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Pathogen-induced pH changes regulate the growth-defense balance in plants

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 5th Apr 2019

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received four referee reports on your manuscript, which are included below for your information.

As you can see from the comments, all referees express interest in the topic of the study. However, they also raise a number of substantive concerns that would need to be addressed before they can support publication of the manuscript. From my side, I judge the referee comments to be generally reasonable, therefore, based on the reviewers' positive opinions on the manuscript, I would like to invite you to submit a revised manuscript in which you address the issues raised in referees' comments, particularly focusing on the following points:
1) Provide additional data to strengthen the link between cc1cc2 mutation and resistance to Fusarium infection and address the potential effect of cell wall integrity sensing mechanism due to cell wall weakening (reviewer #1, reviewer #2)
2) Please add the necessary controls as requested by reviewers #2, #3 and #4
3) Address the questions on the Fusarium-induced apoplast acidification and the properties of the pH sensor raised by reviewers #3 and #4

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss how to proceed.

REFEREE REPORTS:

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Referee #1:

Pathogen-induced pH changes regulate the growth-defense balance of plants

Kesten et al.

This is a potentially significant paper, which seeks to explain the mechanism of cell wall-related sensing in plants in response to biotic stimuli, such as pathogen attack. It is well known that pathogens can induce acidification of the apoplast of plants and that this is linked to host defence. However, the precise mechanism of this acidification, and the role of plant cell wall re-modeling, is unclear. This study provides evidence that apoplastic acidification via proton pump activation leads to acidification of the cortical side of the plasma membrane in response to infection by the filamentous fungal pathogen Fusarium oxysporum. This appears to lead to a reduction in cellulose synthesis and cell growth. The paper also provides evidence for Companion of Cellulose Synthase (CC) proteins as key regulators of the balance between plant growth and plant defense processes. There are potentially important consequences of this study for understanding the interplay between pH control (by both plant and pathogen) and the trade-offs and balance between plant immunity and growth.

In general, I am supportive of publication of this paper in EMBO Journal. The study is carefully executed and the work is novel in the uses of new pH sensors that are superior to previously reported sensors allowing spatial localisation of pH changes that are necessary to implicate processes acting at the plasma membrane and affecting cell wall re-modelling. The paper also attempts to use quantitative data to support the microscopy observations, which is often missing from such studies. There is, as a consequence, a better sense of the reproducibility of the findings and the level of variation observed.

My main concern about the paper in its current form is that it is just so hard to follow. There is an excessive use of abbreviations that sometimes makes the sense of sentences really difficult to understand. This is made worse by the fact that the Figure Legends are very long and also not always clear. I think the legend to Figure 1, for example, could be much shorter and clearer in stating the main observations.

My only query regarding the data is the extent of the role of CC in the rate of vascular penetration by Fusarium. This is shown in Figure 4, but not well explained by the rather inpenetrable description on page 10. I really would like to know more about how the experiment was carried out leading to the graph shown in Figure 4 C. There is a clear effect in the cc1cc2 line on root growth, but the effect on rates of pathogen vascular penetration are actually very small, given the scale on the graph. The difference is never more than 1 penetration event and the scale bars are obscured by the background bar chart. The number of observations is not clear (is it 103 from 3 biological reps?) and although the ANOVA says the results are significant, I remain sceptical, given such small differences. I think that other readers will also be sceptical and therefore the experiment needs explaining in greater detail and I would really like to see an alternative method used to verify these observations, which are quite critical to the conclusions of the paper. There must be a biological read-out in terms of disease symptoms or fungal biomass that could also be used to test this idea more thoroughly.

I am also concerned about the autofluorescence observed in Figure 4A. Why is this so high? A higher resolution image of xylem invasion by Fusarium would be useful here, especially if this could be an example of the observations used to provide the data for Figure 4C. This would help interpretation of the experiment as described above.

In terms of clarity, I am also a little disappointed by the Discussion as I had hoped this would be more in depth in terms of the implications of the observations and how these relate to our knowledge of pH manipulation and adaptation by pathogens (with the recent work of Di Pietro more fully described), coupled with a wider analysis of the balance between growth and immunity revealed by the results here. This would make it much more interesting and thought-provoking. Apart from the last paragraph it is hard to see the wider implications of this study, even though I think they are pretty significant.

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Referee #2:

The manuscript by Kesten et al aims to investigate cellular processes during infection of Arabidopsis by Fusarium with a particular focus on the role of pH and the functions of CC1 and CC2 during the process. The authors describe the development of new reporters to measure pH in different sub-cellular compartments (apoplast, cortex and cytoplasm), proceed to characterize pH changes in these areas, how this affects activity/localization of CESAs; organization of microtubules and show data that the pH changes in the apoplast are induced by a Fusarium-derived elicitors, which seems to lead to activation of AHA via phosphorylation. They then continue by investigating the function of CC1 and CC2 in these events and suggest that loss of cc1cc2 activity causes enhanced resistance to fusarium infection. In this context they perform infection assays using a transgenic fusarium line and quantify cellulose in cc1cc2 plants. In parallel their data suggest that the apoplastic pH levels in cc1cc2 are slightly reduced compared to wildtype in mock conditions and do not drop upon infection like in wildtype.

I think the results regarding the elicitor-induced pH changes in the apoplast are quite exciting, compelling and provide significant novel insights into processes taking place during plant-pathogen interaction, which are of general interest the wider plant science community.
In contrast I think the results regarding the function of cc1cc2 in this context and how direct or indirect the effects observed on Fusarium resistance are not clear. The authors measure cellulose levels (figure 4d) in wildtype and cc1cc2 seedlings and while not actually doing stats on the comparison mock cc1cc2 versus Wildtype the differences are quite pronounced. Similar differences have been reported for other mutants and they affect responses to biotic or abiotic simply because the cell wall is weakened, which has global consequences. One example could be the isoxaben resistant mutant ixr1-1, which grows pretty good under lab conditions, while having reduced cellulose levels. Phytohormone measurements have shown that ixr1-1 seedlings have elevated phytohormone levels under mock conditions Engelsdorf et al, 2018. These results suggested that the ixr1-1 plants are constantly slightly stressed, which will have all kinds of effects, making the interpretation of phenotypes observed difficult. So to me it is not clear if the effects observed with cc1 cc2 are really direct or are caused by changes in the cc1cc2 cell walls, which induce responses that in turn affect pathogen defence. Since, FER implicated in cell wall integrity signalling, has been shown to regulate AHA2 activity I suspect we may see here actually some cell wall integrity effects....

Minor comments:
Calibration of sensors: pHcortical is described in some detail in the main text, this is not the case for pHapo. I think this should be corrected since pHapo seems to exhibit some differences compared to pHcortical.

Sequence of figures: The sequence/organization of the figures should be reflected in the text. Supplementary figure 2 exemplifies this problem.

Figure 3: I cannot find any mock control data for cc1 cc2 with respect to PM CSC density and MT coverage.
If one compares Figures 3G and 3B the complemented cc1cc2 GFPCC1 looks different with respect to PM CSC density compared to Col-0 (looks more like cc1cc2 in figure 3B actually). I assume there may be some experimental variability but this suggests to me that proper Col-0 controls have to be included regularly in each of these figures.

Supplementary figure 3 C, D: Mock controls in the same figure would be really helpful. Comparing with data in figure 1b and g suggests that buffer A has no effect while buffer B has. The wording in the text does not reflect this.

General comment: Not sure if I´m missing something but I cannot find any data on CSC movement and microtubules in mock- treated cc1cc2 seedlings.

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Referee #3:

In this comprehensive study Kesten et al. describe the effects of fungal hyphae and elicitors on the density and speed of CSC and MTs by the use of fluorescence markers, and their impact on root growth. They show that a pH change occurs upon treatment in the apoplast and that this change correlates with the effects on CSC/MTs and growth. The topic is highly relevant in the field of plant-microbe perception and although it is known that pH changes occur in the apolast no direct link to the growth retardation was made before. Thus, the present study has the potential to significantly advance this field. Overall the manuscript is well written and the figures are organized in a logical sequence. The first result section describing the effect of CSC density by the presence of the fungus and the elicitor is solid and impressive (Fig. 1). My major concerns with this work begin with the functional analysis using the double mutants and the measuring of the pH. I find it excellent that they have produced plants with pH sensors linked to the plasma membrane and this is surely a tool that will be used further in the field. I find it interesting that they find an acidification after treatment. This is in contrast with several reports where an increase of pH is reported after treatment with elicitors or microbes. Is this specific to Fusarium? Or could this be due to the different tissues used in the different studies? a better discussion would be required. In this chapter the authors suggest that the depletion of CSC is linked to PM pH changes. Here a possible problem could be the effect of the pH on the GFP fluorescence. It is known that the fluorescence signal of GFP is sensitive to pH changes. Could it be that the observed reduction of CSC density is indeed just a reduced GFP signal at acidic pH (buffer A)? Can the authors exclude this possibility? I think that what the authors see here is a correlation between pH changes and CSC density and growth retardation and I would be carefully in writing that there is a link. I also do not think that by the use of these two buffers they corroborate that a delta pH across PM caused the effects, in fact buffer A, where they see a reduction of the fluorescence, is buffering inside as well as outside so that there no delta pH can be formed anymore. Or what are the authors trying to say here? I still think that this is a good experiment, but I would be more careful in writing this chapter.
In the next chapter the authors started to use a different read out for CSC density, the YFP-CesA6. Is there any specific reason why CesA3 was used in the previous chapter instead of CesA6?
Here my major concern is the missing controls Col-0 and cc1cc2 without treatments. I would expect a difference between the WT and the mutant line to start with. Why are these differences (if any) not taken into consideration or shown statistically? Later the authors show that in the mutant lines there is less cellulose (is this difference significant?). How this fits with the increased CSC density upon fungal treatment in fig3?

Additional points:
There are no numbers for the lines so I cannot refer to them here.
Abstract: proton chemical gradient. Is this correct wording?
Introduction: "internal signals" is strange for a cell wall which is outside
pH at across the PM. At does not belong there
"degrading enzymes depending on the apoplastic pH (pH apo) of their host (Li et al , 2012)" This is not correct and I find it misleading. In that paper the authors have used artificial media buffered at different pHs. There is no link at all to the apoplastic pH and in the apoplast can be many different signals that could overwrite the pH effects/signal. I am concern about the overinterpretation of results by the authors in the introduction and overall in the paper.
Chapter results:
Fig1. Here a representative figure for the WT without treatment should be shown
Fig3. Here the control Col-0 and cc mutant without the fungus are missing. This is an important control.
Fig4b. control without fungus is missing. Also an important control.
Fig4d. the authors do not explain why there is less cellulose in the cc and what this means.
Supplementary figure 2d. Were these pictures taken under the same settings? The FM4:64 is also looking different between the two roots, which should not be the case.
Is cortical the correct description for the PM localization here? Corticalplasma?
Figure 5g and h does not exist

Referee #4:

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In this manuscript, the authors have used novel pH sensors to show that the fungus Fusarium oxysporum induces changes in the apoplastic and cortical pH, accompanying induced changes in root growth. They have shown that this response is accompanied by changes in the abundance of Cellulose Synthase A (CesA) foci at the plasmamembrane, depolymerization of cortical microtubules, and a change in the phosphorylation status of proton-pumps (AHAs). Proteins that regulate cellulose synthesis and microtubule dynamics, such as CC proteins, seemed involved as double mutants in CC1 and CC2 showed not the obvious pH response after elicitor treatment. In addition, the mutant constitutively exhibits an elevated pH-difference across the plasmamembrane.

