised by the two reviewers.

---------- Reviewer comments:

[Provided below along with author responses]

TPC2018-00512-RAR1 1st Revision received Oct. 15, 2018

Reviewer comments on previous submission and **author responses**:

Reviewer #1

This revised study concerns the role of the dual-localized PAPST2 transporter and its complementary role to plastid PAPST1. The revised manuscript includes several new and improved experiments, and the overall quality of the study is much improved. The study now convincingly shows the dual plastid and mitochondrial localization of PAPTS2, the effect on subcellular concentrations of PAP, ATP, ADP and AMP through non-aqueous fractionation in the different mutant backgrounds. The study provides in-depth insight in PAP metabolism and the complementary roles of PAPST1,2 in regulating PAPS fluxes in and out of the cytosol, PAP degradation, and the consequences for plant growth and stress response. However, there are a number of issues that still need to be addressed - most relate to specific information in figures, tables and methods, missing words & spelling mistakes in the text. Also, the figure layouts need systematic improvement. The list of comments above can surely be expanded but I ran out of time.

Point 1. Page 7 and Figure 2. I don't understand this section - if PAP is so efficient at driving ATP uptake, why is ATP uptake (%) so low? And I don't follow the rest of this figure/text either.

RESPONSE: We have slightly rewritten this section to improve the meaning. Three different types of experiments on page 7 aim at describing that: i) PAPST2 is capable of transporting various adenylates (e.g. ATP, ADP, PAPS - Figure 1); ii) PAP is capable of competing with ATP for uptake, as it is taken up by the PAPST2 transporter equally well (that's why ATP uptake is reduced) –Table 1. iii) If ATP and PAP are offered in experiments with pre-loaded liposomes, measuring the antiport, the import of ATP is stimulated/moved by the export of PAP (but also by ATP, ADP and PAPS), Figure 2. This third experiment demonstrates the nearly identical ability of ATP and PAP (when present in the liposomal lumen) to drive the import of ATP via PAPST2.

Point 2. Page 9 Figure 5A. Please provide some quantitative information as to the increase in size of the *papst2* mutant (rather than just 'grew larger').

RESPONSE: Information on fresh and dry weights of shoots of *papst2* and *amiRNA* plants (Figure 4E, Supplemental Figure S1B) as well as plants complemented by *PAPST2* and *mitPAPST1* is now provided (Figure 5B).

Point 3. Page 9 - Figure 5C - three of the five plants of line *amiPAPST2-1* are MUCH smaller than wt. Yet the text states that they are the same than wt. Please explain. Make Figure 5D smaller - half of the x-axis is empty

RESPONSE: Done. Only one *amiPAPST1-1* line out of five independent transgenic lines does not show increased biomass and improved plant growth. This one line (*amiPAPST1-1*) has only marginally (statistically not significant) decreased levels of PAPST2 expression. We added this information to the text.

Point 4. Methods lack a number of details, making in very hard for others to reproduce - some are indicated below. Please check the Methods section throughout and make sure all details are provided. Also, if you state 'predicted', explain what predictor was used, etc.

RESPONSE: Required details are now provided.

Reviewer #2:

In this resubmitted manuscript, Gigolashvilli et al. identified and characterized a PAPS/PAP transporter, PAPST2, which is dual-localized to both chloroplasts and mitochondria. The authors nicely show here that cytosolic PAP levels are indeed elevated in *papst2* and that mitPAPST1 is unable to rescue the *papst2* mutant. The authors propose that this reflects PAPST2's primary role in shuttling PAP from the cytosol into organelles, and that PAPST2 plays a relatively minor role in PAPS supply to the cytosol compared to PAPST1. There are also potentially two different

effects of PAP on growth, depending on its levels. These findings can be an important contribution to the field of PAP metabolism/signalling. Detailed comments are listed below.

Point 1. Most of my major comments are questions regarding the *fry1papst2* mutant that require clarification/reconsideration.

