

Peer Review File

Manuscript Title: Cell surface and intracellular auxin signalling for H⁺-fluxes in root growth

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Review

The manuscript "Antagonistic cell surface and intracellular auxin signalling regulate plasma membrane H⁺-fluxes for root growth" by Lanxin Li et al. explores the molecular mechanisms of auxin mediated root growth rate in the context of pH dependent growth. The authors beautifully show the links and time-scales of root growth regulation and pH. They furthermore uncover that two branches of early auxin signaling, plasma membrane TMK1 signaling and intracellular TIR/AFB1 signaling, act antagonistically on pH and pH dependent root elongation. Moreover, they demonstrate that pH decreasing regulatory action of TMK1 is mediated by specific phosphorylation of plasma-membrane H⁺ - ATPases. Overall, this is very nice and comprehensive work for which I commend the authors. It constitutes a notable advance in our understanding of how auxin regulates cellular expansion in the root and thereby controls root growth. The manuscript is well written and in a concise manner. The conclusions are generally justified by the data, but I have a couple of concerns outlined below. There is a question regarding the level of advance that the work will provide to the field, as outlined below as well.

Major points:

1. While the authors convincingly identify the branch of auxin signaling that leads to acidification, the main question is still open: What is the mechanism that explains the alkalinization that is responsible for the fast growth repressing effect of auxin in the root? In my mind this question would be the major question in the area and it seems also in the minds of the authors as in the first part of the manuscript the authors clearly set up this fast growth response to IAA as a major question.
2. The authors don't comprehensively weave in what has already been demonstrated in this area. For instance, while the Barbez et al. 2017 PNAS paper has been referenced, specific findings corroborating the findings from this manuscript are not. Barbez et al. showed that apoplastic alkalinization precedes apoplastic acidification and that these pH changes are accompanied by changes in root cell length. Other overlaps are the effects of Fusicoccin and AHA hyperactivation in the ost2 mutant. While the authors elegantly provide more mechanistic and comprehensive data for this here, many of their conclusions and observations are supported by findings in Barbez et al. and should be appropriately noted in the manuscript.
3. L111: Several key conclusions regarding phosphorylation sites are made based on an unpublished manuscript (Han et al.); data for these conclusions need to be presented or references to an accessible paper need to be made.
4. The data for the downregulation of AHAs in Extended Data Figure 2i is inconclusive as it is not clear what the WT levels are.
5. How can it be excluded that the growth defects of the TMK1 mutants are indirectly leading to the AHA phosphorylation patterns? In its entirety the data seems to be convincing, but the authors need to discuss this issue.
6. Statistical support: For many of the assays, the number of biological replicates is in the low range; it will be important to have rigorous statistical tests for each of the conclusions then. This is not always the case. For instance, Fig. 3d,f miss statistics; data from Fig 3h needs to be analyzed using 2-way ANOVA ; Fig 4e,f – is the effect of the double mutant really intermediate?

Minor:

1. L51: “Besides intracellular TIR1/AFB auxin signaling, a cell surface-based pathway 50 mediated by TRANSMEMBRANE KINASE 1 (TMK1) has been shown to regulate differential growth in 51 the apical hook10, while its contribution to a general growth regulation, remains unclear. “ TMK1 has also been implicated in temperature responsive root growth regulation in Gailloch et al. 2020.
2. Fig 4g is really hard to read because very similar colors are used for the line plots.
3. Fig. 4g: The colors need to be explained in the figure legend; are the activation/repression arrows the best way to indicate the effect on pH (would arrow up and arrow down not be better?)?

Referee #2 (Remarks to the Author):

Acidic extracellular (apoplastic) pH can promote growth in shoot tissues, but the situation is known to be more complicated in roots. Here the authors have explored how root apoplastic pH is regulated, and find that the TMK class of membrane receptor kinases can activate the H⁺-ATPases that in turn regulate growth. They also find that the more established TIR1/AFB auxin response pathway alkalinises the apoplast and inhibits root growth.

Figure 1 shows assays of root growth using time-lapse imaging of root tip location and a ratiometric pH indicator. They find that auxin application causes alkalinisation of the apoplast within a minute or so, and that this correlates with slowed growth. Manipulations of the pH of the medium also affect growth rate, suggesting that the rapid pH change causes the growth changes. They might obtain further support for this idea by trying to block the auxin effect by buffering the pH. This data parallels previous work from Monshausen and Gilroy and Barbez et al. The authors should discuss these precedents.

Figure 2 shows that auxin treatment can increase phosphorylation of the plasma membrane H⁺-ATPase. This is counterintuitive in the sense that this should cause greater acidification of the apoplast, the opposite of the effect shown in Figure 1. They also show using various mutants and pharmacologically that H⁺-ATPase activation promotes root growth. They propose that there are in fact two effects of auxin that counteract each other. The H⁺-ATPase phosphorylation and resultant apoplast acidification apparently attenuates the alkalinisation that is induced in parallel.

Figure 3 presents data showing interactions of TMK1 with H⁺-ATPases, through co-IP assays and BiFC. They then argue that TMKs increase H⁺-ATPase phosphorylation. The initial data is with the *tmk1* single mutant (Fig. 3d,e,f), but the auxin effect is modest and the difference between wild type and *tmk1* is not that large (and not repeated or statistically tested in Figure 3f). There seems to be a larger decrease in Thr947 phosphorylation in the *tmk1 tmk4* double mutant (Fig. 3g), but this also needs to be repeated and quantitated and has not been done with or without auxin treatment. The assay in Fig. 3g also differs from 3ef in being done with whole seedlings rather than root samples. The overall impression here is of a rather incomplete dataset. So, I suggest that they try the anti-pThr947 blots with and without auxin treatment in the *tmk1 tmk4* double mutant. Since that double is apparently stunted, they could perhaps do this experiment in a *tmk1 tmk4 tir1* triple mutant, which may have longer roots.