This nice study is written very well and describes much data (both as regular and supplemental data). I have, however, some critical remarks.

Major remarks:

Figure 1:
- This figure shows only the marker-lines that are treated, why are the control images shown in the suppl data? I feel they should be included in the actual figure to allow visual comparison.
- Only very few cells seem analyzed. For the microtubules n is close to 8 from 8 roots. Does that mean that only one cell could be imaged? If the response to the fungus is general, naively one would say that many more to all cells close to the hyphae should show the exact same response.
- The effect of the fungus/elicitor is a clear reduction of root growth, but it is unclear whether this is caused by reduced rates of cell division, cell elongation or both. This should be included in the analysis.
- The difference in PM CSC density after elicitor-mix treatment seems very small, whereas one would that this treatment is more effective than the actual hyphae. Can this be explained?
- In addition, the response to the hyphae and the elicitor-mix on root growth shows a different behavior, although the trend of being slightly inhibiting holds. Can this difference be explained/discussed?

Figure 2:
- Panel d and g show clear effects on the output of the novel pH sensor by elicitors or hyphae, but could the root be overlaid on the fluorescence picture? It is difficult to judge where the effect is clearest on this representation. In the same line, it is mentioned that the average pH is 5.30, but is there a difference at the beginning and end of the elongation zone? There were measurements of proton-fluxes around the root that show this could be the case (Staal et al., 2011 Plant Physiol).
- Supplemental figure 2e/g shows the calibration of the pH sensor, but the values of the X-axis are in log-scale. Please use normal scale to better understand the response of the sensor to changing pH values.
- Fig. 2e and f show that upon acidification of the apoplast, the cortical side shows a 1-min lag before it acidifies too. How do the authors explain that the cortical pH returns to normal whereas the apoplast stays acidified?

Figure 5:
- The text mentions panels 5g and h, but I seem to miss them in the current version of the manuscript.

Discussion:
- How do the authors link the absence of CC1 and CC2 to elevated proton pump activity? Could it be that a problem with the CCs affect cellulose synthesis, which changes the cell wall integrity and thus is sensed by the cell wall integrity sensors, feeding back into the cytoplasm and potentially acting through kinases on the proton pumps?
- The pH sensor has a linear output from pH 5.5-8. Does this mean the sensor is nut useful for examining processes where the cell wall is acidified? In addition, one can expect that the sensor, close to the PM and therefore potentially next to an active proton-pump, encounters a very acidic environment. Could this be discussed?

1st Revision - authors' response 26th Jul 2019

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Editor's' comments:

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2) Please add the necessary controls as requested by reviewers #2, #3 and #4

3) Address the questions on the Fusarium-induced apoplast acidification and the properties of the pH sensor raised by reviewers #3 and #4

We thank the reviewers for many useful comments and suggestions that we believe have improved the quality of the manuscript. Below, we address their concerns point by point. We have also added a bibliography section of all the works cited in the responses to the reviewers.

Referee #1:

Pathogen-induced pH changes regulate the growth-defense balance of plants

Kesten et al.

This is a potentially significant paper, which seeks to explain the mechanism of cell wall-related sensing in plants in response to biotic stimuli, such as pathogen attack. It is well known that pathogens can induce acidification of the apoplast of plants and that this is linked to host defence. However, the precise mechanism of this acidification, and the role of plant cell wall re-modeling, is unclear. This study provides evidence that apoplastic acidification via proton pump activation leads to acidification of the cortical side of the plasma membrane in response to infection by the filamentous fungal pathogen Fusarium oxysporum. This appears to lead to a reduction in cellulose synthesis and cell growth. The paper also provides evidence for Companion of Cellulose Synthase (CC) proteins as key regulators of the balance between plant growth and plant defense processes. There are potentially important consequences of this study for understanding the interplay between pH control (by both plant and pathogen) and the trade-offs and balance between plant immunity and growth.

In general, I am supportive of publication of this paper in EMBO Journal. The study is carefully executed and the work is novel in the uses of new pH sensors that are superior to previously reported sensors allowing spatial localisation of pH changes that

are necessary to implicate processes acting at the plasma membrane and affecting cell wall re-modelling. The paper also attempts to use quantitative data to support the microscopy observations, which is often missing from such studies. There is, as a consequence, a better sense of the reproducibility of the findings and the level of variation observed.

Response: We thank the reviewer for the comments and for highlighting the value of microscopy data quantification, which we agree is essential to make conclusions.

My main concern about the paper in its current form is that it is just so hard to follow. There is an excessive use of abbreviations that sometimes makes the sense of sentences really difficult to understand. This is made worse by the fact that the Figure Legends are very long and also not always clear. I think the legend to Figure 1, for example, could be much shorter and clearer in stating the main observations.

Response: We agree with the reviewer and removed the following abbreviations: MT (microtubules), PM (plasma membrane), CW (cell wall), Fo (*Fusarium Oxysporum*). Additionally, we tried to shorten the figure legends as much as possible while keeping all relevant information (e.g. number of replicates, statistics, etc.).

My only query regarding the data is the extent of the role of CC in the rate of vascular penetration by Fusarium. This is shown in Figure 4, but not well explained by the rather inpenetrable description on page 10. I really would like to know more about how the experiment was carried out leading to the graph shown in Figure 4 C. There is a clear effect in the cc1cc2 line on root growth, but the effect on rates of pathogen vascular penetration are actually very small, given the scale on the graph.

Response*: We agree on the need of describing a new method in detail, but we aim to not overcomplicate the text. Therefore, we described the experiment in the methods section under "Plate infection assay", which you can also find here:

"1x10 cm Whatman paper strips were heat sterilized, and two strips placed on 12x12 cm square plate containing 50 ml solid half MS media. 6-10 sterilized and stratified *A. thaliana* seeds were placed on each strip and grown under the conditions indicated in the "Plant material and growth" section. After 8 days, the paper strip was transferred to a mock or infection plate. The infection plate was generated by equally spreading 100 µl of a Fo5176 or Fo5176 pSIX1::GFP spore suspension containing 10^7 spores/ml in water on a 12x12 cm square plate containing 50 ml solid half MS media. Each plate was scanned daily to assess root growth. Root length was measured with Fiji with a macro employing the above described method on whole plates (see "Root growth analysis"). Fo5176 pSIX1::GFP penetration of the root vasculature was assessed with a Leica M205 FCA fluorescent stereo microscope, equipped with a long pass GFP filter (ET GFP LP; Excitation nm: ET480/40x; Emission nm: ET510 LP). Vascular penetration/infection was counted when clear GFP signal was observed. In brief,

we scanned each plate manually with the stereo microscope for GFP signal that showed a clear, linear, root-central pattern (see Fig. 4a), which is typical for the penetration of the xylem. Then, the number of xylem penetrations per root was calculated by dividing the total, cumulative number of penetrations per day by the total number of plants."

When looking at the root with the maximum amount of xylem penetrations at 7 days post transfer to Fo5176 pSIX1::GFP containing plates, we can also clearly see the difference between WT and *cc1cc2*: WT maximum xylem penetration events = 25, *cc1cc2* maximum xylem penetration events = 8. Additionally, the statistics are repeated measures ANOVAs, since we do not only count all xylem penetrations per plate but we can trace back each individual xylem penetration per plant over time.

In addition, we have included *SIX1* expression data in Fo5176 (WT) infected roots (Fig. 4f and g) that shows significantly more expression in WT infected roots in comparison to *cc1cc2*.

(...) and the scale bars are obscured by the background bar chart.

Response: We agree and changed the color of the error bars to the color of the corresponding line (green).

The number of observations is not clear (is it 103 from 3 biological reps?)

Response: We monitored at least 103 individual plants per genotype. For each of them, the root growth and Fo5176 vascular penetrations were counted from 3 to 7 and after 12 days post transfer to the plate with spores. These ≥ 103 plants were distributed over a multitude of plates. On each plate were 10-12 plants. These plates were distributed over the 3 independent experiments (i.e. different date of sowing, and transfer of plants to Fo5176 spore containing plates and individual fungal spore suspensions from different fungal cultures). The raw data can be found in the corresponding source data file.

(...) and although the ANOVA says the results are significant, I remain sceptical, given such small differences.

Response: The raw data are accessible in the source data file. Bear in mind that we used repeated measures ANOVAs for analysis. This results in very robust statistical analysis.

I think that other readers will also be sceptical and therefore the experiment needs explaining in greater detail and I would really like to see an alternative method used to verify these observations, which are quite critical to the conclusions of the paper. There must be a biological read-out in terms of disease symptoms or fungal biomass that could also be used to test this idea more thoroughly.

Response: The reduction of root growth rate when the plants are exposed to Fo5176 (vs mock) is a consequence of the fungal infection process and

therefore constitutes a disease symptom. Our measurements show a clear inhibition of root growth for both WT and *cc1cc2* plants in response to the fungus (Fig 4e and Supplementary Fig. 4a), indicating that the microbe infects the plants and the assay indeed works properly. Additionally, we now have quantified the expression of the *SIX1* gene in roots of plants that have been infected with Fo5176 (WT). In agreement with our results using the pSIX1::GFP expressing Fo5176 strain, we observed a significant upregulation of *SIX1* in WT plants in comparison to *cc1cc2* plants, both relative to a plant and a Fo5176 referencegene (*AtGAPDH* and *F*oβTU*B, respectively;* Fig. 4f and g). This confirms that Fo5176 is less virulent in *cc1cc2* than in WT roots..

We also agree with the reviewer that the fungal biomass inside the plant was not directly addressed and, therefore, followed two complementary methods to quantify it. Using HPLC analyses, we measured the amount of N-acetylglucosamine within infected roots, which is proportional to the amount of fungal cell wall, as N-acetylglucosamine is derived from chitin (Supplementary Fig. 4b and c). To assess the amount of alive fungus inside infected roots, we surface sterilized them, ground and plated the material and subsequently counted developing fungal colonies (Supplementary Fig. 5d). Both analyses showed no differences between total fungal biomass in WT and *cc1cc2* roots, indicating that the *cc1cc2* mutation confers resistance to Fo5176 vascular penetration, but not to the colonization of other root cell layers. The fungal growth in *cc1cc2* resembles that of non-pathogenic endophytic *F. oxysporum* strains, which colonize root cell layers, but cannot reach the xylem (Brader et al., 2017). We directly incorporated these results and discussed them in the new version of the manuscript.

I am also concerned about the autofluorescence observed in Figure 4A. Why is this so high? A higher resolution image of xylem invasion by Fusarium would be useful here, especially if this could be an example of the observations used to provide the data for Figure 4C. This would help interpretation of the experiment as described above.

