The Role of Abscisic Acid Signaling in Maintaining the Metabolic Balance Required for Arabidopsis Growth under Non-stress Conditions

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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.)

TPC2017-00347-RA 1st Editorial decision – *declined* June 6, 2017

All three reviewers are positive about multiple aspects of your work, in particular, your focus on examining the involvement of ABA in basal non-stressed growth conditions. This is an interesting and understudied concept in the field, and you have used a series of state-of-the-art methods to examine this question. However, some critical points were raised by the Reviewers. Multiple Reviewers felt that some of the key conclusions were overstated and ignored some of the limitations inherent in your analyses. I am of the opinion that most of the points raised are ones of interpretation and that they can be addressed in the writing without extensive new experimental data. The one exception to this is the analysis of growth rates in the *srk2* triple mutant, raised by Reviewer 3. Reviewer 2 has suggested that you complement the *srk2* mutant using a guard cell-specific promoter, which would help to disentangle complications arising from the altered stomatal conductance of the mutants you characterized, which echoes concerns raised by Reviewer 3. I agree that this would be an elegant experiment that could add a lot to the paper, but it will also add a long time to your publication timeline.

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

[Reviewer comments shown below along with author responses]

TPC2018-00766-RA Submission received Oct. 11, 2018

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

This manuscript describes a nicely designed series of experiments to analyze the metabolic consequences of ABA-related mutants and extends our understanding of ABA responses at the level of primary metabolism. The methods are excellent and the data logical and well analyzed for the most part.

Point 1. The typescript needs some improvement in English language usage in some places.

RESPONSE: We have carefully checked the manuscript with the help of co-authors, including one of the corresponding authors, who are native English speakers, and revised the typescript accordingly.

Point 2. My only scientific critique is the use of the term 'flux analyses' to describe a series of experiments using labeled sugars. Since individual compounds were not measured, the procedures did not account for uptake, the feeding appeared to be continuous and not pulsed, and no attempts to apply absolute quantitative measurements, I would be reluctant to call this 'flux analysis' in modern metabolomic descriptions. Not that the studies are not important and they did make the point, but they are simply measures of the relative rates respiratory activity on a supplied substrate.

RESPONSE: In response to the comment, we often used the term 'isotope-labelling experiments' for estimation of metabolic flux in this revised manuscript in all but one case, which we discuss in detail within the text, and for which we strongly believe the terminology is justified.

Point 3. Similarly, sugars also function as signaling molecules as well as substrates, so some discussion of the metabolomic consequences of sugar feeding would be a useful control.

RESPONSE: In response to the comment, we have added a sentence regarding the limitation of our isotope-labelling experiments as follows (page 12, lines 332-338): There was no significant increase or decrease in the Glc content of the plants supplied with [U-^{13}C]-Glc (Supplemental Figure 10, 11), suggesting that there was no major perturbation of metabolism from glucose feeding. Although we cannot exclude the possibility that uptake of exogenous glucose affected glucose signaling, it seems likely that any effects of this kind would occur in both the mutant and WT control plants. Thus, any differences in the distribution of ^{13}C-label in the mutant and WT plants should reflect the inherent metabolic differences between the genotypes.

Reviewer #2:

Yoshida et al. takes an "omics" approach to compare the behavior under non-stressed conditions of Arabidopsis wild type (WT) with ABA-related mutants, namely *aba2* (affected in ABA biosynthesis), and *srk2d/e/i* and *areb* (both affected in ABA signaling). The authors counted the number of leaves in seedlings grown in agar plates and found that it was increased in *aba2* and *srk* mutants compared to WT, while in the *areb* mutant, it was similar to WT. This information was already available in the literature for the *srk* mutant. However, here the authors expanded on this and performed a series of 14C-Glc and 13C-Glc experiments using leaf discs to examine the underpinning metabolic changes and fluxes. Based on their findings, they conclude that SnRK2s have a role in the maintenance of metabolism and leaf growth under non-stressed conditions, mostly via an effect on the TCA cycle, and that this action is likely independent from the activity of the SnRK downstream targets AREB/ABF transcription factors.

