THE
EMBO
JOURNAL

Single cell damage elicits regional, nematode-restricting ethylene responses in roots

Peter Marhavý, Andrzej Kurenda, Shahid Siddique, Valerie Dénervaud Tendon, Feng Zhou, Julia Holbein, M. Shahim Hasan, Florian M. W. Grundler, Edward E. Farmer and Niko Geldner

Review timeline:

Submission date:	21st Oct 2018
Editorial Decision:	13th Dec 2018
Revision received:	29th Jan 2019
Editorial Decision:	19th Feb 2019
Revision received:	1st Mar 2019
Accepted:	7th Mar 2019

Editor: Deniz Senyilmaz Tiebe

Transaction Report:

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

1st Editorial Decision 13th Dec 2018

Thank you for submitting your manuscript 'Single cell damage elicits regional, nematode-restricting ethylene responses in roots' for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you can see, referees - and an external expert from whom I sought advice - express interest in your study investigating the response elicited by single cell damage in the root. However, they also raise concerns that need to be addressed in full before we can consider publication of the manuscript here, which I outline below:

• Further characterization of the single cell wound response signals in the root (referee #1, point 1)
• Functional relevance of ethylene signaling on success of H. schachtii reproduction (referee #1, point 3)
• Applicability of the SA markers in the root tissue, which were mostly used in leaves so far (referee #1, point 4)
• The technical concerns raised by the referee #3

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing all comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Please feel free to contact me if you wish to discuss the revision process further.

REFEREE REPORTS:

Referee #1:

Marhavy et al. report single-cell ablation experiments in Arabidopsis roots and describe various local and regional responses triggered by this danger signal. In response to this defined cell damage, ethylene-production and -responses appear to dominate, but not jasmonate responses. Using ethylene signalling mutant plants (ein2 and ein3 genotype) evidence is then presented that nematode infection partially recapitulates the cell ablation-induced damage response. The latter conclusion is based on a reduced incidence of nematode feeding in roots of ein2 and ein3 plants.

There is no doubt that this complex work has been carried out with technical competence. This concerns both the acquisition of cell biological data and their analysis as well as the surface potential recordings. Despite this technical mastery and despite a large amount of data presented, I have doubts that the present study reports a major advance in root biology or provides novel biological insight(s) as explained below. I have also difficulties in extracting a clear 'take-home message' from the current manuscript.

1. A major shortcoming of the present study is that the molecular signal(s) underlying the elicitation of the wound response in roots following laser ablation remain unclear. Are these cell wall-derived damage signals, danger peptides, or metabolic perturbation signals? This is relevant because the molecular basis of plant responses to damage is in parts already well understood. It is known that the perception of danger-associated molecular patterns (DAMPs) that represent altered-self can lead to immune activation. Upon damage, the so-called Pep epitopes derived from plant-encoded PROPEPs are perceived by the two closely related Arabidopsis transmembrane receptors PEPR1 and PEPR2 and trigger an immune response that resembles immunity mediated upon non-self sensing of conserved microbe-derived peptide epitopes (Yamaguchi et al., Plant Cell 2010). Prior work has also provided genetic evidence for ethylene signalling in the PEPR pathway in leaves (Liu et al., PNAS 2013; Tintor et al., PNAS 2013). Thus, in this wider context it is not surprising that laser ablation of single root cells induces ethylene-production and -responses (Fig. 3 and Fig. 5). It is a missed opportunity that in the current study the authors have not tested the (likely?) contribution of the PEPR receptors to regional responses inflicted by single cell damage.

2. Despite the re-invention of single-cell laser ablation technology, pioneered originally in the context of root development (Berg et al., Nature 1997), I remain sceptical whether the striking depolarization spikes observed opposite to the site of cell ablation (Fig. 1) provide truly novel physiological insight in root biology. Unlike root growth on agar or liquid media, Arabidopsis plants grown in natural soil are subject to continuous and extensive mechanical shear stress and other mechanical forces, leading to the abrasion of large parts of the root epidermis. This raises doubts whether single-cell laser ablation reported here is a meaningful proxy for nematode feeding as mechanical wounding and adaptation to cell damage is expected to be a continuous and integral component of root growth in soil in the absence of nematodes or necrotrophic root-colonizing microbes.

3. Fig. 6F. The authors find that interference with the ethylene pathway increases nematode success rate as indicated by a 'faster establishment of the ISC stage". I find this weak evidence because it remains unclear whether 'faster establishment of the ISC nematode stage' in Arabidopsis ein2 and ein3 mutants translates into a robust increase of H. schachtii reproductive success. This is technically feasible as shown in previous work by some of the authors (Siddique et al., 2014), which would involve assessing the average number of nematodes per plant present in WT, ein2, and ein3 mutant plants. Thus, it remains to be shown whether ein2 and ein3 mutants are more susceptible to H. schachtii infection.

4. Non-responsiveness of the pPR1::NLS-3xVenus 'SA marker line' to SA treatment and inconsistent induction of pJAZ10::NLS-3xVenus is both interesting and alarming as it highlights another major caveat of the current work, i.e. an extrapolation of knowledge on defence phytohormone responses and defence phytohormone crosstalk from leaves (Pieterse et al. Nat Chem Biology, 2009) to the root organ. 'Markers of defense phytohormone biosynthesis and signalling' have been extensively used in plant interactions with pathogenic leaf microbes, but rarely in root biology. Moreover, sparse information is available regarding transcriptome-wide responses of root tissue to pathogen challenge or treatment of roots with defence phytohormones. Thus, I am not

convinced that the defence 'marker lines' used in the present study serve as suitable tools to make physiologically relevant conclusions on phytohormone signalling and/or crosstalk in Arabidopsis roots. What is missing in the present study are complementary quantitative measurements of the respective phytohormone levels in roots, at least SA, ET, and JA. This is especially important in ein2 and ein3 mutant to directly examine potential compensatory changes in the defence phytohormone network in roots.

5. The application of pharmacological compounds known to interfere with chloride or potassium or proton pumps upon single-cell laser ablation provides preliminary evidence indicating that these channels contribute to an electrochemical signal propagating across the horizontal root axis (Fig. 2A, B). However, this must be considered preliminary evidence given the known off-target effects of the drugs applied and lack of supporting genetic evidence whether specific family members of these channels or pumps are necessary for propagation of depolarization upon ablation.

Referee #2:

This manuscript describes studies of signaling and (reporter) gene-expression resulting from damage to a single root cell, first by laser ablation and then from nematode attack. The responses include [Ca2+] changes, electrical potentials, generation of reactive oxygen species - all components that have been characterized during more-extensive wounding of leaves and other plant organs. With single-cell ablation the responses are localized; systemic signaling seems not to occur. The authors report that ablation led to the induction of ethylene-regulated genes but did not significantly induce jasmonate-responsive reporter genes.

The experiments with single-cell ablation are comprehensive with good controls. However, the nematode work seems to be a bit of an afterthought and leaves the story with an unsatisfying ending and rather weak discussion - particularly with respect to the (non-)role of jasmonate signaling. Additional experiments are needed to strengthen the authors' conclusions. Presentation of the results and the text also need to be improved.

Main issues:

1. P8, l.231-234. "Interestingly, corroborating our observations upon laser ablation - but in contradiction to earlier studies (Kammerhofer et al., 2015), nematode invasion did not appear to induce a consistent jasmonate response, based on the lack of our JAZ10::NLS-3xVenus jasmonate reporter expression." This is a key finding that leaves the authors hand-waving through much of the Discussion (pp.8/9 l.250-280). The authors need to challenge jasmonate mutants with nematodes to provide another test of their conclusion, here. The prediction is that jasmonate mutants will provide ISC Selection times (cf Fig. 8F) that are as high (or higher? p9 l.280-281) than the wild-type, Col-0 control.

2. The nematode results also need to be strengthened by quantifying and analyzing the results in Fig.6 A&B (and relevant supplementary figures) like the results for the ablation experiments (Fig.1D, etc)

3. The stronger results will allow the discussion of jasmonate (non-)signaling to be presented strongly and briefly, so that more of the Discussion can be spent on the laser-ablation technology and its comparison to nematode infection, and on the signaling events and ethylene-regulated induction of defense genes. This last aspect will benefit from more-extensive reading of the literature on ethylene in plant defense, and incorporation of this information into the Discussion.