In Figure 4 they show that plants deficient in TIR/AFB signaling lack the fast apoplast alkalinisation response, and present additional evidence that TMK and TIR/AFB pathways act antagonistically on pH and growth rate. One particularly nice experiment uses an orthogonal system to activate auxin response with a synthetic auxin analog and produce alkalinisation.

line 59 and a couple of other spots – they cite reference 9 as showing TIR1/AFB effects on root growth independent of transcription, but the cited paper does not seem to address that question. Is there in fact good previous evidence for this? More generally, it seems that an altered baseline gene expression state of *tir1 afb* mutant roots might in fact cause altered fast alkalinisation responses indirectly, without having to invoke putative non-transcriptional control activity.

Previous work has shown that the alkalinisation response requires a different receptor kinase, Feronia (Barbez et al., 2017). How do the authors reconcile that finding with the present results? Might TIR/AFB activation affect Feronia activity? That work also showed the effect of manipulating external pH on root cell elongation rate (although with less precise kinetics than used in the current ms). It is surprising that the authors have not discussed this previous work.

In the last model figure (4g), the arrows converge on apoplast pH, either activating or inhibiting. This depiction is hard to understand at a glance because it is unclear what it means to activate or inhibit pH. I suggest to redraw this in terms of the increase or decrease in pH, or perhaps change "pH" to "acidic pH".

Referee #3 (Remarks to the Author):

Despite decades of intense research into the molecular mechanisms underpinning auxin action in Arabidopsis, significant gaps in our knowledge still exist. One of these relates to the acid-growth hypothesis postulated at the start of the 1970s. We know that auxin can affect root elongation exceedingly rapidly, and that this must occur via a non-transcriptional mechanism. However, the mechanism through which this occurs in roots has proved elusive, and as such it has not been possible to understand how this integrates with the much better studied TIR1 dependent response.

In this manuscript, the authors use an elegant combination of microfluidics and confocal microscopy to show that inhibition of root elongation occurs after just 2 minutes of auxin treatment. They show that the apoplast pH increases within tens of seconds of an auxin treatment. This is done at concentrations of auxin that are likely to be physiologically relevant, and supports a mechanism by which alkalinisation of membranes is likely to rapidly reduce root elongation.

Employing a phosphoproteomics screen (published elsewhere) they identify two H⁺ ATPases that are rapidly phosphorylated following auxin treatment, and by developing a set of tissue-specific knockdown mutants show that reducing levels of these two ATPases were hypersensitive to auxin in terms of both apoplast alkalinisation and root elongation. The authors showed that these ATPases were phosphorylated by TMK1 to acidify the apoplast. This is counter to the previously observed role of TIR1 in promoting H⁺ influx into cells. Through a very elegant experiment in which they exploited an engineered TIR1 receptor only capable of perceiving a synthetic auxin, they demonstrated that these form two separate mechanisms, a TIR1-dependent mechanism acidifying cells and a TMK1-dependent mechanism alkalinising them, which collectively coordinate root growth.

This work is exciting, and fundamentally changes the way in which we understand the role of auxin in regulating organ growth. It uses a wide combination of approaches, and the findings go beyond roots. Given that they pave the way for similar studies to be performed in shoots, they provide key insights into the age old question of how and why auxin response differs in the two organs. As such this is a highly relevant paper for almost all plant biologists. We have a few minor aspects relating to this work that would benefit from further clarification.

Firstly, the authors talk about growth rate throughout the paper. But are they really looking at growth rate or are they looking at root elongation? Auxin regulates cell division via PLT in a completely different process, which affects root growth.

The data showing that auxin triggers rapid alkalinisation of cells is convincing, but I question whether the interpretation of which other factors are involved is robust. In all 3 cases the authors comparing cellular processes (alkalinisation, MT alignment, vacuolar morphology) with previous

data that - as far as I can see - looks at root growth (as in the whole organ). We feel this would be stronger if the authors compared these processes at the same scale (i.e. cell scale) and ideally in the same cell type/position. The timescales investigated are also different "We visualized vacuolar morphology using tonoplast marker pSYP22::SYP22-YFP. By live imaging the same cells, we could not detect obvious changes in vacuolar morphology even after 30 minutes of 100 nM IAA treatment."

In the section titled, Auxin rapidly phosphorylates and activates plasma membrane H⁺ -ATPases in the root, The authors write "After 1 hour 100 nM IAA treatment, we detected increased ATP hydrolysis activity in root protein extracts". Given that AHA1&2 were identified as being phosphorylated within 2 minutes and the growth changes can be observed in this timescale, the changes in ATP hydrolysis activity appear rather slow. By the rationale of the opening experiment where it was noted that changes to the vacuole were not relevant as they could not be observed within 30 mins, would you conclude that hydrolysis activity is not involved in this process as changes were not observed on the same timescale as changes in root elongation? This would benefit from either using an earlier timepoint or a clearer description of why this time point was appropriate.

In the section entitled, TMK1 activity mediates auxin effect on H⁺ -ATPases phosphorylation and activation. It is not clear if the 35S expression of TMK and the kinase dead TMK is done in the tmk1-1 mutant background. If not, then I would expect to see some phosphorylation of AHA2 after auxin treatment due to the endogenous gene. Please explain further.

One issue that makes this data hard to understand is the lack of spatial information about TMK. Which cells is it expressed in? There are publications showing TMK1 localisation in the apical hook, but I am not familiar with where it is present in the root. This should really be here, either as data or a reference to the original data if it has been published elsewhere.

I like the part comparing the acidification/alkalisation of apoplasts, but I think these pathways could be separated further, in a temporal way. We would hypothesise that TIR1/AFB mutants are unaffected by the short (i.e. 30 seconds to 2 mins) auxin treatment (in terms of apoplast pH and if possible to measure root elongation). Is this the case? The experiments on tir mutants seem to be on longer timescales.

In summary, the manuscript main message is exciting, and it's insights fundamentally change the way we understand the role of auxin in regulating organ growth. However, further experimental evidence is required to fully justify its conclusions.

