The Kinase CIPK23 Inhibits Ammonium Transport in *Arabidopsis thaliana*

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00535-RA 1st Editorial decision – *declined* Aug. 8, 2016

Both reviewers felt that the work is original but not sufficiently complete for publication at this stage. Both reviewers indicated that the claims/conclusions made in the abstract are not fully supported by the results presented. In particular, to prove that AMT1.2 is an in vivo target of CIPK23, the immunoblot needs to be improved to show clearly that the AMT1.2-GFP phosphorylation band is stronger in the wild type compared to the *cipk23* mutant. The reviewers also raised the concern that ammonium-dependent phosphorylation of AMT1.2 may not account for the observed decline in low-affinity ammonium uptake in planta, especially given the fact that AKT1 is a known target of CIPK and has low-affinity ammonium transport activity. Is AKT1 phosphorylated in response to ammonium treatment? Is ammonium-induced decline of low-affinity ammonium uptake still apparent in *akt1* mutants? Answers to these questions will help to establish the importance of CIPK23-dependent phosphorylation of AMT1.2 in ammonium-feedback regulation of ammonium uptake. The reviewers provide additional constructive criticism in their reports, which we believe will be useful to you as you bring your work to conclusion. Finally, we would encourage you to hire a professional editor to help improve the organization, structure and presentation of the manuscript, in addition to the language as well.

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

[Reviewer comments shown below along with author responses]

TPC2016-00806-RA Submission received Oct. 24, 2016

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

Reviewer #1:

Feedback inhibition of ammonium transporter by the phosphor-dependent allosteric mechanism among subunits of the trimer reported in 2007 is one of the landmark findings of transporter regulation. Therefore, identifying the kinase responsible for AMT regulation reported in this manuscript is certainly of great interest and importance in the membrane transport field. Authors cleverly use ammonium and MeA toxicity screen to identify CIPK23. However, there are some issues that need to be addressed.

Point 1. Study of Lanquar (Plant Cell 2009, 21:3610, Figure 4) showed that T460 phosphorylation is induced by ammonium but not MeA. If phosphorylation is not triggered by MeA, how can the mutant of the kinase responsible for the T460 phosphorylation showed different MeA sensitivity, compared to wild type? Please provide additional evidence or explanation for this.

RESPONSE: This is right, but in our hypocotyl screen, we do not investigate the effect of shocking plants with high concentrations of MeA. What we apparently quantify is not the result of a MeA-dependent phosphorylation, but the result of overall less phosphorylation in AMTs in *cipk23*. As a result, more toxic MeA enters the plants and inhibits their elongation.

Point 2. It is inconclusive which of the AMTs is the primary target of CIPK23. Because only low-affinity but not high -affinity uptake is increased in the *cipk23* mutant (figure 2), the authors suggest that the high affinity systems AMT1;1 and AMT1;3 are not the prime targets of CIPK23 regulation; instead, the lower affinity AMT1;2 or the molecularly unknown LATS may be the target of CIPK23. Nevertheless, the western analysis results of protein phosphorylation are not consistent with this conclusion. *AMT1.1* and *AMT1.3* count for ~70% of the total AMTs in the root. In Figure 5b, AMTs phosphorylation level in *cipk23* mutant is reduced to 50% of wild type level. This suggests that AMT1.1 and/or AMT1.3 is the target of CIPK23. If AMT1.2 is the primary target, the phosphorylation level in the *cipk23* mutant is expected to be reduced to ~80% of the wild-type level at most.

RESPONSE: It is true that concluding from our western blot results, AMT1;2 might not be the only AMT1 targeted by CIPK23. This is why we spend more effort on investigating the AMT1–CIPK23 interaction. We used a yeast two hybrid approach, which showed that CIPK23 interacts with AMT1;1 and AMT1;2 but not with AMT1;3. Consistently, for these two transporters, we could as well show interaction with CIPK23 by BiFC in planta. We included the yeast two-hybrid experiment in our new Figure 5.