Point 1. The fact that ABA might be involved in the regulation of growth under non-stressed conditions is certainly a novel concept in the field, which merits attention. Unfortunately, however, many of the conclusions of this paper are based on observations correlative in nature, and often supported by only minor differences between WT and *srk* mutant at the metabolic level. In some cases, such as for the gene expression levels, it is even difficult to draw a conclusive picture, due to lack of correlation between gene expression and metabolite amounts.

RESPONSE: In response to the comment, we have revised the manuscript and addressed the concerns as described below.

Point 2. The choice of using the *srk* mutant to investigate the effect of ABA signaling on plant growth creates some doubts. The triple mutant *srk2d/e/i* is known for being unable to grow under normal RH due to totally impaired stomatal transpiration, specifically caused by the lack of SnRK2E (OST1), which has a prominent and well-established role in guard cell physiology. Because of the highly wilting phenotype of *srk* mutant, the authors had to perform the labelling experiments with plants grown under high RH.

RESPONSE: According to the suggestions by editors' and reviewers' concerns, we have assessed the *srk2d/e/i* mutant expressing *SRK2E* under the control of the guard cell-specific promoter as well as a series of single and double mutants of the SnRK2s. Collectively, our results suggest that the impaired stomatal closure in the *srk2d/e/i* mutant has little effect on leaf number. In addition, we have addressed the concerns about our feeding experiments as described below. We acknowledge that this suggestion has greatly improved the solidity of our manuscript and thank the reviewers for these suggestions accordingly.

Point 3. Page 9, metabolic fluxes with radiolabeled 14C-Glc. In general, there are major differences in $14CO_2$ evolution between WT normal RH (control) and WT high RH, which suggests that WT plants have significantly modified their metabolism to adapt to the unusual growth condition. Therefore, I wonder, from a physiological point of view, how relevant actually is making a comparison between WT high RH and the *srk* mutant? Additionally, the differences in emission from [3:4-14C] between the *srk* mutant and WT high RH are only minor, as the authors themselves state on page 9, lines 243-244. The same holds true in the case of [U-14C]-Glc labeling: the differences in the label redistribution into the different fractions between WT and *srk* are only minor. Again here, the redistribution of label into cell wall between WT control and WT high RH shows marked differences, and makes me question the overall comparison between WT high RH and the *srk* mutant if the conclusions were to support a role for SnRKs in plant growth under normal growth conditions.

RESPONSE: We recognize the difficulties in assessing physiological roles of the SnRK2s by using the *srk2d/e/i* triple mutant. To minimize the possible effects caused by impaired stomatal regulation in the *srk2d/e/i* triple mutant, we used the plant material grown under high relative humidity conditions. Given that reassimilation of released $^{14}CO_2$ is negligible in the experimental system (Harrison and Kruger, 2008), the potential change in respiration ratio in the *srk2d/e/i* mutant was considered to have little effect on estimated metabolic flux. As we observed, wild-type plants grown under high relative humidity conditions showed altered metabolic profiles compared with those grown under standard conditions. However, since we performed appropriate statistical tests with enough numbers of biological replicates, the changes between wild-type plants and the *srk2d/e/i* mutant grown under the same conditions were considered to be significant.

To carefully interpret the results of the isotope-labelling experiments, we have also performed a series of phenotypic and metabolic analyses. Please also see our detailed responses to the associated concerns raised by the reviewers.

Point 4. To circumvent this inconvenience, it would have been advisable to work with an *srk2d/e/i* mutant in which the *srk/e* mutation was complemented specifically in guard cells. Alternatively, it would have been possible to examine in parallel other mutants impaired in ABA signaling, but showing a less strong phenotype. Examples are the *abi1* mutant, or the more recently published multiple mutant combinations of the RCAR gene family.

RESPONSE: According to the suggestion, we have generated and examined the *srk2d/e/i* triple mutant expressing *SRK2E* under the control of the guard cell-specific promoter of *MYB60*, which has been well characterized (Nagy et al., 2009 J. Biol. Chem. 284: 33614-22). Although ABA responses were partially complemented in the transgenic lines (Supplemental Figure 7), the number of leaves and the leaf emergence rate were comparable with those in the empty vector lines (Figure 2). Given the single and double mutants harboring the T-DNA insertion in *SRK2E* did not show increased leaf numbers, unlike the *srk2* triple mutant (Figure 2), we suggest that the impaired stomatal closure has little effect on the increased number of leaves in the *srk2* triple mutant. As mentioned above, this idea was an excellent one and we thank the reviewer for it.