Author Rebuttals to Initial Comments:

Referees' comments:

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constitutes a notable advance in our understanding of how auxin regulates cellular expansion in the root and thereby controls root growth. The manuscript is well written and in a concise manner. The conclusions are generally justified by the data, but I have a couple of concerns outlined below. There is a question regarding the level of advance that the work will provide to the field, as outlined below as well.

Major points:

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Response: The main mechanistic insight of this work is the identification of auxin-TMK-AHA-acidification mechanism, which is entirely novel. Since we analyzed this mechanism during auxin-triggered root growth inhibition, where alkalinization occurs, the additional TIR1-related work was necessary to place the TMK/AHA-triggered acidification in the context of TIR1-dependent alkalinization, despite this not being the main focus of the work. It was necessary to verify, under strictly controlled conditions and most importantly, with a rapid dynamics, within which the auxin-triggered growth regulation occurs, whether auxin indeed triggers alkalinization immediately and whether this is causal for root growth regulation. We entirely agree that there was previous work suggesting this, but the time scales used (typically several hours) were unlikely to capture events connected to the fast growth inhibition within 30 seconds. We trust that the data set as presented shows beyond doubt that auxin, via a TIR1-dependent pathway, triggers very rapid apoplast alkalinization and that this is causal for root growth inhibition. In our opinion, this is an additional important step to understand the whole process, but not the main finding of this work.

We fully agree with the analysis of Reviewer 1 that to understand the auxin-triggered root growth inhibition, it is necessary to understand the mechanism underlying the TIR1-mediated alkalinization. To elucidate this, however, is a major undertaking clearly outside of the scope of this work. Nonetheless, in the revised manuscript we provided additional insights into this mechanism. In short:

(A) we tested three possible cellular mechanisms of alkalinization: 1) interconnection of membrane potential (MP) versus apoplastic pH changes (Extended Data Fig. 5b-d), 2) a possibility of H⁺-symport with IAA by AUX1/LAX auxin importers (Extended Data Fig. 5a) and 3) involvement of other ion channels that symport protons (Extended Data Fig. 1i, 5d, 6a).

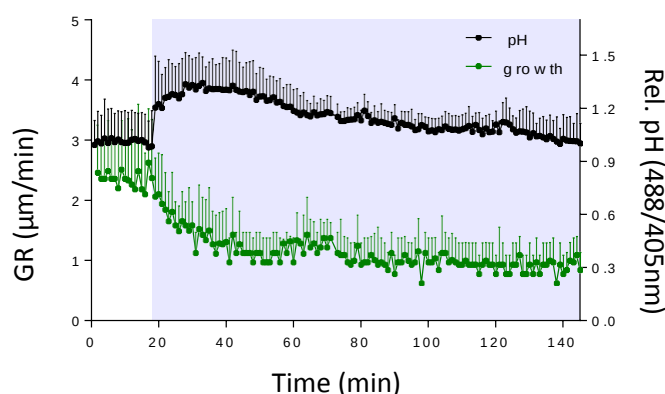
(B) we elaborated on the relationship between auxin-induced H⁺ and Ca²⁺ net influx (Extended Data Fig 6).

(C) we examined contributions of TIR1 and AFB1, two representative family members showing a contrasting nuclear or cytosolic localization (Extended Data Fig. 4h-j).

In summary, we have added investigation of some potential mechanisms that could contribute to the auxin-induced apoplast alkalisation. Auxin has to enter the cell and be perceived by intracellular TIR1/AFB receptors, which induces Ca^{2+} influx, which appears to be required for triggering H^+ influx possibly by opening some unknown H^+ -channels for apoplast alkalization.

2. The authors don't comprehensively weave in what has already been demonstrated in this area. For instance, while the Barbez et al. 2017 PNAS paper has been referenced, specific findings corroborating the findings from this manuscript are not. Barbez et al. showed that apoplastic alkalization precedes apoplastic acidification and that these pH changes are accompanied by changes in root cell length. Other overlaps are the effects of Fusicoccin and AHA hyperactivation in the *ost2* mutant. While the authors elegantly provide more mechanistic and comprehensive data for this here, many of their conclusions and observations are supported by findings in Barbez et al. and should be appropriately noted in the manuscript.

Response: Thank you for pointing this out. We agree entirely that some observations of Barbez et al. support our conclusions, whilst addressing very different time windows (see response to reviewer 2) and perhaps different mechanisms. It was not our intention to omit this work and the publication was cited. We are just not sure how these observations after prolonged times were relevant for a 30 seconds growth inhibition response. We have now integrated the previous results more meticulously. This is where our data differ from the published results: we observe alkalization within 30 s of a low dose of auxin, which is much earlier than in Barbez et al. (Barbez, Dünser et al. 2017) observed. When we tracked the pH for 2 h, our pH dynamics agree with Barbez et al. on the re-establishment of the initial pH in the long term (Data shown below). We did not look at the 8 h time point for which Barbez et al. observed acidification, as we cannot imagine that this is relevant for the rapid response that we study.



3. L111: Several key conclusions regarding phosphorylation sites are made based on an unpublished manuscript (Han et al.); data for these conclusions need to be presented or references to an accessible paper need to be made.

Response: That manuscript is available through bioRxiv now <https://doi.org/10.1101/2021.04.13.439603>, and we included this reference.

4. The data for the downregulation of AHAs in Extended Data Figure 2i is inconclusive as it is not clear what the WT levels are.

Response: We now replotted including the WT expression levels (Extended Data Figure 2k).

5. How can it be excluded that the growth defects of the TMK1 mutants are indirectly leading to the AHA phosphorylation patterns? In its entirety the data seems to be convincing, but the authors need to discuss this issue.