Issues relating to presentation: the manuscript needs to be carefully and comprehensively reworked to make the results more-accessible and to eliminate minor errors. These are the issues I noted, but there are likely to be more.

4. The figure legends (and text) use many abbreviations that are only spelled out in the M&M section (will this be on-line only?). They should also be explained in the main text, at first mention (or Figure legends).

5. The authors use wild type, Col-0 and Col in different figure legends. Use the same term throughout the figures and text.

6. In Fig.4 C&E, the reader is forced to assume "a" means ablation. Make it easy for them and spell out (or include in legend)

7. In Fig.2, the red arrowheads will be difficult to see in the printed journal (and online). Make them white.

8. Make clear that the R-GECO1 reporter line expresses the R-GECKO Ca2+ reporter.

9. The Introduction and Results sections need paragraph breaks added to delineate the ideas and arguments.

10. If the supplementary material is going to appear in PDF format online, I recommend putting the figure legends (single-spaced) immediately below each figure. This will make it much easier for readers to view and assess the results.

11. P4, l.102-104: "Interestingly, ACS6 responses were not exclusive to, but very much biased towards stele tissues, while PR4 responses were largely confined to the endodermis." Tell us if these observations are consistent with previously reported expression of the endogenous genes.

12. P4, l.116: "we found that our single cell ablations upregulated ACS6 and PR4 in a regional, but non-systemic fashion, encompassing a region of about 500µm for ACS6 (Supplementary Figure 6 and data not shown). If the 'data not shown' is needed to support this statement, it needs to be added to the Supplement, otherwise remove the phrase.

13. P6, l.178. Quote the quantitative results on GdCl3 attenuation from Fig.3 to give the reader an accurate measure of this effect.

14. There are a modest number of typographical and grammatical errors through the text, Figure legends and supplement that need to be corrected.

15. In the References the entry for Rojo etal needs initials added.

Referee #3:

Comments on the manuscript "Single cell damage elicits regional, nematode-restricting ethylene responses in roots"

<B>General summary and opinion, question and finding:</B>
The authors address the important and interesting question of how a primary wound performed on root is perceived and communicated to the neighboring cells and tissues. For this purpose, the authors developed a very elegant single cell wounding technique (laser ablation) mimicking a nematode attack. Combining this microsurgery technique with imaging measurement on plant lines expressing different markers the authors showed a predominant role of ethylene-in stress response while induction of jasmonate or salicylic acid is not observable. In this study, the authors also studied the electric potential variation induced by laser ablation and look for its involvement as a response involved in early signaling.

<B>Specific major concerns.</B>
This study based on live imaging performed on reporter lines provide new information on the kinetics and localization of calcium, ethylene and jasmonate responses induced by a laser cell ablation. My main concern concerns the electrophysiological response that I have difficulty to understand and to link to the other cellular responses observed.
That is to say:
(1) My first interrogation concerns the experimental design used in this paper to measure potential

variation. More precisely the type of derivation (position of Ag/AgCl wires) used by the authors. Looking at the literature, very few measurements have been performed on root using contact electrode because roots have to be maintained in a wet condition. Therefore the film of water at the surface of the root create an electrical shunt and short circuit the electrical signal to be measured. Indeed, the situation in roots is much different than in aerial organs (leaf, shoot) for which the cuticle and air make an insulator. To summarize for the aerial part it is possible to record potential variations between electrodes disposed at the surface of the plant and a reference electrode. Then it is assumed (it is an approximation) that potential variation mirror the transmembrane potential variation of cells neighboring measuring (also reference in some situation) electrodes. Then this technique which allows to "follow" an electrical signal give information mostly on the velocity, intensity, path of propagation, ... but not (or very few) on the nature of the signal observed in aerial part.
In the light of the above; did the experimenter make some basic measurements to have an idea of the equivalent circuit between their reference and measuring electrode? For example, what is the background voltage between the reference and measuring electrodes (for two positions of the reference: close to the measuring (5 to 10 mm) and far from the measuring) and what is the electrical resistance between these two electrodes in these two conditions?
As information, they are serval papers on root membrane potential recorded with extracellular electrodes and vibrating probes. In these paper (Katou, Ichino, 1982; Meyer, Weisenseel, 1997, Hejnowicz et al., 1991) authors propose an explanation on the origin of potential variation measured.
(2) My second interrogation concerns the shape of the potential variation. The potential variation shown in fig;1 F present a very deep rising phase and a decrease similar to a discharge of capacitor. The duration of the whole signal is about 5 second. No information is provided on the velocity of the electrical signal and on the duration of the lag between cell ablation and starting of the potential variation. To my point of view this ddp variation in not an AP. I would hypothesis.(1) the sudden collapse of membrane potential is transmitted via electrocoupling (mainly via plasmodesmata) to neighboring cells inducing sharp membrane depolarization then membrane potential recover, or (2) the micromovement produce by laser ablation could suddenly modify the junction potential at the electrode tip, or (3) we have also to keep in mind that an Ag/AgCl wire measure both electric potential and Cl- concentration making this electrode intrinsically a chloride-sensitive electrode (60 mV for a tenfold variation in Cl-). Therefore if the cell ablation induces a release of Cl- this local concentration variation should be sensed by the voltage electrode, or (4) other scenarios .....!

(3) My third interrogation concerns the interpretation of potential variation. If I understand well, the authors assume that variation potential they measure represent the variation of cellular transmembrane potential. In the paragraph "The short-distance electric signaling depends on multiple ion channel activities" (line 146 to 154) and Fig.2A the authors show that blockers targeting various channels and transporter have all the same effect of abolishing the potential variation. That would mean that all these blockers with different mode of action and different target lead to a same and single result. The interpretation given by the authors "a complex interplay of the known major ions driving plant cell electrochemical gradients drives the observed propagation of depolarization upon ablation" is not clear to me and not sustained by the data (simplistic explanation). That leads me to ask two questions/remarks: (1) is there any inactive inhibitors that could be considered as control?, (2) it would be convincing to record (at least in some experiment) the potential variation induced by the inhibitor itself in order to show the efficiency of the blockers and also this would validate the recordings.

I didn't find any comment and mention on the electrical variation in the discussion part (line 243 to 302) of the manuscript. How the electrical signal could be involved in early responses and its link with calcium wave?

Line 91 to 95 (and corresponding figures). The authors wrote, "we mechanically crushed large population of root cells, similar to standard woundings done on leaves by crushing of root tips". Since the wounding performed on leave largely affect the vasculature I wonder whether also a large portion of the vessel is affected by wounding the root tip? This represents an important point in order to compare responses in two different organs.

Figure 2 A and H: the potential is completely restored after 5 sec while it takes 40 sec to the calcium to reach its maxim. Do the authors think that there is a link between potential and Calcium? This

could be discussed in the view of recent results obtained on leaves by the authors (recently published).

<B>Minor concerns:</B>
Surface potential: the authors use the term "surface potential" (already used in previous paper and by some other authors). Surface potential in biophysics reefer to a precise definition related to fixed charges (linked to electrostatic potential). It is a bit misleading since potential variation in this paper is interpreted by the authors in term of transmembrane potential (involving potential generated by diffusion and pump activity) and not in term of "surface potential". In fact in the present experiments, the experimenters use surface electrodes (or contact electrode or extracellular electrode) and they record potential variation in-between the two electrodes (measuring and reference). The potential variation can be theoretically generated anywhere in the (closed) circuit and not specifically at the surface of the measuring electrode.

Regional potential: I don't clearly understand this term. In the present context, it appears too much unprecise. In electrophysiology, it already exists a more objective parameter call "space constant" to quantify over which distance a passive electrical signal is propagated in a cell/tissues. This quantitative parameter λ (in mm or µm) is the distance over which the steady-state voltage decays 37 % of its value at the origin.

I think (I might be wrong) that the authors force (in the abstract and later on in the manuscript) the comparison between the electrical responses in aerial part (propagation from leave to leave) and root. To my point of view, the electrical transmitted response studied in 8 week-old plant growing in a pot and elicited by wounding a large population of cell including the vessel as very little to do with the potential variation induced by wounding a single cell of a 5 day-old agar grown plant.

Additional essential suggestions
In order to be in a clear and safe experimental condition, transmembrane potential should be recorded with an intracellular electrode. Microelectrophysiology is a well-established technique and they are many reports on transmembrane potential measured on different root cell type.

Conclusion:
It is an interesting paper. The electrophysiological response is worth to consider as early signal in root but results provided does not correspond to the outflow standard.