RESPONSE: We greatly appreciate this reviewer's attention towards *fry1papst2*. We agree that the *fry1papst2* mutant need to be more thoroughly analysed in the future in order to understand how different PAP levels affect plant growth and which target genes are affected by these concentrations. As this work is devoted to biochemical and physiological analyses of PAPST2, we would like to avoid making hasty conclusions on the potentially different functions of PAP concentrations in the cell. In revised version of the manuscript, we provide some new data on *fry1papst2*, which allows us to stay within the scope of this manuscript and to make grounded discussion on questions raised in this study.

Point 2. Are the PSK genes similarly up-regulated in *fry1papst2* compared to *mitPAPST1::papst2*, i.e., is the growth retardation related to suppression of sulfotransferase activity?

RESPONSE: We agree that the *fry1papst2* and *mitPAPST1papst2* mutants need to be more thoroughly analysed in the future, in order e.g. to distinguish whether growth inhibition is caused by PSK or sulfotransferases. However, PSK2 and PSK4 precursor genes are not the only genes that can be attributed or are potentially responsible for growth promotion of plants. Other hormone-like peptides, including RGF1-RGF9, PSK1-PSK5, PSY or signalling tRNA are among the potential targets. We will be absolutely thrilled to do more work on the analysis of PAP-mediated growth promotion, the identification of stimulated genes, and unravelling the corresponding growth promotion/inhibition mechanisms and present these data in the future.

Point 3. Why would *fry1papst2* have higher total PAP levels compared to *fry1* if the PAP degradation capacity is similarly impeded between the two mutants? I would expect that the PAP concentration in the cytosol is higher in *fry1papst2* compared to *fry1* (indeed shown in Fig 10B), but total leaf levels would have been expected to be the same. Similarly, since there are higher cytosolic PAP levels in *fry1papst2*, then increased inhibition of sulfotransferase activity would have been expected - so where is the increased PAP coming from?

RESPONSE: We agree with this reviewer that it is not logical to expect more PAP in *fry1papst2* vs. *fry1*. In the revised version of the manuscript, we discuss the unknown (or not well studied) components of PAP synthesis, degradation and regulation. We added the following text: *"It was counterintuitive finding to observe that PAP levels in crude extracts of fry1papst2 were even slightly higher than in fry1 alone. On one side, this can further substantiate important role of PAPST2 in PAP import, but it also indicated that more components beyond enzymatic activities of FRY1/SAL1 can be additionally contributing PAP status in the cell. These components could be PAP catabolizing enzymes and SAL1/FRY1 homologues remaining less studied, yet unknown golgi-localised PAP transporters and sulfotransferases. The golgi-localised sulfotransferase TPST (Komori et al., 2009), is a central player in the sulfation of hormone-like peptides, however as the phenotype of this mutants is not as dramatic as of the APK mutants (Mugford et al., 2010), more sulfotransferase activities are expected to be found in the cell. Furthermore, modulation of PAP by regulatory or signalling proteins, by redox-mediated structural and biochemical modifications of the SAL1/FRY1 enzyme (Chan et al., 2016) is an emerging topic in the plant cell. All these components can additionally contribute to PAP accumulation in the cell, and, ways this is achieved will hopefully get more attention in future".*

Point 4. The accumulation of anthocyanin in *fry1papst2* was interpreted as the double mutants being more vulnerable to stress (Lines 373-374). However, given that the *fry1* mutation causes a constitutive up-regulation of stress responses, and PAP levels are even higher in *fry1papst2* compared to *fry1*, an alternative explanation is that the anthocyanin accumulation in *fry1papst2* reflects constitutive up-regulation of stress responses rather than vulnerability to stress. Does *fry1* at the same growth stage also accumulate anthocyanins? In my view, the stress tolerance of *fry1papst2* needs to be properly tested before this claim is made - in particular, the inability of *fry1papst2* to set seed could well be related to the inhibition of SOTs rather than enhanced stress sensitivity, so it is misleading

to place these two statements next to each other in text.

RESPONSE: We removed our previous statement that *fry1papst2* is more vulnerable to stress and added that that anthocyanin accumulation in *fry1papst2* could reflect constitutive up-regulation of stress responses. As suggested by this reviewer, we also mentioned that the reduced ability to build seeds could be related to inhibited sulfotransferase activity. All *fry1papst2* plants accumulate anthocyanins, whereas in *fry1* this happens only rarely (Supplemental Table S3).

