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TOR acts as a metabolic gatekeeper for auxin-dependent lateral root initiation in Arabidopsis thaliana

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Transaction Report:

(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 **27th Apr 2022**

Dear Alexis,

Thank you again for the submission of your manuscript entitled "TOR acts as metabolic gatekeeper for auxin-dependent lateral root initiation in Arabidopsis thaliana". We have now received three reports from the referees, which I copy below.

As you can see, overall the referees were generally supportive of publication and have produced a list of very constructive comments.

Therefore, based on the interest that is expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. This will probably require additional lab work; here, the report of referee 3 contains the most detail. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, I recommend we organize a brief Zoom chat to discuss the referee comments and go through any potential problems there may be in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual three month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

William

William Teale, Ph.D.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is

needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is 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 this revised version.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Regulation of root branching by plant hormones represents a major focus for plant biology. However, few lateral root studies have addressed the important role played by metabolic status and particularly carbohydrates. The current manuscript addresses this novel angle and reports how TOR kinase regulates lateral root initiation through controlling the translation of several key auxin response components. This new mechanistic information is likely to be of wide interest to readers of EMBO J.

The authors initially observed that auxin response mutants defective for lateral root (LR) development hyper-accumulated starch in their leaves. To determine whether this was a general effect of reduced auxin response or specifically due to a disruption in LR formation, the authors employed elegant genetic tools that could steroid-inducibly repress auxin response in just root pericycle cells (and therefore LR initiation), demonstrating a clear association with blocking root branching and the accumulation of starch in leaves.
NOTE: What is the temporal relationship between repression of LR development and starch accumulation in leaves? How is this impacted by time of day (given plants are grown 16 hours light: 8 hours dark)?

Next, the authors take advantage of a well-established synchronised (auxin inducible) LR initiation system to generate materials for GC-MS-based metabolic profiling. Selected metabolites were discovered to be associated with either wildtype or auxin

mutant slr (such as sucrose and glucose/fructose, respectively), leading them to conclude that LR formation is associated with a switch to glycolysis. LR formation was blocked in the presence of a glucose analog disrupting glycolysis termed 2-deoxy-d-glucose, demonstrating carbohydrate metabolism is a prerequisite for LR formation.
NOTE: Please remove speculation about trehalose-6-P (as this is not detected in their analysis).
It was also not clear in Fig 2E & F at what stage LR initiation was arrested after 2-deoxy-d-glucose treatment?

The authors hypothesised LR induction could lead to activation of the TOR complex (TORC) as it can be activated by glucose and auxin and is required for root meristem activation (Xiong et al, 2013).
Several lines of evidence (e.g. inhibitor, reporter-based) revealed auxin-induced activation of glycolysis promotes the local activation of TORC in the pericycle and LR. An elegant steroid-inducible RNAi downregulation of TOR mRNA, either in every tissue or just root xylem-pole pericycle cells, blocked LR formation - even when attempting to induce branching with auxin - and triggered starch accumulation in shoot tissues.
NOTE: Despite the disruption of LR initiation, the DR5:GUS auxin response reporter continued to be expressed in pericycle, suggesting the auxin responsiveness of this tissue remained intact. This begs the question, at what stage was LR initiation arrested following TOR knockdown? This is unclear from Fig. 4H & I.

To determine how TOR controls LR initiation, the authors investigated the impact of TOR down-regulation on the transcription and translation of key LR regulators. Elegant profiling experiments provide evidence that TOR controls translation (rather than transcription) of ARF7 and ARF19, revealing a new level of regulation of the auxin response machinery during LR initiation. This discovery represents an important new insight, highlighting the importance of post-transcriptional regulatory mechanisms that integrate metabolic and hormone signalling during new organ formation.

Referee #2:

The authors of the study provide strong evidence for a direct and permissive role of TOR signaling in the regulation of lateral root (LR) development. While it has been known for quite some time that the availability of energy is crucial for the establishment of an efficient root system, in this study a mechanistic model is provided how this interference with auxin signaling could work. Considering that formation of lateral roots is based on re-programming of existing pericycle cells leading to the onset of new meristems, in my mind the presented novel findings and their interpretation are even more interesting and deserves recognition also in non-plant communities.

The authors applied a number of methodical approaches, like metabolomics, transcriptomics and specific analyses of the translatome. Together with additional advanced genetic, biochemical, and (limited) physiological techniques, they present the hypothesis that energy availability, provided by the photosynthetic shoot, is sensed by TOR specifically in pericycle cells of the root. Interestingly, the study provides evidence that subsequently the translational efficiency of well characterized and specific factors required for lateral root formation is modulated in these cells.

I have to admit that I am not familiar with the bioinformatic approaches to identify clusters - I guess that other reviewers may comment on these aspects in an informed manner. But in general, I find the presented data conclusive and significant enough to justify the presented hypotheses. Please note my specific comments below. Regarding published data, it could be interesting to comment on recent work of the Laxmi group (e.g. DOI: 10.1111/pce.14290), if possible.

In my mind, the ambitious study spans and addresses quite a wide range of aspects in the framework of lateral root development - providing a novel but also "broad" picture about the process. In consequence, and as a matter of course, a lot of new and further leading questions become obvious. Considering the sequence of well-defined steps in LR development, starting from pre-branch-site formation towards LR primordia formation, at which point exactly acts energy supply/TOR as a "gatekeeper"? When sugars act as long distance (shoot-to-root) signals in root development, is there any relation to the widely accepted role of the hormone auxin in this aspect? But in my mind these and other questions like dose-response relations could be addressed in subsequent studies.

Specific comments

I. Concerning figures
1. Please check scale bars in Fig. 1 - insets vs. representative images.
2. Orange arrow heads in Figs. 3, 4, and 5 - explain in legends.
3. Consistently give concentrations of SUC, IAA, 2D in all figure legends.
4. Fig. S1, according to the legend plus signs assign specific differences in the slr case, but are not visible in the graph.

II. Concerning methods
5. Please give exact information about lateral root number determination. That's missing in the method section.
6. Statistics - I have a problem with calculating and presenting errors derived from very low population sizes (n); e.g. Fig. 5 M,

n=2; or Fig 3 B, n=3!
In this line, please give information about n consistently in all figure legends, and the number of independent exp./replicates in legends or method section.

III. Concerning data
7. The Lugol staining in Fig. 1 D is much brighter compared to the other plants accumulating starch. Is the accumulation in the arf7/arf19 double mutant significantly different compared to the control (Fig. 1 A)? Could you comment on that in the main text?
8. For the metabolomics studies 10 µM Auxin has been applied - could you comment on such an extremely high (and physiologically toxic) concentration besides the goal of full pericycle activation, especially considering potential side effects at later time points of the analysis?
9. The bending assays (Fig 2D,E,F; n=4 and Fig 4 H,I; n=7) are quite crucial in the framework of the study. Please provide a better data basis, like n=12, for these experiments.
10. Lateral root density: please give consistently for all experiments information about potential impact on root elongation growth.
11. Fig. 3 shows no difference in S6K1 phosphorylation comparing Suc and Suc+2D treatments (see also for some sort of additive effect in IAA+Suc+2D treatment). Please comment on that in the main text.