1st Revision - authors' response 29th Jan 2019

Referee #1:

Marhavy et al. report single-cell ablation experiments in Arabidopsis roots and describe various local and regional responses triggered by this danger signal. In response to this defined cell damage, ethylene-production and -responses appear to dominate, but not jasmonate responses. Using ethylene signalling mutant plants (ein2 and ein3 genotype) evidence is then presented that nematode infection partially recapitulates the cell ablation-induced damage response. The latter conclusion is based on a reduced incidence of nematode feeding in roots of ein2 and ein3 plants.

There is no doubt that this complex work has been carried out with technical competence. This concerns both the acquisition of cell biological data and their analysis as well as the surface potential recordings. Despite this technical mastery and despite a large amount of data presented, I have doubts that the present study reports a major advance in root biology or provides novel biological insight(s) as explained below. I have also difficulties in extracting a clear 'take-home message' from the current manuscript.

REPLY: We have significantly revised our manuscript to makes our take home message even more evident: Using pertinent, cellular-resolution markers and techniques, we describe for the first time how single cell damage is perceived in plants and demonstrate that there are significant differences from the intensively described, organ-wide damage in leaves (the response we describe is regional, not systemic; induces ethylene, not jasmonate production). Moreover, we provide data using actual small invaders (nematodes), visualize that they cause single cell damage and very similar associated

defense responses, and then show that these responses make a measurable difference during early stages of infection.

It is possible that the reviewer differs from us in how valuable and interesting it is to describe single cell damage as compared to organ/multiple-tissue damage. To us, the difference in dimension is as profound and relevant as is the difference between single cell RNA sequencing and standard RNA profiling of tissues.

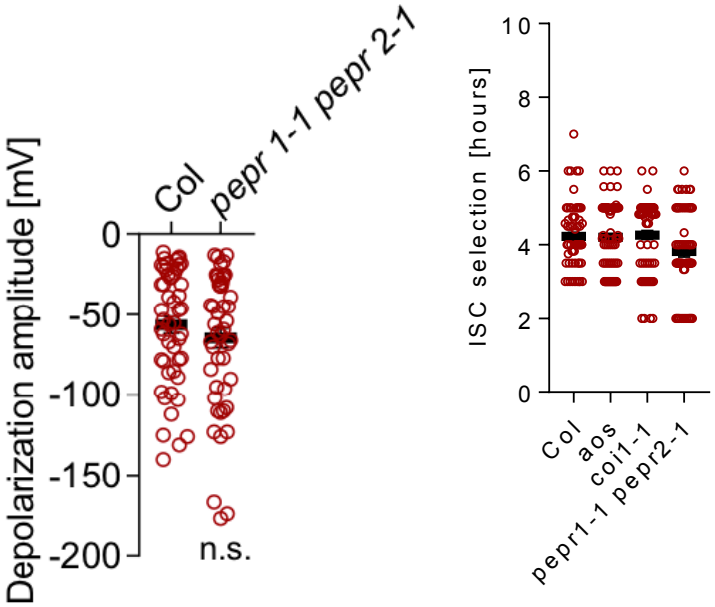
1. A major shortcoming of the present study is that the molecular signal(s) underlying the elicitation of the wound response in roots following laser ablation remain unclear. Are these cell wall-derived damage signals, danger peptides, or metabolic perturbation signals? This is relevant because the molecular basis of plant responses to damage is in parts already well understood. It is known that the perception of danger-associated molecular patterns (DAMPs) that represent altered-self can lead to immune activation. Upon damage, the so-called Pep epitopes derived from plant-encoded PROPEPs are perceived by the two closely related Arabidopsis transmembrane receptors PEPR1 and PEPR2 and trigger an immune response that resembles immunity mediated upon non-self sensing of conserved microbe-derived peptide epitopes (Yamaguchi et al., Plant Cell 2010). Prior work has also provided genetic evidence for ethylene signalling in the PEPR pathway in leaves (Liu et al., PNAS 2013; Tintor et al., PNAS 2013). Thus, in this wider context it is not surprising that laser ablation of single root cells induces ethylene-production and -responses (Fig. 3 and Fig. 5). It is a missed opportunity that in the current study the authors have not tested the (likely?) contribution of the PEPR receptors to regional responses inflicted by single cell damage.

REPLY: We differ from the referee in that we don't consider that "*the molecular basis of plant responses to damage is in parts already well understood*". A number of molecular patterns are established or proposed to be involved in reporting damaged self (ATP, OGAs, cellobiose, AtPEPs, amino acids, most recently, L-Glu), yet most researchers in this field would not claim that any one of them faithfully reproduces what happens during actual cellular damage. The role of AtPEPs is particularly unclear in this context, as they are induced upon PAMP, jasmonate and other treatments and appear to have effects in the absence of damage itself, upon simple overexpression, for example (Huffaker et al., 2006), questioning its role as a damage reporter (it might act as a paracrine enhancer of PAMP signals, rather than a reporter of cellular damage).

Surely, the actual cellular damage that we induce will release an entire bouquet of DAMPs, together with varying degree of cell wall fragments, massive amount of vacuolar solutes, proteases and will additionally generate mechanical stresses on the neighboring cells. All of these factors are probably participating in signaling cellular damage to neighbors. The point of our paper was NOT to try and generate a simulacron of damage using known DAMPs.
Moreover, external application of DAMPs is very different from our single cell laser ablations, because ablations should cause a highly localized release of a cocktail of endogenous DAMPs at endogenous concentrations, impossible to recreate by the more or less systemic external DAMP treatments, mostly at concentrations that are very different from endogenous levels.

EXPERIMENTS TO ADDRESS THE REFEREE'S CONCERNS:
We have done treatments with high amounts of a number of different DAMPs, including AtPEP1. As you can see in Fig. 7A-G all of them lead to some degree of ethylene induction, but none of them to the same degree as actual damage of a single cell. Because of the multitude of known and potential DAMPs, we did not assume that knock-out of a single DAMP perception pathway would lead to any significant difference. We unfortunately cannot generate the triple pepr1 pepr2 mutant; ACS6 marker line in time for this revision. We have, however, measured surface depolarisations in the *pepr1 pepr2* double mutants and also obtained nematode performance pepr1 pepr2 double mutants (and jasmonate mutants in addition). As referee 1 can see below, neither surface depolarization, nor nematode performance are measurably affected by the *pepr1 pepr2* double mutant. We have now added this data as Fig. 7H and Fig. 9D in the revised manuscript.

POINT-BY_POINT REPLY FIG. 1 (Fig. 7H and 9D in manuscript):



2. Despite the re-invention of single-cell laser ablation technology, pioneered originally in the context of root development (Berg et al., Nature 1997), I remain sceptical whether the striking depolarization spikes observed opposite to the site of cell ablation (Fig. 1) provide truly novel physiological insight in root biology. Unlike root growth on agar or liquid media, Arabidopsis plants grown in natural soil are subject to continuous and extensive mechanical shear stress and other mechanical forces, leading to the abrasion of large parts of the root epidermis. This raises doubts whether single-cell laser ablation reported here is a meaningful proxy for nematode feeding as mechanical wounding and adaptation to cell damage is expected to be a continuous and integral component of root growth in soil in the absence of nematodes or necrotrophic root-colonizing microbes.

REPLY: We disagree with referee's contention that "*natural soils are (...) leading to the abrasion of large parts of the root epidermis.*" We would be very happy about any reference in support of this claim. It is generally considered that the root cap is continuously producing root cap cells that are providing a mucosal layer and undergo programmed cell death, precisely to protect the meristem and developing epidermis from such a fate (Sievers et al., 2002, for example).