Response: The autofluorescence only stems from the used filter of the microscope, which is a long range GFP filter as described in the methods section. Using this filter makes the assessment of the assay easier since it clearly separates real signal (in green) from the background (in red) signal of the plant when scanning the plates for vascular penetration. Basically, this filter allows us to still see the outline of plant roots without the need to switch to the brightfield channel and therefore makes assessment of a clear, root central and linear GFP pattern (i.e. vascular colonization) more easy. We of course also have images with a narrow range GFP filter that excludes almost all background signal. We have now exchanged Fig. 4a with such an image and a zoom in of an event of vascular penetration and overlaid it with a brightfield image. In this new figure, one can clearly identify the vasculature of the root and see how far the vascular colonization already progressed.

In terms of clarity, I am also a little disappointed by the Discussion as I had hoped this would be more in depth in terms of the implications of the observations and how these relate to our knowledge of pH manipulation and adaptation by pathogens (with the recent work of Di Pietro more fully described), coupled with a wider analysis of the balance between growth and immunity revealed by the results here. This would make it much more interesting and thought-provoking. Apart from the last paragraph it is hard to see the wider implications of this study, even though I think they are pretty significant.

Response: We thank the reviewer for recognizing our study as so significant for the community. Following the reviewer's suggestions, we have now rewritten the discussion and added a deeper analysis of our data in context with published information and their implications. We also added various hypothesis that we find relevant to be considered by the plant community.

Referee #2:

The manuscript by Kesten et al aims to investigate cellular processes during infection of Arabidopsis by Fusarium with a particular focus on the role of pH and the functions of CC1 and CC2 during the process. The authors describe the development of new reporters to measure pH in different sub-cellular compartments (apoplast, cortex and cytoplasm), proceed to characterize pH changes in these areas, how this affects activity/localization of CESAs; organization of microtubules and show data that the pH changes in the apoplast are induced by a Fusarium-derived elicitors, which seems to lead to activation of AHA via phosphorylation. They then continue by investigating the function of CC1 and CC2 in these events and suggest that loss of cc1cc2 activity causes enhanced resistance to fusarium infection. In this context they perform infection assays using a transgenic fusarium line and quantify cellulose in cc1cc2 plants. In parallel their data suggest that the apoplastic pH levels in cc1cc2 are slightly reduced compared to wildtype in mock conditions and do not drop upon infection like in wildtype.

I think the results regarding the elicitor-induced pH changes in the apoplast are quite exciting, compelling and provide significant novel insights into processes taking place during plant-pathogen interaction, which are of general interest the wider plant science community.

In contrast I think the results regarding the function of cc1cc2 in this context and how direct or indirect the effects observed on Fusarium resistance are not clear. The authors measure cellulose levels (figure 4d) in wildtype and cc1cc2 seedlings and while not actually doing stats on the comparison mock cc1cc2 versus Wildtype the differences are quite pronounced. Similar differences have been reported for other mutants and they affect responses to biotic or abiotic simply because the cell wall is weakened, which has global consequences. One example could be the isoxaben resistant mutant ixr1-1, which grows pretty good under lab conditions, while having reduced cellulose levels. Phytohormone measurements have shown that ixr1-1 seedlings have elevated phytohormone levels under mock conditions Engelsdorf et al, 2018. These results suggested that the ixr1-1 plants are constantly slightly stressed, which will have all kinds of effects, making the interpretation of phenotypes observed difficult. So to me it is not clear if the effects observed with cc1 cc2 are really direct or are caused by changes in the cc1cc2 cell walls, which induce responses that in turn affect pathogen defence. Since, FER implicated in cell wall integrity signalling, has been shown to regulate AHA2 activity I suspect we may see here actually some cell wall integrity effects....

Response: We thank the reviewer for valuing our method to measure dynamic pH changes across the plasma membrane.

Indeed, the cellulose content of *cc1cc2* roots under mock conditions is significantly lower than in WT (Fig. 4i). We agree that the function of CCs in the plant response to Fo5176, the potential influence of *cc1cc2* cellulose deficiency

on its AHA hyperactivation and its resistance to the fungus needs more clarification. We addressed this with the following experiments:

a) To determine if deficiencies in cellulose per se lead to AHA hyperactivation, we measured the acidification process of an alkaline growth media by two other cellulose deficient mutants: *prc1-1*, impaired in one of the CSC subunits (Fagard et al., 2000) and *pom2-4,* affected in the cytosolic protein linking the CSC to microtubules (Bringmann et al., 2012). None of them acidified the media faster than WT plants (see new Supplementary Fig. S5a). The *prc1-1* mutant showed no difference to WT while *pom2-4* acidified the media slower than the other genotypes. Therefore, we can conclude that general cellulose deficiency does not have a direct effect on AHA activity, as each cellulose deficient mutant (*cc1c2*, *prc1-1* and *pom2-4*) acidified the media in a different way. The reason for AHA upregulation in *cc1cc2,* and also in the *fer* mutant, should be further analyzed. Our new data points towards an opposite situation to that suggested by the reviewer, in which the pH directly influences cellulose content and/or structure, as the amount of glucose in the crystalline fraction of *cc1cc2* cell walls was similar to WT levels when buffering the media with MES (see response to reviewer 3 on page 14 and new Supplementary Fig. 4g). Furthermore, growing *cc1cc2* on buffered media inoculated with Fo5176 did not further improve root growth, cellulose levels or vaculare penetration rates, as the pH at the plasma membrane is already non-responsive to Fo5176 in this genotype (Fig. 5a and b; new Supplementary Fig. 4e-h). The total amount of Fo5176 in WT and *cc1cc2* roots grown on buffered media is not significantly different (Supplementary Fig. 4i). Moreover, the cellulose reduction upon fungal infection in WT plants was not altered by buffering the media and is similar to that observed in *cc1cc2* plants grown on MES, despite a reduction in vascular colonization rate in WT roots under these conditions (Supplementary Fig. 4e-i). This observation clearly highlights that pH regulation rather than cellulose-based effects cause the *cc1cc2* resistance to Fo5176 vascular colonization.

b) To address the possibility of a defence priming state in *cc1cc2* mutants, we quantified the transcript levels of the immune response marker genes *At1g51890*, *WRKY45* and *WRKY53* (Roux et al, 2011; Masachis et al, 2016; Souza et al, 2017; Wang et al, 2018). The constitutive upregulation of *WRKY45* and *WRKY53* in *cc1cc2* mutant (see new Fig. 4h) might, at least partially, explain its resistance phenotype to Fo5176. Additional research is needed to determine if these transcriptional changes are a consequence of cellulose deficiency or AHA hyperactivation.

We have included the new figures and commented on them in the discussion of the revised version. In summary, our data indicate that the pH at the plasma membrane influences both the cellulose amount and plant defence to *F. oxysporum* through independent pathways.

Minor comments:

Calibration of sensors: pH cortical is described in some detail in the main text, this is not the case for pHapo. I think this should be corrected since pHapo seems to exhibit some differences compared to pHcortical.

Response: We added the following sentence into the main text: "*In vivo* calibration revealed a sigmoidal correlation between pH and the EGFP/RFP ratio of the sensor with a linear range between pH 5.2 to 6.8 (Supplementary Fig. 2b)." The whole process is also described in detail in the methods section under the paragraph "Live cell ratiometric pH sensor imaging including flat and dark field correction and data processing".

Sequence of figures: The sequence/organization of the figures should be reflected in the text. Supplementary figure 2 exemplifies this problem.

Response: We carefully checked the manuscript and think that the order of the figures was in chronological sequence and also received no comments by the other reviewers. We are happy to change the order if a concrete example is given.

Figure 3: I cannot find any mock control data for cc1 cc2 with respect to PM CSC density and MT coverage.

Response: Our reasoning behind not showing these controls in the first manuscript version was that they were published before showing no difference in CesA and microtubule behaviour (Endler et al., 2015). However, we agree that the previous measurements were done in dark grown hypocotyl cells and that a repetition of these controls therefore is necessary for the current manuscript. Thus, we now included them as Fig. 3a-d and Supplementary Fig. 3a. WT and *cc1cc2* root cells show no difference in regards to CSC/CesA speed and density nor in microtubule density, confirming the published data on etiolated hypocotyl cells.

If one compares Figures 3G and 3B the complemented cc1cc2 GFPCC1 looks different with respect to PM CSC density compared to Col-0 (looks more like cc1cc2 in figure 3B actually). I assume there may be some experimental variability but this suggests to me that proper Col-0 controls have to be included regularly in each of these figures.

Response: We agree with the reviewer that the non-treated (not only Col-0) controls need to be included in the mentioned figures (please also refer to the other answers to the same comment). We now added these in all figures and the controls are consistent across different experiments throughout the study. Indeed when compared to controls, the truncated version of CC1, missing the N-terminal, microtubule interaction domain, fully restores CSC density at the plasma membrane while the full *cc1cc2* knockout only partially recovers CSC density. This is in agreement with previous reports, which showed that the N-terminal truncation can restore plant growth in response to cellulose synthesis inhibiting drugs (Endler et al., 2015).

Supplementary figure 3 C, D: Mock controls in the same figure would be really helpful. Comparing with data in figure 1b and g suggests that buffer A has no effect while buffer B has. The wording in the text does not reflect this.

Response: While working in the new version of the manuscript, we realized that the experiments with these buffers do not add relevant information to the current work. On the contrary, the experiment adds complexity to the text and might distract and/or confuse the readers. We therefore have removed this figure and believe that the experiments with MES showing a recovered CSC-microtubule pattern and root growth upon fungal contact (Fig. 2a-c, e and f; Supplementary Fig. 1c and d; Supplementary Fig. 2f and g) support the proposed role of the pH gradient across the plasma membrane in regulation of cellulose synthesis and root growth (also refer to the answer above and responses to the other reviewers describing the long term effects of buffering the media).

General comment: Not sure if I´m missing something but I cannot find any data on CSC movement and microtubules in mock- treated cc1cc2 seedlings.

Response: As indicated in our response to a previous comment ("Figure 3: I cannot find any mock control data for *cc1cc2* with respect to PM CSC density and MT coverage"), we added these to the new version of the manuscript (Fig.3a-d, 3f-i and Supplementary Fig. 3), confirming the previously reported lack of differences between the genotypes under mock conditions (Endler et al., 2015).

Referee #3:

In this comprehensive study Kesten et al. describe the effects of fungal hyphae and elicitors on the density and speed of CSC and MTs by the use of fluorescence markers, and their impact on root growth. They show that a pH change occurs upon treatment in the apoplast and that this change correlates with the effects on CSC/MTs and growth. The topic is highly relevant in the field of plant-microbe perception and although it is known that pH changes occur in the apolast no direct link to the growth retardation was made before. Thus, the present study has the potential to significantly advance this field. Overall the manuscript is well written and the figures are organized in a logical sequence. The first result section describing the effect of CSC density by the presence of the fungus and the elicitor is solid and impressive (Fig. 1). My major concerns with this work begin with the functional analysis using the double mutants and the measuring of the pH. I find it excellent that they have produced plants with pH sensors linked to the plasma membrane and this is surely a tool that will be used further in the field. I find it interesting that they find an acidification after treatment.