Referee #3:

This work shows that disruption of LR formation causes hyperaccumulation of starch in the leaf, while blocking glycolysis-dependent pathway by 2D completely inhibits the LR primordium formation stimulated by gravistimulation. The authors further shows that TOR kinase is locally activated in the root pericycle. Chemical and genetic inhibition of TOR leads to a block of LR initiation. RNA-Seq and TRAP-Seq analyses indicate that TOR does not control transcription of auxin response genes, instead, TOR controls LR formation by regulating translation of auxin-responsive key transcription factors, e.g. ARF19, LBD16. Based on these complicated data, the authors draw a conclusion that TOR acts as a metabolic gatekeeper for LR formation by locally integrating the availability of shoot derived sugars with the auxin-mediated LR developmental program through controlling the translation of key transcriptional factors. Although this concept is very attractive, the data in this version cannot sufficiently support their conclusion. The textual issues and data interpretation should be seriously taken care of, which were somehow misleading. Overall, I would consider this finding has the potential general interest, but the authors have to fully address the major issues, which are listed below.

1. Figure 1. There is much less starch accumulation in the arf7/arf19 mutant (Fig. 1D) compared to other mutants, e.g. slr, LBD16-SRDX (Fig. 1C and E), although they are all lack of LR. And Figure 1 only showed the starch accumulation of the leaf at the end of the dark period, it will be better to show that starch content of leaves before the dark period for comparison.
2. The authors tried to use pGATA23::shy2-2-GR and pGATA23::slr1-GR plants to prove that increased accumulation of starch was caused by the lack of LRs and not a systemic effect of interfering with auxin signaling. But please note that the expression of GATA23 is not only absolutely limited in the root pericycle, but also is detected in the stem tissue (Plant Physiology, 143, 941-958, 2007. Fig. 4 and Fig. 5).
3. Figure 2. Metabolites analysis, the authors should use other mutants, e.g. pGATA23::shy2-2-GR, but not slr, to support that the obtained results are not caused by the disruption of general auxin signaling, but specifically contributed by LR formation.
4. Figure 3A and B, based on the 2D results, the authors claimed that "Whereas in source tissues TOR activity is promoted by auxin (Schepetilnikov et al, 2017), our results indicate that in sink tissues such as the root, TOR activity is primarily promoted by auxin-induced promotion of sugar breakdown" (lines 279-281). Schepetilnikov et al, 2017 only reported that TOR can be activated by auxin, but did not examined whether this activation could be blocked by 2D or not. Li et al, 2017 PNAS reported that both auxin and glucose energy signal are essential for TOR activation. Therefore, it is not unexpected to see that 2D can inhibit TOR activation even with the presence of IAA in roots. Based on these data, it is hard to conclude that in the root, TOR activity is primarily promoted by auxin-induced promotion of sugar breakdown.
5. Fig. 3A, ponceau staining was not good to be used as the loading control. The pictures are very fuzzy and unclear. Better to use HA antibody for detecting the expression of S6K1.
6. Fig.3 C-D, RAPTOR1B is expressed in the stele, LR founder cells and LR primordia, S6K1 is expressed in LR founder cells and LR primordia. These expression data cannot fully support the conclusion "these data suggest that the auxin-induced activation of glycolysis required for LR formation promotes the local activation of the TORC1 in the pericycle and the LR" (lines 294-296). Since the authors have generated the pS6K1:gS6K1-CFP lines and found that S6K1 is mainly located in the dividing LR founder cells, it will be nice to detect TOR activity using this line, therefore directly confirming this local TOR activation.
7. Fig. 4B, LR density was reduced in rpt1b, how about LR density in the lst8 mutant?
8. Fig. 4C, AZD treatment blocked LR initiation upon gravitropic stimulation. But why not to test the AZD effect on LR density directly, just as they did in the Fig. 4A-B for the rpt1b and TOR-oe lines. It would be easier for comparison using the same treatments.
9. Fig. 4F, G, again, the LR density was used to examine the phenotype in pXPP>>amiR-TOR, but not use the gravitropic stimulation. Need to keep the consistency. Also in Fig. S6, the authors used the starch accumulation in shoot vasculature to confirm the tissue specificity of the TOR knockdown in pXPP>>amiR-TOR. This assay cannot be guaranteed that in the root vasculature tissue, TOR expression is also efficiently silenced.
10. Line 344-345 TOR is locally required in the pericycle to licence the auxin-induced formation of LR. But in Fig. 4F,G, there is

no auxin-related data shown.
11. Fig. 4H, I. Upon TOR knockdown, only a faint GUS signal was detected in the pericycle. However, the author claimed that "while TOR is required for LR initiation it does not compromise the formation of an auxin signalling maxima in the pericycle" (Lines 352-353).
12. Fig.5 A-B. To get the transcriptional auxin response associated with LR formation, pXPP>>amiR-TOR, but not in UB10pro>>amiR-TOR would be the better experimental materials.
13. Fig. S8, It is strange that auxin and AZD have similar promoting effect on ARF7 expression.
14. Fig.5 I-J. Both pARF19-5'UTR::mVENUS and gLBD16-GUS are not the absolute translation reporters. These reporters can also reflect the transcription level/activity. RNA-seq and qRT-PCR (Fig. 5E-H) were performed after 6 h treatment, while Fig. 5I-J were performed after 24 h treatment after bending. It is hard to compare the results among these experiments. TOR is known to regulate translation, additional experiments are needed to prove such observation (Fig. 5I-J) was not caused by the overall translation repression by the TOR inhibition.
15. It is known that ARF7 and ARF19 regulate the transcriptional activation of LBD16. If the translation of ARF7 and ARF19 is compromised by TOR inhibition, why LBD16 gene expression is still not changed between WT and tor mutant?

TOR acts as a metabolic gatekeeper for auxin-dependent lateral root initiation in Arabidopsis thaliana

Point-by-point response to the referee's comments

Dear Editor and reviewers,
We appreciate your precious suggestions and comments that we have integrated into the accompanying revised manuscript. In the followings, we detail our responses to your comments.

Referee #1:
Regulation of root branching by plant hormones represents a major focus for plant biology. However, few lateral root studies have addressed the important role played by metabolic status and particularly carbohydrates. The current manuscript addresses this novel angle and reports how TOR kinase regulates lateral root initiation through controlling the translation of several key auxin response components. This new mechanistic information is likely to be of wide interest to readers of EMBO J.

We thank the reviewer for these comments on our manuscript.

The authors initially observed that auxin response mutants defective for lateral root (LR) development hyper-accumulated starch in their leaves. To determine whether this was a general effect of reduced auxin response or specifically due to a disruption in LR formation, the authors employed elegant genetic tools that could steroid-inducibly repress auxin response in just root pericycle cells (and therefore LR initiation), demonstrating a clear association with blocking root branching and the accumulation of starch in leaves.
NOTE: What is the temporal relationship between repression of LR development and starch accumulation in leaves? How is this impacted by time of day (given plants are grown 16 hours light: 8 hours dark)?

