# A coherent feed-forward loop drives vascular regeneration in damaged aerial organs of plants growing in a normal developmental context

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**Original submission**

First decision letter

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MS TITLE: A coherent feed forward loop drives vascular regeneration in damaged aerial organs growing in normal developmental-context

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

The authors found the PLT-CUC2 module is necessary for vascular regeneration after injuries in Arabidopsis thaliana. Their finding is quite interesting and give an impact on the research field of developmental biology.

*Comments for the author*

The authors found the PLT-CUC2 module is necessary for vascular regeneration after injuries in Arabidopsis thaliana. Their finding is quite interesting and give an impact on the research field of developmental biology. However, the present manuscript includes the following revised portions.

1. Abstract
The authors show no evidence of the conservation of PLT function across the plant species.

2. Fig1
The scale bars should be more narrow. The scale bar in panel J should be straight. All insets should be the same size and be displayed side by side because the present insets are too small. Why do the authors show green dot lines with unequal spacing. Add the explanation to the figure legend. The size of green and blue dots close to the size of signals on nuclei seem to be confusing. The white dotted line in Fig 1H should be revised because the red fluorescence can be detected within the wound region. The audience may misunderstand the timelapse images with 0, 12 and 24 hr. Add the explanation of the image from different samples at each time point to the figure legend.

3. line 130
The higher expression in the upper end in Fig 1D is obscure.

4. line 136-139
The authors claim that PLT3 and PLT5 expression patterns show similar to that of PLT7 with some differences. However, the expression pattern of only PLT5 is detected in the region apart from the wound site as shown in Fig. S1L. The expression pattern of PLT5 is clearly different from those of PLT3 and PLT5.

5. line 141-142
The authors claim that no expression of PLT1 or PLT2 and WOX5 at 12 or 24 hr after the injury, respectively, as shown in Fig. S1U-R. However, the expression of PLT3, 5 and 7 start from 12h after the injury. There is a possibility that PLT1, PLT2 and WOX5 express at more later time point because they are downstream factors of PLT3, 5 and 7.

6. line 171
57/85 (67%) is not “fully penetrant”.

7. Materials in the section of vascular regeneration in “a growing leaf”
The developmental stage and the position of leaf including days after sawing are extremely important in the regeneration competency. Add the material information to the text. Only a growing leaf is obscure.

8. line 208-209
40% can be calucurated from Fig. 2I’. (78-47)/78=0.4
How did the authors get ~45%?

9. line 220-222
The regeneration rates of plt3/5/7 and plt3/7 are 60% and 64%, respectively. Is it really that PLT5 is required for the regeneration? Moreover, this description is inconsistent with the data in Fig. S5A, showing that the regeneration defect in plt3; plt7 is not significant whereas that in single plt7 is significant.

10. Figure 2
The width of scale bars in panels A to F should be unified. The inset of panel K should be added.

11. line 267-278
Add the data of PLT3 overexpression. PLT3 or PLT5 overexpresion suggests that only PLT5 functions in reprogramming. Add the discussion on the interpretation of overexpression data.

12. Figure S7C-F Add the information of samples and how to do the injury to the figure legends.

13. “wound repair” and “vascular regeneration”
Two words, “wound repair” and “vascular regeneration” should be used clearly and properly.
(1) line 369
It seems better that “wound repair” may be “wound repair and vascular regeneration”.
(2) line 504
It seems better that “wound repair” may be “vascular regeneration”.

14. Figure 4
CUC2 imaging data in plt3/5/7 is inconsistent with the expression data as shown in Fig. S6L. In Figure 4, the CUC2 expression seems to be upregulated at 24h after the injury. But the graph of Fig. S6L demonstrates the downregulation. Add the explanation. Moreover, add the time information after the injury in panel D to the figure legend.

15. Figure 5
ATHB8-YFP signals are indistinguishable from PIN1-GFP. Although the authors claimed that ATHB8-YFP can be detected in developing procambium, there are only GFP fluorescence in A and A’.

16. Figure 6
Perform the statistical test in panel A.
In panel N, PLT before Step1 and Step2 should be PLT3, 5, 7 and PLT1, 2.
There is no evidence on “YUC4→CUC2” in panel O.

17. line 500-501
There is no data of CUC2 in vascular regeneration of stems.

18. line 504
The authors should add PLT7 to “Activation of YUC4 by PLT5’’.

19. Supplementary Figure 6L
Perform the statistical test. Add the time information after the injury.

Reviewer 2

*Advance summary and potential significance to field*

The manuscript follows up on previous work by the Prasad group looking at the role of the plethora genes during regeneration (Kareem et al 2015). Here, they undercover a role for PLT3,5,7 during vascular reconnection and callus formation in wounded leaves and stems. They go onto to show that CUC2 also plays a role, and together, these genes upregulate auxin biosynthesis via YUC4. The manuscript provides a nice mechanistic overview for how increases in plethora levels might lead to wound healing.