This is in contrast with several reports where an increase of pH is reported after treatment with elicitors or microbes. Is this specific to Fusarium? Or could this be due to the different tissues used in the different studies? a better discussion would be required.

Response: We were also surprised by the data, as they challenge the paradigm of the plant-microbe field about rapid host apoplastic alkalinization in response to MAMPs or microbes. Thus, we reinforced the apoplastic pH data with AHA phosphorylation studies in response to live fungus (Fig. 2j-k and Fig. 5d-g) and have included the response of our apoplastic pH sensor to chitin. As shown in the new figure (Supplementary Fig. 2h), we could measure a chitin-induced rapid alkalinization of the apoplast in roots, as previously reported in leaves (Felle et al., 2009). We therefore believe that our data helps to broaden the paradigm of "apoplastic alkalinization" in response to microbes, which should rather be referred to as "apoplastic pH alterations". Indeed, root surface acidification was previously reported in response to the mycorrhizal fungus Piriformospora indica (Felle et al., 2009).

In addition, one should consider that many studies actually measured the pH of the growth media many hours after microbe inoculation and not directly the pH of the apoplast shortly after microbe/elicitors contact, as we do in this work. Indeed, we also observed an alkalinization of the growth media in response to Fo5176 in a timeframe of 1 day after inoculation (Supplementary Fig. 5g). This alkalinization was significantly slowed down in c*c1cc2* plants, as a result of their more acidic apoplast. In conclusion, it might be beneficial to re-investigate plant responses to microbes/elicitors with higher cellular and time resolution, with new tools at hand.

In this chapter the authors suggest that the depletion of CSC is linked to PM pH changes. Here a possible problem could be the effect of the pH on the GFP

fluorescence. It is known that the fluorescence signal of GFP is sensitive to pH changes. Could it be that the observed reduction of CSC density is indeed just a reduced GFP signal at acidic pH (buffer A)? Can the authors exclude this possibility?

Response: We agree that GFP is indeed pH sensitive and therefore use it as a pH sensor. However, we do not detect changes of CSC density in the *cc1cc2* mutant lines that already exhibit a more acidic apoplastic and cortical pH under mock conditions (Fig. 3a-d; Fig. 5a and b). Additionally, all the CSC marker lines used in the study (GFP-CesA3, YFP-CesA6, GFP-CC1) are tagged at their cytosolic N-terminus. Thus, the pH, regardless of treatment, is in the range of 6.0-7.5 where fluorescence of the GFP/YFP is reliably detectable with a spinning disk microscope equipped with an EMCCD camera.

I think that what the authors see here is a correlation between pH changes and CSC density and growth retardation and I would be carefully in writing that there is a link. I also do not think that by the use of these two buffers they corroborate that a delta pH across PM caused the effects, in fact buffer A, where they see a reduction of the fluorescence, is buffering inside as well as outside so that there no delta pH can be formed anymore. Or what are the authors trying to say here? I still think that this is a good experiment, but I would be more careful in writing this chapter.

Response: We agree with the reviewer about the data generated with the buffer treatments and have removed the figure (see answer to referee 2 on page 9). We also agree with the reviewer in the lack of connection between fast changes in CSC density and growth retardation upon fungal/elicitors contact. We find it unlikely that the fast growth inhibition upon pH changes is a consequence of the reduction of CSC density and speed, as the cellulose content at the cell wall does not change so fast. Our data indicate that the pH changes are the actual cause for both CSC response and growth retardation shortly after fungal contact, as MES blocks both responses (Fig. 2a-f; Supplementary Fig. 1c-d; Supplementary Fig. 2f and g).

In the long-term, the apoplastic pH does influence cellulose content (quantified as glucose contained in the crystalline fraction of the cell wall). See new Fig. 4i and Supplementary Fig. 4g and comments below to your question about old Fig 4d (now 4i; on page 14).

In the next chapter the authors started to use a different read out for CSC density, the YFP-CesA6. Is there any specific reason why CesA3 was used in the previous chapter instead of CesA6?

Response: We used a different marker line to image available lines in *cc1cc2* background that already have been described before (Endler et al., 2015). We always included the corresponding control in WT background. The dynamics of GFP-CesA3 and YFP-CesA6 were shown to be the same (compare Col-0 mock in Fig.1b and c, Supplementary Fig.1a and b, Fig. 3c and d, and Supplementary

Fig. 3a) as both are part of the same complex (Paredez et al., 2006; Desprez et al., 2007).

Here my major concern is the missing controls Col-0 and cc1cc2 without treatments. I would expect a difference between the WT and the mutant line to start with. Why are these differences (if any) not taken into consideration or shown statistically?

Response: Following also the advice of the reviewer 2, we have included the mock controls in all the new figures. We observed no difference in CSC nor microtubule behaviour between WT and *cc1cc2* under mock conditions, as described earlier (Endler et al., 2015, Kesten et al., 2019). Please also refer to our answers to your question about decreased cellulose levels in *cc1cc2* under mock conditions that we could fully restore by buffering the media (page 14).

Later the authors show that in the mutant lines there is less cellulose (is this difference significant?).

Response: Yes, the *cc1cc2* mutant has significantly less cellulose (glucose in the crystalline fraction of the cell wall) than WT when grown in non-buffered media. We have included the statistics into the new figure (Fig. 4i). Please refer to the comments below regarding Fig 4d (now 4i; page 14) for further discussions on the topic of *cc1cc2* cellulose content.

How this fits with the increased CSC density upon fungal treatment in fig3?

Response: There is no increased CSC density upon fungal treatment in *cc1c2*, but less reduction than in WT0 compared to their respective mocks. We hope that by including the mock data in the same figure, this point is more clear (Fig. 3c).

Additional points:

There are no numbers for the lines so I cannot refer to them here.

Response: This is due to the upload as a pdf through bioRxiv. We are sorry if this caused any inconvenience.

Abstract: proton chemical gradient. Is this correct wording?

Response: The expression "proton chemical gradient" is frequently used to describe ∆pH across the plasma membrane (Angel et al., 1981; Michelet et al 1995, and Haruta et al., 2012).

Introduction: "internal signals" is strange for a cell wall which is outside

Response: We agree and deleted internal signals.

pH at across the PM. At does not belong there

Response: We thank the reviewer for pointing out this mistake, which we have corrected.

"degrading enzymes depending on the apoplastic pH (pH apo) of their host (Li et al , 2012)" This is not correct and I find it misleading. In that paper the authors have used

artificial media buffered at different pHs. There is no link at all to the apoplastic pH and in the apoplast can be many different signals that could overwrite the pH effects/signal.

Response: We agree that the mentioned publication does not specifically address apoplastic pH and therefore removed "apoplastic". The sentence now reads:

"Plant microbes were shown to secrete various cell wall degrading enzymes depending on the ambient pH (Li *et al, 2012).*"

Li *et al* clearly show that the secretome of microbes is dependent on pH (even though by using artificial culture conditions). The publication established this on a rather broad, tissue specific range (e.g. whole root, whole leaves, fruits). We address the dependence of microbes on pH of their host on a much more detailed, cellular range by measuring the pH of Arabidopsis epidermis cells in the apoplast, cortex and cytosol individually.

I am concern about the overinterpretation of results by the authors in the introduction and overall in the paper.

Response: We actively aimed to avoid overinterpretation and to distinguish between direct conclusions from our and published data and hypotheses arising from our results. If the reviewer considers that this was not clear enough, we would be happy to rewrite any indicated sentence that can be read as an overinterpretation.

Chapter results:

Fig1. Here a representative figure for the WT without treatment should be shown

Response: We agree (please also see the questions of the other reviewers) and have modified the figure accordingly.

Fig3. Here the control Col-0 and cc mutant without the fungus are missing. This is an important control.

Response: We agree (please also see the questions of the other reviewers) and added the controls to Figure 3.

Fig4b. control without fungus is missing. Also an important control.

Response: The control was shown in Supplementary Fig. 5a and b in the original manuscript. As with all the other figures, we now moved the control to the corresponding main figure (Fig. 4) to make the structure of the manuscript more clear.

Fig4d. the authors do not explain why there is less cellulose in the cc and what this means.

Response: Our data show that, when grown on plates without sucrose and MES, *cc1cc2* roots contain less cellulose (measured as glucose contained in the crystalline fraction of the cell wall) than WT. This deficiency was restored by adding MES to the media (see new Supplementary Fig. 4g), confirming previous

data of *cc1cc2* etiolated hypocotyls that showed no differences in cellulose content in comparison to WT (again, quantified as glucose contained in the crystalline fraction of the cell wall) when grown on media supplemented with sucrose and MES (Endler et al., 2015; Kesten et al., 2019). These data indicate that the pH at the plasma membrane influences the structure of cellulose. We hypothesize that the glucan chain assembly into crystalline structures is pH dependent, as the polysaccharide interactions and proteins activity at the apoplast are also dependent on pH. This idea should be supported with cellulose structural analysis by X-ray in the future. Our theory would explain why CSC density and speed is not affected in *cc1cc2*, while the mutant has less crystalline cellulose than WT plants in media without MES (Figure 3a-d; Fig. 4i and Supplementary Fig. 3a, 4g and h).

In addition, we might have to reconsider the current hypothesis in the field of cellulose synthesis that links speed of the CSCs at the plasma membrane proportionally to their catalytic activity; i.e. their ability to incorporate glucose into glucan chains. The amount of cellulose (in its paracrystalline structure) is therefore also supposed to be proportional to the density and speed of CSCs at the plasma membrane. Following this theory, we expected to detect a reduction of CSC density and/or speed in *cc1cc2* cells without MES, which would explain the lower cellulose content of the mutant under these growth conditions. The increased plasma membrane ΔpH of *cc1cc2* might have an impact on the plasma membrane ionic state, CSC enzymatic activity and shape as well as on the charge state of its substrate UDP-glucose, which in consequence might alter the activity of the complex (Colombani et al, 2004; Cho et al, 2017). In addition, the upregulated ΔpH might influence the speed of the CSCs by changing the plasma membrane lipid composition. Therefore, further research is required to fully understand the mechanism of cellulose synthesis and assembly and to elucidate the influence of apoplastic ionic state on cell wall architecture.

In any case, the *cc1cc2* cellulose deficiency does not seem to be the reason for its resistance to Fo5176 infection (Supplementary Fig. 4e-i).

We have included these new data and discussion into the new version of the manuscript.

Supplementary figure 2d. Were these pictures taken under the same settings? The FM4:64 is also looking different between the two roots, which should not be the case.

Is cortical the correct description for the PM localization here? Corticalplasma?

Response: Expression of apo-pHusion (Gjetting et al., 2012) is driven by the 35S promoter from CaMV while expression of SYP122-pHusion is driven by UBQ10 promoter from Arabidopsis. Consequently, these two lines cannot be imaged side by side with identical microscope settings, as more laser power is needed to get detectable signal for SYP122-pHusion. However, except for the excitation power of the lasers, imaging settings for Fig. S2d have been identical (please

see used settings below). No FM 4-64 staining was used for Fig. S2d, the red signal represents the pH independent-mRFP-moiety of the pH sensor pHusion.