Response: Thanks you for the comment. First of all, it is unlikely that the growth defects of TMK1 mutants have an indirect effect on AHA phosphorylation, since *tmk1-1* mutants have no obvious root growth phenotype in mock conditions, whereas Western blot, MS and ATP hydrolysis data already show a significant effect on the AHA T⁹⁴⁷ phosphorylation state. Secondly, we have included in the revised manuscript a direct phosphorylation data from the *in vitro* [γ -³²P]-ATP kinase assay. We detected the phosphorylation on the C-terminal domain of AHA2 in presence of intracellular kinase domain of WT TMK1 but not kinase-dead TMK1^{K616E} (Fig. 3e and Extended Data Fig. 3i), indicating that TMK1 directly phosphorylates AHA2. Besides, a direct interaction and phosphorylation on AHA2 by TMK1 is also shown in the back-to-back submitted paper on TMK1-AHA regulation in shoot growth (https://assets.researchsquare.com/files/rs-203621/v1_stamped.pdf).

6. Statistical support: For many of the assays, the number of biological replicates is in the low range; it will be important to have rigorous statistical tests for each of the conclusions then. This is not always the case. For instance, Fig. 3d,f miss statistics; data from Fig 3h needs to be analyzed using 2-way ANOVA ; Fig 4e,f – is the effect of the double mutant really intermediate?

Response: Thank you for pointing this out. We have added the missing statistical analysis where possible. As for the sample size, especially for imaging the rapid responses of growth and pH simultaneously in the vRootchip, the time interval of 1 min fundamentally limits the sample size. The vRootchip was designed to only contain 8 roots, so when comparing WT and mutants, each genotype has a maximum of 4 roots in a single experiment. For each experiment, we have however 2-3 independent replicates with similar results. All other assays were done with higher sample numbers. As for the Western blots, quantification was done on a single blot and can hence not include more statistics. We now removed this type of quantification to avoid confusion, instead we provide data of 3 independent blots to indicate reproducibility of the result. For Fig. 3d, we now displayed both WT and *tmk* instead of their ratios, so we can now include statistics between these two. For Fig. 4e,f, we could repeatedly see the intermediate trend in both pH and growth response, though the double mutants are more inclined to the phenotype of *tir1-1*, suggesting a dominant role of TIR1 over TMK1.

Minor:

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Response: Thanks you. We have included the reference in our manuscript.

2. Fig 4g is really hard to read because very similar colors are used for the line plots.

Response: We think this refers to Fig. 4d and have adjusted the colorscheme.

3. Fig. 4g: The colors need to be explained in the figure legend; are the activation/repression arrows the best way to indicate the effect on pH (would arrow up and arrow down not be better?)?

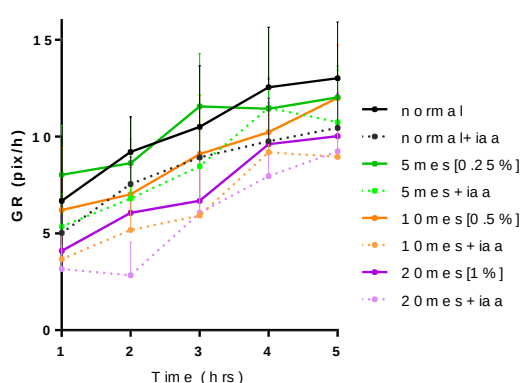
Response: Thanks a lot! We now explained the colors appropriately and made the arrows more indicative.

Referee #2 (Remarks to the Author):

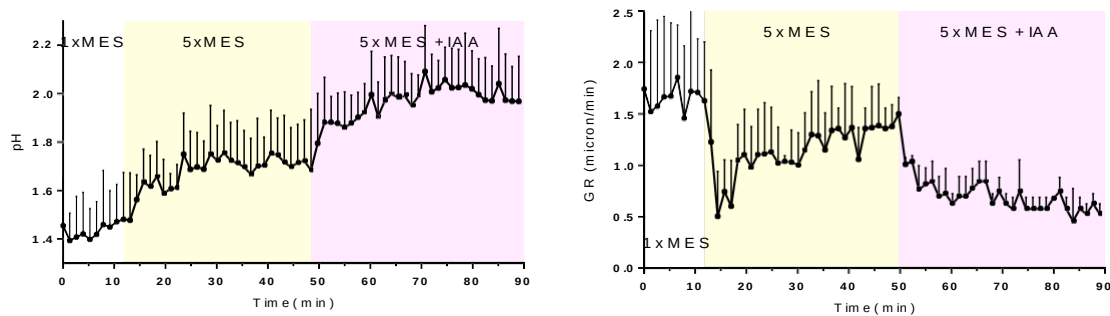
Acidic extracellular (apoplastic) pH can promote growth in shoot tissues, but the situation is known to be more complicated in roots. Here the authors have explored how root apoplastic pH is regulated, and find that the TMK class of membrane receptor kinases can activate the H⁺-ATPases that in turn regulate growth. They also find that the more established TIR1/AFB auxin response pathway alkalises the apoplast and inhibits root growth.

Figure 1 shows assays of root growth using time-lapse imaging of root tip location and a ratiometric pH indicator. They find that auxin application causes alkalinisation of the apoplast within a minute or so, and that this correlates with slowed growth. Manipulations of the pH of the medium also affect growth rate, suggesting that the rapid pH change causes the growth changes. They might obtain further support for this idea by trying to block the auxin effect by buffering the pH. This data parallels previous work from Monshausen and Gilroy and Barbez et al. The authors should discuss these precedents.

Response: Thank you for this comment. Please, see our explanation on this topic in the response to reviewer 1. Based on your suggestions we incorporated the previous work better and compared our data with the known precedents throughout the manuscript. Thank you for the idea of buffering the pH. We now tried higher concentration of MES to buffer the medium. However, higher concentration of buffering agents strongly inhibited root growth (Data shown below) either due to buffering agent itself (Kagenishi, Yokawa et al. 2016) or due to excess of KOH, which was added to set the pH.



When using 0.25% (w/v) MES in vRootchip, we observed a normal response in pH and growth after auxin (Data shown below). We therefore concluded that this ‘buffered medium’ is still not sufficient to buffer the apoplast.