Point 5. It is not clear from Fig 9B whether *fry1* itself already has partially bleached cotyledons? The authors need to provide more substantive evidence e.g. showing pictures of multiple seedlings, better quality photos, or quantification of the extent of bleaching in the different genotypes. Otherwise, this claim should be removed because the single image provided is not convincing.

RESPONSE: We provide new Figure 9B (now Figure 7B) allowing better visualisation of the pale areas in cotyledons of *fry1papst2*, as indicated by arrows. Chlorophyll content of young *fry1* seedlings is not affected, however an "eagle's eye" can recognise tiny pale areas in cotyledons (indicated by arrow). Bleaching of cotyledons along the vein, as observed in *fry1papst2,* was not detected in any other mutant, including *fry1*. In Supplemental Table S3, we provide a table that quantifies the number of plants with bleached cotyledons in *fry1, fry1papst1* and *fry1papst2* mutants.

Point 6. Comments 1(iii) and 1(iv) above have already been raised in the previous round of review, yet not addressed. Similarly, there were a few other specific concerns raised by the previous reviewers that were not addressed in this current version. This is disappointing.

RESPONSE: We apologise, as it was not our intention to ignore your concerns. As this paper was originally not recommended for publication, we were urged to significantly revise the manuscript, generate new data addressing comments asked by all three reviewers and the editors. We hope that the improvements made in the revised version of the manuscript address all essential concerns raised by all three reviewers.

Point 7. Statistical analyses are missing in some results. For example, Line 188 states that only ADP, PAPS, PAP and dATP significantly reduced the uptake of labelled substrate, but no statistical analysis was done to verify this claim. Looking at Table 1, the trends are indeed clear, but there are some compounds that might also show significant reduction, for example Acetyl CoA. Similarly, Fig. S6 lacks statistical analysis.

RESPONSE: Statistical analysis has been added to former Supplemental Figure S6 (now Supplemental Figure S7). As for Table 1, we slightly rewrote this section and exchanged "significantly" with "substantially". As this table describes screening for the potential substrates of PAPST2, we believe that t-test analysis is dispensable here. For more details, previous studies on mitochondrial carrier family (MCF) from the Haferkamp group and other labs working with MCF carriers (e.g. see publications from the group of Palmieri) can be considered.

Reviewer #3:

The new submission of the manuscript is greatly improved. The differential role of PAPST2 is now well recognizable and based on several independent lines of evidence. However, several major and minor issues still need attention:

Point 1. Figure 2 - Uptake into liposomes. The results are now presented much more clearly and provide a direct comparison between PAPST2 and PAPST1. Unfortunately, the data still only suggest that PAPST2 preferentially mediates the exchange of PAP/ATP. This weak biochemical difference needs to be more critically discussed.

RESPONSE: We agree with the reviewer that the biochemical differences as measured in liposomes are minimal. We stressed this fact in the Discussion and added the following sentence: *"Transport studies also revealed relatively high affinity of PAPST2 for ATP. However, ATP was transported with lower maximal velocity than ADP or PAPS. Furthermore, analysis of binding affinities indicated that PAP can successfully compete with the high affinity import substrate ATP. Altogether, these data suggest that PAPST2 may preferentially mediate a PAP/ATP antiport, but PAPS and ADP might compete with these two substrates, dependent on actual concentrations in the respective plant compartments. Altogether, biochemical data reveal that the differences in the uptake and kinetics of potential substrates are not very striking in vitro. That is why the comparative analysis of papst2 and papst1 Arabidopsis mutants and measurements of potential substrates in different plant compartments were carefully addressed here".*

Point 2. Figure 3 - Dual localization of PAPST2. No scale bars are shown in any photo or a note in the legend. The localization is really crucial for the function of PAPST2 compared to chloroplast localized PAPST1 and needs to be done in a state-of-the-art manner. The images in 3C, D have been improved, but the others are the same weak photos. It is confusing what kind of cells were used in 3C to F. Legend: 'Cultured root cells' - what is this, a hairy root culture? 'dark grown mesophyll cells' - how is this done and how were cells isolated? Results: 'cultured Arabidopsis cells'. Methods: ' dark-grown cultured Arabidopsis cells'. Can it be that a cell suspension culture was used? If so, no reference of origin is provided.