Image settings	**Apo-pHusion**	**SYP122-pHusion**
Resolution	**1024 x 1024 pixel**	**1024 x 1024 pixel**
Pinhole	**1 AU**	**1 AU**
488 nm laser	**16 %**	**35 %**
561 nm laser	**15 %**	**20 %**
HyD gain GFP	**260**	**260**
HyD gain RFP	**243**	**243**

Figure 5g and h does not exist

Response: We apologize for the figure numbering error. The referenced figures should actually be Figure S6f and g in the original manuscript version. We now corrected this.

Referee #4:

In this manuscript, the authors have used novel pH sensors to show that the fungus Fusarium oxysporum induces changes in the apoplastic and cortical pH, accompanying induced changes in root growth. They have shown that this response is accompanied by changes in the abundance of Cellulose Synthase A (CesA) foci at the plasmamembrane, depolymerization of cortical microtubules, and a change in the phosphorylation status of proton-pumps (AHAs). Proteins that regulate cellulose synthesis and microtubule dynamics, such as CC proteins, seemed involved as double mutants in CC1 and CC2 showed not the obvious pH response after elicitor treatment. In addition, the mutant constitutively exhibits an elevated pH-difference across the plasmamembrane.

This nice study is written very well and describes much data (both as regular and supplemental data). I have, however, some critical remarks.

Major remarks:

Figure 1:

- This figure shows only the marker-lines that are treated, why are the control images shown in the suppl data? I feel they should be included in the actual figure to allow visual comparison.

Response: We agree with this and other reviewers who asked for the controls. We have included them in the main figure. We had to remove the single frames of the image series for this but think that the time averages are more informative for the following analysis.

- Only very few cells seem analyzed. For the microtubules n is close to 8 from 8 roots. Does that mean that only one cell could be imaged? If the response to the fungus is general, naively one would say that many more to all cells close to the hyphae should show the exact same response.

Response: We made a mistake in the figure legends of the CSC and microtubule density measurements and are thankful that the reviewer questioned these figure legends. The macro analyzing these image series is already producing an average density per analyzed image, which we took to define the amount of cells analyzed. This is wrong, as an image usually contains more than one cell. We now counted the analyzed cells of each individual image and reported these in the figure legends. In the given example of the reviewer (Fig. 1c), the actual N $\geq$ 20 cells from 8 roots and 3 independent experiments.

For statistical analysis, the acquired density averages of each image (and therefore cells) are then further averaged per corresponding seedling (please have a look at the source data). The actual mock treatment is the same for all shown experiments depicting microtubule or CSC density/speed (regardless of used marker line, i.e. GFP-CesA3, YFP-CesA6, GFP-CC1, or hyphae/elicitor treatments, which do not significantly differ between experiments. This means

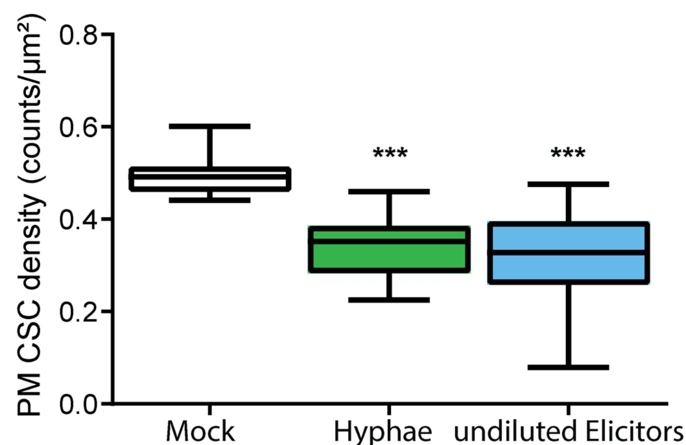
that the actual N for the mock treatments in the whole study is substantially higher and from more than 3 independent experiments.

- The effect of the fungus/elicitor is a clear reduction of root growth, but it is unclear whether this is caused by reduced rates of cell division, cell elongation or both. This should be included in the analysis.

Response: We agree with the reviewer that we can of course not precisely separate actual cell growth and cell division. However, the timeframe of our experiments is 30 min. Multiple studies of cell division in the root meristem showed that cells in this tissue separate in a timeframe of 10-40 h (e.g. Campilho et al., 2006; Rhani et al., 2019) We can therefore almost exclude that our measured root growth data is skewed by actual cell division.

- The difference in PM CSC density after elicitor-mix treatment seems very small, whereas one would that this treatment is more effective than the actual hyphae. Can this be explained?

Response: The response to the elicitor mix is concentration dependent whereas we only imaged cells in direct contact with hyphae. Since we are still unsure about which component of the elicitor mix induces the observed response, we cannot actually determine the concentration of this molecule. The elicitor mix used throughout the manuscript is diluted in a factor of 1:3.66 as this was the final dilution for the ratiometric pH imaging (as indicated in the methods section). The undiluted elicitor mix induces the same, if not an even more severe response, on root cells in comparison to hyphae treatment (please see graph below). We are happy to include this graph and a corresponding image in the final version of the manuscript if the reviewers and editor consider that necessary.



CSC density at the plasma membrane of roots treated with undiluted elicitors (blue). Mock and hyphae treatment was replotted as in the manuscript as comparison. * indicates $p < 0.001$ in comparison to mock treatment. Hyphae and undiluted elicitors treatment is not significantly different ($p = 0.51$). Welch's unpaired t-test.**

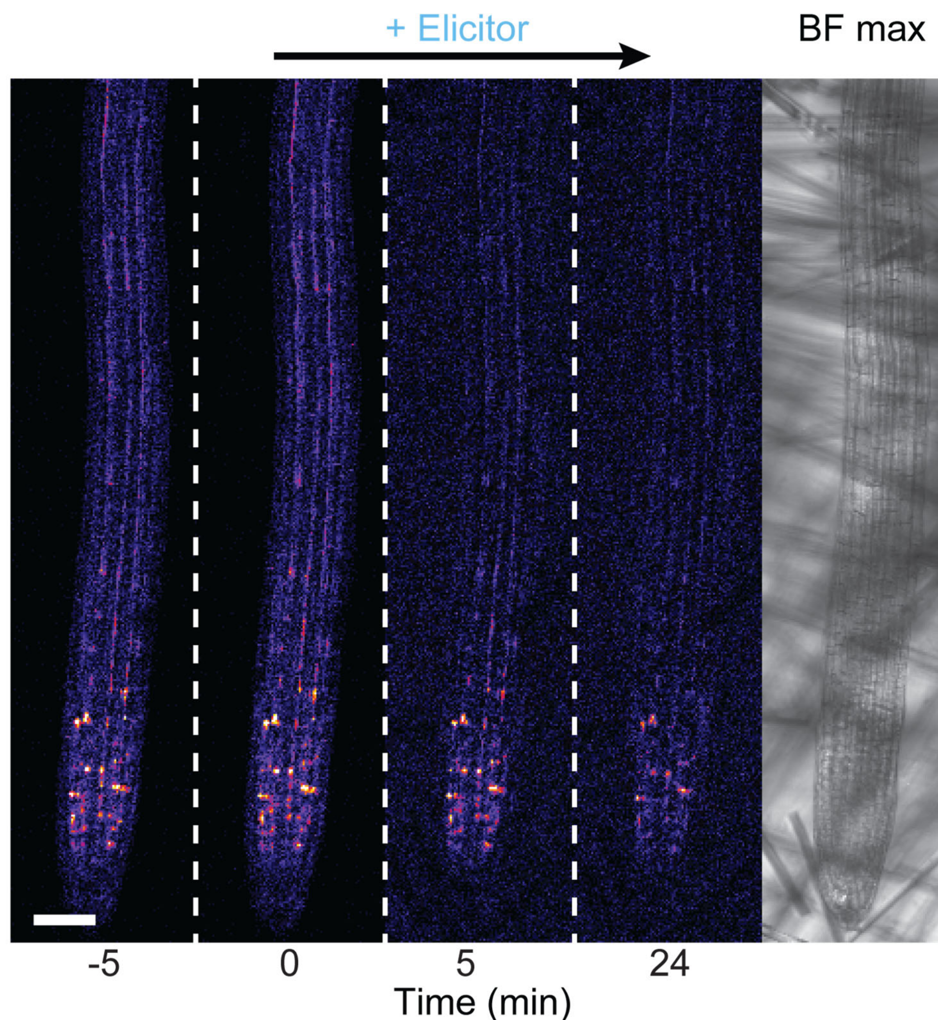
- In addition, the response to the hyphae and the elicitor-mix on root growth shows a different behavior, although the trend of being slightly inhibiting holds. Can this difference be explained/discussed?

Response: The elicitor treatment is much more uniformly distributed, meaning that more cells are directly affected by the treatment over time. When treated with hyphae, only few cells are in direct contact with a hyphae in our experimental setup. This is reflected by the fluctuations in growth rate (Figure 1e). The more cells are in direct contact with hyphae, the more the inhibition of growth rate. But, due to the growth of the root, the number of cells in direct contact with hyphae varies significantly during the assay.

Figure 2:

- Panel d and g show clear effects on the output of the novel pH sensor by elicitors or hyphae, but could the root be overlaid on the fluorescence picture? It is difficult to judge where the effect is clearest on this representation.