Thanks for pointing out these crucial elements.
- Temporal relationship between the repression of LR development and starch accumulation in leaves.
Although the onset of LR formation begins as early as 3-4 DAG, a substantial effect on starch accumulation was only observed once the resources of the cotyledons were consumed. In our conditions, we observed pronounced differences from 12DAG onward. This is illustrated in a new supplemental Figure S1 monitoring the accumulation of starch in leaves of Col, *arf7/19*, *slr, pGATA23::slr1-GR* and *pGATA23::shy2-2-GR* seedlings from 9 to 21 DAG.

- Impact of the time of day on starch accumulation.

Daytime is of crucial importance. Plants accumulate starch in their leaves to supply carbohydrates to sink tissues during the dark period. Consequently, in the wild-type, starch accumulation is maximal at the end of the light period and minimal at the end of the dark period. We verified that, independently of whether lateral roots are formed, foliar starch accumulation is comparable at the end of the light phase. The data are presented in a new supplemental Figure S2.

Next, the authors take advantage of a well-established synchronised (auxin inducible) LR initiation system to generate materials for GC-MS-based metabolic profiling. Selected metabolites were discovered to be associated with either wildtype or auxin mutant slr (such as sucrose and glucose/fructose, respectively), leading them to conclude that LR formation is associated with a switch to glycolysis. LR formation was blocked in the presence of a glucose analog disrupting glycolysis termed 2-deoxy-d-glucose, demonstrating carbohydrate metabolism is a prerequisite for LR formation. NOTE: Please remove speculation about trehalose-6-P (as this is not detected in their analysis).

We agree with the reviewer that this aspect was ectopic and has now been removed.

It was also not clear in Fig 2E & F at what stage LR initiation was arrested after 2-deoxy-d-glucose treatment?

2DG blocks LR formation very early before any pericycle division could be observed. We agree with the reviewer that this was not clearly explained. We have added confocal microscopy pictures in Fig 2E that emphasize the absence of formative divisions in the pericycle upon 2DG treatment and clarified this in the text.

The authors hypothesised LR induction could lead to activation of the TOR complex (TORC) as it can be activated by glucose and auxin and is required for root meristem activation (Xiong et al, 2013). Several lines of evidence (e.g. inhibitor, reporter-based) revealed auxin-induced activation of glycolysis promotes the local activation of TORC in the pericycle and LR. An elegant steroid-inducible RNAi downregulation of TOR mRNA, either in every tissue or just root xylem-pole pericycle cells, blocked LR formation - even when attempting to induce branching with auxin - and triggered starch accumulation in shoot tissues.
NOTE: Despite the disruption of LR initiation, the DR5:GUS auxin response reporter continued to be expressed in pericycle, suggesting the auxin responsiveness of this tissue remained intact. This begs the question, at what stage was LR initiation arrested following TOR knockdown? This is unclear from Fig. 4H & I.

We thank the reviewer for this question of central importance. TOR knockdown blocks LR initiation before any division of the pericycle. This is now illustrated in Fig. 4F with better images and clarified in the text. Panels 4G & 4H illustrate that despite this very early block, we detect in ~60% of the LR a faint DR5::GUS signal indicating that despite auxin signaling taking place, the pericycle is not dividing.

To determine how TOR controls LR initiation, the authors investigated the impact of TOR down-regulation on the transcription and translation of key LR regulators. Elegant profiling experiments provide evidence that TOR controls translation (rather than transcription) of ARF7 and ARF19, revealing a new level of regulation of the auxin response machinery during LR initiation. This discovery represents an important new insight, highlighting the importance of post-transcriptional regulatory mechanisms that integrate metabolic and hormone signalling during new organ formation.

Referee #2:

The authors of the study provide strong evidence for a direct and permissive role of TOR signaling in the regulation of lateral root (LR) development. While it has been known for quite some time that the availability of energy is crucial for the establishment of an efficient root system, in this study a mechanistic model is provided how this interference with auxin signaling could work. Considering that formation of lateral roots is based on re-programming of existing pericycle cells leading to the onset of new meristems, in my mind the presented novel findings and their interpretation are even more interesting and deserves recognition also in non-plant communities.

We thank the reviewer for these appreciative comments on our findings and for providing helpful and constructive comments.

The authors applied a number of methodical approaches, like metabolomics, transcriptomics and specific analyses of the translatome. Together with additional advanced genetic, biochemical, and (limited) physiological techniques, they present the hypothesis that energy availability, provided by the photosynthetic shoot, is sensed by TOR specifically in pericycle cells of the root. Interestingly, the study provides evidence that subsequently the translational efficiency of well characterized and specific factors required for lateral root formation is modulated in these cells.

I have to admit that I am not familiar with the bioinformatic approaches to identify clusters - I guess that other reviewers may comment on these aspects in an informed manner. But in general, I find the presented data conclusive and significant enough to justify the presented hypotheses. Please note my specific comments below. Regarding published data, it could be interesting to comment on recent work of the Laxmi group (e.g. DOI: 10.1111/pce.14290), if possible.

Thanks for drawing our attention to this paper which is relevant to the focus of the manuscript and has been incorporated.

In my mind, the ambitious study spans and addresses quite a wide range of aspects in the framework of lateral root development - providing a novel but also "broad" picture about the process. In consequence, and as a matter of course, a lot of new and further leading questions become obvious. Considering the sequence of well-defined steps in LR development, starting from pre-branch-site formation towards LR primordia formation, at which point exactly acts energy supply/TOR as a "gatekeeper"? When sugars act as long distance (shoot-to-root) signals in root development, is there any relation to the widely accepted role of the hormone auxin in this aspect? But in my mind these and other questions like dose-response relations could be addressed in subsequent studies.

Specific comments

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1. Please check scale bars in Fig. 1 - insets vs. representative images.

2. Orange arrow heads in Figs. 3, 4, and 5 - explain in legends.
3. Consistently give concentrations of SUC, IAA, 2D in all figure legends.
4. Fig. S1, according to the legend plus signs assign specific differences in the slr case, but are not visible in the graph.

We thank the reviewer for pointing out these.
1. The scale bars are now consistent.
2. The meaning of the arrowheads is now clearly mentioned in the figures' legends.
3. The concentrations are now consistently reported in the legends.
4. Fig. S1 is now S3. We apologize for the confusion, the ‘+’ signs referred to a previous version of the figure. The legend has been corrected.

II. Concerning methods
5. Please give exact information about lateral root number determination. That's missing in the method section.

This was indeed missing from the methods section, we apologize for this. A description of the method used is now present.

6. Statistics - I have a problem with calculating and presenting errors derived from very low population sizes (n); e.g. Fig. 5 M, n=2; or Fig 3 B, n=3!
In this line, please give information about n consistently in all figure legends, and the number of independent exp./replicates in legends or method section.

We agree with the reviewer that statistically sound conclusions can not be drawn from small samples. Our presentation of the data was confusing.
In Figure 3B, the boxplot summarizes measurements of three independent biological replicates (all blots are provided as source data), each corresponding to ~200 individual seedlings.
Similarly, in Figure 5M the data corresponded to two independent biological replicates, each with ~200 seedlings run in three technical replicates. A third replicate was analyzed during the revision, and the consolidated data (N=3 biological replicates) are now presented. This has been clarified in the figure legend.
The data presented in Figure 2E & F were repeated, and the results now represent 5 to 16 replicates. The legend has been updated to clarify this point.