*Comments for the author*

I have some concerns or suggestions for improvement:

- The authors state that PLT3,5,7 is necessary for leaf vein regeneration, and mutants fail to form regenerative veins (lines 41, 182-186, 248, 255). This in only partially true since regeneration decreases from 80% to 40% (Figure 2) but still occurs. These statements should be rephrased to indicate that PLT3,5,7 contributes to regeneration, but is not absolutely required.
- The rice PLT experiments are fine, but too much is interpreted from these data. Simply because rice PLTs can rescue the Arabidopsis phenotype doesn’t mean their function is conserved in rice. Instead, similar experiments would need to be conducted in rice to show function in rice (are PLTs induced by wounding in rice? Are the PLT mutants in rice unable to heal wounds?). The abstract (line 47-48) and this section (line 286-288) need rephrasing. The discussion also (593-598).
- It’s not clear how the PLT7-GR overexpression experiments (Figure 3) are examples of vascular regeneration or wound healing. They appear more like de novo organ formation (Kareem et al

2015). Phenotypes associated with callus formation at the wound site, or an increase in vascular regeneration, could be measured instead.
- The authors state that “The PLT-CUC2 module is required for vascular regeneration, but is dispensable for vascular tissue development per se” (line 48) but they don’t measure vascular development in the PLT, CUC2 quadruple mutants. Measurements could be made to test their statement. The vascular measurements used in the manuscript are measuring the number of loops in early leaf development. Other examples of vascular development or vascular anatomy changes are not tested so it’s tough to say whether their statement is accurate. A more specific statement would be more appropriate

Minor points

- PLT induction in Figure 1 and S1 is not always clear or obvious for intermediate time points. The green, blue and white circles and dotted lines are not always helpful and distract from the images. Blue and green arrows are too small to see. Incision or abrasion could be labelled on the relevant panels. Can panels 1H-K include zoom ins to better see the area of interest? Panels 1B and E are difficult to see signal – is there a clearer way to see the signal? PLT5 induction is not clear in S1.
- Maximum intensity projections (ie Figure 1) for confocal stacks create an image where each pixel contains the maximum value over all images in the stack at the particular pixel location, and is not the best way to show data since it is not quantitative. Average intensity projections should be used instead and are more representative of the data in the stack.
- Figure 5 is tough to see. PIN1 and AtHB8 signal are not very clear – can some of the excessive arrows and markings be removed? Why does DR5 signal decrease so much in Figure 5G, 5G’.
- YUC4-PLT ChIP or Dapseq data would be useful to show
- Quantifying vein loops in the yuc1yuc4 mutant would be a useful control
- Figure 6 model is drawn to make it appear that CUC2 is dependent on PLTs. The data in the paper suggest that CUC2 appears only partially dependent on PLTs. It would make more sense to have both CUC2 and PLT at the top of the diagram, and both regulating YUC4. A horizontal arrow could be drawn from PLT to CUC2 to indicate that CUC2 is partially regulated by PLTs.
- What is “AND gate” (line 609)?
- Figure S9 – R, S, T are not clear differences. Can these differences be quantified?

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**First revision**

Author response to reviewers' comments

**RESPONSE TO COMMENTS OF REVIEWER1**

1. Abstract: The authors show no evidence of the conservation of PLT function across the plant species.
Thank you very much for pointing this out. We agree that at present our data do not lead to the conclusion that the function of PLT genes is conserved across plant species. Currently our data only demonstrate that rice PLT-like gene can repair the wound and regenerate vascular tissue in Arabidopsis. Therefore, we have removed the sentence related to “conservation of PLT function across the plant species” from the abstract.

2. Fig1: The scale bars should be more narrow. The scale bar in panel J should be straight. All insets should be the same size and be displayed side by side because the present insets are too small. Why do the authors show green dot lines with unequal spacing. Add the explanation to the figure legend. The size of green and blue dots close to the size of signals on nuclei seem to be confusing. The white dotted line in Fig 1H should be revised because the red fluorescence can be detected within the wound region. The audience may misunderstand the time lapse images with 0, 12 and 24 hr. Add the explanation of the image from different samples at each time point to the figure legend.
Thank you for these very useful suggestions. We have edited the scale bar to a narrow size and ensured that the style remains uniform throughout the manuscript. The scale bar in panel J has been straightened. We have added enlarged views of the YFP channels below the corresponding image

panels for clarity in the case of inflorescence abrasion and incision. We have arranged the panels and maintained the uniformity in panel size as per your suggestion. To avoid confusion, we have removed all the dotted lines and other markings in the image panels. Currently we use white asterisks to mark wound regions. The dotted line in Fig1H has been revised. We have added the explanation for sample corresponding to each time point to the figure legend (lines 809-810).

3. line 130:The higher expression in the upper end in Fig 1D is obscure.
Fig.1D represents the control sample at 0 hour post incision. Fig1E refers to the sample at 6hours which shows higher expression marked within the dotted rectangle at the upper end of partially incised inflorescence stem. To avoid confusion we no longer refer to Fig.1D in line 129. Also we have added a separate panel showing only YFP signal (in YFP channel) showing the expression in the upper end of the partially incised inflorescence (Fig1E').