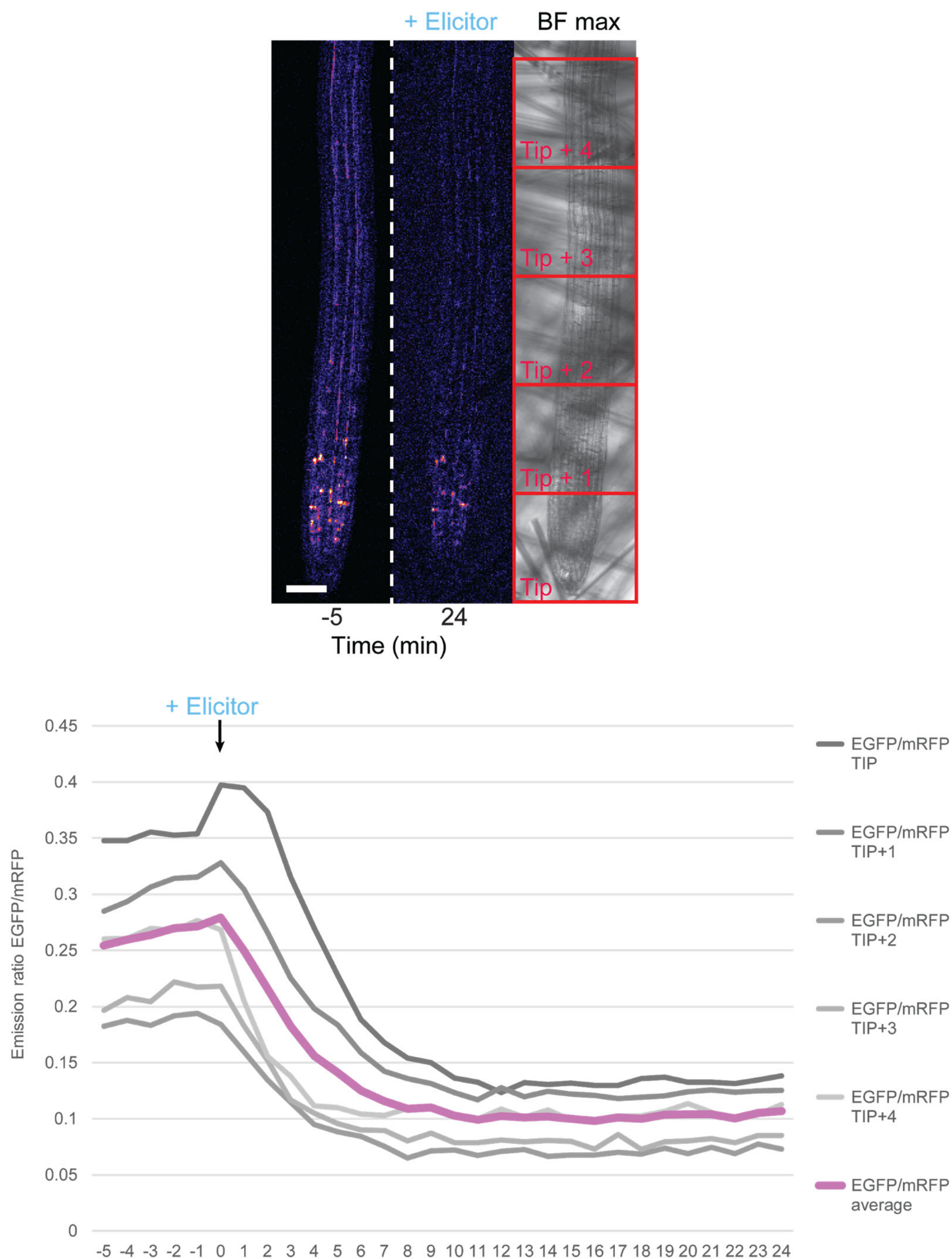
Response: An overlay of the brightfield images is difficult to add for the chosen surface plot over time. Since the bright field intensity does not change over time, a resulting surface plot would just be a gray image. Furthermore, to create the surface plot, Fiji/imageJ scales the images to a square image using nearest neighbor sampling, which makes it difficult to just add a non-modified brightfield image underneath. Below, you can find the same root used in the manuscript without a surface plot including a maximum projection of the brightfield channel. If desired, we can of course add this representation to the paper, but we think that a surface plot, in which one can see signal intensity by peak size, facilitates data visualization. As you can also see, the detected pH drop is evident and in the same magnitude in each part of the root (please also refer to next answer).



Signal of the EGFP channel of a SYP122-pHusion expressing root. An Elicitor mix was added after 5 min, which induces a rapid loss of signal intensity (i.e. decrease of pH). Scale bar = 100 µm.

In the same line, it is mentioned that the average pH is 5.30, but is there a difference at the beginning and end of the elongation zone? There were measurements of proton-fluxes around the root that show this could be the case (Staal et al., 2011 Plant Physiol).

Response: Indeed, we measured the intensity ratio between EGFP and mRFP of SYP122-pHusion in different areas of the root elongation zone and tip. Please have a look at the figure below in which we separated the root from the tip to the elongation zone (same root as in the previous answer).

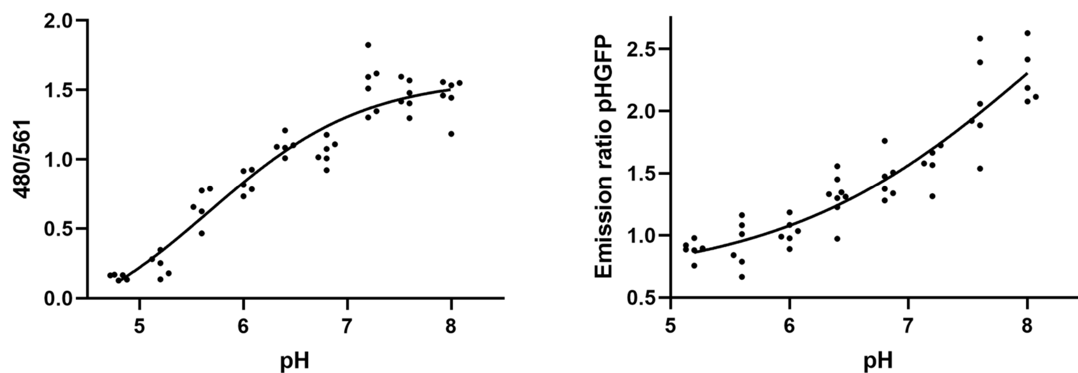


The ratio between EGFP and mRFP is indeed initially higher in the root tip (TIP) and the following region (TIP+1), which would fit with the data acquired in the above mentioned study. The standard curve, which is used to calculate actual pH values, was derived from analyzing intensity ratios of whole roots, not just specific zones. This means that one cannot directly correlate these higher intensity ratios in the tip zone to actual higher pH. To do this, one would also have to acquire the standards in different areas of the root. This is not feasible with the used objective and imaging system as the magnification is too low to reliably count cells, which means that we could never reliably select the same

zones in different roots. We observed the same response to the treatment in all visible root zones; i.e. a drop of intensity ratios once elicitors were added. Thus, we decided to show an overall average of the measured pH per root, rather than in different zones individually.

- Supplemental figure 2e/g shows the calibration of the pH sensor, but the values of the X-axis are in log-scale. Please use normal scale to better understand the response of the sensor to changing pH values.

Response: The 4PL regression analysis requires the x-axis to be in log scale (https://www.graphpad.com/guides/prism/7/curve-fitting/index.htm?reg_example_ria.htm). However, please find the same calibration graphs with normal scale below. These show the same trend and shape in comparison to log scale, as the log of the used numbers (4.8, 5.2, … 7.6, 8.0) results in an almost linear range.



Left panel: In vivo calibration of SYP122-pHusion in 6 day-old roots. Dots represent individual samples. Data points were fitted using sigmoidal regression.
Right panel: In vivo calibration of pHGFP-Lti6b in 6 day-old roots. Dots represent individual samples. Data points were fitted using sigmoidal regression.

- Fig. 2e and f show that upon acidification of the apoplast, the cortical side shows a 1-min lag before it acidifies too. How do the authors explain that the cortical pH returns to normal whereas the apoplast stays acidified?

Response: Fig. 2f shows an initial alkalization of the cortical side of the plasma membrane for 1 min in response to apoplastic acidification. Afterwards, the cortical pH recovered to control levels within 7 min after elicitor application. Our interpretation is that plant cells have a rheostat of redundant biochemical and biophysical mechanisms to control cytosolic pH. The plasma membrane proton pumps create both a pH gradient and an electrical difference across the plasma membrane. Therefore, enhanced H^+ efflux hyperpolarizes the plasma membrane, which then gate-opens inward-rectifying K^+ channels and chloride-permeable channels that release cytosolic anions. Together, these channels dissipate the electrical charge and prevent the backflow of protons. The operation of these biophysical effectors (H^+ and Cl^- efflux, K^+ influx) may help to explain the initial increase of cortical pH in the vicinity of the plasma membrane

upon elicitor sensing. However, in addition to proteins and solutes that buffer pH in the cytosol, ion transporters at the tonoplast also respond to changes in cytosolic pH. These transporters exchange H^+ and other inorganic (K^+, Na^+, Cl^-, NO_3^-) and organic (malate, citrate) ions in and out of vacuoles to counteract deviations in cytosolic pH. Moreover, considerable amounts of organic acids are synthesized in response to cytoplasmic alkalinization. Hence, any significant deviation of cytosolic pH is immediately counter-acted by these redundant mechanisms that enable the cell to return to neutrality within minutes, as observed in Fig. 2f. The oscillating behavior of the cortical pH during the approach to reach the set-point at pH 7.2 reflects the typical operation of a rheostat.

Figure 5:

- The text mentions panels 5g and h, but I seem to miss them in the current version of the manuscript.

Response: We thank the reviewer for identifying this mistake. The referenced figures should actually be Supplementary Fig. 6f and g in the old version of the manuscript. We now changed it in the new version.

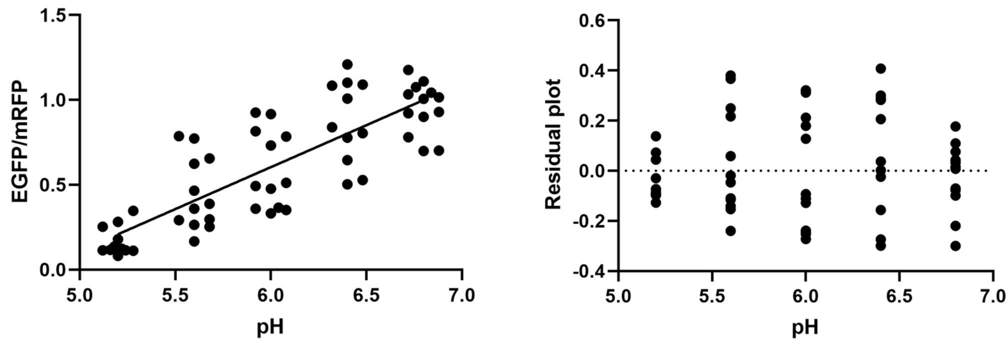
Discussion:

- How do the authors link the absence of CC1 and CC2 to elevated proton pump activity? Could it be that a problem with the CCs affect cellulose synthesis, which changes the cell wall integrity and thus is sensed by the cell wall integrity sensors, feeding back into the cytoplasm and potentially acting through kinases on the proton pumps?

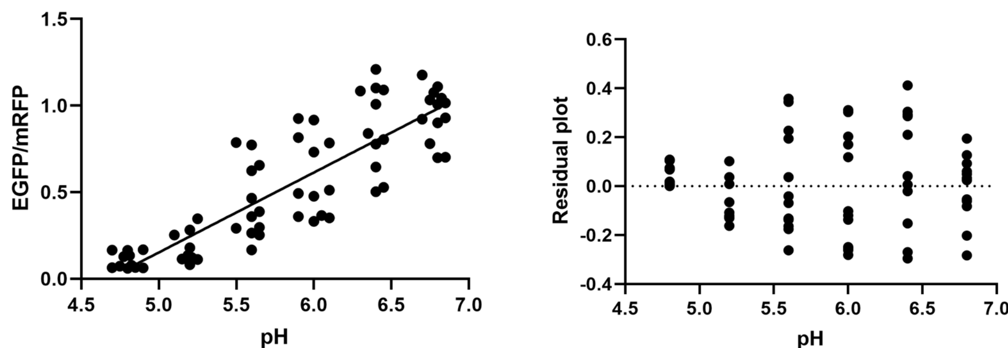
Response: Reviewer 2 suggested the same explanation for AHA hyperactivity in *cc1cc2*. We addressed this comment above (see page 7 and 14) and our data indicate that alterations of cellulose levels do not per se lead to AHA hyperactivation.