Point 3. For Figure 5b, to demonstrate that phosphorylation of AMT1.2 is reduced in the *cipk23* mutant, a better western image of AMT1.2-GFP protein needs to be provides. It is clear that phosphorylation of 45 KD band is reduced *in cipk23* mutant, but the phosphorylation of 80 Kd (AMT1.2-GFP) is difficult to see from the image provided.

RESPONSE: As you stated, even for wild type, the maximum share of AMT1;2 of root AMT1s is about 20-30% and even less for the GFP-fusion. We repeatedly and consistently only observed a weak band for AMT1;2 and always only a mild reduction of phopho-band in *cipk23*. However, all the other data also show that the effect is significant, but not an all-or-none effect. Furthermore, in our experience, the amount of wild type AMT1;2 in protein extracts is always very low and hard to detect by MS analysis. Finally, we included a western blot with AMT1;1-GFP and clearly show a (again mild) reduction in phosphorylation of AMT1;1-GFP. This is highly consistent with all functional uptake measurements, as all-or-none effects are expected to change the ammonium transport by a much larger amount.

Point 4. The uptake study shown in Figure 2 is the key evidence of CIPK23 being involved in phosphor-dependent feedback regulation of AMT transporter. However, the description in the text is different from that of the figure legend. In the text, Figure 2F is 0.5 mM for 30 min, but in figure legend, Figure 2F is 5 mM for 6 min. Assuming that text is correct, the uptake rate measured by 30 min is lower than that of 6 min, suggesting the 30 min is not in the linear region and is less reliable.

RESPONSE: We are very sorry for the confusion and the wrong labeling in the text. We have revised Figure 2 and it is now correctly labeled as well as mentioned in the text. We expect to have a mix of several different steps of uptake.

- High affinity uptake of ammonium at the root surface, which may be almost completely blocked after ammonium shock in all plants, since AMT1;3 is not targeted by CIPK23 and other kinases may regulate this AMT.

- Passive mass flux of ammonium into the apoplast, which should as well be similar in all plants and explains the high background uptake.

- High affinity uptake of ammonium into the endodermis and further transport into the vascular tissue, which is abolished by phosphorylation in WT that cannot be blocked in *cipk23* plants.

Since the 30 min 5 mM value represents a mix of mass flux into the apoplast and further high affinity low capacity transport into the endodermis, the uptake cannot be expected to be linear to uptake time. But this data point

highlights the importance of AMT1;2 in blocking further transport of ammonium into the vasculature. This data is completely in line with data presented by N. von Wirén at the NITROGEN 2016 meeting in Monpellier, as well showing the importance of AMT1;2 in blocking superfluous ammonium flow over the Casparian strip.

Reviewer 2:

The work presented by Straub et al. identified CIPK23 as a most promising kinase mediating phosphorylation of AMT1-type transporters in response to ammonium shock. Using a hypocotyl elongation screen of >50 kinase mutants at high ammonium/MeA supply, CIPK23 turned out to be involved in ammonium sensitivity. The *cipk23* mutant was hypersensitive to high ammonium/MeA, which coincided well with the de-repression of $^{15}NH_4^+$ uptake in the low-affinity range. The complementation of this mutant with wild-type CIPK23 restored $^{15}NH_4^+$ uptake. The interaction between AMT1 proteins and CIPK23 was analyzed by BiFC, which indicated a direct interaction of AMT1;1 and 1;2-CIPK23 pairs in Arabidopsis roots. By co-expressing CBL1, CIPK23 with AMT1;2 in oocytes, the authors showed that the CIPK23 kinase in combination with CBL1 can inactivate AMT1;2-dependent ammonium currents.