In a “compromise” assay that does not affect root growth as negatively, we limited the availability of H^+ by a 30 min pretreatment with a more alkaline pH 6.1 medium and compared the auxin-response in this buffer to the 5nM IAA response in basal pH 5.8 medium. The auxin-triggered apoplast alkalinization and growth inhibition was delayed (shown in the dotted window in two upper graphs below). In contrast, when increasing the availability of H^+ by pretreating the roots with more acidic pH 5.4 medium, followed by the application of 5 nM IAA we saw an over-responsive apoplast alkalinization and growth inhibition followed by a recovery (shown in the dotted window in two lower graphs below).

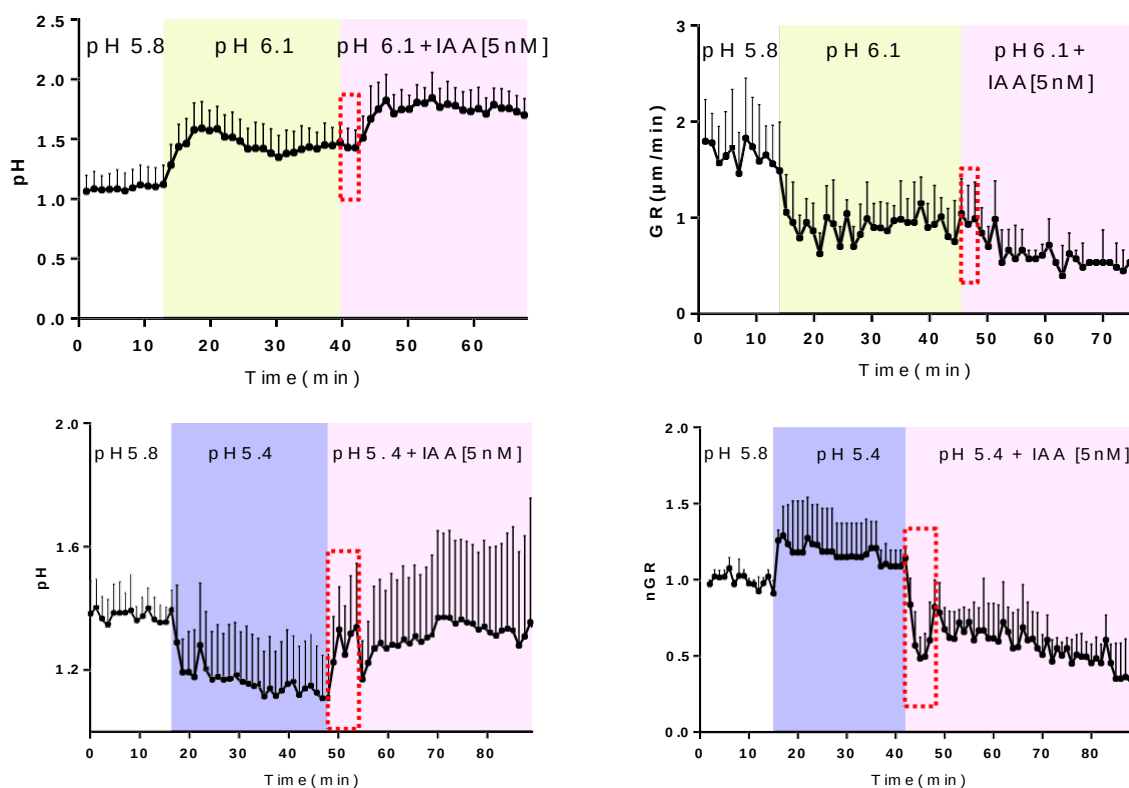


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apoplast, the opposite of the effect shown in Figure 1. They also show using various mutants and pharmacologically that H⁺-ATPase activation promotes root growth. They propose that there are in fact two effects of auxin that counteract each other. The H⁺-ATPase phosphorylation and resultant apoplast acidification apparently attenuates the alkalinisation that is induced in parallel.

Figure 3 presents data showing interactions of TMK1 with H⁺-ATPases, through co-IP assays and BiFC. They then argue that TMKs increase H⁺-ATPase phosphorylation. The initial data is with the *tmk1* single mutant (Fig. 3d,e,f), but the auxin effect is modest and the difference between wild type and *tmk1* is not that large (and not repeated or statistically tested in Figure 3f). There seems to be a larger decrease in Thr947 phosphorylation in the *tmk1 tmk4* double mutant (Fig. 3g), but this also needs to be repeated and quantitated and has not been done with or without auxin treatment. The assay in Fig. 3g also differs from 3ef in being done with whole seedlings rather than root samples. The overall impression here is of a rather incomplete dataset. So, I suggest that they try the anti-pThr947 blots with and without auxin treatment in the *tmk1 tmk4* double mutant. Since that double is apparently stunted, they could perhaps do this experiment in a *tmk1 tmk4 tir1* triple mutant, which may have longer roots.

Response: Thank you for these valuable points. We now managed to obtain more seeds of *tmk1tmk4* and updated Fig. 3g with and without auxin treatment, shown in Fig. 3f.

Regarding to creating a *tmk1,4 tir1* triple mutant, we did not manage to generate it and we also doubt - due to the extremely strong growth phenotype in the *tmk1,4* mutant - whether it would be informative.

In Figure 4 they show that plants deficient in TIR/AFB signaling lack the fast apoplast alkalinisation response, and present additional evidence that TMK and TIR/AFB pathways act antagonistically on pH and growth rate. One particularly nice experiment uses an orthogonal system to activate auxin response with a synthetic auxin analog and produce alkalinisation.