Point 5. I am also not convinced about the importance of the last paragraph about the transcriptomic reanalysis. Here are my specific concerns: Page 12. Transcriptome data re-analysis. It is not clear if the data from *aba2* are reanalyzed or originally produced by the authors.

RESPONSE: To avoid ambiguity, we have revised the descriptions (page 13, lines 378-382; page 26, line 763-767).

Point 6. Anyhow, I find this paragraph too speculative and not very informative. Assuming that movement of sucrose into cellular compartments is altered based on changes in expression levels of sugar transporters is a quick way to go. We know well that the expression levels are often not correlated with protein levels or activity, and, in many cases, sugar transporters are regulated post-transcriptionally. Similar observation can be made regarding the expression of genes involved in amino acid metabolism. In this case, there is no correlation between levels of amino

acids and gene expression. Finally, there seems not to be agreement in the expression of *TRE1* between transcriptomic data and qPCR. In fact, the reduction observed in the *srk* mutant upon ABA treatment compared to WT is really minor. So, I am not sure what we actually learn from this analyses. And I do not agree with the final statement on page 13, lines 361-363.

RESPONSE: To prevent over speculation, we have more carefully interpreted and discussed the results in the paragraph. We have also revised the conclusion as follows (page 15, lines 428-431): When taken in combination these data suggest that transcriptional regulation by subclass III SnRK2s is important for several metabolic pathways, such as raffinose synthesis and the TCA cycle, and therefore plays a role in the maintenance of primary metabolism under non-stressed growth conditions.

Furthermore, to clarify our claim in this context, we have revised the descriptions regarding *TRE1* expression as follows (page 15, lines 425-427): However, given that reduction of *TRE1* in the *aba2-1* mutant was not statistically significant (*P* > 0.05), these results imply that ABA signaling via subclass III SnRK2s is partially involved in transcriptional regulation of *TRE1*. This critique of the review was also much appreciated as we believe it allowed us to improve the manuscript.

Point 7. Page 13, lines 345-346. What is the relevance for this manuscript that FUM2 is regulated by Snrk2-AREB/ABF in response to ABA? According to this, FUM2 should be then involved in regulating metabolism in response to stress (not under control conditions). This was indeed recently suggested in a paper where FUM2 was implicated in plant acclimation to low temperature.

RESPONSE: To clarify our claim in this context, we have moved the associated descriptions from the Result to the Discussion (page 17, lines 495-499).

Point 8. I have one more comment about the potential targets of SnRK in the regulation of growth under nonstressed conditions. While it is clear from the data presented here that AREB/ABF are not part of this signaling cascade, the paper leaves the readers with many hypotheses but no real answers. The model is therefore too speculative and not supported by the experiments.

RESPONSE: To clarify our conclusion, we have distinguished transcriptional regulation from other regulation in the model (Figure 9). We have also added the associated discussion as follows (from page 19, line 575 to page 20, line 580): Currently, little is known about the transcriptional factors downstream of the SnRK2s, except for the AREB/ABFs, and little attention has been paid to the transcriptional regulation of primary metabolism under non-stressed conditions. Future studies focusing on the candidate metabolism-associated genes, such as *FUM2* and *TRE1*, will aid in understanding how primary metabolism under non-stressed conditions is transcriptionally regulated.

Point 9. Page 3, lines 52-53. I disagree with the statement “plant growth under water-limiting conditions is likely to be actively suppressed than actually limited by resources”. In fact, it has been demonstrated by several studies that, under stress, plant growth is adjusted, not suppressed. For example, shoot growth can be reduced to favor root growth, allowing the plant to increase nutrient and water uptake. In other cases, grain filling can be promoted during stress to accelerate the life cycle in the hope to produce vital seeds before the plant may die.

RESPONSE: To avoid ambiguity, we have revised the sentence as follows (page 3, lines 53-55): Furthermore, plant shoot growth under water-limiting conditions is likely to be actively suppressed rather than merely limited by resources, with the available resources being diverted to enhance root growth.