RESPONSE: Scale bars were added. The confusion about 'cultured root cells' (= suspension of Arabidopsis cells derived from roots) has been avoided. The section "Arabidopsis Suspension Cell Culture. Cultivation and Transformation" has been added to the Methods section.

Point 3. The finding is that PAPST2only localizes to chloroplasts in mesophyll protoplasts but not to mitochondria (3A,B). PAPST2 fusion signal is found in small and large dots (mitochondria and plastids? No markers shown) in 3C,D using 'cultured root cells'. PAPST2-GFP signal arise solely from mitochondria in 3E,F in 'culture Arabidopsis cells grown in darkness', but not non-green plastids. A plastid control is missing. These severe inconsistencies are only superficially addressed in the text.

RESPONSE: As pointed out by this reviewer, different expression systems provided varying results. In the revised version, we discuss different localization patterns in different expression systems by suggesting that in photosynthetic and non-photosynthetic tissues, PAPST2 might be differentially localized. This can also imply that PAPST2 may have different functions in different tissues. While mesophyll cells may have greater demand for a chloroplastic PAPS/PAP antiporter (chloroplastic localization is preferred), root-cells or other photosynthetically non-active cells may rely on PAPST2. Alternatively, it could be also possible that using different expression systems is the only option to get dual localization results on PAPST2, because of the known limitation of all expression systems in general (Yin et al., 2007). We also provided a plastid localization control (Figure 3C), as requested by this reviewer. More information on this type of protein expression system for MCF proteins and use of different controls using the same expression system can be found (Koroleva et al., 2005; Berger et al., 2007; Gigolashvili et al., 2012).

In the revised version, we also stressed the fact that MCF proteins are known to be typically localized to mitochondria, but also to chloroplasts. Furthermore, bioinformatic tools applied to PAPST2 predicted that this protein is localized in both organelles, and proteomics data had previously confirmed the dual localisation (Supplemental Table S2; Ferro et al., 2003; Millar and Heazlewood, 2003; Kleffmann et al., 2004).

Point 4. Figure 5: Scale bars are missing throughout the figure. The growth differences between mutant *papst2* and *papst1* lines in Fig. 5A really should be better documented by a growth curve based on biomass. The larger cells in *papst2* could simply be based on larger vacuoles containing more water. Fig. 5C also requires proper quantification of the growth of amiRNA lines. This could even be blotted against remaining RNA levels to make this more convincing.

RESPONSE: Scale bars were added, and data on plant biomass (see also response to comment N.4 of reviewer #1) in the form of new Figure 4E (shoot fresh weight) and Supplemental Figure S2B (shoot dry weight) are provided.

Point 5. Fig. 9 again shows no scale bars. The pale look of the *fry1 papst2* line is never analysed in a quantitative manner, e.g. by chlorophyll determination.

RESPONSE: Scale bars are added. The portion of young *fry1papst2* seedlings having cotyledons with pale veins was quantified and is now presented in Supplemental Table S3. We also provide a new Figure 9 (now Figure 7B), where one can visualise pale areas in cotyledons, as indicated by arrows. Furthermore, we mentioned in the text that the chlorophyll content of adult *fry1papst2* plants is not significantly changed (If needed, we can provide chlorophyll quantification). However minimal chlorophyll changes would be expected in cotyledons of young *fry1* seedlings.

TPC2018-00512-RAR1 2nd Editorial decision – *acceptance pending* **Oct. 26, 2018**

We are pleased to inform you that your paper entitled "PAPST2 plays a critical role for PAP removal from the cytosol

and subsequent degradation in plastids and mitochondria" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. The editors concluded that you had met the concerns of the reviewers and the editorial board and consider the article to be a significant contribution to the field.

We do have one more request - that you do complete the statistical analysis of Figure 2, as you make conclusions about differences based on that figure. Also, please check again for errors - for example in the sentence "It was counterintuitive finding to observe that PAP levels in crude extracts of fry1papst2 were even slightly higher than in fry1 alone" the mutants were not in italics.

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 **Nov. 15, 2018**