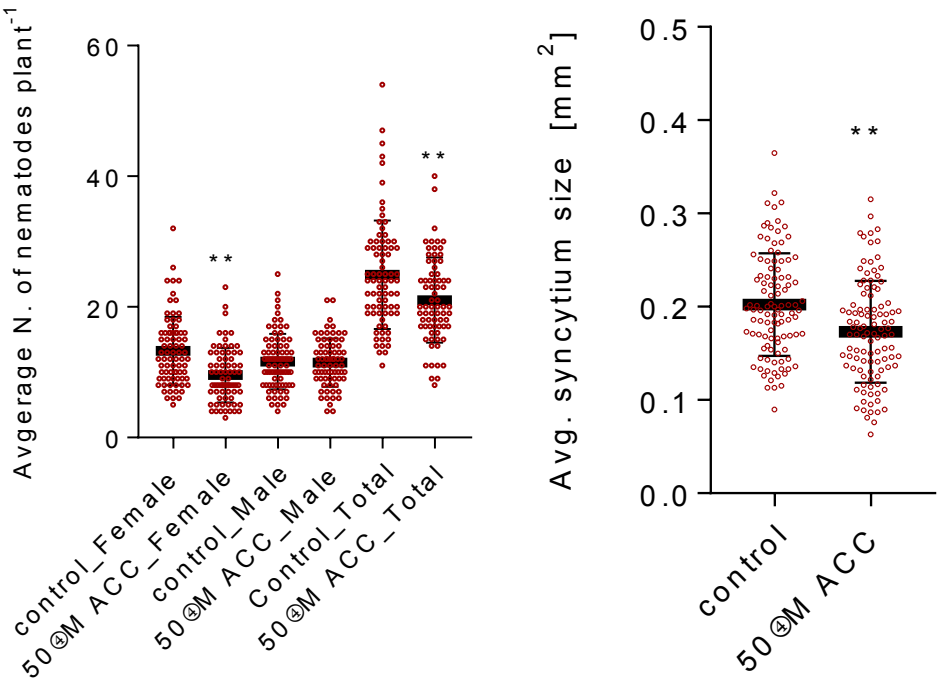
3. Fig. 6F. The authors find that interference with the ethylene pathway increases nematode success rate as indicated by a 'faster establishment of the ISC stage". I find this weak evidence because it remains unclear whether 'faster establishment of the ISC nematode stage' in Arabidopsis ein2 and ein3 mutants translates into a robust increase of H. schachtii reproductive success. This is technically feasible as shown in previous work by some of the authors (Siddique et al., 2014), which would involve assessing the average number of nematodes per plant present in WT, ein2, and ein3 mutant plants. Thus, it remains to be shown whether ein2 and ein3 mutants are more susceptible to H. schachtii infection.

REPLY: A successful cycle of nematode infection from initial attraction to invasion to ISC establishment to eventual reproduction is highly complex, and ethylene, jasmonate and other hormones might have various, sometimes opposing, effects on nematode success (Goverse et al., 2000; Wubben et al., 2001; Piya et al., 2018; reviewed in Gheysen and Mitchum, 2018). Assessing overall reproductive success rate in ethylene mutants might therefore confound distinct effects of ethylene on different stages.

We therefore decided to focus on the infection stage relevant to our analysis, i.e. from the first contact of the nematode, when the nematode breaks the first cells, until ISC establishment, at which stage the nematode stops moving and stops causing progressive cell damage.

EXPERIMENTS TO ADDRESS THE REFEREE'S CONCERNS:
In the hope of alleviating the referee's concern, we have nevertheless measured later stages of infection, assessing syncytium size, as well as female-to-male ratio. As the referee can see, in both cases, a significant difference between untreated and ACC-treated plants is maintained. However, we have refrained from integrating this data into the revised version.

POINT-BY POINT REPLY FIG. 2 (not integrated into manuscript):



4. Non-responsiveness of the pPR1::NLS-3xVenus 'SA marker line' to SA treatment and inconsistent induction of pJAZ10::NLS-3xVenus is both interesting and alarming as it highlights another major caveat of the current work, i.e. an extrapolation of knowledge on defence phytohormone responses and defence phytohormone crosstalk from leaves (Pieterse et al. Nat Chem Biology, 2009) to the root organ.

REPLY: We do not extrapolate knowledge from leaves. On the contrary, our entire story should be seen as an effort to provide unbiased, direct experimental data on wound responses in roots – and to even improve on data from leaves, since single cell ablations and cellular resolution imaging of responses would be much harder to do in leaves. We have established in Fig. S1 and S2 (now Figs. 1 and S1) that our markers respond consistently to their respective hormones in roots and are therefore adequate for our experiments. We use three different jasmonate response/production markers (one of them JAS9 being a direct, non-transcriptional sensor), for all of which we demonstrate responsiveness to MeJa in roots. The JAS9 data is now improved by use of a normalised variant, now shown in Fig. 1 I-L). We have also established that the SA reporter pPR1::NLS-3xVenus responds strongly in leaves and therefore pointed out that the non-responsiveness in roots indicates, at the least, that SA responses in roots and leaves are molecularly distinct. Beyond this we have not used the SA reporter for anything and do not see reason for alarm.

'Markers of defense phytohormone biosynthesis and signalling' have been extensively used in plant interactions with pathogenic leaf microbes, but rarely in root biology. Moreover, sparse information is available regarding transcriptome-wide responses of root tissue to pathogen challenge or treatment of roots with defence phytohormones. Thus, I am not convinced that the defence 'marker

lines' used in the present study serve as suitable tools to make physiologically relevant conclusions on phytohormone signalling and/or crosstalk in Arabidopsis roots.

REPLY: See our reply above, we urge referee 1 to take note of our controls. In Fig. S1 and S2 (now Figs. 1 and S1), we demonstrate that our markers respond consistently to their respective hormones.

What is missing in the present study are complementary quantitative measurements of the respective phytohormone levels in roots, at least SA, ET, and JA. This is especially important in ein2 and ein3 mutant to directly examine potential compensatory changes in the defence phytohormone network in roots.

REPLY: WE CANNOT measure phytohormones directly with the manipulations and spatial scales we are working at. We analyse damage in a single cortical cell in a region encompassing a few hundred micrometres. Differences in hormone levels in seedling would be almost certainly undetectable on the whole seedling level and dissecting the damaged region for analysis would defy the purpose of our experiments by introducing a much more massive damage than the one being analysed. A recent work from the Gasperini lab in Halle analyses pools of more than 10 entire seedling roots per data point in order to observe increases in jasmonate after large-scale leaf damage (Schulze et al., BioRxiv, 2018).

Our analysis should be compared to the use of DR5 or DII-Venus, TCS etc. for auxin and cytokinin in developmental biology, in which they are extensively used as powerful proxies of hormonal responses. Except for SA, which is a minor aspect of our work, we always based ourselves on two markers, three for jasmonate in order to establish, as well as possible, that we are indeed picking up differences in hormone production or response.

5. The application of pharmacological compounds known to interfere with chloride or potassium or proton pumps upon single-cell laser ablation provides preliminary evidence indicating that these channels contribute to an electrochemical signal propagating across the horizontal root axis (Fig. 2A, B). However, this must be considered preliminary evidence given the known off-target effects of the drugs applied and lack of supporting genetic evidence whether specific family members of these channels or pumps are necessary for propagation of depolarization upon ablation.

REPLY: We describe – for the first time to our knowledge – that single cell damage elicits a regional response in the root of higher plants. The widely-used drugs we have employed were simply used to establish that the rapid depolarisaiton/repolarization event depend on active cellular processes and require the activities of an expected set of ion channel and transporter activities. We did not intend to conclude anything further from our drug experiments and do not consider our evidence preliminary for reaching this basic conclusion.

Also, we would like to insist that we did provide genetic components of the depolarization signal by demonstrating that the depolarization amplitude is strongly affected in NADPH oxidase mutants, which we maintain to be both novel and important (this has not been established for systemic signal propagation in leaves, but is fitting nicely to various observations that ROS is required for cytosolic calcium increases (see Ranf et al., 2011 or Foreman et al., 2003 for example) and we now demonstrate that it applies to single-cell wound-induced signals (see Fig. 5B).

EXPERIMENTS TO ADDRESS THE REFEREE'S CONCERNS:

In order to provide additional genetic data, we have now also provided experiments demonstrating that the *glr3.3 ; glr 3.6* double mutant – required for systemic electrical signaling in leaves – is not required for our regional electric signaling upon single cell wounding. This is now integrated into the revised version as Fig. S5A.

Referee #2:

This manuscript describes studies of signaling and (reporter) gene-expression resulting from damage to a single root cell, first by laser ablation and then from nematode attack. The responses include [Ca2+] changes, electrical potentials, generation of reactive oxygen species - all components that

have been characterized during more-extensive wounding of leaves and other plant organs. With single-cell ablation the responses are localized; systemic signaling seems not to occur. The authors report that ablation led to the induction of ethylene-regulated genes but did not significantly induce jasmonate-responsive reporter genes.