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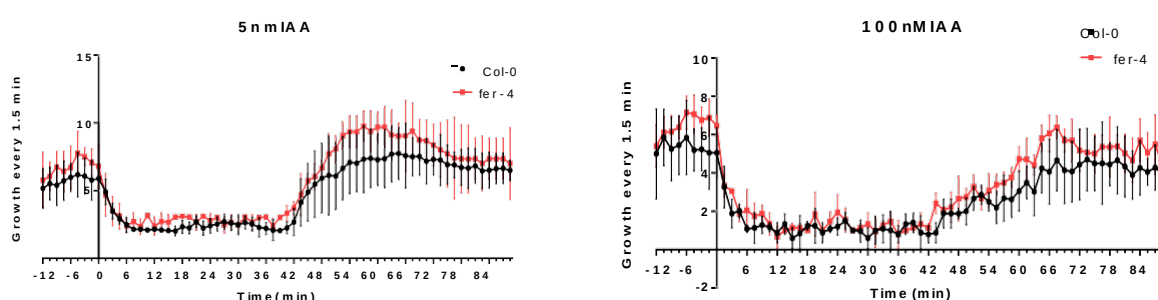
Response: Fendrych et al. used cycloheximide to block protein translation and still observed an auxin-induced rapid growth inhibition. Besides the timing of our observations: we detected apoplast alkalinization within 30 seconds and there is no known transcriptional regulation that occurs this fast, we now included the evaluation of the auxin-induced apoplast alkalinization after blocking protein translation or transcription, by a pre-treatment of cycloheximide (Fendrych, M., M. Akhmanova, et al. 2018) or cordycepin (Robert, S., J. Kleine-Vehn, et al. 2010). In presence of these inhibitors, auxin still triggered rapid apoplast alkalinization (Extended Data Fig. 2a,b), which confirms a non-transcriptional mechanism.

Thank you for the second comment. We also thought about the possibility of altered gene expression and its effect on alkalinization. We hence added PEO-IAA, which blocks TIR1/AFB signaling, directly to IAA without pretreatment, and observed the growth after 1 hour was

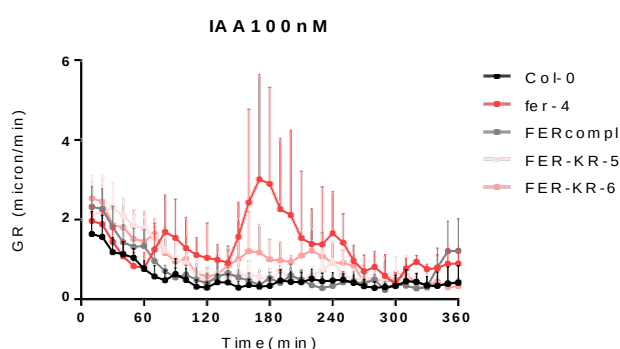
completely rescued (Extended Data Fig. 4f,g). We think this pharmaceutical tool used in a fast time period helps us to avoid the secondary effect of altered baseline genes in the mutants.

Previous work has shown that the alkalinisation response requires a different receptor kinase, Feronia (Barbez et al., 2017). How do the authors reconcile that finding with the present results? Might TIR/AFB activation affect Feronia activity? That work also showed the effect of manipulating external pH on root cell elongation rate (although with less precise kinetics than used in the current ms). It is surprising that the authors have not discussed this previous work.

Response: Thank you for raising this point. We tested fast responses of the *fer-4* mutant after 5 nM or 100 nM IAA in vRootchip and we saw a completely normal growth response (Data shown below, with auxin treatment from time point 0 and washout by basal medium at 42 minutes). We therefore do not think FER plays a role in the early auxin-mediated rapid root growth response.



To understand the discrepancy, we analysed a longer term effect of auxin in *fer-4* by tracking the root growth over 6 h. At low concentration (5 nM IAA), the mutant was inhibited similarly to Col-0 (not shown), whereas at a high auxin concentration (100 nM), we found transient resistance during 2-4 h of treatment (Data shown below). This is in line with Barbez et al (2017), in which a decrease of the total accumulated cell length after 8 h of 250 nM IAA was measured.



In the last model figure (4g), the arrows converge on apoplast pH, either activating or inhibiting. This depiction is hard to understand at a glance because it is unclear what it means to activate or inhibit pH. I suggest to redraw this in terms of the increase or decrease in pH, or perhaps change “pH” to “acidic pH”.

Response: Thank you very much! We adjusted the scheme according to your suggestions and hopefully it is more intuitive now.

Referee #3 (Remarks to the Author):

Despite decades of intense research into the molecular mechanisms underpinning auxin action in Arabidopsis, significant gaps in our knowledge still exist. One of these relates to the acid-growth hypothesis postulated at the start of the 1970s. We know that auxin can affect root elongation exceedingly rapidly, and that this must occur via a non-transcriptional mechanism. However, the mechanism through which this occurs in roots has proved elusive, and as such it has not been possible to understand how this integrates with the much better studied TIR1 dependent response.

In this manuscript, the authors use an elegant combination of microfluidics and confocal microscopy to show that inhibition of root elongation occurs after just 2 minutes of auxin treatment. They show that the apoplast pH increases within tens of seconds of an auxin treatment. This is done at concentrations of auxin that are likely to be physiologically relevant, and supports a mechanism by which alkalinisation of membranes is likely to rapidly reduce root elongation.

Employing a phosphoproteomics screen (published elsewhere) they identify two H⁺ ATPases that are rapidly phosphorylated following auxin treatment, and by developing a set of tissue-specific knockdown mutants show that reducing levels of these two ATPases were hypersensitive to auxin in terms of both apoplast alkalinisation and root elongation. The authors showed that these ATPases were phosphorylated by TMK1 to acidify the apoplast. This is counter to the previously observed role of TIR1 in promoting H⁺ influx into cells. Through a very elegant experiment in which they exploited an engineered TIR1 receptor only capable of perceiving a synthetic auxin, they demonstrated that these form two separate mechanisms, a TIR1-dependent mechanism acidifying cells and a TMK1-dependent mechanism alkalinising them, which collectively coordinate root growth.

This work is exciting, and fundamentally changes the way in which we understand the role of auxin in regulating organ growth. It uses a wide combination of approaches, and the findings go beyond roots. Given that they pave the way for similar studies to be performed in shoots, they provide key insights into the age old question of how and why auxin response differs in the two organs. As such this is a highly relevant paper for almost all plant biologists. We have a few minor aspects relating to this work that would benefit from further clarification.