- The pH sensor has a linear output from pH 5.5-8. Does this mean the sensor is nut useful for examining processes where the cell wall is acidified?

Response: Indeed, calibration becomes unreliable when values are close to the end of the linear range of the used sensor (Benjaminsen et al., 2011). The linear range mentioned by the reviewer is actually the range of the Lti6b-pHGFP sensor as mentioned in the text. The apoplastic sensor SYP122-pHusion has a linear range between 5.2 to 6.8 as indicated in the manuscript. Linear regression analysis (see left panel below) of the data points of two individually acquired standard curves (separated by several months) between pH 5.2 and 6.8, shows a clear linear correlation and a replicates test for lack of fit (runs test) indicates no significance ($p = 0.3759$). Furthermore, a residual plot (right panel below) shows a random pattern and no apparent “shape” formation. Hence, we still operate in the linear range of the sensor with our measurements and the sensor itself is therefore an ideal tool to measure pH in the apoplast.



Even when one includes the data points at pH 4.8, the resulting graph still follows a linear pattern (left panel below) and a replicates test for lack of fit indicates no significance (p = 0.2772). However, the residual plot becomes rather "cone shaped", indicating a non-linear pattern (right panel below). This is due to the fact that EGFP fluorescence is totally quenched at pH values <5 (Gjetting et al., 2012), hence this is also the case for our sensor Syp122-pHusion. In consequence, this leads to signal intensities close to zero and basically no error in between replicates, which then leads to the cone shaped residual plot. Therefore, we did not report the definitive pH of the apoplast after addition of elicitors or hyphae, only that it dropped below 5, since we were aware of the fact that we are outside of the linear range (i.e. below pH 5). This does not mean that one should not use our apoplastic sensor to measure pH close to pH=5.0, one just has to be aware of the fact that the measurement becomes slightly more error prone.



In addition, one can expect that the sensor, close to the PM and therefore potentially next to an active proton-pump, encounters a very acidic environment. Could this be discussed?

Response: The apoplastic pH that we quantified with our sensor (approx. apoplastic pH 5.4) is in good agreement with previously reported values obtained with fluorescent dyes (Barbez et al, 2017) or surface electrode measurements in the root elongation zone of Arabidopsis (Staal et al, 2011), i.e. methods that are either more dispersed (dyes) or not in direct contact with the membrane (surface electrodes). This highlights that our genetically encoded, membrane bound sensor is indeed working properly with the advantage that we

do not observe high cell-internal background signals in comparison to free apo-pHusion expressed only with a signaling peptide (Supplementary Fig. 2a). This allows for more precise measurements of the apoplastic pH especially in response to stress (biotic or abiotic), since these stresses affect both extracellular and intracellular pH at the same time.

The pH at the apoplastic side of the plasma membrane is indeed acidic (average pH of 5.4), considering the pH in other cell compartments (7.2 at the cortical side of the PM (this manuscript), 6.6 at cis-Golgi (Luo, Scholl & Doering et al. 2015), 6.3 at trans-Golgi (Luo, Scholl & Doering et al. 2015), 5.6 at TGN/EE (where the VHA-a1 proton pumps are active; Luo, Scholl & Doering et al. 2015), and 5.8 at the vacuole (Krebs et al., 2010).

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2nd Editorial Decision 17th Sep 2019

Thank you for submitting a revised version of your manuscript. I sincerely apologise for the delay in communicating the decision due to belated submission of referee reports. Your manuscript has now been seen by all original referees, who find that their main concerns have been addressed and are now broadly in favour of publication of the manuscript. However, reviewers #2 and 3 raise a number of remaining concerns that should be addressed textually in the manuscript. Regarding the major point by referee #2, please include the AHA phosphorylation data or tone down the conclusions as appropriate.

Additionally, I would like to ask you to fix a few editorial issues before I can extend formal acceptance of the manuscript.

REFEREE REPORTS:

Referee #1:

I am happy with the changes made by the authors to my previous comments. They have made a very commendable revision in response to the comments of all three reviewers, in my view, and the paper should be accepted for publication. It is a potentially significant contribution

Referee #2:

Review of manuscript by Kesten et al., EMBOJ 2019

This manuscript represents the revised version of previously submitted manuscript. The content has not changed fundamentally therefore I will not summarize it again but focus on the modified bits and the responses to my previously raised concerns.
I think overall the quality and readability of the manuscript has improved. Particularly the discussion is much more informative and coherent. This also ensured that the significance and mechanistic insights presented in this manuscript have become clearer. I´m also glad to see that the authors have supplied the necessary mock controls, which were missing throughout the manuscript before. Overall it has become much clearer / more convincing that cc1 cc2 influence apparently primarily pH levels and not cellulose biosynthesis.

Major point:
I appreciate the attempt by the authors to address my concern about direct or indirect effects / involvement of CWI. However, I´m not convinced that the acidification assays with cc1cc2, pom 2-4 and prc1-1 are the solution since the differences between wt controls in acidification induced in Figures 5c and S5a are larger than between the mutants and the controls tested in the respective assays. That makes me wonder if these assays have the appropriate resolution / reproducibility level to be useful here. If the authors want to make the point that cellulose deficiency does not automatically activate AHAs then they should probably do phosphorylation assays like they performed for cc1cc2 with pom2-4 and/or prc1-1, which is not perfect either but would be closer to the actual processes and better comparable to the cc1cc2 situation.....

Minor points:
Line 32-33: The authors have not really investigated "cellulose structure" and I think there are more pertinent points to be made in the abstract so I recommend changing this. They do "hypothesize" though (line 428)....
Line 214: Am not sure if "partially maintained" is the appropriate description for the cc1cc2 data in figure 3c. The data also suggest that the effects of CC1CC2 loss are more profound on microtubules than on CSC density. Last but not least, I wonder if an ANOVA based statistical test is more appropriate here than all the pairwise tests?
Lines 245-251: I would recommend rephrasing the description of the cc1cc2 root growth phenotypes after infection. Since the mutant root growth is also reduced (compare fig4.c vs. e),

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which is also nicely correlated with the reduced but not absent vascular penetration in the mutant.
Line 263: "to better understand" should be rephrased, because you don´t "understand" vascular colonization by a pathogen by quantifying gene expression changes alone....
Lines 304-307: The authors talk about "significant differences" but I don´t see any stats and I suspect if they compare pHapo cc1cc2 to wildtype and pHcortical cc1cc2 to wt there won´t be any.
Line 403: Replace "we quantified" with "we detected".
Line 419: The statement is not supported by figure 4. I suspect the authors mean Fig. S4g and 5d, f.
Figure S4g: is there a whisker missing for Col-0 + MES?
Discussion: seems the fact that elicitor treatment is sufficient for induction of responses and the relevant molecular mechanism has not been discussed....

Referee #3:

The very well written and notably improved manuscript provides new information about early events at the plasma membrane, microtubule rearrangement and regarding cellulose synthase activity in root - microbe interactions.
Few minor last comments/suggestions:
Fig. 1 shows an epidermal cell of a 5 day old root. Were the analyzed cells in the elongation zone or the root hair zone? Felle et al. demonstrated for barley that the pH is highly affected by the developmental stage. Is this also true for Arabidopsis? This information could be given e.g. at line 109.

Line 193: The crude elicitor (which I guess contains chitin) has an opposite effect compared to the MAMP chitin. Do the authors speculate a Fo specific compound in the elicitor preparation? This could be pointed out in the discussion.

Line 262: Why the cc1cc2 mutant with altered epidermal response to Fo shows no difference in colonization except reduced entry into the xylem?
Line 277: Glucan is the major cell wall component in most fungi. Being able to distinguish glucose from N-acetylglucosamine does not proof that the measured signal is only coming from the plant. (But the glucose content was anyhow reduced after Fo infection).

Line 304f: cc1cc2 plants already showed significantly different pHapo and pHcortical when compared to WT plants under mock conditions. Is the difference between 5.21 {plus minus} 0.20 and WT pHapo 5.42 {plus minus} 0.32 really significant? Please mention test and p-values as there is no figure where the data are directly compared.
Line 305ff: Could the authors please check the pH values mentioned? WT pHapo 5.42 {plus minus} 0.32 in Fig. 2h compared to 5.44 {plus minus} 0.33 in line 188 and WT pHcortical 7.07 {plus minus} 0.64 in Fig. 2i and 6.99 {plus minus} 0.73 in line 188.
Line 464: "Rapid, chitin-mediated apoplastic alkalinization (Supplementary Fig. 2h, (Felle et al, 2009) might therefore be a plant evolutionary response to counteract the initial pHapo drop caused by the fungus": The crude elicitor preparation causes the same effect as the living fungus which is opposite to the chitin oligomers (which are components of the elicitor preparation I guess). Recognition of a MAMP like chitin or flg22 triggers a very robust and common defense response including alkalinization. Evolutionary it would make more sense for me to speculate that the pathogenic fungus evolved mechanisms to prevent alkalinization.

Referee #4:

After reading the rebuttal to the editor and reviewers comments and the revised version of the paper, I feel the authors have made a good case by answering to the comments both text-wise and by performing extra experiments.
Therefore, the paper could be accepted for publication.

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2nd Revision - authors' response 11th Oct 2019

We thank the reviewers and editors for supporting our work to be published in EMBO J. We also acknowledge their contribution to the manuscript with their suggestions and comments.

Below, we address point by point the last concerns from Reviewers #2 and #3.

Referee #1:
I am happy with the changes made by the authors to my previous comments. They have made a very commendable revision in response to the comments of all three reviewers, in my view, and the paper should be accepted for publication. It is a potentially significant contribution

Referee #2:
Review of manuscript by Kesten et al., EMBOJ 2019.
This manuscript represents the revised version of previously submitted manuscript. The content has not changed fundamentally therefore I will not summarize it again but focus on the modified bits and the responses to my previously raised concerns.
I think overall the quality and readability of the manuscript has improved. Particularly the discussion is much more informative and coherent. This also ensured that the significance and mechanistic insights presented in this manuscript have become clearer. I´m also glad to see that the authors have supplied the necessary mock controls, which were missing throughout the manuscript before. Overall it has become much clearer / more convincing that cc1 cc2 influence apparently primarily pH levels and not cellulose biosynthesis.

Major point:
I appreciate the attempt by the authors to address my concern about direct or indirect effects / involvement of CWI. However, I´m not convinced that the acidification assays with cc1cc2, pom 2-4 and prc1-1 are the solution since the differences between wt controls in acidification induced in Figures 5c and S5a are larger than between the mutants and the controls tested in the respective assays. That makes me wonder if these assays have the appropriate resolution / reproducibility level to be useful here. If the authors want to make the point that cellulose deficiency does not automatically activate AHAs then they should probably do phosphorylation assays like they performed for cc1cc2 with pom2-4 and/or prc1-1, which is not perfect either but would be closer to the actual processes and better comparable to the cc1cc2 situation.....
Response: We agree with the reviewer about the limitations of both methods (media acidification and AHAs phosphorylation in their penultimate amino acid) to determine changes in AHA activity and PM ΔpH. Indeed, the more accurate technique is measuring the pH at each side of the PM using the pH sensors we have generated, which was not feasible due to time limitations. As the aim of the experiment was to determine whether the enhanced proton efflux observed in cc1cc2 was induced by its the cellulose deficiency under non-buffered conditions, we have: (a) Included all the genotypes (WT, *cc1cc2*, *pom2-4* and *prc1-1*) in a new media acidification assay. As it can be observed in Figure EV5a, only *cc1cc2* acidifies the media faster than WT. (b) Substituted "confirming" by "suggesting" (see line 311 "suggesting that the observed ΔpH alteration in *cc1cc2* is not purely based on its cellulose deficiency").