The experiments with single-cell ablation are comprehensive with good controls. However, the nematode work seems to be a bit of an afterthought and leaves the story with an unsatisfying ending and rather weak discussion - particularly with respect to the (non-)role of jasmonate signaling. Additional experiments are needed to strengthen the authors' conclusions. Presentation of the results and the text also need to be improved.

REPLY: We are sorry for the issues with data presentation in figures and text and have worked a lot on improving this. As referee 2 can see the structure of the figures has been considerably re-worked and we have done our best to straighten-out our data presentation and make is more coherent and intelligible. The text has also been entirely re-read for inconsistent namings, typos, grammatical errors etc.

Main issues:

1. P8, l.231-234. "Interestingly, corroborating our observations upon laser ablation - but in contradiction to earlier studies (Kammerhofer et al., 2015), nematode invasion did not appear to induce a consistent jasmonate response, based on the lack of our JAZ10::NLS-3xVenus jasmonate reporter expression." This is a key finding that leaves the authors hand-waving through much of the Discussion (pp.8/9 l.250-280). The authors need to challenge jasmonate mutants with nematodes to provide another test of their conclusion, here. The prediction is that jasmonate mutants will provide ISC Selection times (cf Fig. 8F) that are as high (or higher? p9 l.280-281) than the wild-type, Col-0 control.

REPLY: We have now provided additional data concerning nematodes. Specifically, we have added data showing that nematode ISC stage establishment is indeed unaffected in jasmonate mutants, corroborating our data. This has been added as Fig. 9D in the revised version.

2. The nematode results also need to be strengthened by quantifying and analyzing the results in Fig.6 A&B (and relevant supplementary figures) like the results for the ablation experiments (Fig.1D, etc).

REPLY: We have now done these quantifications, presented in Figs. 8 and 9 of the revised manuscript.

3. The stronger results will allow the discussion of jasmonate (non-)signaling to be presented strongly and briefly, so that more of the Discussion can be spent on the laser-ablation technology and its comparison to nematode infection, and on the signaling events and ethylene-regulated induction of defense genes. This last aspect will benefit from more-extensive reading of the literature on ethylene in plant defense, and incorporation of this information into the Discussion.

REPLY: We are indeed not experts on ethylene responses, but have tried our best to read and cite the relevant literature and have now added a new paragraph treating ethylene responses. As suggested by the referee - and after further in-depth reading of the available data on jasmonate responses during nematode infection – we now conclude that jasmonate responses appear to have a significant role only after ISC establishment.

Issues relating to presentation: the manuscript needs to be carefully and comprehensively reworked to make the results more-accessible and to eliminate minor errors. These are the issues I noted, but there are likely to be more.

4. The figure legends (and text) use many abbreviations that are only spelled out in the M&M section (will this be on-line only?). They should also be explained in the main text, at first mention (or Figure legends).

REPLY: As stated above, we have entirely re-worked out text. We have also done our best to spell out all abbreviations either in the main text or figure legends.

5. The authors use wild type, Col-0 and Col in different figure legends. Use the same term throughout the figures and text.

REPLY: This is now corrected

6. In Fig.4 C&E, the reader is forced to assume "a" means ablation. Make it easy for them and spell out (or include in legend)

REPLY: We are sorry for this condensed notation, we have now spelled it out as suggested.

7. In Fig.2, the red arrowheads will be difficult to see in the printed journal (and online). Make them white.

REPLY: We thank the referee to pointing this out. We have changed the red for yellow arrowheads, because we use white arrowheads to indicate position of ablation.

8. Make clear that the R-GECO1 reporter line expresses the R-GECKO Ca2+ reporter.

REPLY: We are sorry for the confusion. This was a spelling mistake in the main text. We have used the R-GECO1 Ca2+ reporter lines as described in Keinath et al. and cited in the legends and Material and Methods section.

9. The Introduction and Results sections need paragraph breaks added to delineate the ideas and arguments.

REPLY: We have introduced these now.

10. If the supplementary material is going to appear in PDF format online, I recommend putting the figure legends (single-spaced) immediately below each figure. This will make it much easier for readers to view and assess the results.

REPLY: We have now done this in the revised version.

11. P4, l.102-104: "Interestingly, ACS6 responses were not exclusive to, but very much biased towards stele tissues, while PR4 responses were largely confined to the endodermis." Tell us if these observations are consistent with previously reported expression of the endogenous genes.

REPLY: It is a bit difficult to find high resolution data for differentiated root tissue – but our reported expression is entirely consistent with the reported expression of GUS marker lines in Tsuchisaka and Theologis, 2004 (now cited in the manuscript).

12. P4, l.116: "we found that our single cell ablations upregulated ACS6 and PR4 in a regional, but non-systemic fashion, encompassing a region of about 500µm for ACS6 (Supplementary Figure 6 and data not shown). If the 'data not shown' is needed to support this statement, it needs to be added to the Supplement, otherwise remove the phrase.

REPLY: We have removed “data not shown”.

13. P6, l.178. Quote the quantitative results on GdCl3 attenuation from Fig.3 to give the reader an accurate measure of this effect.

REPLY: The graph and legend of Fig. 4E,F now shows the average, 95% confidence interval, plots all the data point and mentions the significant threshold and precise n-values in the legends.

14. There are a modest number of typographical and grammatical errors through the text, Figure legends and supplement that need to be corrected.

REPLY: As stated above, we have entirely re-read and re-worked the manuscript text and hope to have eliminate most typographical and grammatical errors.

15. In the References the entry for Rojo etal needs initials added.

REPLY: Thank you for pointing this out. This has been corrected.

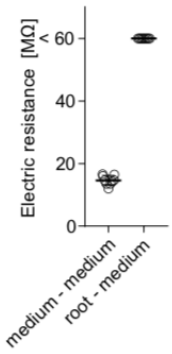
Referee #3:

Comments on the manuscript "Single cell damage elicits regional, nematode-restricting ethylene responses in roots"

General summary and opinion, question and finding:
The authors address the important and interesting question of how a primary wound performed on root is perceived and communicated to the neighboring cells and tissues. For this purpose, the authors developed a very elegant single cell wounding technique (laser ablation) mimicking a nematode attack. Combining this microsurgery technique with imaging measurement on plant lines expressing different markers the authors showed a predominant role of ethylene-in stress response while induction of jasmonate or salicylic acid is not observable. In this study, the authors also studied the electric potential variation induced by laser ablation and look for its involvement as a response involved in early signaling.

Specific major concerns.
This study based on live imaging performed on reporter lines provide new information on the kinetics and localization of calcium, ethylene and jasmonate responses induced by a laser cell ablation. My main concern concerns the electrophysiological response that I have difficulty to understand and to link to the other cellular responses observed.
That is to say:
(1) My first interrogation concerns the experimental design used in this paper to measure potential variation. More precisely the type of derivation (position of Ag/AgCl wires) used by the authors. Looking at the literature, very few measurements have been performed on root using contact electrode because roots have to be maintained in a wet condition. Therefore the film of water at the surface of the root create an electrical shunt and short circuit the electrical signal to be measured. Indeed, the situation in roots is much different than in aerial organs (leaf, shoot) for which the cuticle and air make an insulator. To summarize for the aerial part it is possible to record potential variations between electrodes disposed at the surface of the plant and a reference electrode. Then it is assumed (it is an approximation) that potential variation mirror the transmembrane potential variation of cells neighboring measuring (also reference in some situation) electrodes. Then this technique which allows to "follow" an electrical signal give information mostly on the velocity, intensity, path of propagation, ... but not (or very few) on the nature of the signal observed in aerial part.
In the light of the above; did the experimenter make some basic measurements to have an idea of the equivalent circuit between their reference and measuring electrode? For example, what is the background voltage between the reference and measuring electrodes (for two positions of the reference: close to the measuring (5 to 10 mm) and far from the measuring) and what is the electrical resistance between these two electrodes in these two conditions?

REPLY: We measured a resistance of > 60 MOhms between the electrodes positioned in the same way as for all experiments shown. 60 MOhms is the limit of the resistance we can measure and the actually resistance along the root may be far higher. The average resistance between electrodes placed at a similar distance in the root growth medium was 14-16 MOhms.



As information, they are serval papers on root membrane potential recorded with extracellular electrodes and vibrating probes. In these paper (Katou, Ichino, 1982; Meyer, Weisenseel, 1997, Hejnowicz et al., 1991) authors propose an explanation on the origin of potential variation measured.