Point 10. Page 3, line 64-67. I don’t see how the statement of this sentence about control of gene expression in an ABA-dependent or –independent manner can lead to the conclusion that ABA acts as a negative regulator of plant growth.

RESPONSE: In response to the comments from two reviewers, we have revised the sentence as follows (page 3, lines 67-70): In vegetative tissues under osmotic stress conditions, ABA-dependent and ABA-independent signaling pathways function co-operatively to induce a large number of genes involved in signal transduction and stress tolerance (Yoshida et al., 2014), diverting resources away from growth to enhance stress tolerance.

Point 11. Page 6, lines 135-136. I don't agree with the statement "*srk* mutant germinated earlier than the *aba2* in terms of radicle emergence". As shown in Supplemental Figure 1A, the pattern of radicle emergence over time of *srk* mutant overlaps almost perfectly with that of *aba2*.

RESPONSE: To precisely describe the result, we have revised the sentence as follows (page 5, lines 142-143): Due to its weak dormancy, the *srk2d/e/i* mutant showed earlier seedling establishment in terms of cotyledon greening than both the *aba2-1* and *areb* mutants and WT (Supplemental Figure 1).

Point 12. Supplemental Figure 3A. It seems that exogenous application of 0.5 µM ABA induces root elongation in all genotypes, except in *srk*. Isn't this inconsistent with other data presented here, i.e. reduced leaf number, reduced biomass etc.?

RESPONSE: The *areb* and *aba2-1* mutants and wild-type plants grown on agar plates with ABA showed greening cotyledons 1 to 2 days later than control conditions (Supplemental Figure 2). This slightly late start of vegetative growth could be the cause of the reduced leaf numbers and weights. As shown in Figure 2B in the revised manuscript, the leaf emergence ratio of wild-type plants in this growth stage was around 0.7 leaves per day. This result was consistent with the difference of leaf numbers between control and ABA conditions (Supplemental Figure 3C). Moreover, the differences of weights between 10 and 12 days after germination (Supplemental Figure 4A, B) were comparable with those between control and ABA conditions (Supplemental Figure 3D). Collectively, the reduced leaf numbers and biomass in these lines grown on agar plates with ABA were well explained by the late start of vegetative growth, and the results were not inconsistent with slight root elongation in these lines.

Reviewer #3:

The study provides a metabolomic characterisation of Arabidopsis altered in ABA responses by comparing wt with mutants deficient in ABA biosynthesis and signaling. The important metabolic aspect of ABA regulation is currently neglected. The study uses state-of-the art analytical tools to elucidate this process.

Point 1. However, the claim of the study, summarized in the title, is overstated. The initial strategy and working hypothesis are questionable.

RESPONSE: According to the editors' suggestions and reviewers' comments, we have the evaluated leaf emergence rates of young seedlings for two weeks after the appearance of true leaves. The new data support our conclusion summarized in the title and the Abstract. Please also see our detailed responses below.

Point 2. For instance, deficiency in ABA signaling generated by a triple *SnRK2* mutant line may have many secondary physiological effects that are not related directly to ABA signaling. Hence, the conclusion drawn in the abstract ' SnRKs…mediate normal proper metabolism and leaf growth...' is not substantiated.

RESPONSE: To exclude the possibility that the increased number of leaves in the triple mutant is a secondary consequence of other phenotypic changes, we evaluated a series of mutants and transgenic lines. These results support the notion that 'the SnRK2s involved in ABA signaling modulate metabolism and leaf growth under non-stressed conditions by fine-tuning flux through the tricarboxylic acid cycle' (Abstract; page 2, lines 36-38).

Point 3.The growth-promotive trait of the *aba2* and triple *SnRK2* mutants is questionable based on the higher number of leaves at an early developmental stage. These mutants are less dormant than wt (seeds of the triple *SnRK2* have been described as viviparous). Arabidopsis at this young stage shows a doubling time of the fresh weight of approx. 2 days. A single day advantage in development makes a big difference in biomass, more than the authors show as a difference between the triple mutant and wt. What is missing is a kinetic analysis of the seedling development in terms of biomass and number of leaves to assess the difference between the mutants and wt. The authors also state that the size of the *SnRK2* mutant is not different from wt but has a higher number of leaves. How can the conclusion be that SnRK2-deficiency is growth-promotive? Is the effect (if not explained by the non-dormant phenotype) affecting leaf size and number? The leaf number and leaf area need to be determined (also in more adult plants).