III. Concerning data
7. The Lugol staining in Fig. 1 D is much brighter compared to the other plants accumulating starch. Is the accumulation in the arf7/arf19 double mutant significantly different compared to the control (Fig. 1 A)? Could you comment on that in the main text?

We thank the reviewer for drawing our attention to this point. To obtain more accurate measurements of the starch accumulation in the different genotypes and remove bias, we assessed the absolute starch content via enzymatic quantification. These measurements and a new set of Lugol staining are now presented in a revised Figure 1. The starch quantification revealed that *arf7/arf19* accumulate starch at a higher level than Col-0, albeit at reduced levels compared to the other lateral root less genotypes. This could be due to *slr* affecting additional ARFs other than ARF7/19 and/or that other ARFs potentially compensate for the lack of ARF7 and ARF19 and affect the metabolic regulation of starch accumulation. This point is discussed in the main text.

8. For the metabolomics studies 10 µM Auxin has been applied - could you comment on such an extremely high (and physiologically toxic) concentration besides the goal of full pericycle activation, especially considering potential side effects at later time points of the analysis?

We agree with the reviewer that 10µM is a high IAA concentration. Yet, this has been repeatedly used in the field of lateral root research as the concentration of choice for the synchronized induction of LR formation in Arabidopsis without apparent toxicity (see: doi/10.1105/tpc.105.035493. ; doi:10.1038/Emboj.2012.303 or doi: 10.1101/gad.390806.). We confirmed that 72h of treatment with 10µM IAA does not lead to toxic effects (see Fig. S7).

9. The bending assays (Fig 2D,E,F; n=4 and Fig 4 H,I; n=7) are quite crucial in the framework of the study. Please provide a better data basis, like n=12, for these experiments.

We agree with the reviewer; we performed additional replicates of the experiments. The revised panels 2E and F now represent data for five biological replicates (2E) and 16 for 2F.

10. Lateral root density: please give consistently for all experiments information about potential impact on root elongation growth.

We apologize to the reviewer for omitting to report this critical data. The effect on the primary root elongation is now presented in the new Figure S5.

11. Fig. 3 shows no difference in S6K1 phosphorylation comparing Suc and Suc+2D treatments (see also for some sort of additive effect in IAA+Suc+2D treatment). Please comment on that in the main text.

We thank the reviewer for pointing this out and accordingly added the information to the main text: "*Simultaneous provision of external 2D and sucrose did not lower the levels of pS6K1, possibly due to an excess of sucrose.*"

Referee #3:

This work shows that disruption of LR formation causes hyperaccumulation of starch in the leaf, while blocking glycolysis-dependent pathway by 2D completely inhibits the LR primordium formation stimulated by gravistimulation. The authors further shows that TOR kinase is locally activated in the root pericycle. Chemical and genetic inhibition of TOR leads to a block of LR initiation. RNA-Seq and TRAP-Seq analyses indicate that TOR does not control transcription of auxin response genes, instead, TOR controls LR formation by regulating translation of auxin-responsive key transcription factors, e.g. ARF19, LBD16. Based on these complicated data, the authors draw a conclusion that TOR acts as a metabolic gatekeeper for LR formation by locally integrating the availability of shoot derived sugars with the auxin-mediated LR developmental program through controlling the translation of key transcriptional factors. Although this concept is very attractive, the data in this version cannot sufficiently support their conclusion. The textual issues and data interpretation should be seriously taken care of, which were somehow misleading. Overall, I would consider this finding has the potential general interest, but the authors have to fully address the major issues, which are listed below.

1. Figure 1. There is much less starch accumulation in the arf7/arf19 mutant (Fig. 1D) compared to other mutants, e.g. slr, LBD16-SRDX (Fig. 1C and E), although they are all lack of LR. And Figure 1 only showed the starch accumulation of the leaf at the end of the dark period, it will be better to show that starch content of leaves before the dark period for comparison.

We thank the reviewer for raising these points, which are similar to point #7, raised by reviewer #2 (*arf7/arf19* accumulates less starch than other mutants), and point #1, raised by reviewer #1 (effect of time of the day).

→ *arf7/arf19* accumulates less starch than other mutants. To confirm this observation while obtaining more accurate measurements of the starch accumulation in the different genotypes and exclude potential bias in starch staining, we replicated these assays, and further assessed the absolute starch content via enzymatic starch quantification. These measurements and new starch stainings are now presented in a revised Figure 1. The starch quantification revealed that *arf7/arf19* accumulate starch to a higher level than Col-0, albeit at lower levels than the other lateral root less genotypes. This could be due to *slr* affecting more ARFs than ARF7/19 and/or other ARFs potentially compensate for the lack of ARF7 and ARF19 and affect the metabolic regulation of starch accumulation. This point is now discussed in the main text.

→ Effect of time of the day. As plants accumulate starch in their leaves during the light phase to supply carbohydrates to sink tissues during the dark, the time of the day is crucial for the starch content. In the wild type, starch accumulation is maximal at the end of the light period and minimal at the end of the dark period. As suggested, we performed starch staining at the end of the light period (presented in a new supplemental Figure S2). We observed that the lateral rootless mutants all accumulated starch in leaves to a level similar to the wild type. This shows that these mutants are not defective in their ability to produce or store carbohydrates during the light period but in their ability to consume them in the root.

2. The authors tried to use pGATA23::shy2-2-GR and pGATA23::slr1-GR plants to prove that increased accumulation of starch was caused by the lack of LRs and not a systemic effect of interfering with auxin signaling. But please note that the expression of GATA23 is not only absolutely limited in the root pericycle, but also is detected in the stem tissue (Plant Physiology, 143, 941-958, 2007. Fig. 4 and Fig. 5).

We thank the reviewer for bringing this fact to our attention. Indeed, as described in Manfield et al., endogenous *GATA23* transcript can be detected in the aerial part. However, here we use a 0.75Kb fragment of the *GATA23* promoter that leads to a root-specific expression. This promoter was initially described in de Rybel et al. 2010 (10.1016/j.cub.2010.09.007) as root-specific, and we have extensively confirmed that it is not expressed in the aerial part in previous studies (e.g. 10.1016/j.cub.2019.06.039, 10.1073/pnas.1820882116 or 10.1016/j.cub.2015.12.047).

3. Figure 2. Metabolites analysis, the authors should use other mutants, e.g. pGATA23::shy2-2-GR, but not slr, to support that the obtained results are not caused by the disruption of general auxin signaling, but specifically contributed by LR formation.

The reviewer is correct that performing the metabolome profiling with the *pGATA23::shy2-2-GR* or *pGATA23::slr1-GR* would have been more adequate. These lines had, however, only been established long after we conducted the metabolomics experiment. Given the similarity in phenotype (foliar starch accumulation and absence of lateral root) shared between *slr*, *pGATA23::shy2-2-GR*, *pGATA23::slr1-GR,* and *gLBD16:SRDX* it is likely that the perturbation in primary metabolism we observed in our profiling of *slr* results precisely from the inhibition of LR formation. In light of the heavy experimental and analytical work required for conducting the metabolomics approach with the tissue-specific line, we hope that the reviewer will share our opinion that the insights gained by a repetition of the metabolomics dataset would be marginal.