REPLY: Thank you for these useful citations. Meyer and Weisenseel 1997 proposed that the release of negatively charged macromolecules from wounded cells produced what appeared to be an inward current. We did not wound the surface of the root and what we measured is likely to be transmitted changes in membrane potential as have been reported previously in roots (e.g. Mertz and Higinbotham, 1976).

(2) My second interrogation concerns the shape of the potential variation. The potential variation shown in fig;1 F present a very deep rising phase and a decrease similar to a discharge of capacitor. The duration of the whole signal is about 5 second. No information is provided on the velocity of the electrical signal and on the duration of the lag between cell ablation and starting of the potential variation. To my point of view this ddp variation in not an AP.

REPLY: We agree that this is not an AP and we did not use that term in the manuscript. The term 'wound potential' (as used by Simmen, 2001 and Stahlberg and Cosgrove, 1994) is still poorly defined and its shape and duration doesn't match what we observe.

I would hypothesis.(1) the sudden collapse of membrane potential is transmitted via electrocoupling (mainly via plasmodesmata) to neighboring cells inducing sharp membrane depolarization then membrane potential recover,

REPLY: We agree that this is a plausible interpretation, although there are of course alternatives.

or (2) the micromovement produce by laser ablation could suddenly modify the junction potential at the electrode tip,

REPLY: If this were the case we would not expect the signal to correlate with the genetic backgrounds we used. The strong effects of the rboh mutants (or indeed the chemical inhibitors) we used rules this possibility out.

or (3) we have also to keep in mind that an Ag/AgCl wire measure both electric potential and Cl- concentration making this electrode intrinsically a chloride-sensitive electrode (60 mV for a tenfold variation in Cl-). Therefore if the cell ablation induces a release of Cl- this local concentration variation should be sensed by the voltage electrode, or (4) other scenarios .....!

REPLY: Cortical cells were ablated but surface electrical activity is measured. It would be unlikely that Cl- could flow through the apoplast all the way to the electrode without some dispersal which would attenuate the sharp depolarization spike seen. Also, simple diffusion of Cl- from the damaged cell would mean that the chemical inhibitors we used would not function.

(3) My third interrogation concerns the interpretation of potential variation. If I understand well, the authors assume that variation potential they measure represent the variation of cellular transmembrane potential. In the paragraph "The short-distance electric signaling depends on

multiple ion channel activities" (line 146 to 154) and Fig.2A the authors show that blockers targeting various channels and transporter have all the same effect of abolishing the potential variation. That would mean that all these blockers with different mode of action and different target lead to a same and single result. The interpretation given by the authors "a complex interplay of the known major ions driving plant cell electrochemical gradients drives the observed propagation of depolarization upon ablation" is not clear to me and not sustained by the data (simplistic explanation). That leads me to ask two questions/remarks: (1) is there any inactive inhibitors that could be considered as control?, (2) it would be convincing to record (at least in some experiment) the potential variation induced by the inhibitor itself in order to show the efficiency of the blockers and also this would validate the recordings.

REPLY: We were surprised that all inhibitors reduced laser-induced depolarisations. Meyer and Weisenseel 1997 found strong inward ion currents close to quite large surface wounds in maize roots and, in contrast to our results, these currents were not blocked by a number of inhibitors. The authors concluded that a likely source of the currents was simply the release of negatively charged macromolecules from the surface wound. We wounded cells beneath the surface epidermal layer but we monitored on the root surface. What we measured is likely to be transmitted changes in membrane potential as have been reported previously in roots (e.g. Mertz and Higinbotham, 1976).

Please note that none of the well-established inhibitors completely blocked the electrical signal and that the inhibitors do not appear to act identically. For example, please see the different repolarisation profiles observed in the presence of GdCl3 or vanadium.

I didn't find any comment and mention on the electrical variation in the discussion part (line 243 to 302) of the manuscript. How the electrical signal could be involved in early responses and its link with calcium wave? We lack good readouts for early responses with which we can fully address the first part of this question.

REPLY: In the case of slow wave (variation) potentials in leaves the calcium peak occurs many seconds after the major membrane depolarization phase (Nguyen and Kurenda, et al., 2018). Our present observations are consistent with this, even though we do not call the root signals SWPs.

Line 91 to 95 (and corresponding figures). The authors wrote, "we mechanically crushed large population of root cells, similar to standard woundings done on leaves by crushing of root tips". Since the wounding performed on leave largely affect the vasculature I wonder whether also a large portion of the vessel is affected by wounding the root tip? This represents an important point in order to compare responses in two different organs.

REPLY: We expect extensive disruption of provascular tissues. Considering the precocious development of the phloem we would expect at least some differentiated phloem to be damaged. We also did crushings of fully differentiated root tissues, with very similar results. One such damage can be seen in Fig. S2E,F, where crushing was done in differentiated root parts, leading only to a weak and inconsistent (occurrence in only aobut 8 out-of 10 roots) upregulation of the jasmonate biosynthesis LOX6 GUS reporter.

Figure 2 A and H: the potential is completely restored after 5 sec while it takes 40 sec to the calcium to reach its maxim. Do the authors think that there is a link between potential and Calcium? This could be discussed in the view of recent results obtained on leaves by the authors (recently published).

REPLY: Please see the above response concerning the Nguyen and Kurenda paper.

Minor concerns:

Surface potential: the authors use the term "surface potential" (already used in previous paper and by some other authors). Surface potential in biophysics reefer to a precise definition related to fixed charges (linked to electrostatic potential). It is a bit misleading since potential variation in this paper is interpreted by the authors in term of transmembrane potential (involving potential generated by diffusion and pump activity) and not in term of "surface potential". In fact in the present experiments, the experimenters use surface electrodes (or contact electrode or extracellular

electrode) and they record potential variation in-between the two electrodes (measuring and reference). The potential variation can be theoretically generated anywhere in the (closed) circuit and not specifically at the surface of the measuring electrode.

REPLY: We are aware of the use of the term 'surface potential' in physics and biophysics, but the term has unfortunately a somewhat different and accepted meaning among plant physiologists. We are indeed aware that changes in the circuit may occur anywhere in the path between the two electrodes.

Regional potential: I don't clearly understand this term. In the present context, it appears too much unprecise. In electrophysiology, it already exists a more objective parameter call "space constant" to quantify over which distance a passive electrical signal is propagated in a cell/tissues. This quantitative parameter λ (in mm or µm) is the distance over which the steady-state voltage decays 37 % of its value at the origin.

REPLY: The effects we measure are regional i.e., spanning a good number of cellular distances, but not spreading through the entire organ, or crossing into other organs (which we would describe as "systemic" then). Yet, we did not use the term 'regional potential' to avoid coining yet another term.

I think (I might be wrong) that the authors force (in the abstract and later on in the manuscript) the comparison between the electrical responses in aerial part (propagation from leave to leave) and root. To my point of view, the electrical transmitted response studied in 8 week-old plant growing in a pot and elicited by wounding a large population of cell including the vessel as very little to do with the potential variation induced by wounding a single cell of a 5 day-old agar grown plant.

REPLY: We avoided using the terms 'slow wave potential' or 'variation potential'. We have adjusted the text to make it clearer that different signals are generated in the present experiments on roots than in previous experiments with leaves. We have made some comparative statements but we avoid stating that there are strict parallels between electrical signals in leaves and roots. (Please note that most plants used in studies of electrical signaling in the aerial parts are 5 weeks old).

Additional essential suggestions
In order to be in a clear and safe experimental condition, transmembrane potential should be recorded with an intracellular electrode. Microelectrophysiology is a well-established technique and they are many reports on transmembrane potential measured on different root cell type.

REPLY: We would expect these types of experiments to be extremely difficult to conduct and to interpret because an invasive intracellular electrode will puncture cells along the insertion route causing damage additional to that generated intentionally with the laser. In the present study this would add a great deal of unwanted complexity. The Farmer lab has experience with invasive intracellular electrodes and, while highly useful in many cases, these are extremely challenging to use in wounding studies. For example, we are aware that cell contents can bleed onto the organ surface from electrode-caused wounds and this can, in some cases, interfere with electrical circuits.

Conclusion:
It is an interesting paper. The electrophysiological response is worth to consider as early signal in root but results provided does not correspond to the outflow standard.

2nd Editorial Decision 19th Feb 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see, both referees recommend the manuscript for publication. However, before I can send the official acceptance letter, there are a few editorial issues concerning text and figures that I need you to address.