RESPONSE: In response to the comment, we have counted the numbers of leaves in the *srk2d/e/i* triple mutant for two weeks and compared the result with those of a series of mutants, including an ABA-biosynthesis mutant (*aba2-1*) and an ABA-insensitive and early-germination mutant (the T-DNA mutant of *ABI3*; *abi3*). Although the numbers of leaves in the three mutants, *srk2d/e/i*, *aba2-1*, and *abi3* were increased at certain time points compared with wild-type plants, the leaf emergence rate after the appearance of true leaves were increased in the *srk2d/e/i* and *aba2-1*

mutants, but not in the *abi3* mutant (Figure 2). These results suggest that the early germination has little effects on the leaf numbers in the *srk2d/e/i* and *aba2-1* mutants during vegetative growth.

We have also excluded the effects possibly caused by impaired stomatal closure as described in detail above. Furthermore, we have evaluated biomass and total leaf area (Supplemental Figure 4), and the results indicated that the relative growth rate of *srk2d/e/i* is not changed compared with wild-type plants. Taken together with our data of metabolite profiling, we suggest that the *srk2d/e/i* mutant is not a 'growth-promotive' mutant but that the impaired regulation of primary metabolism can promote leaf emergence. We have added Figure 2, the associated supplemental Figures (Supplemental Figure 4-8), and the descriptions in the Result section (from page, 6 line 159 to page 8, line 211). We have also revised the associated descriptions in the Discussion (from page 20, line 602 to page 21, line 611) and the legend of the model (Figure 9; page 42, lines 1272-1273). As we mention above, we are very thankful for the prompt to perform these experiments, which we believe allowed us to substantially improve our manuscript.

Point 4. Concerning the metabolic analysis, release of labeled CO_2 was assessed as a means of respiration in light. However, a large portion of respired CO_2 is refixed. In fact, current (simplified) models on CO_2 flux assume (and are still fairly accurate, see Farquhar) no CO_2 diffusion resistance between mitochondria and chloroplast. What is critical for the exchange of CO_2 between plant tissue and atmosphere is the stomatal aperture. The stomatal aperture in *aba2* and triple *SnRK2* is more open than wt. Stomatal aperture has not been assessed and the difference in 'release and respiration' considering the presumed differences not calculated.

RESPONSE: In the experiment system we used to estimate metabolic flux (Figure 6), it has been reported that reassimilation of released $^{14}CO_2$ is negligible (Harrison and Kruger, 2008 Phytochemistry 69: 2920-7). However, we could not fully exclude the secondary effects possibly caused by impaired stomatal regulation in the *srk2d/e/i* triple mutant. We were also aware that stomata of the *aba2-1* mutant grown on standard agar medium are likely more open than wild-type plants (Christmann et al., 2007 Plant J. 52: 167-74). We have added a discussion to this effect in the revised manuscript (page 20, line 581-599).

Point 5. Differentially labeled glucose sources were fed to determine the turn-over by different catabolic pathways and to gain insights into (glucose-based) carbon fluxes. A caveat in these analyses is that they measure sort of 'steady-state' levels without determining the flux in and out for a component other than the original label. While those studies are quite interesting (and challenging), the authors fall short to state their limitations.

RESPONSE: In response to the comment, we have added a description about the limitation of tracer experiments using radiolabeled sugars as follows (page 11, lines 323-326): Radio-labelling approaches give a broad overview of metabolic fluxes into major cellular components, but resolution to the single metabolite level is technically challenging and time consuming. Therefore, we also performed stable-isotope labelling experiments with [U-^{13}C]-Glc to investigate the metabolic alterations in the *srk2d/e/i* mutant in more detail (Figure 7).

Point 6. Results from metabolite profiling and flux analyses indicate that the SnRK2s are involved in organic acid and amino acid metabolism'. See above, claim not supported.