4. Figure 3A and B, based on the 2D results, the authors claimed that "Whereas in source tissues TOR activity is promoted by auxin (Schepetilnikov et al, 2017), our results indicate that in sink tissues such as the root, TOR activity is primarily promoted by auxin-induced promotion of sugar breakdown" (lines 279-281). Schepetilnikov et al, 2017 only reported that TOR can be activated by auxin, but did not examined whether this activation could be blocked by 2D or not. Li et al, 2017 PNAS reported that both auxin and glucose energy signal are essential for TOR activation. Therefore, it is not unexpected to see that 2D can inhibit TOR activation even with the presence of IAA in roots. Based on these data, it is hard to conclude that in the root, TOR activity is primarily promoted by auxin-induced promotion of sugar breakdown.

We thank the reviewer for pointing out this critical point. We agree with the reviewer that Schepetilnikov et al, 2017 only reported that TOR could be activated by auxin and did not examine whether 2D could block this activation. We, however, respectfully disagree with the reviewer regarding the data in Li et al. Indeed, the manuscript shows that in roots, TOR activity (pS6K blots) is

promoted by Glucose. Still, it does not present evidence that auxin leads to the same effect. Our data show that in roots, auxin leads to TOR activation and that this activation can be reverted by co-treatment with 2D, indicating that TOR activation requires glycolysis. We agree with the reviewer that our conclusion was too strongly formulated. We have reworded this section of the manuscript to be more moderate: " *glycolysis-dependent promotion of TOR activity could be a specificity of heterotrophic tissues that allows a systemic integration of developmental progression with shoot photosynthetic capacity"*.

5. Fig. 3A, ponceau staining was not good to be used as the loading control. The pictures are very fuzzy and unclear. Better to use HA antibody for detecting the expression of S6K1.

As suggested, we replaced the Ponceau staining with the detection of the HA-tagged S6K1. The new data are presented in panel 3A.

6. Fig.3 C-D, RAPTOR1B is expressed in the stele, LR founder cells and LR primordia, S6K1 is expressed in LR founder cells and LR primordia. These expression data cannot fully support the conclusion "these data suggest that the auxin-induced activation of glycolysis required for LR formation promotes the local activation of the TORC1 in the pericycle and the LR" (lines 294-296). Since the authors have generated the pS6K1:gS6K1-CFP lines and found that S6K1 is mainly located in the dividing LR founder cells, it will be nice to detect TOR activity using this line, therefore directly confirming this local TOR activation.

We agree with reviewer 3 and have accordingly revised our conclusion to "*Together these data suggest that the auxin-induced activation of glycolysis required for LR formation promotes the activation of the TORC in the root tissues contributing to LR formation."*

7. Fig. 4B, LR density was reduced in rpt1b, how about LR density in the lst8 mutant?

We thank the reviewer for this excellent suggestion. We examined LR formation in the *lst8* mutant. Lateral root density was strongly reduced in *lst8* compared to wildtype. These new data have been added in Fig 4C and S5. We also noted that the *lst8* mutant produced many adventitious roots from the hypocotyl; this prompted us to examine whether this was also the case in the *TOR* knockdown (*UB10pro>>amiR-TOR*). Indeed TOR knockdown leads to an increase in the formation of adventitious roots (new Figure EV1). This, together with the reviewer's question related to the transcriptional activation of LBD16 in TOR knockdown (point #15), led us to consider more globally the role of TOR in controlling root branching by the formation of lateral and adventitious roots. We explain in more detail at point #15 the additional data we provide that support a model where TOR, through its regulation of ARF7, ARF19, and LBD16 translation, ensures that canonical (ARF7/ARF19 driven) and non-canonical (WOX11) root branching is only possible if the energy status of the root permits it.

8. Fig. 4C, AZD treatment blocked LR initiation upon gravitropic stimulation. But why not to test the AZD effect on LR density directly, just as they did in the Fig. 4A-B for the rpt1b and TOR-oe lines. It would be easier for comparison using the same treatments.

The reviewer raises an important point and prompts us to clarify the differences in the experimental settings we used. AZD is a potent inhibitor of TOR activity that inhibits root growth at <1µM (Montané et al. 2013 - 10.1093/jxb/ert242). Growing plants on <1µM AZD for several days did not reveal any impact on lateral root formation. Lateral root initiation occurs in the pericycle in a root region where the overlying endodermis has completed the formation of its Casparian strip (Vermeer et al. 2014 - 10.1126/science.1245871). This structure severely limits the diffusion of soluble compounds through the apoplast. We hypothesize that this explains why only higher AZD concentration (10µM) leads to an inhibition of LR formation. However, this concentration blocks cell proliferation in the root apical meristem comparable to the *tor*-null mutants, preventing the quantification of LR density. To circumvent this shortcoming, we turned to the gravitropic LR-induction, a process triggering LR initiation (10.1093/jxb/erm171, 10.1093/jxb/erv541, 10.1073/pnas.0807814105) without relying on the proliferation of the root meristem. We clarified this in the main text.

9. Fig. 4F, G, again, the LR density was used to examine the phenotype in pXPP>>amiR-TOR, but not use the gravitropic stimulation. Need to keep the consistency. Also in Fig. S6, the authors used the starch accumulation in shoot vasculature to confirm the tissue specificity of the TOR knockdown in pXPP>>amiR-TOR. This assay cannot be guaranteed that in the root vasculature tissue, TOR expression is also efficiently silenced.

We could not obtain RT-PCR data showing a reduction of TOR mRNA levels in the *pXPP>>amiR-TOR* line. As the amiR-RNA is only active in a few cells, the TOR mRNA reduction is diluted during tissue lysis by the surrounding tissues where the amiR-TOR is not active, a likely explanation for the observed lack of mRNA reduction. It has been previously firmly established (Caldana et al. 2013) that TORC activity reduction leads to starch accumulation in green tissues. We verified this with our *UB10pro>>amiR-TOR* line that hyper-accumulated starch in their leaves upon Estradiol treatment (Figure S8). We observe a similar starch hyper-accumulation, albeit restricted to the region around the veins where the amiR-TOR is expressed, in the *pXPP>>amiR-TOR* line (Figure S8). We agree with the reviewer that these observations suggest a reduction of TOR expression, albeit not definitive proof. We have thus reworded the text to clarify this: "*Similarly, we observed intense starch accumulation around the leaf vasculature in DEX-treated XPPpro>>amiR-TOR, suggesting TOR knockdown*".
Concerning the induction of LR formation by gravistimulation in the *pXPP>>amiR-TOR* line, we thank the reviewer for this suggestion. We have performed this experiment and observed a reduction in the number of initiation events, albeit not a complete block (Figure EV2). Given that the promoter driving the expression of the amiR-TOR switches off as soon as LR initiation starts (Andersen et al, 2018), this indicates that sustained downregulation of TOR is required in the pericycle to suppress LR formation. This has been added to Figure EV2. We have amended the main text to tone down the claim that TOR is specifically required in the XPP cells, a conclusion that our data do not formally

support. Still, based on the expression of the other TORC components and the nature of the phenotype, the claim that TOR is required in the root for lateral root initiation remains valid.