The study is definitely original and of high relevance for the membrane transporter field, as i) it is the first time to show a functional interaction between a kinase and ammonium transporters in plants and ii) CIPK23 also has with AKT1 and NRT1;1 other prominent targets, suggesting that several nutrient signals may be integrated via CIPK23. The chosen experimental approaches are fine and complementary and provide independent evidence to support the major conclusions. However, at some points, the concept of the study does not appear to be consistent and especially the structure and quality of manuscript require more care and attention:

Point 1. Actually, the real target of CIPK23 has not been identified. Of course, the oocyte data show that CIPK23 can inhibit AMT1;2 activity, and BiFC shows that CIPK23 can interact with AMT1;1 and AMT1;2, and the amiRNA approach shows that loss of AMT activity reverses the *cipk23* phenotype, and the Western suggests that AMTs may be phosphorylated in a CIPK23-dependent manner. I fully agree that this is compelling evidence to suggest CIPK23 as an AMT-phosphorylating kinase. However, even taken together, all of these approaches fail to define the real target in planta. In this regard, the study is somehow not complete.

RESPONSE: We were aware of the problem and tried to gain stronger evidence on functional physiologic interactions of CIPK23 with different AMTs in planta. In collaboration with Prof. Waltraud Schulze at the University of Hohenheim, we isolated crude phospho-proteins and measured unbiased shotgun protein phosphorylation by MS analysis. Unfortunately, three trials with three biological repetitions were unsuccessful in isolating sufficient significant results for individual peptides. Even though the clear tendency is towards a reduced phosphorylation of AMT1;1 and AMT1;2 in the *cipk23* plants, the peptides were not consistently found and, as this is a non-saturating approach, we hesitate including these data in the manuscript. On the other hand, the additional yeast two hybrid assay provides another convincing line of evidence for the interaction of CIPK23 with AMT1;1 and AMT1;2.

Point 2. In relation to point 1, AKT1 is also a target of CIPK23, and AKT1 is likely to act as a low-affinity ammonium transporter (ten Hoopen et al., 2010, J. Exp. Bot.). Thus, maybe this side-activity of AKT1 is responsible for the CIPK-dependent ammonium uptake activity. This would also explain why the de-repression of uptake became evident at millimolar (low-affinity) concentrations. Unfortunately, this very important aspect has even not been properly discussed, although the authors seem to be aware of the ammonium transport activity of AKT1.

RESPONSE: It is right that ten Hoopen et al. show yeast data implying a questionable function of AKT1 in low-affinity ammonium transport. The complementation of the ΔΔΔmep yeast or the ΔΔΔ mepΔtrk yeast is only visible at the very high, not in any way physiologic ammonium concentration of 50 mM, far off the concentrations relevant here. We experience that at external ammonium conditions higher than 7 mM, the passive flow of ammonia over the membrane is sufficient to yield growth even of the ΔΔΔ mep yeast. Assuming a participation of AKT1 in low-affinity ammonium uptake in *Arabidopsis thaliana*, the regulation of AKT1 is, however, opposite (!) to the regulation of AMT1s. AKT1 needs to be phosphorylated to become active. Thus, AKT1 (and potential low affinity NH_4^+ transport) is inactive in the *cipk23* mutant. If AKT1 matters, the low affinity transport of *cipk23* plants for the long 30 min time period should therefore already be strongly reduced. In our experiments, the transport is not significantly different from WT transport, which strongly argues against the participation of AKT1 in low affinity ammonium uptake.

Point 3. While the hypocotyl assay is acceptable for the screening approach, there is a major problem with its subsequent and quantitative use: i) data in Fig.1 are expressed as 'relative normalized hypocotyls length' (also true for 'relative normalized primary root length') without referring to original, absolute values, making it difficult to distinguish whether phenotypes are to the treatment or the genetic background. Please show here measured data. ii) The hypocotyl reduction assay is later used to exclude the root-expressed AMT1;3 from further analysis, because AMT1;3 is not expressed in the hypocotyl. Actually, this way of argumentation represents a conceptual flaw. The scope of this paper is to show CIPK-dependent phosphorylation of root AMTs suppressing ammonium uptake in roots and not in hypocotyl elongation.