RESPONSE: According to a previous comment, we used the term 'isotope-labelling experiments' in this revised manuscript. A series of metabolite profiling and feeding experiments (Figure 3-8; Table 1) support our conclusion that 'the SnRK2s are involved in organic acid and amino acid metabolism, via effects on the tricarboxylic acid (TCA) cycle, as well as on sugar metabolism' (page 5, lines 119-121). To detail, these include changes in both steady state levels of the TCA cycle intermediates, such as citrate, aconitate and isocitrate, and respiratory fluxes.

Point 7. In summary, I think the ms is from an experimental/technical point very interesting and might be very helpful to the community if the presentation is improved. The reasoning of the comparative approach is not sufficiently substantiated. A general problem is that the mutants used are not only deficient at different steps of the ABA response pathway, they also show different degrees of severity, which might affect the quantitative interpretation of the data.

RESPONSE: According to the editors' suggestions and the reviewers' comments, we have revised the manuscript and addressed all of the concerns in a point-by-point manner as described above.

TPC2018-00766-RA 1st Editorial decision – *accept with minor revisions* **Nov. 30, 2018**

Reviewer 1 is still concerned about the semantics of "flux" and whether or not your experiments really measure flux. This issue is outside my sphere of direct expertise but appears to me to a point that can be addressed in the Discussion. Review 2 has concerns about the growth of plants in Petri plates and the distance of this from real-world rates of transpiration (which the *SnrK2* triple mutant could not tolerate anyhow). I too feel that this is a point for discussion and that you need to address this limitation, nonetheless, it is clear from your data that the *SnRK2* triple mutant has many interesting phenotypes in the absence of water stress and that this is an important dimension to the "basal" functions of ABA, which have received surprisingly little attention over the years. We are therefore of the opinion that the remaining concerns are primarily ones of interpretation -- so long as you address them in the discussion, we are satisfied with your revision and cognizant of the lengths you went in generating transgenic materials to satisfy the reviewers' original concerns.

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

[Provided below along with author responses]

TPC2018-00766-RAR1 1st Revision received **Dec. 13, 2018**

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

Reviewer #1

Point 1. I think the authors have done a good job of responding to the reviewer comments. They have chosen, however, to argue the point about metabolic flux. I do not agree to their point but do agree that many in the literature have made the same over-reaching statements (see, for example, Wang et al Plant Methods 2018 doi:10.1186/s13007-018-0318-3). Simply put, metabolic flux is a measure of the flow of matter over time, and thus has the units of "mole/unit time/mass of tissue". There are basically two methods that have been used for this, for a simple system pulse labeling can be applied and used to 1) test that the system is obeying first order kinetics and 2) measure the half-life of the labeled pool using dilution measurements and the first order rate equation. The second approach is to model a metabolic network and to approximate flux by matching measurements of quantity of compounds and isotopic enrichment from steady state labeling (Hasunuma et al. 2010 JEB doi: 10.1093/jxb/erp374; Reviewed by Kruger et al 2011 JEB doi:10.1093/jxb/err382). These authors, however, have chosen a bit different approach - constant labeling, but not at steady state. While they did measure both labeling and pool size, they cannot calculate a true flux from this experimental design. I realize I might be applying a rigorous standard for these authors, but The Plant Cell should, in my opinion, be setting high standards. In the absence of "mole/unit time/mass of tissue", they are not measuring 'Flux" as we typically understand it and they should use terms like 'approximate', 'indicates' and 'suggests' to differentiate their work for MFA.

> **RESPONSE: We acknowledge the suggestion, and we thus decided to use the term 'relative flux' specifically when the results of the positionally labelled glucose feeding were described, in the revised manuscript (page 10, lines 283-284; page 11, lines 315-317, 320, and 321; page 20, line 590; page 21, line 628). In addition, we have revised the term of the y-axis of Figure 6E as well as in the legend. For interpretation and discussion of the results, the term 'flux' was used, as has previously been done in many other articles (we cite some of these) and take the authors suggestions of using the above words to caveat the wording in order to address this concern.**

Reviewer #2:

I read the revised manuscript of Yoshida et al. about the suggested role for SnRK2s kinases in ABA-mediated regulation of Arabidopsis leaf growth and metabolism, particularly the TCA cycle, under non-stressed conditions. I acknowledge the authors effort of taking this long time to generate and characterize Arabidopsis transgenics overexpressing SnRK2E specifically in guard cells.

Point 1. Regarding the experiments in agar plates. Notoriously, transpiration through stomata in seedlings grown on agar plates, which are sealed to avoid contamination, is negligible. Therefore, analysing the phenotype of SnRK2E

CL #1 and #2 under these growth conditions does not directly address the question as to whether or not the impairment in stomatal behaviour influences the triple *snrk* mutant phenotype under non-stressed conditions.

RESPONSE: According to the instructive suggestions after the first round of review, we had cautiously examined whether impaired stomatal regulation in the *srk2d/e/i* triple mutant could be responsible for the increased leaf number using two approaches: the comparative analysis of the single, double, and triple mutants of the SnRK2s and the complementation analysis by guard cell-specific expression of *SRK2E*. These results (Figures 2E-H) suggested that the leaf number phenotype of the *srk2d/e/i* triple mutant is not a secondary consequence of the impaired stomatal regulation. Furthermore, we had carefully discussed the results by citing the paper reporting that stomata of the *aba2-1* mutant grown on standard agar medium are more likely to be open than those of WT (Christmann et al., 2007) (page 20, lines 583-595).

Point 2. Regarding the experiments conducted with plants grown in soil, I still remain seriously concerned about the choice of performing measurements of respiratory parameters and 13C enrichment analyses on plants grown in soil under high RH. This is per se a stressful condition for the plant, which dramatically affects stomatal behaviour and other physiological responses. Not surprisingly, the differences between WT control and WT high RH are often bigger than those between WT high RH and triple *snrk* mutant high RH.

RESPONSE: In response to the comment and the editors' suggestion, we have added further discussion (from page 20, line 600 to page 21, line 613). We agree with the point concerned, and to draw readers' attention, we have described that 'in some cases, such as the $^{14}CO_2$ emission derived from [1-^{14}C]-Glc, the changes between WT under different RH conditions were greater than those between the *srk2d/e/i* mutant and WT under high RH conditions (Figure 6, 7)' in this discussion. Please also see below.

Point 3. SnRK2E CL #1 and #2 lines have not been investigated for their behaviour in soil under control RH. If the stomatal behaviour of triple *snrk* mutant was like that one of WT due to the presence of SnRK2E in guard cells, I would expect these lines to be able to grow under normal RH, allowing the authors to perform 13C enrichment experiments under normal RH. This has not been tested. However, the one single experiment done in soil (complementation of ABA-induced stomatal closure, probably even still done under HR?) shows that the rescue is only partial.

RESPONSE: We appreciate the suggestion, but we did not attempt it using the complementation lines in this manuscript for two reasons. First, the complementation lines showed only partial responses to ABA in stomatal assays (Supplemental Figure 7C), and we therefore assume these plants also show moderate or severe growth defect under standard relative humidity conditions due to their impaired stomatal regulation. We have not verified these growth phenotypes on soil in a quantitative manner, because we only had a limited number of seeds to perform a large number of experiments (Figure 2G, H; Supplemental Figure 7, 8). Given that the phenotypes related to seed maturation and germination were not likely rescued by the guard cell-specific promoter (Supplemental Figure 8A, B), we rationally assume that the dry seeds are lethal and thus not able to be stored. This is another limitation of the complementation lines. We did not expect the partial rescue of stomatal regulation in the complementation lines, but we assume that the result is partially explained by the transcriptome data of guard cells showing that *MYB60* is more specifically expressed in guard cells but less abundant than *SRK2E* (Yang et al., 2008).

Secondly, even if the complementation lines are useful, control lines expressing the empty vector are necessary. In this case, the background is the *srk2d/e/i* triple mutant, and both the complementation lines and the control plants should grow under the same conditions for proper control. Collectively, we think that the mutants and/or transgenics of metabolism, signaling, and transport of ABA, which have a comparable transpiration rate with WT, would be useful to confirm and extend our findings, but that such analyses go beyond the present study. We have added a discussion of this point to the revised manuscript (from page 20, line 600 to page 21, line 613).

Reference: Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S., and Schroeder, J.I. (2008). Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. Plant Methods 4: 6.

Point 4. I therefore do not agree that the collection of data in this manuscript allows the conclusion that SnRKs are involved in ABA-mediated regulation of metabolism under non-stressed conditions by fine-tuning flux through the tricarboxylic acid cycle.

RESPONSE: We thank the review for the intensive review, which we believe led to substantial improvement of our manuscript.

Reviewer #4:

Yoshida et al. report metabolome and phenotype analyses of ABA deficient and insensitive mutants in non-stressed conditions. The authors performed state-of-the-art techniques and careful physiological examinations of these mutants. This research provides useful information in the role of ABA in non-stressed conditions. Nonetheless, the relationship between the metabolic regulation and the growth regulation is unclear, which makes the discussion too descriptive. More specific comments are listed below.

Point 1. The last sentence of Abstract: The authors concluded that SnRK2s modulate metabolism and leaf growth under non-stressed conditions by fine-tuning flux through the TCA cycle. The result showed the citrate levels are correlated with ABA signaling. However, I disagree the authors saying that this result indicate SnRK2 control metabolism and growth by tuning TCA cycle.

RESPONSE: We observed an increased level of citrate in the *srk2d/e/i* and *aba2-1* mutants, both of which showed increased leaf numbers. Furthermore, isotope-labelling experiments indicated that respiration through the TCA cycle was enhanced in the *srk2d/e/i* mutant. In addition, our absolute quantification of organic acids and phosphorylated intermediates revealed that *srk2d/e/i* and *aba2-1* both have higher levels of citrate, aconitate, and isocitrate. Taken together, these results indicated that the TCA cycle was impaired in the mutants of ABA biosynthesis and signaling, and we concluded that the SnRK2s involved in ABA signaling modulate metabolism and leaf growth under non-stressed conditions by fine-tuning flux through the TCA cycle. We feel this is clearly described in the manuscript, even if the concise nature of the Abstract renders it difficult to describe this complex phenotype in full.

Point 2.The last paragraph of the Results: I don't understand why the authors point that ABA signaling is 'partially' involved in transcriptional regulation of TRE1.

RESPONSE: Our transcriptome and qRT-PCR results indicated that expression of *FUM2* was significantly decreased both in the *srk2d/e/i* and *aba2-1* mutants under non-stressed conditions (Supplemental Figures 12B and 15). By contrast, we only observed the clear decrease of *TRE1* expression in the *srk2d/e/i* mutant by qRT-PCR (Supplemental Figures 12B and 18), implying that the SnRK2s are conditionally involved in transcriptional regulation of *TRE1*. We further discussed the upstream regulatory mechanisms of *TRE1*, which remains fragmentary (page 18, lines 535-543).

Point 3. Discussion line 496-499: The authors discussed that AREB has little functions in non-stress conditions. AREB-dependent regulation of FUM2 does not fit the other story in this text.

RESPONSE: This is the point also concerned in the first round of review, and we had moved these sentences in this context to discuss the role of FUM2 under abiotic stress conditions. Because little is known about transcriptional regulation of the genes in primary metabolism, including *FUM2*, regardless of whether or not under stress conditions, we believe it is worth discussing possible transcriptional activators of *FUM2*.

Point 4. The authors sometimes discuss metabolism and growth in parallel, but the other times not. The present data do not provide any insight into whether the changes in metabolism are the cause or consequence of alteration of growth, which makes the overall text too descriptive.

RESPONSE: We showed that the TCA cycle was impaired in the mutants of ABA biosynthesis and signaling, and that both mutants have increased number of leaves. These findings are consistent with previous studies of the mitochondrial TCA cycle that is essential for photosynthesis and plant growth, as discussed in detail (from page 17, line 509 to page 18, line 523). In addition, we suggest that the SnRK2 kinases are involved in maintenance of leaf growth directly and/or indirectly through sugar metabolism such as trehalose metabolism. Although the consequences remain unclear, we carefully discussed these issues (page 18, lines 524-546; from page 21, line 619 to page 22, line 650).

We had split the Discussion by introducing three subheadings after the first round of review. This could be a reason why the overall text looked descriptive. However, we think that introducing the subheadings enables readers, especially those who are not familiar with either ABA signaling or central metabolism, to easily follow the story.

We are pleased to inform you that your paper entitled "The role of ABA signaling in the maintenance of the metabolic balances required for Arabidopsis growth in non-stressed conditions" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor	**Dec. 25, 2018**