10. Line 344-345 TOR is locally required in the pericycle to licence the auxin-induced formation of LR. But in Fig. 4F,G, there is no auxin-related data shown.

The reviewer is correct. We have modified the sentence accordingly: "*(...) indicating that TOR is locally required in the pericycle to license the formation of LR.*"

11. Fig. 4H, I. Upon TOR knockdown, only a faint GUS signal was detected in the pericycle. However, the author claimed that "while TOR is required for LR initiation it does not compromise the formation of an auxin signalling maxima in the pericycle" (Lines 352-353).

The reviewer is correct. Our wording was ambiguous. The conclusion of this paragraph has been reworded to "*(...) these data suggest that while TOR is required for LR initiation, TOR knockdown does not cause a complete inhibition of auxin signaling.*"

12. Fig.5 A-B. To get the transcriptional auxin response associated with LR formation, pXPP>>amiR-TOR, but not in UB10pro>>amiR-TOR would be the better experimental materials.

The reviewer is correct that performing the transcriptional profiling with *pXPP>>amiR-TOR*, instead *UB10pro>>amiR-TOR* could have been done. The *XPPpro>>amiR-TOR* lines were only obtained long after the transcriptome (and translatome) data were obtained. Additionally, given the milder effect of this line on LR initiation (see point #9), performing could lead to erroneous conclusions

13. Fig. S8, It is strange that auxin and AZD have similar promoting effect on ARF7 expression.

Indeed we, too, were puzzled by the observation that AZD promotes the accumulation of ARF7 transcripts. A similar observation can be made for *TOR* mRNA whose abundance is also promoted by AZD treatment (now in Fig S10). We can only speculate about the underlying mechanism: TOR-inhibition negatively regulates either specific suppressor proteins of ARF7/TOR, or the turnover of *TOR/ARF7* transcripts is differentially affected by the TOR inhibition. Although interesting, investigating these aspects is outside the scope of the present manuscript.

14. Fig.5 I-J. Both pARF19-5'UTR::mVENUS and gLBD16-GUS are not the absolute translation reporters. These reporters can also reflect the transcription level/activity. RNA-seq and qRT-PCR (Fig. 5E-H) were performed after 6 h treatment, while Fig. 5I-J were performed after 24 h treatment after bending. It is hard to compare the results among these experiments. TOR is known to regulate translation, additional experiments are needed to prove such observation (Fig. 5I-J) was not caused by the overall translation repression by the TOR inhibition.

We thank the reviewer for raising this point. We verified that the TOR-dependent reduction in ARF19 is the same in the two LR induction schemes we use (6h after IAA or 24h after bending). We

confirmed that *pARF19-5'UTR::mVENUS* expression is reduced in a TOR-dependent manner 6h after IAA treatment. These data are now presented in a new Figure EV3.

15. It is known that ARF7 and ARF19 regulate the transcriptional activation of LBD16. If the translation of ARF7 and ARF19 is compromised by TOR inhibition, why LBD16 gene expression is still not changed between WT and tor mutant?

We thank the reviewer for this insightful comment that prompted us to perform additional experiments and provided new insights on how TOR controls LR formation.
The Arabidopsis root system is determined by the production of lateral roots, which originate from the growing root, and adventitious roots (ARs), which are formed from non-root organs. WOX11 is a crucial trigger of AR formation [1,2]. In standard laboratory conditions, WOX11 does not contribute to LR formation. However, when plants are grown on soil or upon wounding, the primary root can produce both WOX11-mediated roots and non-WOX11-mediated roots [1]. Interestingly WOX11-mediated and non-WOX11-mediated root initiation converges on the activation of LBD16 [1]. This led us to examine whether WOX11 is involved in LBD16 regulation upon TOR knockdown. We observed that *WOX11* transcription increases in the *UB10pro>>amiR-TOR* mutant or upon AZD treatment (new Figure 6 and Figure S12). To know in which tissue WOX11 is expressed upon TOR knockdown, we crossed a *WOX11pro::GUS* line to *UB10pro>>amiR-TOR.* In control conditions, and as previously reported by Sheng et al. [1], *WOX11pro::GUS* signal was not detectable during LR formation; more specifically, WOX11 expression was absent in xylem-pole pericycle cells. Upon *TOR* knockdown, we observed *WOX11pro::GUS* signal in the pericycle. Together, these data suggest that upon TOR inhibition or knockdown, WOX11 expression is upregulated in the root pericycle, promoting the transcription of *LBD16* independently of ARF7/19. Through its control of LBD16 translation, TOR would also control the WOX11-dependent root branching. To test this hypothesis, we cut the root of the *arf7/arf19* double mutant to induce the formation of only WOX11-dependent side roots [1]. On 1/2MS medium, we observed the expected formation of roots near the wounded region, while on 1/2MS medium containing AZD, no roots were formed near the cut side. This indicates that TOR activity is also required for the non-canonical, WOX11-dependent primary root branching.

These data and a schematic model are part of the new Figure 6 and the data discussed in the main text. These results shed additional light on the metabolic control of root branching and the central role TOR plays in controlling it.

WOX11-mediated root initiation is a hallmark of root formation from autotrophic tissues (hypocotyl, leaves) that can be coopted for non-canonical root branching upon stress [1]. By inhibiting WOX11 expression, TOR prevents the activation of this fail-safe branching mechanism in sink tissue. In source tissue such as the hypocotyl, reduction of TOR abundance (amiR-TOR) or its activity (lst8 mutant) may be perceived as a stress by the plant leading to an increased AR formation in the hypocotyl induced by WOX11 up-regulation. How TOR levels modulate the expression of WOX11 and how, in the hypocotyl, TOR regulation of LBD16 translation is alleviated remain to be elucidated. Together, these results shed additional light on the metabolic control of root branching and the central role TOR plays in controlling it.

Literature cited:

1. Sheng, L., Hu, X., Du, Y., Zhang, G., Huang, H., Scheres, B., and Xu, L. (2017). Non-canonical WOX11-mediated root branching contributes to plasticity in Arabidopsis root system architecture. Development *144*, 3126–3133. 10.1242/dev.152132.

2. Liu, J., Sheng, L., Xu, Y., Li, J., Yang, Z., Huang, H., and Xu, L. (2014). WOX11 and 12 Are Involved in the First-Step Cell Fate Transition during de Novo Root Organogenesis in Arabidopsis. Plant Cell *26*, 1081–1093. 10.1105/tpc.114.122887.

Dear Alexis,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees whose comments are enclosed. As you will see, both are broadly in favour of publication, pending satisfactory minor revision. Referee 3 raises points which certainly add to the academic debate over the exact nature of the relationships among glucose, auxin and TOR activity during lateral root initiation. Firstly, I would like you to address the point on the description of your data in a revised version of the manuscript. For the other points, especially those concerning the data shown in Figure 3, it will be enough to respond to the comments once more in a point-by point response. Having this discussion documented in our RPF file will be very helpful for our readership and will allow publication of those discussion points where consensus has not yet been found, without further diluting the conclusions of your manuscript. Referee 2 does, of course, have a valid point about the use of 10uM IAA; however, I also see that your use of established protocols adds by allowing direct comparisons to previous work.