RESPONSE: In the first manuscript, we used normalization to combine data from several experiments and to highlight the *cipk23* effect. Still, we have no reservation to show non-normalized data. We included non-normalized measured data for the hypocotyl length from one representative experiment to show that there is no difference in hypocotyl elongation in control conditions and no general phenotype effect. We as well performed yeast two hybrid experiments, which as well exclude an interaction of CIPK23 with AMT1;3. Therefore, there is no conceptual flaw in our argumentation anymore.

Point 4. The Western in Fig. 5 is not at all convincing: i) the 80kD band is smeary and weak and not reliable as shown there, and ii) the 80kD band might not be specific to AMT1;2. While the AMT1;2-GFP fusion is 30kD larger than the endogenous AMTs, it cannot be distinguished from AMT dimers and trimers, which are also detectable at higher molecular weight.

RESPONSE: It is right that Yuan et al. 2007 show two bands for AMT1;2 when denaturing the protein for 30 min at 50°C, one possibly representing the dimer. We performed a stronger denaturation at 99°C for 15 min with a higher amount of SDS (4% vs. 2%) and (3-Mercaptoethanol (10% vs. 2.5%). We think that this excludes the appearance of dimers or trimers. Nevertheless, we reproduced the mild effect on the phospho-band in AMT1;2, but such a mild effect (not all-or-none) is expected. As you stated, even for wild type, the maximum share of AMT1;2 of root AMT1s is about 20-30%. This is even less for the GFP-fusion. Furthermore, in our experience, the amount of AMT1;2 in protein extracts is always very low and hard to detect by MS analysis. However, we repeated western blots with AMT1;1-GFP and clearly show a (reproducibly mild) reduction in phosphorylation of AMT1;1-GFP, which is highly consistent with the other data. Also here no "all-or-none" effects are expected (compare to the effects on NH_4^+ uptake).

Point 5. In Fig. 4, the AMT1;2-dependent YFP localization is on the epidermis, since root hairs also show fluorescence. However, AMT1;2 should localize to the endodermis. Using *35Spromoter-YN* as a negative control would be more appropriate than using *pCIPK23-YN*, because there might be not enough '-YN' protein expressed and available at the plasma membrane when using *pCIPK23-YN*.

RESPONSE: We agree that the expression under the CIPK23 promoter is relatively weak and we think that this is the reason why we see interaction mainly in the outer root parts. Inner root parts are not transmitted equally by fluorescent light, but simply increasing the light gain is prone to false detections of putative interactions. Even if AMT1;2 is mainly expressed in the endodermis and cortex, we always observed some expression in the epidermis as well. We carefully checked the emission peaks of the signal to verify that the fluorescence is originating from YFP. We further tested all lines by PCR to determine whether the correct AMT-YFP and CIPK23-YFP fusions are present in the genome. We are therefore completely sure that the signal originates from reconstitution of YFP by AMT1;2 – CIPK23 interaction. We think that the *CIPK23* promoter is sufficient for a negative control since it is sufficiently expressed to yield fluorescence for the interaction with AMT1;1 and AMT1;2. Furthermore, our novel yeast two-hybrid data now confirm the interaction as well.

TPC2016-00806-RA 1st Editorial decision – *accept with minor revision* Nov. 20, 2016

Please address the following two main points from Reviewer 1 to either tighten the data that has been presented or provide the right context for the work, in addition to more minor revisions recommended by both reviewers at the end of this letter:

POINT 1. To support the claim that CBL1, CIPK23 and AMT1;2 interact in a functional manner, experiments to demonstrate this using the Xenopus assay are important. At present, this assay results in a reduction of ammonium induced inward currents, surprisingly without a notable change in the resting potential of the oocytes. At a minimum,

we should know that all three proteins are targeted to the plasma membrane with a GFP or similar tag and if feasible, the use of BIFC-YFP or western blots to show evidence of possible interactions or a loss of the trimeric form of AMT1;2. A comparable chemical flux experiment would also help to support the reported change in ammonium influx.