Please address the remaining concerns of the referee #3 as discussed on the phone.

REFEREE REPORTS:

Referee #2:

The authors have added data from new experiments and made changes to the manuscript that largely address the issues raised in my original review.

Referee #3:

Referee #3:

Comments on the manuscript "Single cell damage elicits regional, nematode-restricting ethylene responses in roots"
For this 2nd review of the manuscript I will address questions that seem essential to me related to the manuscript and to my field of expertise.

Still the major point for me is that the authors assume (they admit) that "surface potential" variation represent transmembrane potential variation whilst it is not demonstrated in their system neither in other root system. Surface electrodes are probes that record any potential difference originated by any electrogenic processes. My concern is justified by the fact that roots are growing in wet conditions and the water film at root surface establish an electric shunt that should considerably decrease (attenuate) potential variation initiated at the transmembrane level. This is attested by the fact that the resistance of the biological material (60 MOhms) is only four times higher than the resistance of culture medium (14-16 MOhms). In order to remove any doubt and to prove that extracellular potential variation represent transmembrane potential variation the straightforward demonstration is to record on the same root (example on hypocotyl, Spalding and Cosgrove, Planta 1988) "surface"potential and transmembrane potential at the same time. The author ONLY need, whatever they use as depolarizing signal, to show that "surface" potential variation is following transmembrane potential variation.

(1) In the last answer the authors developed a too much negative argumentation a bout measurement of transmembrane potential with microelectrode and there are some points that I cannot support:
- <I>"invasive intracellular electrode will puncture cells along the insertion"</I> : the diameter of the tip electrode around 1 µm does not damage the cell. Generally for a good impalement the transmembrane potential is even not affected (very negative), criteria indicating the good health of the cell. In order to avoid any wounding transmembrane measurement can be limited to epidermal cell.
- <I>"causing damage additional to that generated intentionally with the laser"</I> the purpose of the present experiment is to show if extr- and intra-cellular potential follow or not the same variation. That does not necessarily imply to perform a laser ablation. Please note that the publication you referred to in one of your answer (Meyer and Weisenseel 1997) describe a potential variation induced by wounding and registered with a microelectrode.
- <I>"extremely difficult to interpret"</I> the microelectrode recorded transmembrane potential is the only way to ascertain that the potential variation is originated by the plasma membrane. Additionally it allow to perfuse the root medium and to exactly show the effect of various pharmacological agent (channel blocker, pump uncoupler, ...) upon transmembrane potential. Then it is allowed to safely use the vocabulary "depolarization", "hyperpolarization".
- <I>"extremely difficult to conduct"</I> transmembrane potential have been recorded in plant cells since three decades. I agree that it is a tricky and time-consuming technique. "extremely difficult" as claimed by the authors is somewhat excessive!
(3) <I>"REPLY: We were surprised that all inhibitors reduced laser-induced depolarisations. Meyer

and Weisenseel 1997 found strong inward ion currents close to quite large surface wounds in maize roots and, in contrast to our results, these currents were not blocked by a number of inhibitors. The authors concluded that a likely source of the currents was simply the release of negatively charged macromolecules from the surface wound. We wounded cells beneath the surface epidermal layer but we monitored on the root surface. What we measured is likely to be transmitted changes in membrane potential as have been reported previously in roots (e.g. Mertz and Higinbotham, 1976).

Please note that none of the well-established inhibitors completely blocked the electrical signal and that the inhibitors do not appear to act identically. For example, please see the different repolarisation profiles observed in the presence of GdCl3 or vanadium."</I>

- I keep on being surprised by this experiment. I CANNOT see any significant difference between different inhibitors. <I>"inhibitors effects .... suggests that a complex interplay of the known major ions underlying plant cell electrochemical gradients drives the observed propagation of depolarization upon ablation"</I> I am puzzled about this sibylline sentence! What do the authors exactly mean? Do they mean that channels/transporter are involved in transmembrane potential? True control are missing in this experiment. I suggest (1) a treatment with KCl 150 µM which will deliver the same quantity of chloride than GdCl3 50 µM, (2) a pump activator such as AIA or fusicoccin or acid load (acetic acid 1mM).

(4) I continue to wonder why the authors did not discuss at all (not even mentioned in DISUSSION) their results about electrical signal induced by single cell root wounding. I assume that these electrophysiological experiments were conducted to test certain hypotheses and to address certain questions about root signalling?

(5) <B>TO BE POSITIVE</B>: for me, the most interesting and the most innovative electrophysiological finding of this paper concern the results in relation with the role of membrane localized NADPH oxidases: rbohA, rbohD, rbohF (line 203 to 209). Indeed authors provide both genetic and pharmacological information (Fig. 5A and B) that the (so called) "regional" potential depend on NADPH-transporter presence/activity. These transporters which transport electrons should provide a transmembrane electronic current which contribute to the membrane polarization. The potential generated by electron-transporter has been evidence in few case using patch clamp or standard electrophysiology (see: Trost et al, Biophysical Journal, dx.doi.org/10.1016/j.bpc.2017.05.006, 2017; Sijmons et al., Plant Physiol, 1984; Picco et al., Plant Physiol, DOI: https://doi.org/10.1104/pp.15.00642; 2015). In experimental approaches described in the literature, electron-transporters constitutively reduce externally added, hydrosoluble electron acceptors such as ferri-cyanide (FeCN).
My plausible interpretation of the results (provided that "surface potential" is an image of the transmembrane potential):
(1) Channels inhibitors used in Fig. 3A and B for some of them collapse the membrane potential (DCCD, vanadium) other block channels (Gd, TEA, ...) leading to the loss of transmembrane potential variation induced by wounding.
(2) NADPH inhibitors and rboh mutant (Fig. 5A and B) lead to the suppression or modification of the shape of the "depolarization". This suggest that the NADPH activity could directly generate the potential variation. The decay of the potential after reaching the peak could be due to the depletion of the natural electron acceptor in the apoplast. Such an hypothesis of the involvement of electron-transporter could be tested by adding an electron acceptor (FeCN, ...) in external medium. In such a case, we could expect a lengthening of the "depolarization" phase.

<B>IN CONCLUSION</B>, in order to validate the electrophysiological data of this paper (to make them believable and great) in is necessary to correlate on few recordings extra- and intra-cellular potential variations. If the authors agree with the hypothesis above, it might also be worth to validate (or invalidate) this hypothesis of an electronic current provided by NADPH-transporter as a electric signal involved in the root immune response.

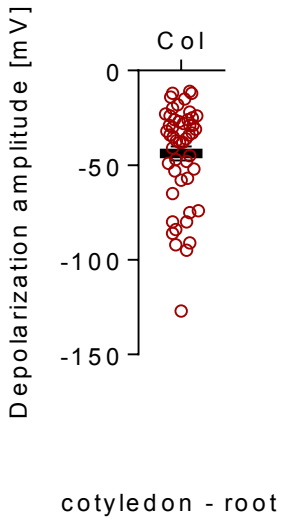
2nd Revision - authors' response 1st Mar 2019

Referee #3:

Comments on the manuscript "Single cell damage elicits regional, nematode-restricting ethylene responses in roots"
For this 2nd review of the manuscript I will address questions that seem essential to me related to the manuscript and to my field of expertise.

Still the major point for me is that the authors assume (they admit) that "surface potential" variation represent transmembrane potential variation whilst it is not demonstrated in their system neither in other root system. Surface electrodes are probes that record any potential difference originated by any electrogenic processes. My concern is justified by the fact that roots are growing in wet conditions and the water film at root surface establish an electric shunt that should considerably decrease (attenuate) potential variation initiated at the transmembrane level. This is attested by the fact that the resistance of the biological material (60 MOhms) is only four times higher than the resistance of culture medium (14-16 MOhms).
REPLY: This is not the case. We have clearly stated in our last reply that the equipment at our disposal does not allow to measure resistances above 60 MOhms and that the resistance between medium and root surface is at least 60 MOhms, but might well be much higher than that. In order to fully exclude the reviewer's concern that the measured depolarization upon ablation are generated by an extracellular shunt (although we still do not understand how, in such a scenario, inhibitors of transmembrane potential or NADPH oxidase mutant could interfere with this depolaristation), we have placed the reference electrode on the cotyledon, which is dry and disconnected from the agar block overlying the root. Ablation in this setup give similar readings of depolarization as in our original setup.

REPLY FIGURE 1:



In order to remove any doubt and to prove that extracellular potential variation represent transmembrane potential variation the straightforward demonstration is to record on the same root (example on hypocotyl, Spalding and Cosgrove, Planta 1988) "surface"potential and transmembrane potential at the same time. The author ONLY need, whatever they use as depolarizing signal, to show that "surface" potential variation is following transmembrane potential variation. (1) In the last answer the authors developed a too much negative argumentation a bout measurement of transmembrane potential with microelectrode and there are some points that I cannot support:
- *"invasive intracellular electrode will puncture cells along the insertion"* : the diameter of the tip electrode around 1 µm does not damage the cell. Generally for a good impalement the transmembrane potential is even not affected (very negative), criteria indicating the good health of the cell. In order to avoid any wounding transmembrane measurement can be limited to epidermal cell.
REPLY: We do not doubt feasibility of such measurements, which might be routinely done in the reviewer's laboratory. Yet, we maintain that it would be extremely difficult for us to set this up in our lab in a reasonable time-scale, especially if we want to maintain our depolarization stimulus

(laser ablation) which is the whole point of our paper. Please consider that our setup requires an inverted confocal microscope and that the surface electrode is on the other side of the root (from top). This setup makes it impossible to microscopically observe the site at which an intracellular electrode would have to be placed. In any case, we hope reviewer 3 agrees that our additional experiments have now sufficiently established that the depolarization we observe at the root surface must, in some way, be caused by changes in transmembrane potential.

- *"causing damage additional to that generated intentionally with the laser"* the purpose of the present experiment is to show if extr- and intra-cellular potential follow or not the same variation. That does not necessarily imply to perform a laser ablation. Please note that the publication you referred to in one of your answer (Meyer and Weisenseel 1997) describe a potential variation induced by wounding and registered with a microelectrode.
REPLY: Yes, but the damage induced was orders of magnitudes more important and done in a completely different setup. We don't know how we could avoid performing laser ablation, since our entire paper is based on investigating the response to this damage.

- *"extremely difficult to interpret"* the microelectrode recorded transmembrane potential is the only way to ascertain that the potential variation is originated by the plasma membrane. Additionally it allow to perfuse the root medium and to exactly show the effect of various pharmacological agent (channel blocker, pump uncoupler, ...) upon transmembrane potential. Then it is allowed to safely use the vocabulary "depolarization", "hyperpolarization".
- *"extremely difficult to conduct"* transmembrane potential have been recorded in plant cells since three decades. I agree that it is a tricky and time-consuming technique. "extremely difficult" as claimed by the authors is somewhat excessive!
REPLY: See our answer above.
(3) *"REPLY: We were surprised that all inhibitors reduced laser-induced depolarisations. Meyer and Weisenseel 1997 found strong inward ion currents close to quite large surface wounds in maize roots and, in contrast to our results, these currents were not blocked by a number of inhibitors. The authors concluded that a likely source of the currents was simply the release of negatively charged macromolecules from the surface wound. We wounded cells beneath the surface epidermal layer but we monitored on the root surface. What we measured is likely to be transmitted changes in membrane potential as have been reported previously in roots (e.g. Mertz and Higinbotham, 1976).*

Please note that none of the well-established inhibitors completely blocked the electrical signal and that the inhibitors do not appear to act identically. For example, please see the different repolarisation profiles observed in the presence of GdCl3 or vanadium."

- I keep on being surprised by this experiment. I CANNOT see any significant difference between different inhibitors. *"inhibitors effects .... suggests that a complex interplay of the known major ions underlying plant cell electrochemical gradients drives the observed propagation of depolarization upon ablation"* I am puzzled about this sibylline sentence! What do the authors exactly mean? Do they mean that channels/transporter are involved in transmembrane potential?
REPLY: We changed this sentence to make is less sibylline (now page 11, line 325-330). But yes, we simply wanted to say that many of the drugs known to inhibit transmembrane potential also inhibit the surface potential changes that we observe after ablation, suggesting that the changes depend on the plant cell transmembrane potential?

True control are missing in this experiment. I suggest (1) a treatment with KCl 150 µM which will deliver the same quantity of chloride than GdCl3 50 µM,
REPLY: Please note that the experiments are done in 0.5 MS medium, which contains chloride ions at a concentration of 1.5 mM already, making it implausible that an addition of an additional 1/10 of this concentration accounts for the effects of GdCl3.
(2) a pump activator such as AIA or fusicoccin or acid load (acetic acid 1mM).
REPLY: We thank the reviewer for this suggestion! We have done fusicoccin treatments and have observed that fusicoccin does not inhibit, but rather enhances the surface depolarization response after ablation (now added as Fig. 3A-C). We hope that this at least alleviates the reviewers concerns about the fact that all inhibitors have similar effects. Fusicoccin at least, does the opposite.

(4) I continue to wonder why the authors did not discuss at all (not even mentioned in DISUSSION) their results about electrical signal induced by single cell root wounding. I assume that these

electrophysiological experiments were conducted to test certain hypotheses and to address certain questions about root signalling?
REPLY: We now discussed the electrophysiological measurements in more detail (see also our reply below)

(5) **TO BE POSITIVE**: for me, the most interesting and the most innovative electrophysiological finding of this paper concern the results in relation with the role of membrane localized NADPH oxidases: rbohA, rbohD, rbohF (line 203 to 209). Indeed authors provide both genetic and pharmacological information (Fig. 5A and B) that the (so called) "regional" potential depend on NADPH-transporter presence/activity. These transporters which transport electrons should provide a transmembrane electronic current which contribute to the membrane polarization. The potential generated by electron-transporter has been evidence in few case using patch clamp or standard electrophysiology (see: Trost et al, Biophysical Journal, dx.doi.org/10.1016/j.bpc.2017.05.006, 2017; Sijmons et al., Plant Physiol, 1984; Picco et al., Plant Physiol, DOI: https://doi.org/10.1104/pp.15.00642; 2015). In experimental approaches described in the literature, electron-transporters constitutively reduce externally added, hydrosoluble electron acceptors such as ferri-cyanide (FeCN).
My plausible interpretation of the results (provided that "surface potential" is an image of the transmembrane potential):
(1) Channels inhibitors used in Fig. 3A and B for some of them collapse the membrane potential (DCCD, vanadium) other block channels (Gd, TEA, ...) leading to the loss of transmembrane potential variation induced by wounding.
REPLY: We concur. This is now explicitly discussed in this way in the discussion section.

(2) NADPH inhibitors and rboh mutant (Fig. 5A and B) lead to the suppression or modification of the shape of the "depolarization". This suggest that the NADPH activity could directly generate the potential variation. The decay of the potential after reaching the peak could be due to the depletion of the natural electron acceptor in the apoplast. Such an hypothesis of the involvement of electron-transporter could be tested by adding an electron acceptor (FeCN, ...) in external medium. In such a case, we could expect a lengthening of the "depolarization" phase.

IN CONCLUSION, in order to validate the electrophysiological data of this paper (to make them believable and great) in is necessary to correlate on few recordings extra- and intra-cellular potential variations. If the authors agree with the hypothesis above, it might also be worth to validate (or invalidate) this hypothesis of an electronic current provided by NADPH-transporter as a electric signal involved in the root immune response.
REPLY: We thank the reviewer for pointing out this possibility, which we had speculated about in our groups, but did not think appropriate to discuss. We have now done so, hopefully prompting future experiments exploring this intriguing possibility that has until now been very much neglected in the plant field.

3rd Editorial Decision 7th Mar 2019

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in The EMBO Journal.

Congratulations on the very nice work!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Geldner
Journal Submitted to: EMBO Journal
Manuscript Number:

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Generally sample size is chosen empirically, based on prior experience of how big a sample size must be to most probably obtain a reproducible, statistically significant result. Expected "size-of-effect" is taken into account.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from this submission
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	blinding was not applied
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	ttest was appropriate justified
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	all individual mesurments are presented in respesctive gapghs
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	in terms of variation coefficient are similar and comparable between groups mesurments

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-tun

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/

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).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* 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.	Heterodera sachtii - widely distributed
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

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

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	Data are provided in the Material and Methods (the mandatory items are not applicable for this manuscript). All published marker lines are in a central laboratory seeds stock and are distributed upon request.
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).	Supplementary data are provided as Expand View
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).	N/A
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.	N/A

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.	N/A