Minor points:
Line 32-33: The authors have not really investigated "cellulose structure" and I think there are more pertinent points to be made in the abstract so I recommend changing this. They do "hypothesize" though (line 428)....
Response: We thank the reviewer for the suggestion, but do not agree, as we used "cellulose structure" as the most general term possible here. Using descriptions like "crystallinity" or "composition" would be an overstatement of what we see and analyzed here. Our biochemical data suggest a reduction on cellulose crystallinity under low apoplast pH, which should be confirmed by X-ray analyses. Thus, we prefer to keep "structure" as a more general term. We believe this is relevant for the community as it might be a way for the pathogen to loosen the host CW while it advances through the root layers (as discussed in lines 452-454).

Line 214: Am not sure if "partially maintained" is the appropriate description for the cc1cc2

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data in figure 3c. The data also suggest that the effects of CC1CC2 loss are more profound on microtubules than on CSC density. Last but not least, I wonder if an ANOVA based statistical test is more appropriate here than all the pairwise tests?
Response: We thank the review for her/his concern, which we hope can clarify here: It is less significantly reduced, i.e. partially maintained in comparison to WT. Partially maintained only refers to CSC density, not to MT density (we write indistinguishable there). An ANOVA without pairwise comparison would only tell us that the whole data is different, which it is. We therefore need a pairwise comparison to say which group is different from which one. I.e. if we do an ANOVA with subsequent pairwise comparison or just compare pairwise from the beginning does not change the outcome.

Lines 245-251: I would recommend rephrasing the description of the cc1cc2 root growth phenotypes after infection. Since the mutant root growth is also reduced (compare fig4.c vs. e), which is also nicely correlated with the reduced but not absent vascular penetration in the mutant.
Response: We have added "and root growth less affected than in WT".

Line 263: "to better understand" should be rephrased, because you don´t "understand" vascular colonization by a pathogen by quantifying gene expression changes alone....
Response: We have changed the sentence to "to gain further insight into".

Lines 304-307: The authors talk about "significant differences" but I don´t see any stats and I suspect if they compare pHapo cc1cc2 to wildtype and pHcortical cc1cc2 to wt there won´t be any.
Response: The tests were included in the first version of the manuscript but removed for the resubmitted version to increase readability as indicated by the reviewer. We have added them again (see lines 298-301). Furthermore, we supplied the source data for any needed confirmation of the statistical analysis.

Line 403: Replace "we quantified" with "we detected".
Response: We do quantify something here: we count the vascular penetrations. So, we will keep the sentence as it is.

Line 419: The statement is not supported by figure 4. I suspect the authors mean Fig. S4g and 5d, f.
Response: Figure 4i shows reduced crystalline cellulose under mock conditions in *cc1cc2* plants. We added an "i" to the Fig. 4 citation to make this more clear.

Figure S4g: is there a whisker missing for Col-0 + MES?
Response: It is not missing. As described, the whiskers show MIN and MAX. There is just no MIN variation outside the box.

Discussion: seems the fact that elicitor treatment is sufficient for induction of responses and the relevant molecular mechanism has not been discussed....
Response: The exact molecular mechanism is yet unknown and we prefer to avoid an excessive number of hypothesis.

Referee #3:
The very well written and notably improved manuscript provides new information about early events at the plasma membrane, microtubule rearrangement and regarding cellulose synthase activity in root - microbe interactions.
Few minor last comments/suggestions:
Fig. 1 shows an epidermal cell of a 5 day old root. Were the analyzed cells in the elongation zone or the root hair zone? Felle et al. demonstrated for barley that the pH is highly affected by the developmental stage. Is this also true for Arabidopsis? This information could be given e.g. at line 109.
Response: We thank the reviewer for the useful comment. We imaged everything in the elongation zone. Other root zones show almost no CesAs as the growth of cells already stopped. We now added "in the elongation zone" into line 109 (now, line 105).

Line 193: The crude elicitor (which I guess contains chitin) has an opposite effect compared to the MAMP chitin. Do the authors speculate a Fo specific compound in the elicitor preparation? This could be pointed out in the discussion.

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Response: We agree with the reviewer and have included it in the discussion (line 371-374).

Line 262: Why the cc1cc2 mutant with altered epidermal response to Fo shows no difference in colonization except reduced entry into the xylem?
Response: We can only image CSC-MT response at the epidermis, but we expect an altered response to the fungus also in deeper layers because the mutant has a more acidic apoplast in all root layers. We currently do not have a definitive reason for the ability of Fo to grow in *cc1cc2* roots (same fungal biomass than in WT roots) but reduced ability to colonize the xylem. We addressed this point and explained our hypothesis in the resubmitted version: "However, the total amount of fungus was the same in *cc1cc2* and WT roots (Fig. 4), indicating that the *cc1cc2* mutations specifically hinder Fo5176 to enter the vasculature. Since *cc1cc2* has a more acidic apoplastic milieu that alkalinizes slower in response to Fo5176 (Fig. 5; Fig. EV5), this is in line with previous reports showing the need of an alkaline environment for Fo5176 to produce invasive hyphae (Masachis *et al*, 2016). Vascular microbes establish a compatible pathogenic interaction with the host only if they can reach xylem vessels. Indeed, non-pathogenic endophytes, like several *F. oxysporum* f.sp, are unable to colonize the xylem and some of them confer protection to vascular pathogens (Brader *et al*, 2017). The fungal growth in *cc1cc2* therefore resembles that of non-pathogenic, endophytic *F. oxysporum* strains, which colonize the root cell layers, but cannot reach the xylem. The resistance to Fo5176 conferred by *cc1cc2* should not interfere with the growth of beneficial endophytes or endosymbionts and might render this mutation an interesting option for agronomical applications in conditions of no salinity stress."

Line 277: Glucan is the major cell wall component in most fungi. Being able to distinguish glucose from N-acetylglucosamine does not proof that the measured signal is only coming from the plant. (But the glucose content was anyhow reduced after Fo infection).
Response: We agree with the reviewer. To clarify the origin of the crystalline glucose in infected plants, we measure fungal-derived glucose in AIR crystalline fraction by processing 1/2MS grown fungus. As shown in Figure EV4b right panel, the amount of fungal glucose is the same in strong- and weak-hydrolyzed AIR samples, indicating a negligible presence of fungal glucose in a crystalline state. Meanwhile, in plants there is a clear difference between the glucose peaks in either hydrolysis method, representing the crystalline cellulose.
Line 304f: cc1cc2 plants already showed significantly different pHapo and pHcortical when compared to WT plants under mock conditions. Is the difference between 5.21 {plus minus} 0.20 and WT pHapo 5.42 {plus minus} 0.32 really significant? Please mention test and p-values as there is no figure where the data are directly compared.
Response: Yes it is. We now re-added the tests to the text (we removed them to increase readability in this second version).

Line 305ff: Could the authors please check the pH values mentioned? WT pHapo 5.42 {plus minus} 0.32 in Fig. 2h compared to 5.44 {plus minus} 0.33 in line 188 and WT pHcortical 7.07 {plus minus} 0.64 in Fig. 2i and 6.99 {plus minus} 0.73 in line 188.
Response: These data are correct but seem to need a clarification. As described in the methods section, we used 2 different methods and therefore used the WT data from the corresponding method to compare with *cc1cc2*. While working with elicitors (i.e. in line 188) we glued the roots onto a cover glass. In line 305 we do hyphae treatment in which the roots are placed on an agarose sandwich covered with spores (or without as mock treatment). Since *cc1cc2* was only tested with alive hyphae, we used this last method and compared the data with those obtained from WT without gluing the roots to the surface. It should be pointed out that even though the techniques were slightly different, there is no statistical significant difference between the mock treatments in both methods.

Line 464: "Rapid, chitin-mediated apoplastic alkalinization (Supplementary Fig. 2h, (Felle et al, 2009) might therefore be a plant evolutionary response to counteract the initial pHapo drop caused by the fungus": The crude elicitor preparation causes the same effect as the living fungus which is opposite to the chitin oligomers (which are components of the elicitor preparation I guess). Recognition of a MAMP like chitin or flg22 triggers a very robust and common defense response including alkalinization. Evolutionary it would make more sense for me to speculate that the pathogenic fungus evolved mechanisms to prevent alkalinization.
Response: In the case of *F. oxysporum* it has been shown that its virulence depends on an

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alkaline environment. Indeed, it induces long-term apoplastic alkalinization (see Masachis *et al*, 2016, among other).

Referee #4:
After reading the rebuttal to the editor and reviewers comments and the revised version of the paper, I feel the authors have made a good case by answering to the comments both text-wise and by performing extra experiments. Therefore, the paper could be accepted for publication.

3rd Editorial Decision 17th Oct 2019

Thank you for implementing the final changes in your manuscript. I am now pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Clara Sanchez-Rodriguez
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-101822

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 justified
- 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.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	There was no specific statistical method used to determine sample size. However, sample size and number of replicates were chosen according to previous studies (Endler et al., Cell 162: 1353–1364,2015; Paredez et al., Science 312: 1491-1495, 2006; Krebs et al., Plant J 69:181-192, 2012; Delgado-Cerezo et al Mol Plant 5(1):98-114, 2012).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	If the fluorescent ratios of the pH sensors (pHusion, pHGFP) were measured to be outside of the standard curve range, they were excluded from the analysis. If samples drifted during image acquisition and this could not be corrected, they were excluded from the analysis.
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.	Plants were always randomly distributed in the growth/treatment chambers. Otherwise, no randomization was necessary for this study.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Data analysis (especially for images) was either done automatically, so independently from the investigator or file names were removed for analysis (e.g. including an indication for genotype or replicate number). For the measurement of plant size, investigators were not blinded since this is not relevant to the study. In this case, data were always collected according to the genotype.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
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.	We only used Welch's unpaired t-test, i.e. always assumend unequal variances and/or unequal sample sizes. For repeated measures two way ANOVA analyses, we did not assume sphericity and used Greenhouse-Geisser method to correct for violations of the assumption.

Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	That depends on each individual experiment and as described above we per se did not assume equal variance and used methods to correct for this.

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).	Hayashi Y, Nakamura S, Takemiya A, Takahashi Y, Shimazaki K-I & Kinoshita T (2010) Biochemical Characterization of In Vitro Phosphorylation and Dephosphorylation of the Plasma Membrane H - ATPase. Plant Cell Physiol. 51: 1186–1196
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used in this study.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	No such data was acquired in this study.
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).	We supplied our raw data in a source data file attached to the manuscript.
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).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA