EMBO reports

Nitrate triggered phosphoproteome changes and a PIN2 phosphosite modulating root system architecture

Andrea Vega, Isabel Fredes, José O'Brien, Zhouxin Shen, Krisztina Otvos, Rashed Abualia, Eva Benková, Steven Briggs, and Rodrigo Gutiérrez
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Corresponding author(s): Rodrigo Gutiérrez (rgutierrez@bio.puc.cl)

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1st Editorial Decision 13th Oct 2020

Dear Prof. Gutiérrez

Thank you for the submission of your research manuscript together with the associated referee reports from Review Commons to EMBO reports. I have meanwhile discussed your study further with an editorial advisor and based on the outcome of these discussions, I would like to invite you to submit a revised version of your manuscript for potential publication in EMBO reports. We will then aim to coordinate the publication with the related manuscript from Otvos et al.

The advisor considered the data interesting but also noted that it would be helpful and informative to report on the "true variability" of the physiological assays. The advisor noted: "The authors apparently chose to give the mean and s.d. of the means of three replicates, which each had 8-15 roots depending on the assay. I wonder about the true variability, i.e. what does a single experiment with 8-15 roots look like?" Since other experts in the field interested in the physiological consequence of PIN2 phosphorylation might have similar concerns, we suggest to provide a measure of the variability between roots within one experiment as well (Figure 6, 7).

I understand that the manuscript you have submitted to us has already been revised according to the reviewer instructions, but the invitation to revise is a formal and technical requirement from our side to be able to resume the review process. Moreover, we routinely perform an initial initial quality control on all revised manuscripts before re- review, for which we require the files to be in a specific format.

Please revise your manuscript according to the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses 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 (). 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 (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. 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 labeled 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.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Specifically, we would kindly ask you to provide public access to the phosphoproteomics datasets.

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should 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 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 .

9) 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 .

10) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but 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.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

The work here is all well described and makes use of appropriate methods. It was sensible to use whole roots for this, to enable key changes to be seen with enough abundance to be measurable. Use of timepoints enabled a rough sequence of phosphorylation events to be determined. It was also good to see was that the multinetwork constructed included genes/protein whose regulation was at different levels - this is a more realistic situation than if only one type of regulation is shown, and enables connectivity/partitioning over the network to be better understood.

This is a well-written manuscript on which I only have a few formatting queries (set out below). All figures and supplementary materials are essential for the data to be properly understood and for further benefits to be gained from it, particularly publication of the phospho-proteomic dataset. I also really liked the way the gravitropism responses are shown.

Major comments:

Based on complementary RNAseq or proteomics data, can you implicate the phosphorylation status changes seen here to any particular kinases whose RNA then protein expression level changes earlier? Or, are any of the kinases here good candidates as regulators of the later changes, based on them being activated by post-translational modification following N-influx? CIPK is mentioned in the introduction and overrepresentation of kinase activity was seen but later in the manuscript there was less on specific genes that could be key regulators.

The majority of proteins found to be differentially phosphorylated according to N status here had not been previously found to be regulated by N at the mRNA level. Some good description of the changes in phospho status are shown but I felt that a more detailed analysis of their mRNA level, based on existing data, would have been helpful. This would help to understand if these proteins are (a) highly expressed and 'waiting' for phosphorylation in order to be activated; or (b) mRNA is regulated by N in a similar way to the protein, but on a different timescale/by different N source than has previously been studied; or (c) mRNA level is very dynamic/noisy and thus changes are undetectable, but phosphorylation status is

more steady. It might be hard to categorise the proteins as such, but taking time to consider these mechanisms would usefully supplement the multinetwork analysis since it might help to connect some of the data types/nodes. It could be added/inserted into the paragraph between lines 482-494, or perhaps the discussion needs to be redrafted to set this out more clearly.

The title does not reflect the novelty of the work - PIN2 phosphorylation is known, albeit not at this level of detail and with work enabling the positioning so precisely in the regulation of RSA shaping by nitrate; the title makes it sound like this was the only thing found/studied. It was examined in great detail but only as an example for validation. It also contrasts to the abstract that spends a lot of time talking about NRT1.1.

Minor comments:

Lines 212-217: This paragraph seems rather vague and do not provide an effective solid conclusion - consider reducing to a single sentence that is more specific and move this to the discussion. The other ends of sections are fine - some a little long and could be more concise, but they are specific and useful.

It would have been helpful to have more references to specific figure panels (and use specific figure panel letters), particularly when describing the cluster patterns (e.g. p10/Figure 2) but I appreciate that the specific journal targetted might have specific requirements that means doing this should come later.

Lines 469-473: The link to ABA is interesting but this section is underdeveloped .... what do each of these pieces of evidence suggest, in the context of the data you have on AREB3?

Gene network analysis: How was the network put together and were all of the edges of equal value? How was the visualisation generated, was this partially manually or does the distance between nodes designate something? Why are some edges thicker/darker than others in Figure 4?

Very minor comments:

Dataset or data-set: decide which term to use

3. Significance:

Significance (Required)

In this manuscript the authors characterise changes in phospho-status for proteins in the Arabidopsis root after nitrate treatment. NRT1.1 was found to be phosphorylated, as seen before, but many novel proteins were implicated in nitrate-signalling according to variation in phospho-status, despite the fact they have not been found to be N-regulated in the past. This makes an exciting resource but the data also enabled some new mechanistic insight to be gained about the role of PIN2 and auxin responses in N-responses in the root.

The datasets here will be of great value to plant scientists, especially those studying nitrogen dynamics, auxin and growth responses - in total this is a very large readership/reach.

REFEREE'S CROSS-COMMENTING:

I think our comments are well in agreement. The questions about protein levels and the questions about the specific figure panels (and replication) all need to be addressed.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

Using phosphopeptide enrichment protocol combined with the mass-spectrometry based identification and quantification, the authors delineated a list of differential phosphoproteins (up and down-regulated) in response to nitrate supplementation, in the Arabidopsis thaliana roots. Furthermore, the authors focused on a specific phosphorylation event in the PIN2 auxin transporter and using phosphonull and phosphomimic mutants of PIN2 (phenotypic characterization and PIN2 localization experiments), they tied together nitrate signaling, auxin transport, and root growth/architecture.

Major comments:

- Are the key conclusions convincing?

Yes, however:

(1) The authors should consider reanalyzing the phosphoproteomics dataset (see below), which can affect some of their conclusions.

(2) The authors delineate a list of differential phosphoproteins (up and down-regulated). It should be noted that, however, obtained differences are likely a consequence of the changes in the phosphorylation status; they can also be a consequence of the changes in the overall protein abundance. The latter cannot be excluded by the lack of the mRNA changes as mRNA and protein levels are not necessarily correlated.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

Please see above.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Phospho-tag Western blot (Fig 5C) and Western blot analysis (Fig 5D) should be replicated (see below).

- Are the suggested experiments realistic in terms of time and resources?

It would help if you could add an estimated cost and time investment for substantial experiments.
Under normal circumstances: one / two months.

- Are the data and the methods presented in such a way that they can be reproduced?

Yes.

- Are the experiments adequately replicated and statistical analysis adequate?

Phosphoproteomics experiment
Replication:
(1) Could the authors specify better what constitutes a replica?
(2) There is an inconsistency between material and methods and supplemental datasets (Table S1 ver. Table S4) regarding the number of replicas for the KNO3, 20min treatment.

Data analysis:

(1) The authors substituted NA values by zero prior data analysis. Such a replacement should only be conducted if there is logical reasoning for converting NA's to zero. Lack of detection in case of the MS measurements is not equivalent to absence, and thus I would be cautious in imputing 0 before proceeding with the quantitative analysis.

(2) The authors mention ANOVA in the material and methods, but ANOVA results are missing in the supplementary tables. Obtained p-values should be subjected to the FDR correction.

(3) The authors decided for the t-test, p-value threshold of 0.1, which is above the traditionally acceptable 5%. Could they justify their decision?

(4) I wondered why the authors decided to conduct their analysis at the level of phosphoproteins rather than phosphopeptides (such as presented in Figure 5B)?

(5) Tables S1-S3 and Table S6, authors should specify the nature of the data. According to Material and Methods ANOVA was done on the log transformed and quantile normalized intensities.

(6) Time 0 (samples T0, Table S1) is not used in the statistical analysis. Is there a particular reason?

Overall, as it stands now, I have doubts about data analysis, and thus the list of differential phosphoproteins.

Data availability:
Proteomics data should be submitted to a public depository such as PRIDE before submission.

Western-blot analysis

Phospho-tag Western blot (Fig 5C) and Western blot analysis (Fig 5D) should be replicated. Western blots should be quantified to remove ambiguity. For instance, looking at the Figured 5D, I would say that PIN2 levels are decreased following nitrate supplementation.

Root growth and PIN2 localisation

Figures 6 and 7; could the authors please specify n and type of the statistic test used (if t-test whether it was paired or unpaired, one- or two-tailed, assuming equal or unequal variance).

To analyze differences in PIN2 localization (Figure 8), authors decided to use ANOVA, while to analyze root traits (Figure 6 and 7) (presumably) t-test. Was there a particular reason for that?

Minor comments:

- Are prior studies referenced appropriately?
In addition to already referenced, authors should consider referring to the nitrate re-supplementation phosphoproteomic dataset (for Arabidopsis roots) published by Wu et al., 2017 Frontiers in Plant Science.

- Are the text and figures clear and accurate?

Yes.

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

Response to nitrate differs between WT and chl1-5 (Table S6). Nevertheless, phosphoproteome of the chl1-5 mutant does respond to nitrate. Could the authors elaborate more on the differences?

3. Significance:

Significance (Required)

-*Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.*

Presented work describes a novel regulatory mechanism that ties differences in the nutrient status (in this case, nitrate) with the auxin transport and plant architecture, which is, in my opinion, both interesting and significant.

- State what audience might be interested in and influenced by the reported findings.

Reported findings would be of interest to the broad audience, particularly plant researchers working in the area of nutrient regulation, development, and hormone signaling.

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

Biochemistry, molecular biology and mass spectrometry

REFEREE'S CROSS-COMMENTING:

I agree. Thank you.

EMBO Reports comments from the editor.

The advisor considered the data interesting but also noted that it would be helpful and informative to report on the "true variability" of the physiological assays. The advisor noted: "The authors apparently chose to give the mean and s.d. of the means of three replicates, which each had 8-15 roots depending on the assay. I wonder about the true variability, i.e. what does a single experiment with 8-15 roots look like?" Since other experts in the field interested in the physiological consequence of PIN2 phosphorylation might have similar concerns, we suggest to provide a measure of the variability between roots within one experiment as well (Figure 6, 7).

RESPONSE: Following the editors advice, we modified Figures 6 and 7 to include all data from three independent biological replicates. The new figures show box plots with the new statistical analysis. Moreover, we now include the source data for both figures as a table file.
As suggested by the advisor, phosphoproteomics raw spectra was deposited at the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository ID MSV000086215.
(https://massive.ucsd.edu/ProteoSAFe/private-dataset.jsp?task=68006a3bdf314832a63d567235850fe8).
We chose this public database instead of PRIDE as it is a standard repository used by our collaborators and there are already many relevant datasets available in MassIVE that would facilitate future analysis or comparisons. We will make the data public as soon as the manuscript is accepted for publication. We included this information in “Data availability” (Material and Methods section).

Review Commons Refereed Preprint #RC-2020-00331 revisions.

Response to Reviewers

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:

The work here is all well described and makes use of appropriate methods. It was sensible to use whole roots for this, to enable key changes to be seen with enough abundance to be measurable. Use of timepoints enabled a rough sequence of phosphorylation events to be determined. It was also good to see was that the multinetwork constructed included genes/protein whose regulation was at different levels - this is a more realistic situation than if only one type of regulation is shown, and enables connectivity/partitioning over the network to be better understood.

This is a well-written manuscript on which I only have a few formatting queries (set out below). All figures and supplementary materials are essential for the data to be properly understood and for further benefits to be gained from it, particularly publication of the phospho-proteomic dataset. I also really liked the way the gravitropism responses are shown.

Major comments:

1) Based on complementary RNAseq or proteomics data, can you implicate the phosphorylation status changes seen here to any particular kinases whose RNA then protein expression level changes earlier? Or, are any of the kinases here good candidates as regulators of the later changes, based on them being activated by post-translational modification following N-influx? CIPK is mentioned in the introduction and overrepresentation of kinase activity was seen but later in the manuscript there was less on specific genes that could be key regulators.

RESPONSE: We cannot implicate a kinase to explain changes in phosphorylation patterns directly based on the data we have. That is the reason why we did not highlight any specific kinase. However, we can suggest some candidate kinase families based on RNA, protein expression level and also based on the motif analysis we performed (Figure 4 and Appendix Figure S4). Moreover and based on the reviewer's comment, we now provide more information about the ten kinases we found differentially phosphorylated in our experiments (MAP kinase Kinase 2, MAPKK-related, transmembrane kinase-like 1, BR-signaling kinase 1, calmodulin-binding receptor-like cytoplasmic kinase CRCK2 and CRCK3, and four protein kinase superfamily proteins). A previous study identified a MAPK kinase cascade involved under nitrate resupply conditions. MKP1 was found phosphorylated under low-affinity nitrate uptake conditions. These results are consistent with our data and suggests the MAPK signaling cascade was affected by nitrate treatments and are interesting candidates to mediate the

nitrate response. We complemented our results and discussion sections to include a more detailed discussion of these kinases (lines 702-709, page 20).

2) The majority of proteins found to be differentially phosphorylated according to N status here had not been previously found to be regulated by N at the mRNA level. Some good description of the changes in phospho status are shown but I felt that a more detailed analysis of their mRNA level, based on existing data, would have been helpful. This would help to understand if these proteins are (a) highly expressed and 'waiting' for phosphorylation in order to be activated; or (b) mRNA is regulated by N in a similar way to the protein, but on a different timescale/by different N source than has previously been studied; or (c) mRNA level is very dynamic/noisy and thus changes are undetectable, but phosphorylation status is more steady. It might be hard to categorise the proteins as such, but taking time to consider these mechanisms would usefully supplement the multinetwork analysis since it might help to connect some of the data types/nodes. It could be added/inserted into the paragraph between lines 482-494, or perhaps the discussion needs to be redrafted to set this out more clearly.

RESPONSE: As shown in Figure RV1, 95% of the genes coding for differentially phosphorylated proteins in our data set are not regulated at the mRNA level in response to nitrate treatments, under a number of experimental conditions (27 experimental datasets corresponding to 131 arrays Canales *et al*., 2014). We analyzed expression levels of these genes coding for differentially phosphorylated proteins in the Canales et al. (2014) dataset and found 63% have greater than average mRNA levels. This result indicates these genes/proteins are relatively highly expressed and susceptible to regulation by phosphorylation as another regulatory layer or mechanism independent of the nitrate-mediated changes in mRNA levels. We modified the text to include this analysis in the new version of the manuscript (lines 731-739, page 21).

The title does not reflect the novelty of the work - PIN2 phosphorylation is known, albeit not at this level of detail and with work enabling the positioning so precisely in the regulation of RSA shaping by nitrate; the title makes it sound like this was the only thing found/studied. It was examined in great detail but only as an example for validation. It also contrasts to the abstract that spends a lot of time talking about NRT1.1.

RESPONSE: We thank the reviewer for this comment as made us rethink the title of our manuscript. While PIN2 phosphorylation was known, our results describe a new phosphorylation site which is important for PIN2 function and root architecture. This is the reason which we highlighted PIN2 phosphorylation. Nevertheless, it is true an important result in our study also refers to the large impact of NRT1.1 in the phosphoproteomics changes. Based on this, we decided to modify the title to: Nitrate triggered phosphoproteome changes and a PIN2 phosphosite modulating root system architecture (following EMBO reports instructions).

Minor comments:

3) Lines 212-217: This paragraph seems rather vague and do not provide an effective solid conclusion - consider reducing to a single sentence that is more specific and move

this to the discussion. The other ends of sections are fine - some a little long and could be more concise, but they are specific and useful.

RESPONSE: We appreciate this comment to improve readability of our manuscript. We reduced this paragraph to a single sentence as suggested: "In summary, our phosphoproteome analysis identified new genes coding for phosphoproteins involved in nitrate responses." (lines 313-314, page 10). We also moved some sentences to the discussion section (lines 684-689, page 20).

4) It would have been helpful to have more references to specific figure panels (and use specific figure panel letters), particularly when describing the cluster patterns (e.g. p10/Figure 2) but I appreciate that the specific journal targeted might have specific requirements that means doing this should come later.

RESPONSE: We added a new Supplementary Figure EV2 for all clusters mentioned in the manuscript. We hope this change easiest understanding the cluster patterns as commented by the reviewer. Moreover, we also incorporated cluster information in Dataset EV2.

5) Lines 469-473: The link to ABA is interesting but this section is underdeveloped .... what do each of these pieces of evidence suggest, in the context of the data you have on AREB3?

RESPONSE: Thanks for pointing this out. It is true the link to ABA is interesting and consistent with earlier studies of nitrate modulation of root system architecture. But it is not central to our story. To avoid confusion, we decided to remove the connection to ABA in this revised version of the manuscript.

6) Gene network analysis: How was the network put together and were all of the edges of equal value? How was the visualisation generated, was this partially manually or does the distance between nodes designate something? Why are some edges thicker/darker than others in Figure 4?

RESPONSE: We included more details about how the network was constructed and visualized. In response to the queries, all edges are equal in terms of the visualization. Visualization was done using the community cluster (GLay) algorithm in the ClusterMaker Cytoscape tool (Morris et al., 2011). This algorithm recognizes functionally related groups and find densely connected regions in a network that aggregate spatially, which results in clusters with good visual separation (Su et al., 2010) (lines 484-487, Page 13). The legend for Figure 4 now contains additional details regarding how the network was constructed and visualized. (lines 953-956, Page 27). However, not all edges are qualitatively the same. To improve network readability, we now distinguish different types of edges as indicated in the revised Figure 4 and corresponding legend (lines 957-958, Page 27).

Very minor comments:

7) Dataset or data-set: decide which term to use

RESPONSE: This issue has been corrected.

Reviewer #1 (Significance (Required)):

In this manuscript the authors characterise changes in phospho-status for proteins in the Arabidopsis root after nitrate treatment. NRT1.1 was found to be phosphorylated, as seen before, but many novel proteins were implicated in nitrate-signalling according to variation in phospho-status, despite the fact they have not been found to be N-regulated in the past. This makes an exciting resource but the data also enabled some new mechanistic insight to be gained about the role of PIN2 and auxin responses in N-responses in the root.

The datasets here will be of great value to plant scientists, especially those studying nitrogen dynamics, auxin and growth responses - in total this is a very large readership/reach.

REFEREE'S CROSS-COMMENTING:

I think our comments are well in agreement. The questions about protein levels and the questions about the specific figure panels (and replication) all need to be addressed.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

Using phosphopeptide enrichment protocol combined with the mass-spectrometry based identification and quantification, the authors delineated a list of differential phosphoproteins (up and down-regulated) in response to nitrate supplementation, in the Arabidopsis thaliana roots. Furthermore, the authors focused on a specific phosphorylation event in the PIN2 auxin transporter and using phosphonull and phosphomimic mutants of PIN2 (phenotypic characterization and PIN2 localization experiments), they tied together nitrate signaling, auxin transport, and root growth/architecture.

Major comments:

- Are the key conclusions convincing?

Yes, however:

(1) The authors should consider reanalyzing the phosphoproteomics dataset (see below), which can affect some of their conclusions.

RESPONSE: We have reanalyzed the dataset as requested. The conclusions were not affected.

(2) The authors delineate a list of differential phosphoproteins (up and down-regulated). It should be noted that, however, obtained differences are likely a consequence of the changes in the phosphorylation status; they can also be a consequence of the changes in the overall protein abundance. The latter cannot be excluded by the lack of the mRNA changes as mRNA and protein levels are not necessarily correlated.

RESPONSE: We agree with the reviewer in that mRNA and protein changes are not necessarily correlated. This has been widely documented. However, it has been also documented that proteome and phosphoproteome do not correlate (Huttlin et al., 2010; Walley et al., 2013; Park et al., 2016; Walley et al., 2016). Phosphorylation changes are dynamic and independent of protein abundance. Therefore, it is common practice nowadays to only use phosphoproteomics data as having the corresponding proteome data does not improve the quality of the conclusions (Umezawa et al., 2013; Hou et al., 2015; Wu et al., 2017; Zadora et al., 2019). However, and for the sake of clarity, we modified the text to make clear that we always refer to phosphoprotein abundance.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

Please see above.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Phospho-tag Western blot (Fig 5C) and Western blot analysis (Fig 5D) should be replicated (see below).

- Are the suggested experiments realistic in terms of time and resources?

It would help if you could add an estimated cost and time investment for substantial experiments.
Under normal circumstances: one / two months.

- Are the data and the methods presented in such a way that they can be reproduced?

Yes.

- Are the experiments adequately replicated and statistical analysis adequate?

Phosphoproteomics experiment
Replication:

(1) Could the authors specify better what constitutes a replica?

RESPONSE: In the "Material and Methods" section, we clarify what constitutes a biological replicate: "Each independent biological replicate consisted of a pool of approximately 4.500 roots collected from *Arabidopsis* plants grown independently under the same experimental conditions" (lines 825-826, page 24).

(2) There is an inconsistency between material and methods and supplemental datasets (Table S1 ver. Table S4) regarding the number of replicas for the KNO3, 20min treatment.

RESPONSE: We apologize for this unintentional mistake. We performed three biological replicates for all experimental conditions. However, one of the replicate experiments (nitrate treatment at 20 min) failed and we decided to eliminate it from the data analysis. We mistakenly included this failed replicate in Dataset EV4. We have now removed this experiment from the manuscript and clarified the experimental design in the Materials and Methods section (lines, 823-824, page 24).

Data analysis:

(3) The authors substituted NA values by zero prior data analysis. Such a replacement should only be conducted if there is logical reasoning for converting NA's to zero. Lack of detection in case of the MS measurements is not equivalent to absence, and thus I would be cautious in imputing 0 before proceeding with the quantitative analysis.

RESPONSE: Missing values is common in proteomics and phosphoproteomics experiments using MS/MS methodology mainly due to sensitivity issues. And it is routine practice to impute NA values. Different methods for imputation have been used to replace these missing values. It is common practice to substitute NA's by zero prior to data analysis as we did or an arbitrary minimal number near zero (Facette et al., 2013; Marcon et al., 2015). More recently, another approach has being used where missing values are replaced by random draws from a Gaussian distribution centered in the minimal value of the sample (Lazar et al., 2016; Roustan et al., 2017). In this new version of the manuscript, we used this second method to impute values for the NA cases with similar results as to the first version. We modified Dataset EV1 and it now includes the NA cases to avoid confusion and facilitate future analysis of the raw data. We also modified the "Material and Methods" section accordingly (page 24, 840-841 lines).

(4) The authors mention ANOVA in the material and methods, but ANOVA results are missing in the supplementary tables. Obtained p-values should be subjected to the FDR correction.

RESPONSE: Thanks for pointing this out. We performed a two-way ANOVA analysis and these results are now provided in a new Supplemental Dataset EV2 ($p < 0.05$). We considered nitrate (N), time (Ti), and the interaction between nitrate and time (N-Ti) as the factor for the ANOVA models. We identified 120 (N), 197 (Ti) and 106 (N-ti) phosphoproteins that were significantly affected. We selected the group of phosphoproteins significantly affected by N or N-Ti (176, $p < 0.05$). We used this group to identify changes at 5 and 20 min in response to nitrate.

FDR or multiple testing corrections are not widely used in (phospho)proteomic experiments. The scale of (phospho)proteomic experiments is significantly smaller as compared to transcriptome experiments where it is routinely used. Moreover, changes in (phospho)proteome experiments are typically very small. Finally, it is still expensive to perform a large number of replicate experiments in phosphoproteomics due to high reagent cost, and instrument time availability, among other issues (Pascovici et al., 2016). In this scenario, FDR correction is too stringent.

There are a number of published studies where using multiple testing corrections method with conventional thresholds fail to detect any true positives even when many exist. Handler and Haynes (2020) examined the statistical analyses from 100 proteomics articles published in 2019 and multiple testing corrections (FDR) were employed in just under a fifth of these papers (17/100). For example, Wu et al. (2017) performed an ANOVA analysis (without FDR correction, $p < 0.05$) to identify up- and down-regulated phosphopeptides under low or high nitrate supply. Menz et al. (2016) discussed their results in phosphoproteomic experiments using *t-test* without multiple testing correction because they detect zero changes with BH corrections ($p < 0.05$). In our case, if we were to use FDR we would only detect 12 changes in phosphoprotein levels which would remove cases we have independently validated such as PIN2. Therefore, we decided not to use FDR correction in our analysis.

(5) The authors decided for the t-test, p-value threshold of 0.1, which is above the traditionally acceptable 5%. Could they justify their decision?

RESPONSE: We apologize for the confusion. As mentioned in comment 4, we performed a two-way ANOVA analysis ($p < 0.05$). We considered nitrate treatment (N), time (Ti), and the interaction between nitrate and time (N-Ti) as the factor for the ANOVA models. We used a model with abundance Y of a given phosphoprotein *i* calculated as $Yi = \beta0 + \beta1N + \beta2Ti + \beta3N\text{-}Ti + \varepsilon$, where β0 is the global mean, and where β1, β2, and β3 are the factor effects. The variable ε corresponds to the unexplained variance. Then, we selected the group of phosphoproteins significantly affected by N or N-Ti (176, $p < 0.05$) and we organized them based on changes at 5 or 20 min in response to nitrate. In the previous version of the manuscript, we used a Tukey's post-hoc analysis with $p < 0.1$ as cutoff to identify the significant factors of the model in pairwise comparison. However, for simplicity, more stringency and consistency we now only use the ANOVA model with 0.05 for the overall fit as well as to identify significant factors in the model. We added this description in "Material and Method" section (page 24-25, 843-845 lines) and "Results" section (page 9, 273-280 lines).

(4) I wondered why the authors decided to conduct their analysis at the level of phosphoproteins rather than phosphopeptides (such as presented in Figure 5B)?

RESPONSE: We performed the phosphoproteomic analysis using label-free quantification. Phosphoprotein levels were quantified using spectral counting, as described by Walley *et al* 2013, 2016, where they analyzed the phosphoprotein abundance changes in seed and maize development. We selected this approach to identify global changes in phosphoproteins in response to nitrate treatments and used the combined values obtained for all phosphopeptides in our experiments (Zhang et al., 2006). Moreover, in our data set 50% of the analyzed phosphoproteins are represented by only one phosphopeptide, as is the case for PIN2 (Figure 5B). And 42% of nitrate-regulated phosphoproteins are represented by one phosphopeptide. Finally, we believe it is also easier for readers to refer to the phosphoproteins rather than phosphopeptides for biological interpretation.

(5) Tables S1-S3 and Table S6, authors should specify the nature of the data. According to Material and Methods ANOVA was done on the log transformed and quantile normalized intensities.

RESPONSE: As indicated, we now include a data description in all supplemental Datasets.

(6) Time 0 (samples T0, Table S1) is not used in the statistical analysis. Is there a particular reason?

RESPONSE: Time 0 was initially included to look at the time effect. However, and as correctly pointed out by the reviewer, we do not discussed this aspect in our study and did not use this data point. We decided to focus our story on the nitrate effect at both 5 and 20 min and the corresponding KCl sample is the best control for this analysis. Several studies in nitrate-responsive genes at transcriptomic level compare nitrate- and KCl-treated roots (Wang et al., 2003, Vidal et al., 2010, Alvarez et al., 2014, Riveras et al., 2015, Xu et al., 2019). Engelsberger et al. (2012) also considered the addition of KCl as the control conditions, where nitrate and ammonium resupply (3, 5, 10, and 30 min) were analyzed at phosphoproteomic levels. Because we do not use this data point, we decided to remove it from the manuscript to avoid confusions.

Overall, as it stands now, I have doubts about data analysis, and thus the list of differential phosphoproteins.

RESPONSE: We clarified all concerns and comments and also made improvements based on the reviewer's comments. We regret some mistakes in the first version that conveyed problems with the data analysis. The list of differential phosphoproteins has been rigorously defined and in this version we are even more stringent than before. We have three independently validated cases, one of which, PIN2, is analyzed extensively in this manuscript and was independently verified by collaborators.

(7) Data availability: Proteomics data should be submitted to a public depository such as PRIDE before submission.

RESPONSE: Phosphoproteomics raw spectra was deposited at the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository ID MSV000086215. (https://massive.ucsd.edu/ProteoSAFe/private-

dataset.jsp?task=68006a3bdf314832a63d567235850fe8). Moreover, phosphoproteomic data is also submitted to PhosPhat4.0 database (http://phosphat.uni-hohenheim.de/index.html). This database contains information on *Arabidopsis* phosphorylation sites which were identified by mass spectrometry in large scale experiments by different research groups. We selected PhosPhat because is specific for *Arabidopsis* phosphoproteomics experiment and it is easy to compare an specific phosphoprotein and/or phophopeptide between diverse experiments. Likewise, the phosphoproteomics experiments performed by Engelsberger et al., 2012, Menz et al., 2016 and Wu et al., 2017 were dropped in this database. We included this information in "Material and Methods" section.

Western-blot analysis

(8) Phospho-tag Western blot (Fig 5C) and Western blot analysis (Fig 5D) should be replicated. Western blots should be quantified to remove ambiguity. For instance, looking at the Figured 5D, I would say that PIN2 levels are decreased following nitrate supplementation.

RESPONSE: As suggested by the reviewer, we now include additional Western blot replicates (Fig 5D) and new pictures to demonstrate we have replicated these experiments. In addition, we quantified the blots to remove ambiguity. We added these results in a new Supplemental Figure EV4. These results are in agreement with *PIN2* mRNA levels not being regulated by nitrate treatments as indicated.

Phos-tag Western blot is useful for separating a phosphorylated protein from its unphosphorylated counterpart by a slower moving rate. Therefore, we conducted a Phos-tag SDS–PAGE followed by Western analysis anti PIN2 to detect differences between the bands' migration more than their quantification. In Figure 5C, phosphorylated PIN2 in the gel (time 0 and KCl conditions) are visualized as slower migration bands (white asterisk) compared with corresponding less phosphorylated proteins (red asterisk) observed in nitrate treatments. To facilitate data interpretation, we now distinguish in Figure 5C fast- and slow-mobility PIN2 specific bands and we described them accordingly in the revised manuscript.

Root growth and PIN2 localisation

(9) Figures 6 and 7; could the authors please specify n and type of the statistic test used (if t-test whether it was paired or unpaired, one- or two-tailed, assuming equal or unequal variance).

RESPONSE: Thanks for pointing this out. The number of roots (denoted by n) used for the analysis were included in Figures 6 and 7 legends. The statistical information for the t-test analysis are now provided in the revised manuscript (lines 997-999 and lines 1009-1011, page 28).

(10) To analyze differences in PIN2 localization (Figure 8), authors decided to use

ANOVA, while to analyze root traits (Figure 6 and 7) (presumably) t-test. Was there a particular reason for that?

RESPONSE: In the phenotypic root analysis (Fig. 6 and 7), we determined the difference between means from nitrate- and KCl-treated roots by t-test in each genotype. The reason is that in this assay, we were testing a known response to the nitrate treatment (primary root inhibition at 3 days in the presence of nitrate) in each genotype (Vidal et al., 2010). We were not interested in genotype differences. As the reviewer indicated, the impact of nitrate-regulated phosphorylation of PIN2 on cellular localization in the different genotypes was analyzed using ANOVA analysis. We used ANOVA for these experiments because we did not know *a priori* what the impact of the phosphosite was on localization.

Minor comments:

- Are prior studies referenced appropriately?
(11) In addition to already referenced, authors should consider referring to the nitrate re-supplementation phosphoproteomic dataset (for Arabidopsis roots) published by Wu et al., 2017 Frontiers in Plant Science.

RESPONSE: Thanks for this suggestion. We now include the reference to Wu et al. (2017) in the comparison of phosphoproteomics experiments related to nitrate starvation or resupply. We also modified Figure 1 accordingly (283-288 lines, page 9). The overlap between all experiments continues to be quite small.

- Are the text and figures clear and accurate?

Yes.

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

Response to nitrate differs between WT and chl1-5 (Table S6). Nevertheless, phosphoproteome of the chl1-5 mutant does respond to nitrate. Could the authors elaborate more on the differences?

RESPONSE: It is known CHL1-5 mutant does not abolish all nitrate responses. While the nature of the NRT1.1-independent nitrate signaling is unknown, some candidates include NRT2.1. Disruption of NRT1.1 signaling may affect the balance between alternative nitrate signaling pathways that result in anomalous changes in the mutant. We have elaborated this aspect in the Discussion and Results section. (lines 448-456, page 12; line 694-698, page 20).

Reviewer #2 (Significance (Required)):

-*Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.*

Presented work describes a novel regulatory mechanism that ties differences in the nutrient status (in this case, nitrate) with the auxin transport and plant architecture, which is, in my opinion, both interesting and significant.

- State what audience might be interested in and influenced by the reported findings.

Reported findings would be of interest to the broad audience, particularly plant researchers working in the area of nutrient regulation, development, and hormone signaling.

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

Biochemistry, molecular biology and mass spectrometry

REFEREE'S CROSS-COMMENTING:

I agree. Thank you.

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1st Revision - Editorial Decision 11th Dec 2020

Dear Prof. Gutiérrez

Thank you for the submission of your revised manuscript to EMBO reports. It has been evaluated again by former referee 2 and we have now received the report copied below.

As you will see, while the referee acknowledges that you have addressed most of the concerns, s/he remains concerned about the strength of the dataset and the abundance of imputed N/A values. Please address the remaining concerns from referee 2, provide access to all values generated by the MinProb method, justify the choice of this method and be transparent about the extent of its use.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Please add up to five keywords.

- Please add paragraphs/statements on Conflict of Interest and Author Contributions.

- Figure EV4B displays the mean and SD from 2 biological replicates. Please either use scatter blots or at least add the individual measurements as data points to the bar graph.

- Appendix: You currently display the figure legends in duplicate, once on the first page and then again underneath each figure. Please change page 1 into a table of content, only list the name and titles of the figures and add page numbers.

- Please update the callout to table S1 to Appendix table S1 in the legend of Appendix Figure S4.

- Appendix figure S5: Please specify the number of replicates (biological, technical) and the statistical test used in the legend.

- Please correct the following figure callouts:
+) Add a callout to Fig 1B wherever appropriate.
+) There is a callout to Fig 4A, but figure 4 has no panels.
+) Fig EV2 many panel callouts are missing.
+) Add a callout to Dataset EV4+EV5 wherever appropriate
+) There is a callout to a Table EV1, which doesn't exist. Please update it.

- Please correct the header 'Methods' to 'Materials and Methods'.

- Please move the figure legends to the end of the Article file. The EV figure legends need a heading called 'Expanded View Figure Legends'.

- I have also taken the liberty to make some changes to the Abstract (copied below my signature). Could you please review it?

- Please make sure to cite the related manuscript from Otvos et al in your manuscript.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of

the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #2:

I want to thank you, the authors, for addressing the requested revisions. However, I still have some reservations about data analysis (see below), and phospho-tag Western blot (Fig 5C) requires replication.

Could the authors specify better what constitutes a replica?
RESPONSE: In the "Material and Methods" section, we clarify what constitutes a biological replicate: "Each independent biological replicate consisted of a pool of approximately 4.500 roots collected from Arabidopsis plants grown independently under the same experimental conditions".

Thank you. Could the authors also clarify what do they mean by "grown independently"? In independent experiments, or in one experiment but independent Phytatrays?

The authors substituted NA values by zero prior data analysis. Such a replacement should only be conducted if there is logical reasoning for converting NA's to zero. Lack of detection in case of the MS measurements is not equivalent to absence, and thus I would be cautious in imputing 0 before proceeding with the quantitative analysis.
RESPONSE: Missing values is common in proteomics and phosphoproteomics experiments using MS/MS methodology mainly due to sensitivity issues. And it is routine practice to impute NA values. Different methods for imputation have been used to replace these missing values. It is common practice to substitute NA's by zero prior to data analysis as we did or an arbitrary minimal number near zero (Facette et al., 2013; Marcon et al., 2015). More recently, another approach has being used where missing values are replaced by random draws from a Gaussian distribution centered in the minimal value of the sample (Lazar et al., 2016; Roustan et al., 2017). In this new version of the manuscript, we used this second method to impute values for the NA cases with similar results as to the first version. We modified Dataset EV1 and it now includes the NA cases to avoid confusion and facilitate future analysis of the raw data. We also modified the "Material and Methods" section accordingly (page 24, 840-841 lines).