Firstly, the authors talk about growth rate throughout the paper. But are they really looking at growth rate or are they looking at root elongation? Auxin regulates cell division via PLT in a completely different process, which affects root growth.

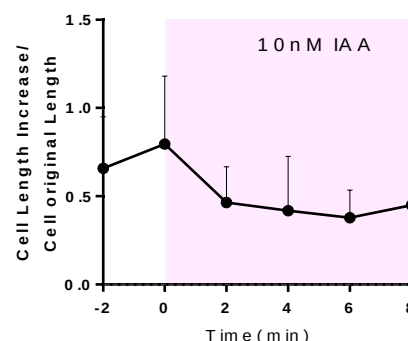
Response: Thank you for your positive comments about our work.

Thank you for raising the issue of cell division versus elongation. Within the time scale we measured typically within few minutes (longest 6 h) the contribution of cell divisions can be excluded and hence the presented root growth rate primarily represents cell elongation. Previous publications have shown that 30 nM IAA does not change the cell division rate within 7 h and there was no rapid regulation of auxin on PLT expression as the earliest upregulation was observed after 24 h

(Mähönen, Ten Tusscher et al. 2014). Therefore, we think the effects of auxin on cell division is not relevant in this context.

The data showing that auxin triggers rapid alkalisation of cells is convincing, but I question whether the interpretation of which other factors are involved is robust. In all 3 cases the authors comparing cellular processes (alkalinisation, MT alignment, vacuolar morphology) with previous data that - as far as I can see - looks at root growth (as in the whole organ). We feel this would be stronger if the authors compared these processes at the same scale (i.e. cell scale) and ideally in the same cell type/position. The timescales investigated are also different "We visualized vacuolar morphology using tonoplast marker pSYP22::SYP22-YFP. By live imaging the same cells, we could not detect obvious changes in vacuolar morphology even after 30 minutes of 10 nM IAA treatment."

Response: Thank you for the comments. Indeed, cell length will be more exact than root growth to compare the other cellular processes. However, the imaging resolution does not allow the analysis of cell length in the same time interval (30 s or 1 min) as used for the pH analysis. There is in that time interval just not sufficient cellular growth to ensure reliable measurements without too much variation. To definitely confirm that cell length also changes fast after auxin treatment, we have now included the evaluation of individual cell length every 2 min after 100 nM IAA treatment. With greater variability for the reasons mentioned before, we still observed rapid cell growth inhibition (Data shown below). This confirms that individual cell length and total root growth correlate and we are hence convinced that the presented growth rate is measure of cumulative elongation changes of individual cells.



Regarding the different time scale of vacuole morphology visualization, we actually saw no changes at earlier time points. Therefore, we opted to show the results after a prolonged incubation of a high concentration of IAA treatment to strengthen the conclusion that changes of vacuolar morphology are not at the same time and concentration scale as auxin-triggered root growth inhibition.

In the section titled, Auxin rapidly phosphorylates and activates plasma membrane H⁺-ATPases in the root, The authors write "After 1 hour 100 nM IAA treatment, we detected increased ATP hydrolysis activity in root protein extracts". Given that AHA1&2 were identified as being phosphorylated within 2 minutes and the growth changes can be observed in this timescale, the changes in ATP hydrolysis activity appear rather slow. By the rationale of the opening experiment where it was noted that changes to the vacuole were not relevant as they could not be observed within 30 mins, would you conclude that hydrolysis activity is not involved in this process as changes

were not observed on the same timescale as changes in root elongation? This would benefit from either using an earlier timepoint or a clearer description of why this time point was appropriate.

Response: Thanks for noticing this. This is a standard way how these ATP hydrolysis assays were established for Arabidopsis roots. This was originally due to some time required for products of the enzymatic activity to reach the threshold detection level but it does not mean that the activation does not occur faster. With other more sensitive methods like MS-phosphoproteomics and Western blots, we detect activation as early as 2 min after the treatment.

In the section entitled, TMK1 activity mediates auxin effect on H⁺-ATPases phosphorylation and activation. It is not clear if the 35S expression of TMK and the kinase dead TMK is done in the *tmk1-1* mutant background. If not, then I would expect to see some phosphorylation of AHA2 after auxin treatment due to the endogenous gene. Please explain further.

Response: We are sorry for not making this sufficiently clear. The 35S-driven expression of TMK1 and kinase-dead TMK1 were done transiently in tobacco leaves for which no genetic mutant material is available. We saw the wilting phenotype in these TMK1 overexpressing leaves, which indicates an effect on the endogenous H⁺-pumps. Additionally, we used Arabidopsis transgenic lines harboring a DEX-inducible TMK1 and kinase dead TMK1. We observed an increased phosphorylated AHA2 after induction of WT TMK1 but not in the kinase-dead version (Extend Data Fig. 3h) in both steady state and auxin-treated conditions. This indicates that for both endogenous and auxin-treated condition, AHA2 phosphorylation depends on TMK1. Lastly, we think the kinase dead result is caused by a dominant-negative effect of overexpressing inactive TMK1.

One issue that makes this data hard to understand is the lack of spatial information about TMK. Which cells is it expressed in? There are publications showing TMK1 localisation in the apical hook, but I am not familiar with where it is present in the root. This should really be here, either as data or a reference to the original data if it has been published elsewhere.

Response: Thank you for this question. We have included a confocal image of pTMK1::TMK1-GFP expressing roots taken in our exact growing conditions in the vRootchip to demonstrate the expression pattern and localization of TMK1 (Extended Data Fig. 3a).

I like the part comparing the acidification/alkalisation of apoplasts, but I think these pathways could be separated further, in a temporal way. We would hypothesise that TIR1/AFB mutants are unaffected by the short (i.e. 30 seconds to 2 mins) auxin treatment (in terms of apoplast pH and if possible to measure root elongation). Is this the case? The experiments on *tir* mutants seem to be on longer timescales.