POINT 2. As also noted by referee 2, the data to suggest CIPK23 influences NH_4^+, K^+ and NO_3^- transport in a coordinated way is at best preliminary. I doubt the authors want to tackle that relationship in detail as it's not the intent or focus of this manuscript. This is an exciting hypothesis that should be constructed and communicated for what it is until more detailed analysis is completed. The cartoon model in Figure 8 is fine but could be presented with context which recognizes what is still not known rather than a fait accompli.

Please see the attached file for suggested changes to the figures. In general, the font sizes need to be larger and in proportion to the size of the images throughout the paper and the supplemental. Please apply the individual comments to all the figures as applicable.

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

[Provided below along with author responses]

TPC2016-00806-RAR1 1st Revision received Jan. 20, 2017

RESPONSE TO REVIEWING EDITOR: We agree that showing interaction of the proteins in oocytes would support the reported reduction of ammonium influx into the oocytes. We therefore cloned the respective genes coding for the interaction partners into oocyte vectors containing split YFP tags. Unfortunately, a meaningful chemical flux experiment is not feasible with oocytes coexpressing several genes, even though we agree that this would be very supportive. We routinely conduct this kind of experiment, but typically only with groups of oocytes expressing single genes. Expression of more than one gene (especially CBL genes) may stress the oocytes, increases oocyte batch variability and decreases oocyte survival. Dead oocytes, however, have large tracer uptake, which masks real uptake from active ammonium transporters. It is technically impossible to visually determine well expressing but still stable oocytes from oocytes that are already broken. Thus, tracer uptake with small groups of oocytes is always obscured by the intrinsic high variability in the oocytes (this is not a problem in the experiments with individual oocytes that are shown). Therefore, the large background and variability in individual oocytes precludes the dissection of real AMT-mediated transport from unspecific background.

Still, the results of the split YFP experiments are very nice and fully confirm not only the results of our electrophysiology experiments but as well the CBL1-dependent interaction of CIPK23 with AtAMT1;1 and AtAMT1;2, but not with AtAMT1;3, in the membrane of Xenopus oocytes. We are thankful for the reviewers and editor for suggesting these further experiments, as they further strengthen our conclusions. We discuss our model and its limitations now in more detail and with greater care to highlight that a coordinated regulation is still to be proven.

Reviewer #1

Point 1. In Figure 7, there is no AMT1;1 control due to technical difficulties. Although the result is quite clear, with AMT1;2, it would be nice to see that each of these proteins actually interact or at least show co-localisation on the Xenopus PM. If co-expressed with AMT1;3, do you mirror the experiments observed in yeast cells? I would imagine cRNA fusions with GFP or partial YFP tags could help reveal these interactions.

RESPONSE: We are very appreciative for this suggestion. Even though the constructs were not present in the lab, we decided to conduct this experiment with split YFP tags and could nicely confirm our results from the split ubiquitin assay in yeast and split YFP in plants. Our results show CBL1-dependent interaction of CIPK23 with AMT1;1 and AMT1;2, but interestingly not with AMT1;3. Furthermore, CIPK23-dependent interaction of CBL1 with AMT1;1 and AMT1;2, but not with AMT1;3 was observed. The interaction takes place at the membrane of the oocyte, as visualized by the fluorescent markers. This strongly supports our conclusion that AMT1;1 and AMT1;2 might be the target of CIPK23 and that CBL1 and CIPK23 activity are crucial for the AMT regulation.

We changed the oocyte part (and added the new results) in the manuscript as follows (Line 240 – 250):

"We used our split YFP system to confirm the interaction of CIPK23, CBL1 and the single AMT1s at the plasma membrane of Xenopus laevis oocytes (Fig. 7 A). These experiments repeatedly show that CIPK23-YN interacts with AMT1;1-YC and AMT1;2-YC in a CBL dependent way. Again we could not detect interaction of CIPK23-YN

with AMT1;3-YC even though AMT1;3 was expressed in the oocytes as shown by the positive interaction of AMT1;3-YN and AMT1;3-YC. The coexpression of the tagged proteins further shows a CIPK23 dependent interaction of CBL-YN with AMT1;1-YC and AMT1;3-YC. This indicates that the tree proteins build a trimeric regulatory complex since YFP reconstitution could only be seen in oocytes expressing all three interaction partners. To finally prove direct functional interaction of the proteins without tags, *CIPK23, CBL1* and *AMT1;2* were co-expressed in *Xenopus laevis* oocytes."

We further added to the methods (Line 462 – 466):

"Split YFP constructs were obtained by amplifying AMT1;1, AMT1;2 and CIPK23 with split YFP tags attached from the respective plant expression vectors using gene specific primers containing suitable restriction sites for cloning into the pOO2 plasmid. AMT1;3 and CBL1 were as well amplified by PCR and cloned into pOO2-C-YFP and pOO2-N-YFP by adding suitable restriction sites into the primers. The electrophysiological..."

Point 2. Why do both WT and *cipk23* hypocotyl lengths respond equally to toxic levels of NH_4^+? In theory, native CIPK23 activity in the WT should partially alleviate NH_4^+ toxicity relative to that observed with cipk23 (See Figure 1A). Do other AMTs that are not responsive to CIPK23 have a greater role in ammonium homeostasis?

RESPONSE: Thank you for this observation. In the graph, the difference between WT and *cipk23* in toxic ammonium concentrations is really not nicely visible, but this difference is highly significant. Given the very high concentration of 15 mM NH_4^+, which is extremely toxic for these small dark-grown seedlings, as well as the fact that it is very likely that there is a redundant kinase, even a small but significant effect is already remarkable.

This effect is much more visible with the toxic ammonium analog methylammonia, constraining the ammonium results. For certain, these concentrations are well in the range of the so far unidentified LATS for ammonium which will be responsible for most of the ammonium uptake and toxicity, while methylammonia transport is restricted only to AMT1 transporters. This explains why the effect for MeA is higher, since the LATS might not be influenced by CIPK23.

Point 3. Is CBL1 co-expressed in the same cell types and tissues that of AMT1;1, AMT1;2 and CIPK23? Although not essential for this manuscript, the same question would have to extend to NPF6.3 and AKT1;1 for the model to take shape and these four transport pathways to operate as suggested.

RESPONSE: Referring to ammonium uptake from the soil, the expression of AtAMT1;1 and AtAMT1;2 has been shown in the plasma membrane of root cells in the root hair zone of *Arabidopsis thaliana*. While AtAMT1;1 expression is more pronounced in the epidermis and cortex cells, AtAMT1;2 expression is highest in the endodermis and cortex cells (Neuhäuser et al. 2007), but in older root regions is also detected in the rhizodermis and root hairs. Using GUS-fusion constructs, the expression of CBL1 and CIPK23 was shown in the root hair zone as well (Cheong et al. 2007). CBL1 and CIPK23 interact at the plasma membrane. This interaction recruits CIPK23 to the plasma membrane, which was shown by split YFP experiments in protoplasts (Cheong et al. 2007, Xu et al. 2006). The cell-specific localization of CBL1 and CIPK23 has not been shown in plants, but the combined gene expression experiments in the Arabidopsis eFP Browser indicate expression of both genes in all root cell layers. Therefore, AMT1;1, AMT1;2, CBL1 and CIPK23 seem to be co-expressed in the same cell types as well as tissue. AKT1 and NPF6.3 are as well ubiquitously expressed in the root hair zone, with a preference for the epidermis, and therefore overlap in expression as well.

Reviewer #2

The resubmitted manuscript by Straub et al. has been substantially improved. Direct protein-protein interactions between CIPK23 and AMT1;1, as well as with AMT1;2, but not AMT1;3, have been verified by yeast-two hybrid assays. Improved immunoblotting images of the phosphorylation status of AMT1;1-GFP and AMT1;2-GFP fusion proteins under ammonium supply have also been provided. Thus, this revised manuscript demonstrates that the CIPK23-CBL1 complex is responsible for the C-terminal phosphorylation of AMT1 proteins, which acts as a crucial inactivation mechanism to prevent excess ammonium uptake into plant cells. The characterization of CIPK23's function in inactivation of AMT1s not only fills an important missing piece of information regarding the regulation of ammonium transport processes, but also proposes a CIPK23-dependent signaling network balancing the uptake of nitrate, ammonium and potassium via phosphorylation of the corresponding target proteins. This work represents a

substantial advancement in this field and is expected to draw the attention of the scientific community. However, the organization of the manuscript and the accuracy of the conclusions still have some of space for improvement. Major points:

Point 1. Line 133- Line 135: A more significant difference between WT and *cipk23* was observed after either long-term ammonium treatment (Fig. 2F;2H) or higher ammonium supply (Fig. 2G;2H), which is consistent with the finding that ammonium induces C-terminal phosphorylation in a time- and concentration-dependent manner (Lanquar et al., 2009, Plant Cell). A more pronounced effect in *cipk23* observed at high ammonium (5 mM) is therefore not sufficient to draw the conclusion that AMT1;2 rather than AMT1;1 or AMT1;3 is the primary target of CIPK23.

RESPONSE: All our results document that your statement is correct. AMT1;2 is not the only target of the CBL/CIPK complex, since the complex interacts with AMT1;1 as well and also changes its phosphorylation state. This is why we formulated this statement very carefully. Since we do not want to speculate, we have removed this statement from the text:

"... potentially suggesting that the high affinity primary rhizodermal AMT1;1 and AMT1;3 are not the primary targets of CIPK23, but the lower affinity AMT1;2, located in cortical and endodermal root cells."

Point 2. Line 319- Line 320: This part of the discussion is not clear and needs revision: The weaker impact of cipk23 under low ammonium concentrations cannot be explained by CIPK23-independent phosphorylation of AMT1;3. C-terminal phosphorylation of AMT1;3 upon ammonium resupply has not been detected by either using a phospho-specific antibody (Yuan et al., 2013) or by a phosphoproteomics approach (Engelsberger and Schulze, 2012), suggesting AMT1;3 appears to escape from ammonium-induced trans-inactivation of C-terminal phosphorylation. Instead, an endocytic shut-off mechanism affects AMT1;3-mediated ammonium uptake. In particular, the residence time of AMT1;3 on the plasma membrane is decreased due to protein clustering and endocytosis upon elevated ammonium supply (Wang et al., 2013).

RESPONSE: You are correct about the differential regulation of AMT1;3; our novel data in oocytes also support that conclusion. However, it has recently been shown that AMT1;3 is also differentially phosphorylated in the C-terminus due to changes in the nitrogen status (Menz et al. 2016). It was shown that AMT1;1 forms heterotrimers with AMT1;3. Therefore the phosphorylation of AMT1;3 might be one reason for the inactivation of rhizodermal ammonium uptake. Still, we agree that this might not be the only reason. This is why we added the following part to the discussion (Line 328 – 329): "Furthermore rhizodermal AMT1s might be internalized upon high external ammonium concentrations as shown for AtAMT1;3 (Wang et al. 2013)."

Point 3. This manuscript tackles a very important issue, as CIPK23-dependent ion transporter phosphorylation seems to serve as a fine-tuning and balancing mechanism for the uptake of nitrate, ammonium and potassium in response to the fluctuating nutrient availabilities. Unfortunately, this has not been clearly worked out in the discussion part of the manuscript. Therefore, we would like to motivate the authors to provide a more careful description of the CIPK23-dependent regulatory network of nutrient uptake. a) For instance, the expression of CIPK23 is upregulated under low K^+ and/or high NH_4^+ conditions, which leads to an increase in K^+ and a decrease in NH_4^+ transport, resp., indicating that CIPK23 affects the K^+-to-NH_4^+ ratio. b) In addition, the cell-type specific localization of NPF6;3, AKT1, AMT1;1, AMT1;2 together with CIPK23 as well as CBL1/CBL9 has not been considered at all in the revised manuscript. c) Finally, Fig. 8A needs clarification regarding the availability of nitrate and potassium in Fig. 8, because there should be more substrate symbols for nitrate and potassium outside the cell.

RESPONSE: We changed the Discussion and added Line 344–362:

"Here we postulate that CIPK23 and CBL1 might regulate the balance between NO_3^- and its counter ion by antipodal regulation of AKT1, AMT1;1 and AMT1;2 (Fig. 8). Low availability of nitrate leads to the phosphorylation of Thr101 in NPF6;3 by the CIPK23/CBL complex and a corresponding change in affinity. Recently it was shown that nitrogen starvation after exclusive nitrate supply reduces AMT1;1 Thr461 phosphorylation (Menz et al., 2016). This contradicts with the idea of nitrate as a common signal for phosphorylation of N-ion and K^+ transporters. Lacking phosphor-proteomic approaches comparing potassium sufficient and deficient plants, we can only hypothesize whether potassium deficiency might not only induce the phosphorylation of AKT1 but nitrogen ion transporters as well. CIPK23 expression increased due to low external potassium conditions (Cheong et al., 2007). Combined with

the fact that CIPK23 expression is increasing due to high external ammonium concentrations this indicates that CIPK23 regulates the K^+/NH_4^+ uptake ration according to the external nutrient concentrations. Future research must clarify the role of single ions in this regulation. In our postulated model, Arabidopsis balances NO_3^- uptake with AMT1 mediated NH_4^+ uptake under high nitrogen demand and high nitrate conditions (Fig. 8 A). By contrast, under low nitrogen demand or potential NH_4^+ toxicity, NO_3^- uptake is balanced by K^+ uptake through activation of AKT1 and simultaneous inactivation of AMT1s (Fig. 8 B). Potential NH_4^+ toxicity might be given by high external NH_4^+ concentrations or low nitrate concentrations increasing the toxic effect of NH_4^+."

We further changed Fig. 8 as suggested.

Point 4. In Fig 4C, ammonium uptake under short-term conditions (6 min, 0.5 mM) is significantly higher in cipk23 and cbl1 compared with the WT, whereas in Fig. 2E, ammonium uptake shows no difference between *cipk23* and WT under the same conditions (6 min, 0.5 mM). What is the explanation for this apparent contradiction?

RESPONSE: Thank you for this observation. We re-checked the data. The data in 4C are from initial uptake data, were we grew plants with 1 mM K^+. During the several years of study, we later realized that supplying 5 mM K^+ results in better plant growth and from that time on, we therefore used higher potassium. Consistently also with other control experiments over the years, these differences are thus due to slightly altered culture conditions, which makes sense in the context of the results. However, we do not try to explain these differences, as several explanations seem possible. It seems to make sense that CIPK23 is more important when overall nutrient and potassium supply is low, so bigger differences are seen with low K^+ supply. We now mention the different K^+ concentration in the figure legend:

"Plants were grown for 6 weeks in standard HL medium with low (1 mM) potassium."

TPC2016-00806-RAR1 2nd Editorial decision – *acceptance pending* **Jan. 25, 2017**

We are pleased to inform you that your paper entitled "The Kinase CIPK23 Inhibits Ammonium Transport in *Arabidopsis thaliana*" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **Feb. 7, 2017**