To perform a statistical analysis of their data, the authors imputed missing values using the Probabilistic Minimum Imputation method discussed in Lazar et al., 2016. Considering that the dataset contained > 50 % of missing values and only 2-3 replicas per condition / time-point I remain skeptical about the strength of the presented dataset. Unfortunately, this is also the case for authors' focal candidate PIN2, which, when looking at the normalized nSPC, clearly does not pass

significance criteria at 5 min (T-TEST, p-value = 0.17). Of the 17 data points used to create Figure 5b seven had to be imputed (based on data available in Table EV1). However, imputation is discussed as a possibility in proteomics data; the usual datasets have many more replicas. Experimental design and data structure must guide the imputation method's choice. I do understand that the authors are not prepared to perform extra experiments. However and at an absolute minimum, they should give access to all values generated by MinProb method (additional table), compare different imputation methods to justify the choice of MinProb, and make it very clear in the results sections that > 50% of the data-points were imputed and address (in the text) the associated limitations.

Phospho-tag Western blot (Fig 5C) and Western blot analysis (Fig 5D) should be replicated. Western blots should be quantified to remove ambiguity. For instance, looking at the Figured 5D, I would say that PIN2 levels are decreased following nitrate supplementation.
RESPONSE: As suggested by the reviewer, we now include additional Western blot replicates (Fig 5D) and new pictures to demonstrate we have replicated these experiments. In addition, we quantified the blots to remove ambiguity. We added these results in a new Supplemental Figure EV4. These results are in agreement with PIN2 mRNA levels not being regulated by nitrate treatments as indicated. Phos-tag Western blot is useful for separating a phosphorylated protein from its unphosphorylated counterpart by a slower moving rate. Therefore, we conducted a Phos-tag SDS-PAGE followed by Western analysis anti PIN2 to detect differences between the bands' migration more than their quantification. In Figure 5C, phosphorylated PIN2 in the gel (time 0 and KCl conditions) are visualized as slower migration bands (white asterisk) compared with corresponding less phosphorylated proteins (red asterisk) observed in nitrate treatments. To facilitate data interpretation, we now distinguish in Figure 5C fast- and slow-mobility PIN2 specific bands and we described them accordingly in the revised manuscript.

Phospho-tag Western blot (Fig 5C) requires independent replication, especially that the phosphoproteomics data for PIN2 suffer from a high rate of NA (see above).

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Abstract
Nitrate commands genome-wide gene expression changes that impact metabolism, physiology, plant growth and development. In an effort to identify new components involved in nitrate responses in plants, we analyze the Arabidopsis thaliana root phosphoproteome in response to nitrate treatments via liquid chromatography coupled to tandem mass spectrometry. 268 phosphoproteins show significant changes at 5 min or 20 min after nitrate treatment. Proteins identified by 5 min include signaling-components such as kinases or transcription factors. In contrast, by 20 min, proteins identified were associated with transporter activity or hormone metabolism functions, among others. The phosphorylation profile of NITRATE TRANSPORTER 1.1 (NRT1.1) mutant plants was significantly altered as compared to wild-type plants, confirming its key role in nitrate signaling pathways that involve phosphorylation changes. Integrative bioinformatics analysis highlights auxin transport as an important mechanism modulated by nitrate signaling at the post-translational level. We validate a new phosphorylation site in PIN2 and provide evidence that it functions in primary and lateral root growth responses to nitrate.

Response to Reviewer

Reviewer #2

I want to thank you, the authors, for addressing the requested revisions. However, I still have some reservations about data analysis (see below), and phospho-tag Western blot (Fig 5C) requires replication.

Could the authors specify better what constitutes a replica?
RESPONSE: In the "Material and Methods" section, we clarify what constitutes a biological replicate: "Each independent biological replicate consisted of a pool of approximately 4.500 roots collected from Arabidopsis plants grown independently under the same experimental conditions".

Thank you. Could the authors also clarify what do they mean by "grown independently"? In independent experiments, or in one experiment but independent Phytatrays?

RESPONSE: We thank the reviewer for pointing this out. The biological replicates were performed as independent experiments, that is repeating the entire procedure starting from independently grown plants. In the "Materials and Methods" section, we clarify this point: "Each biological replicate consisted of a pool of approximately 4.500 roots collected from *Arabidopsis* plants grown in independent experiments under the same experimental conditions" (lines 694-695, page 23).

To perform a statistical analysis of their data, the authors imputed missing values using the Probabilistic Minimum Imputation method discussed in Lazar et al., 2016. Considering that the dataset contained > 50 % of missing values and only 2-3 replicas per condition / time-point I remain skeptical about the strength of the presented dataset. Unfortunately, this is also the case for authors' focal candidate PIN2, which, when looking at the normalized nSPC, clearly does not pass significance criteria at 5 min (T-TEST, p-value = 0.17). Of the 17 data points used to create Figure 5b seven had to be imputed (based on data available in Table EV1). However, imputation is discussed as a possibility in proteomics data; the usual datasets have many more replicas. Experimental design and data structure must guide the imputation method's choice. I do understand that the authors are not prepared to perform extra experiments. However and at an absolute minimum, they should give access to all values generated by MinProb method (additional table), compare different imputation methods to justify the choice of MinProb, and make it very clear in the results sections that > 50% of the data-points were imputed and address (in the text) the associated limitations.

RESPONSE: Missing values are expected in proteomics and phosphoproteomics experiments using MS/MS methodology mainly due to sensitivity issues. Moreover, biological factors in phosphoproteomic, including a low abundance of phosphorylated proteins and their transitory nature, also generate measurements with a large proportion of missing data that complicate data analysis (normalization, statistical analysis, comparison, quantification, among others). These missing values must be dealt with in some fashion. In the first version of our manuscript, we arbitrarily assigned a value of 0 to missing data, a method used in many publications. In our revision, we used a more sophisticated statistical approach, the MinProb method, to impute missing values. We favored this second method because it was more rigorous and statistically sound than our first approach. The final list of phosphoproteins was essentially a subset of the list in the first submission and the main conclusions remain regardless of the method we used to deal with missing values.
As suggested by the reviewer, we now provide a more detailed description of the data analysis pipeline utilized and highlight the limitations: We used the data processing tools and followed the pipeline described in Differential Enrichment analysis of Proteomics data (DEP, Zhang *et al*., 2018) and MSnBase (Gatto *et al*., 2012) packages using R/Bioconductor (Huber *et al*., 2015). First, the raw data set was filtered eliminating reverse hits and proteins quantified in only one experimental condition.