In addition to these changes, I also have a few minor editorial changes for you to address. In this regard, would you please:

- include funding information for INST 35/1314-1 FUGG, INST 35/1503-1 FUGG in our online submission portal,
- remove the author accreditation section from the text of the manuscript,
- include callouts for Figure 4G, 5G, 5H, 5K and 6C-6H; your text includes a callout for Figure 4I but no panel I exists,
- rename Table EV1 and EV2 as Dataset EV1 and EV2 with the corresponding callouts and legends uploaded as a separate tab in each Excel file,
- include page numbers to the appendix file in Appendix 1, and
-reorganise the Source Data so one zipped file is uploaded per figure.

We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.
We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (15th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #2:

I read and checked the response to reviewers, and concerning my
comments I am almost satisfied.
One critical aspect is still the usage of these extremely high auxin concentrations. Even if also others in the field use e.g. 10 µM IAA for certain experiments - in my mind it should be clear that such conditions of course are extremely artificial, and bear a high risk of side effects unrelated to the processes under analysis. Therefore, please deal with this aspect/problem in the text of your ms.

Referee #3:

In this revised manuscript, the authors didn't address several of my previous comments (See below). More importantly, to interpret the potential discrepancy why LBD16 gene expression is not changed between WT and tor mutant, they investigated the WOX11-mediated and non-WOX11-mediated root formation, and found that "upon TOR knockdown, formation of AR from the hypocotyl was increased (Figure EV1), as previously reported (Deng et al., 2017)". However, please note, Deng et al 2017 actually showed the OPPOSITE results in which inhibition of TOR indeed strongly suppresses the AR formation.

For the metabolic data, there are several descriptions are not correct/accurate.
page 8 "They remained high in Col-0 while it declined over the day in control-treated samples and remained most unchanged in the presence of IAA in the slr mutant" But based on Appendix Figure S3, there are similar patterns between WT and slr mutant.

page 9 "Strikingly, root levels of glucose and fructose derived from sucrose cleavage, which did not build up in Col-0 upon IAA, probably due to their catabolism by glycolysis, were strongly increased by the IAA treatment in the slr mutant (Fig. 2C)". However, in fact, in Col-0, IAA treatment also increase glucose and fructose levels in 6 hour treatment, and more importantly, levels of glucose and fructose are all decline to similar low level in both Col-0 and slr mutant after 24 hour, the time of end of night, suggesting that glycolysis is not real impaired in the root of the slr mutant in the night.

Related to previous comments 4: Page 10 and 11, Based on Fig. 3, the authors conclude "TORC activation by auxin depends on carbohydrate catabolism in roots. This glycolysis-dependent promotion of TOR activity could be a specificity of heterotrophic tissue....". "Together, these data suggest that the auxin-induced activation of glycolysis required for LR formation promotes the activation of TORC....". As discussed in my previous comments, Schepetilnikov et al, 2017 EMBO J have reported that TOR can be activated by auxin, Xiong et al, 2013 Nature found that TOR can be activated by glucose, and Li et al 2017 PNAS further revealed that auxin and glucose are two independent and parallel upstream signals for TOR activation. Absence of either signals causes TOR inactivation. 2D treatment could disrupt sugar-dependent TOR activation, it is unexpected to see that 2D treatment can inhibit TOR activation even with the presence of IAA in roots. Therefore, based on the current data, it cannot draw such conclusion that auxin-induced activation of glycolysis promotes the activation of the TORC. Also, the glycolysis-dependent promotion of TOR activity is not a specificity of heterotrophic tissue, which is also required in the photosynthetic tissues, e.g. leaf (Brunkard et al., PNAS 2020).

Related to previous comment 6: the author did not address this comment.

Related to previous comment 8: The authors also only examined LR initiation upon gravitropic stimulation in UB10pro>>amiR-TOR plants, but examined the LR density in XPPpro>>amiR-TOR plants. Again, it would be very hard to draw conclusion by using these different treatments in different genetic materials.

Related to previous comment 9: in page 12, "in the root, induction of amiR-TOR in the XPP cells led to a severe reduction in the number and density of emerged LR formed compared to mock-induced plants (Figure EV2A, B). However, induction of LR by gravistimulation did not impair initiation event (Figure EV2C, D)". The authors explained this difference by" the promoter driving the expression of the amiR-TOR switches of as soon as LR initiation starts". How can this XPPpro>>amiR-TOR plants cause a severe reduction in the number and density of emerged LR? Please clarify this discrepancy.

TOR acts as a metabolic gatekeeper for auxin-dependent lateral root initiation in Arabidopsis thaliana

A point-by-point response to the referee's comments

Dear Editor and reviewers,
We appreciate your precious suggestions and comments that we have integrated into the accompanying revised manuscript. We think that your comments significantly improved our manuscript. In the following, we detail our responses to your comments.

Referee #2:

I read and checked the response to reviewers, and concerning my comments I am almost satisfied. One critical aspect is still the usage of these extremely high auxin concentrations. Even if also others in the field use e.g. 10 µM IAA for certain experiments - in my mind it should be clear that such conditions of course are extremely artificial, and bear a high risk of side effects unrelated to the processes under analysis. Therefore, please deal with this aspect/problem in the text of your ms.

We thank the reviewer for the opportunity to clarify this point.
Treatment with 10 µM IAA triggers all pericycle cells to be synchronously activated and initiate the LR developmental program. Several previous works have used this treatment to decipher the transcriptomic response of the pericycle during LR initiation and validated that it recapitulates the activation of the pericycle seen during physiological LR initiation (Vanneste *et al*, 2005; Lavenus *et al*, 2013; Ramakrishna *et al*, 2019; Vilches Barro *et al*, 2019; Ursache *et al*, 2021). The first motivation to use this protocol is to circumvent that at any given time, only a few pericycle cells are activated, making the analysis difficult as the non-activated cells would pre-dominate. To ensure that the response we analyse and the changes we highlight are genuinely linked to pericycle activation and not to generic auxin effects, we analysed the metabolites in wild-type (Col-0) and *slr* mutant seedlings in which pericycle activation is prevented (Vanneste *et al*, 2005). The second motivation is to allow direct comparisons to previous works. We have clarified these points in the text, and to avoid ambiguity, we refer to pericycle activation and not LR formation.

Referee #3:

In this revised manuscript, the authors didn't address several of my previous comments (See below). More importantly, to interpret the potential discrepancy why LBD16 gene expression is not changed between WT and tor mutant, they investigated the WOX11-mediated and non-WOX11-mediated root formation, and found that "upon TOR knockdown, formation of AR from the hypocotyl was increased (Figure EV1), as previously reported (Deng et al., 2017)". However, please note, Deng et al 2017

actually showed the OPPOSITE results in which inhibition of TOR indeed strongly suppresses the AR formation.