Response: Thank you for the stimulating discussion. In our analysis of fast responses, *tir1-1afb2-1afb3-1* triple mutants were partially resistant to auxin for both rapid apoplast alkalinization (Fig. 4a) and root growth inhibition (Fendrych, Akhmanova et al. 2018). We found the *tir1-1*, *tir1-10* and *afb1-3* single mutants to also be resistant to auxin treatment after 6 h (Fig. 4e,f and Extended Data Fig. 4-2 a). Therefore, for both rapid and long-term effects, TIR1/AFB receptors are necessary. However, we exclude that the rapid TIR1/AFB-mediated alkalinization occurs through the TIR1/AFB canonical transcriptional pathway, as our additional data using transcriptional and translation inhibitors show that the response does not depend on *de novo* protein synthesis (Extended Data Fig. 2a,b).

Additionally, we now include a comparison between the roles of different TIR1/AFB family members, particularly TIR1 and AFB1, which are localized mostly in nuclear and cytosol, respectively (Prigge, Platre et al. 2020). We found that both TIR1 and AFB1 contribute with *tir1-10* being more auxin-resistant in the long term (Extended Data Fig. 4h, i) while *afb1-3* showing more resistance to rapid auxin effects in vRootchip (Extended Data Fig. 4j, k). This supports the idea that cytosolic TIR1/AFB receptors represent the dominant auxin perception mechanism for the rapid auxin-induced apoplast alkalinisation and root growth inhibition. Together with our ccvTIR-cvxIAA data showing that TIR1 is able to trigger the rapid responses (Fig. 4b and Fendrych, M., M. Akhmanova, et al. 2018), we propose that cytosolic TIR1/AFB mediates a non-transcriptional signaling branch which mediates rapid H⁺-influx to alkalinize apoplast and growth inhibition, while TMK1-AHA activates H⁺-efflux to counterbalance this.

In summary, the manuscript main message is exciting, and it is insights fundamentally change the way we understand the role of auxin in regulating organ growth. However, further experimental evidence is required to fully justify its conclusions.

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Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors have addressed all concerns that I have raised. I commend them for their excellent work.

Referee #2 (Remarks to the Author):

This work reports antagonistic effects of two auxin response pathways on apoplastic (cell wall space) pH and growth in Arabidopsis roots. They have used a microfluidics setup to monitor root growth along with apoplastic pH at very fast time scales, as well as several other biochemical and genetic approaches. The results bring together previous observations that seemed contradictory, and also reveal action of a new pathway by which auxin can activate the plasma membrane H⁺-ATPase, through TMK receptor kinases.

In response to my earlier suggestions, they did some experiments to explore how altered pH in the medium affects the responses, and also looked at responses in a feronia mutant, and included data on these questions in the responses to reviews. As other readers may have the same questions, why not put some of this information in the supplementary figures?

lines 149, 290 - the authors refer to the slower pathway as a feedback on the first one, but they have not actually shown that the proton pump activation causes the alkalinisation, in a classic feedback loop. Rather, the results suggest that the two pathways likely act independently. Therefore, it would be more accurate to refer to them as parallel pathways rather than one feeding back on the other.

Using each half-YFP construct is not really a useful negative control for the BiFC experiments. Better would be a fusion of the other half of YFP to a protein that fails to interact but is expressed.

In Figure 1a, does "MZ" refer to maturation zone? Please indicate this in the figure legend.

Referee #3 (Remarks to the Author):

In summary, the manuscript main message is exciting, and it is insights fundamentally change the way we understand the role of auxin in regulating organ growth.

The authors have provided additional experimental evidence (in response to all 3 reviewers comments) which I felt now fully justify the manuscripts conclusions.

I am confident that this (and the accompanying) paper will serve to rewrite the textbooks about our understanding of rapid auxin action which has remained unresolved for many decades.

Author Rebuttals to First Revision:

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supplementary figures?

Thanks for your interest in our data. We have included the growth response in *fer-4* in the discussion of the manuscript and the data is available now in Extended Data Figure 8.

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That is true. Thank you for the more precise expression. Indeed, we meant that two pathways TMK-AHA and TIR1/AFB-mediated H⁺-influx converge on net H⁺-flux, and that the regulation comes from the same signal: auxin. Auxin acts positively in one branch and negatively in the other pathway. Now, we removed the word “feedback” from the text.

Using each half-YFP construct is not really a useful negative control for the BiFC experiments. Better would be a fusion of the other half of YFP to a protein that fails to interact but is expressed.

As we have several independent methods (IP-MS, Co-IP) confirming the interaction, we did not include a negative control for the interaction between TMK1 and AHA2. However, we could use the lack of interaction with AUX1 to demonstrate the specificity of our findings. AUX1 (AUXin resistant 1) is a PM located auxin influx transporter. Using the same plasmids (*pSPYNE* and *pSPYCE*) no interaction in BiFC between AUX1 and AHA2 was observed. This is nicely shown in Ren et al. (2018 – Suppl. Fig. 7) and Wong et al. (2019 – Figure 2C). We have included these references now in the Methods section. Since overexpression of TMK1, even transiently, always gave a strong wilting phenotype in *N. benthamiana* leaves, we tried BiFC using YPF^N-TMK1^{K616R}, a kinase-inactive mutant, together with AHA2-YFP^C or AUX1-YFP^C. We respectively observed complementation at the PM and no complementation. A representative image of this experiment is now included in Extended Data Figure 4.

In Figure 1a, does “MZ” refer to maturation zone? Please indicate this in the figure legend.

Sorry for the confusion. We now changed this abbreviation in Figure 1a to “RH” for root hair zone, in order to have it distinct from the “MZ”, which was used for meristematic zone in Extended Data Figure 4a.

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I am confident that this (and the accompanying) paper will serve to rewrite the textbooks about our understanding of rapid auxin action which has remained unresolved for many decades.

Editorial Note

Reviewers were consulted about the authors’ rebuttal and were satisfied with the responses at this stage.