After these quality filters, the resulted data set shows only 34% of missing values in 1177 phosphoproteins (Dataset EV2). This group of phosphoproteins was analyzed to

visualize the pattern of missing values. We analyzed the densities and accumulative fractions for proteins with and without missing values. Previous published studies found that missing values are associated to proteins levels that were below or around the detection limit (Zhang *et al*. 2018). In our dataset, we observed that proteins with missing values have low average intensities, with a distribution with median 2.36 (base log_2). On the contrary, proteins without missing values show a distribution with median 3.89 (base log_2). These results suggest missing values in our data set are likely due low protein levels near the detection limit. Based on these results and to deal with missing values, we used a left-censored imputation method (Probabilistic Minimum Imputation method) to impute missing values. The limitation of this approach is that missing values can also occur throughout the entire range of values and left-censored methods had been described as less effective with this type of missing values (Lazar *et al*., 2016). Thus, imputing below a detection limit may inappropriately take values too small and influence statistical analysis. While are aware there is no perfect solution to deal with this issue, our results are robust, and we experimentally validated in detail the case for PIN2.

As suggested by the reviewer, we include a new Dataset with all imputed values (Dataset EV2, 1177 phosphoprotein). We modified the "Results" and "Materials and Methods" sections to incorporate this new information. We now clarify that our imputation method was performed in a data set with 36% of missing values (not 50% if we considered the raw-data set) and better justify the selection of this imputation method (241-247 lines, page 9 and 710-720 lines, page 24). Moreover, this analysis workflow assumes that most proteins are not differentially expressed and a correct result of the fraction of differentially proteins should not exceed 10–15% of the quantified proteins (Zhang *et al*, 2018; Gatto & Lilley, 2012). Our data analysis identified 176 phosphoproteins affected in response to nitrate (15% of the filtered dataset), consistent with this assumption.

We have experimentally validated three phosphoproteins identified with our dataset. One of which, PIN2, was extensively analyzed in this manuscript and was independently verified by our collaborators in independent experiments and with different experimental strategies (Ötvös *et al*., 2021). Concerning PIN2, and as mentioned before, we analyzed its role in nitrate responses using different experimental approaches to corroborate the phosphoproteomic analysis and describe this novel phosphosite's role. Moreover, our collaborators also verified this phosphosite's function in root growth depending on nitrogen source and availability (Ötvös *et al*., 2021). In Figure 5b, we analyzed PIN2 values using multiple t-tests without multiple corrections (GraphPad software) and we identified statistical differences ($p < 0.05$) between nitrate and control conditions only at 5 min in Col-0 roots. We incorporated this analysis as source data of Figure 5.

Phospho-tag Western blot (Fig 5C) requires independent replication, especially that the phosphoproteomics data for PIN2 suffer from a high rate of NA (see above).

Response: As suggested by the reviewer, we now include two additional Phospho-tag Western blot replicates (Fig EV4B). Our laboratory and our collaborator (Eva Benkova's laboratory) performed these blots independently, including Time 0, nitrate, and KCl control treatment at 5 min to demonstrate changes in PIN2 phosphorylation status. In these new experiments, we also identified two sub-populations of PIN2 depending on their phosphorylation levels, one more phosphorylated (blue asterisk, at

Time 0 or in KCl treatments) and another less phosphorylated (red asterisk, under nitrate conditions). This band with faster mobility corresponding to a less phosphorylated PIN2 isoform was mainly observed after nitrate treatments. We quantified these bands in one experiment using Image J. We found that nitrate treatment enhances the accumulation of the less phosphorylated form of PIN2 by 3.5- and 6-fold, as compared with time 0 or control conditions (KCl treatment), respectively. These results are consistent with the phosphoproteomics data.

To complement these results, we, and our collaborators (Ötvös *et al*, 2021), also confirmed the function of the PIN2 phosphorylation status in response to nitrate by phosphomimicking analysis using two different pin2 mutants (*eir1.1* and *eir 1.4*, respectively). Our work demonstrated that this new phosphosite (S439) is essential for a correct subcellular localization in response to nitrate treatments that influence primary and lateral root architecture.

Gatto L & Lilley KS (2012) MSnbase-an R/Bioconductor package for isobaric tagged mass spectrometry data visualization, processing and quantitation. *Bioinformatics* 28: 288–289

Lazar C, Gatto L, Ferro M, Bruley C & Burger T (2016) Accounting for the Multiple Natures of Missing Values in Label-Free Quantitative Proteomics Data Sets to Compare Imputation Strategies. *J Proteome Res* 15: 1116–1125

Ötvös K, Marconi M, Vega A, O'Brien J, Johnson A, Abualia R, Antonielli L, Montesinos JC, Zhang Y, Tan S, *et al* (2021) Modulation of plant root growth by nitrogen source-defined regulation of polar auxin transport. *Embo J* 40: e106862

Zhang X, Smits AH, Tilburg GB van, Ovaa H, Huber W & Vermeulen M (2018) Proteome-wide identification of ubiquitin interactions using UbIA-MS. *Nat Protoc* 13: 530–550

2nd Revision - Editorial Decision 23rd Jun 2021

Prof. Rodrigo Gutiérrez
Pontificia Universidad Católica de Chile
Santiago
Chile

Dear Prof. Gutiérrez,

Thank you for your patience while we have editorially reviewed your revised manuscript. I am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Martina Rembold, PhD
Senior Editor
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Corresponding Author Name: Rodrigo A. Gutiérrez
Journal Submitted to: EMBO reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In this study, each experiment was examined by three biological replicates using adequate numbers of Arabidopsis seedling that have been included in the Figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not Applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	Not Applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not Applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. In ANOVA analysis, the Brown-Forsythe test and Bartlett's test were performed to test the equality of variance in different samples. In Student's t-test analysis, F-test was used to test that variances are equal for all groups. These analysis was carried out with GraphPad8.0.
Is there an estimate of variation within each group of data?	Yes, this is provided through standard errors of the means.

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http://ClinicalTrials.gov
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies used in this study are all commerial antibodies and their information was included in the Method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not Applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not Applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not Applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not Applicable.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not Applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not Applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Phosphoproteomic datasets (raw spectra) had been deposited at the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository with the MassIVE ID MSV000086215 (ftp://massive.ucsd.edu/MSV000086215).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Phosphoprotein normalized spectral counts (nSPC), nitrate regulated phosphoproteins in Col-0 and chl1-5 mutant roots are provided in the manuscript as Expanded view Dataset.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not Applicable.
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G- Dual use research of concern

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