Thank you for the opportunity to clarify this point. Deng et al. used an engineered rapamycin system (potato plants over-expressing BP12) to study the inhibitory effect of Rapamycin on adventitious root (AR) formation. This effect was only observed in this background; rapamycin did not affect AR number in WT potato plants (Deng et al., Fig 2 B, C). The authors also studied the impact of AZD or KU on AR branching in Arabidopsis (Deng et al., Fig 6A, B) and, as mentioned by the reviewer, see a reduction in AR formation. However, AR formation was, in their case, induced in dark-grown hypocotyl where the root had been removed. These conditions differ from the ones we used, making a direct comparison difficult. We agree with the reviewer that our formulation was not accurate. We have modified the sentence to "*Notably, we observed that upon TOR knockdown, while LR formation was inhibited, AR formation from the hypocotyl was not (Figure EV1)*" and discuss the differences with the results of Deng et al. in the last paragraph of the manuscript.

For the metabolic data, there are several descriptions are not correct/accurate.
page 8 "They remained high in Col-0 while it declined over the day in control-treated samples and remained most unchanged in the presence of IAA in the slr mutant" But based on Appendix Figure S3, there are similar patterns between WT and slr mutant.

We thank the reviewer for spotting this and apologize for the error in our description. While the initial increase (within 2h) in shoot sucrose level is identical between Col-0 and *slr*, it keeps increasing up to 12h in *slr* while it plateaus in Col-0. The sentences have been removed and replaced by "*Upon shift to IAA, levels of shoot sucrose quickly increased within two hours, and while they kept increasing for up to 12 hours in slr, it plateaued in WT.*"

page 9 "Strikingly, root levels of glucose and fructose derived from sucrose cleavage, which did not build up in Col-0 upon IAA, probably due to their catabolism by glycolysis, were strongly increased by the IAA treatment in the slr mutant (Fig. 2C)". However, in fact, in Col-0, IAA treatment also increase glucose and fructose levels in 6 hour treatment, and more importantly, levels of glucose and fructose are all decline to similar low level in both Col-0 and slr mutant after 24 hour, the time of end of night, suggesting that glycolysis is not real impaired in the root of the slr mutant in the night.

We did not intend to claim glycolysis is globally impaired in *slr,* and we amended our text accordingly. Rather than for up to 12 hours after IAA treatment, levels of fructose and glucose keep increasing in *slr* while their levels slightly decrease in WT, suggesting that in the absence of pericycle activation (first 12h), fructose and glucose accumulate probably due to the lack of glycolytic consumption. To clarify this point, we have reformulated this section: "*The root levels of glucose and fructose derived from sucrose cleavage increased for 6 h upon IAA in Col-0 and then decreased until the end of the night period. Noticeably, glucose and fructose levels were strongly increased by the IAA treatment in the* slr *mutant and peaked at 12h (Fig 2C). This suggests that upon pericycle activation, glucose and*

fructose derived from sucrose cleavage do not build up, probably due to their catabolism by glycolysis, the latter being reduced in the absence of pericycle activation (slr mutant)."

Related to previous comments 4: Page 10 and 11, Based on Fig. 3, the authors conclude "TORC activation by auxin depends on carbohydrate catabolism in roots. This glycolysis-dependent promotion of TOR activity could be a specificity of heterotrophic tissue....". "Together, these data suggest that the auxin-induced activation of glycolysis required for LR formation promotes the activation of TORC....". As discussed in my previous comments, Schepetilnikov et al, 2017 EMBO J have reported that TOR can be activated by auxin, Xiong et al, 2013 Nature found that TOR can be activated by glucose, and Li et al 2017 PNAS further revealed that auxin and glucose are two independent and parallel upstream signals for TOR activation. Absence of either signals causes TOR inactivation. 2D treatment could disrupt sugar-dependent TOR activation, it is unexpected to see that 2D treatment can inhibit TOR activation even with the presence of IAA in roots. Therefore, based on the current data, it cannot draw such conclusion that auxin-induced activation of glycolysis promotes the activation of the TORC. Also, the glycolysis-dependent promotion of TOR activity is not a specificity of heterotrophic tissue, which is also required in the photosynthetic tissues, e.g. leaf (Brunkard et al., PNAS 2020).

Like the reviewer, we were surprised that 2D treatment could inhibit TOR activation even in the presence of IAA in roots. We repeated the experiment three times, obtaining the same result. This led us to consider that TOR activation per auxin requires glycolysis in roots. While the reviewer is right that Li et al 2017 showed that auxin and glucose are two independent and parallel upstream signals for TOR activation, this was only shown in shoots and not in roots. To our knowledge, our data are the first to examine the interplay between auxin and glycolysis on TOR activation in roots. While we stand by our conclusions, we thank the reviewer for pointing out the manuscript by Brunkard et al. and adjusted the wording of our conclusion to explicitly refer to the auxin-dependent activation of TOR: "*This glycolysis-dependent promotion of TOR activity <u>by auxin</u> could be a specificity of heterotrophic tissues that allows a systemic integration of developmental progression with shoot photosynthetic capacity.*"

Related to previous comment 6: the author did not address this comment.

We thank the reviewer for suggesting to use the pS6K1:gS6K1-CFP line for TOR activity analysis. It was unfortunately not possible to detect S6K1 activation with this line. We have further revised the conclusion of this paragraph to "*These results suggest that TORC is turned on in the activated pericycle cells but does not exclude that it could also be activated in the stele tissue important for pericycle activation such as the protoxylem.*"

Related to previous comment 8: The authors also only examined LR initiation upon gravitropic stimulation in UB10pro>>amiR-TOR plants, but examined the LR density in XPPpro>>amiR-TOR

plants. Again, it would be very hard to draw conclusion by using these different treatments in different genetic materials.

The point #8 raised by the reviewer was related to assaying LR density upon AZD treatment. We explained that the amount of AZD (10µM) necessary to reach the pericycle and observe the inhibition of LR formation blocks cell proliferation in the root apical meristem comparable to the *tor*-null mutants, terminates root growth and thus prevents the quantification of LR density. This is why we had to resort to gravitropic stimulation. Upon gravitropic stimulation, both reduction of TOR activity (AZD treatment) and TOR knock-down (pUB10>>amiR-TOR) lead to the absence of pericycle activation and hence LR formation. In point #9 the reviewer asked to use consistent assays when comparing pUB10>>amiR-TOR and pXPP>>amiR-TOR. These experiments were performed, included in a new Figure EV2 and discussed in the main text of the revision. As suggested by the reviewer, we have thus used coherent assays for both genetic materials.

Related to previous comment 9: in page 12, "in the root, induction of amiR-TOR in the XPP cells led to a severe reduction in the number and density of emerged LR formed compared to mock-induced plants (Figure EV2A, B). However, induction of LR by gravistimulation did not impair initiation event (Figure EV2C, D)". The authors explained this difference by" the promoter driving the expression of the amiR-TOR switches of as soon as LR initiation starts". How can this XPPpro>>amiR-TOR plants cause a severe reduction in the number and density of emerged LR? Please clarify this discrepancy.

We can only speculate about this. As the XPPpro becomes active in the newly formed lateral root primordium, this second wave of amiR expression may be sufficient to arrest its emergence and account for the severe reduction in the number of emerged LR. We added the following sentence: "*The severe reduction in the number and density of emerged LR may be a consequence if the re-activation of the XPPpro>>amiR-TOR in the newly formed LR primordium.*"

2nd Revision - Editorial Decision 3rd Mar 2023

Dear Prof. Maizel,

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Congratulations on a really careful set of experiments - I'm delighted that we can publish them!

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Yours sincerely,

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