4. line 136-139:The authors claim that PLT3 and PLT5 expression patterns show similar to that of PLT7 with some differences. However, the expression pattern of only PLT5 is detected in the region apart from the wound site as shown in Fig. S1L. The expression pattern of PLT5 is clearly different from those of PLT3 and PLT5.
Thank you for pointing it out. We have edited the sentence to : "In response to injury, PLT3::PLT3-vYFP and PLT5::PLT5-vYFP also showed upregulation of expression in the vicinity of the would albeit with some differences in the timing of their activation and in spatial distribution. In response to leaf incision, while both PLT3 and PLT7 are expressed in close proximity to the wound, PLT5 is expressed predominantly in the vascular tissue near the damage" (lines 135-140).

5. line 141-142:The authors claim that no expression of PLT1 or PLT2 and WOX5 at 12 or 24 hr after the injury, respectively, as shown in Fig. S1U-R. However, the expression of PLT3, 5 and 7 start from 12h after the injury. There is a possibility that PLT1, PLT2 and WOX5 express at more later time point because they are downstream factors of PLT3, 5 and 7.
Thank you for evoking the possibility of PLT1, PLT2 and WOX5 detection at more later points. In the revised version of the manuscript we have now included the later time points including 3days after injury. However we did not detect the expression of PLT1, PLT2 and WOX5 even at the later time points (Fig. S4 U,X,A').

6. line 171:57/85 (67%) is not "fully penetrant".
Thank you very much for the correction. We have edited the text.

7. Materials in the section of vascular regeneration in "a growing leaf". The developmental stage and the position of leaf including days after sawing are extremely important in the regeneration competency. Add the material information to the text. Only a growing leaf is obscure.
Thank you for the valuable advice. Because of the space constraints we had restricted the description to method section. We have now elaborated the details in the main text of revised version of the manuscript (lines 180-192).

8. line 208-209: 40% can be calculated from Fig. 2I'. (78-47)/78=0.4.How did the authors get ~45%?
Thanks for the correction. We have edited the text to read 40% (line 210).

9. line 220-222:The regeneration rates of plt3/5/7 and plt3/7 are 60% and 64%, respectively. Is it really that PLT5 is required for the regeneration? Moreover, this description is inconsistent with the data in Fig. S5A, showing that the regeneration defect in plt3; plt7 is not significant whereas that in single plt7 is significant.
Thank you for raising this point. We guess you meant plt 5,7 (not single plt7). We revisited our data. In FigureS8A, plt3/5/7 shows only ~40 per cent regeneration while plt3/7 shows ~64 per cent regeneration and plt3/5 and plt5/7 double mutants showed ~70 per cent regeneration. We performed the Pearson's chi squared test again for evaluating the significance and we found the same result. The statistical analysis shows that the difference between wildtype and plt3/7, and between wildtype and plt3/5 is not significant. We noticed that error bar in plt3/7 is larger while plt5/7 has a shorter error bar compared to wildtype. The statistical significance analysis reflects the differences in the size of error bars among these samples. We believe that such variations for plt3/7 and plt3/5 may be related the genetic backgrounds. We have corrected the text to read consistent with results (lines 219-222).

10. Figure 2:The width of scale bars in panels A to F should be unified. The inset of panel K should be added.
Thank you for the suggestion. We have made the thickness of scalebars uniform and we have also added inset in panel K.

11. line 267-278: Add the data of PLT3 overexpression. PLT3 or PLT5 overexpresion suggests that only PLT5 functions in reprogramming. Add the discussion on the interpretation of overexpression data.
To study the consequences of PLT5 and PLT7 overexpression we used 35S::PLT5-GR and 35S::PLT7-GR lines as only these lines were available with us. In the revised manuscript, we have replaced the *de novo* organogenesis data with phenotypes associated with leaf vascular regeneration and callus formation at the wound site upon inducible over expression of PLT5 and PLT7 (Fig3 A-D' and S8 D-F'). Consistent with our data on loss of function of PLT genes, we find that inducible over expression of PLT5 as well as of PLT7 promotes the wound repair and leads to increase in vascular regeneration. We have added the new data, described and interpreted in the revised manuscript (lines 237-248).

12. Figure S7C-F:Add the information of samples and how to do the injury to the figure legends.
Thank you for the suggestion. We have added the sample number and details of the injury to the figure legend (new Fig. S10 C-F) in addition to the methods section.

13. "wound repair" and "vascular regeneration".Two words, "wound repair" and "vascular regeneration" should be used clearly and properly.(1) line 369;It seems better that "wound repair" may be "wound repair and vascular regeneration".(2) line 504;It seems better that "wound repair" may be "vascular regeneration".
We have edited the text (new lines 320 and 407) as per your suggestion. Thank you for pointing it out.

14. Figure 4:CUC2 imaging data in plt3/5/7 is inconsistent with the expression data as shown in Fig. S6L. In Figure 4, the CUC2 expression seems to be upregulated at 24h after the injury. But the graph of Fig. S6L demonstrates the downregulation. Add the explanation. Moreover, add the time information after the injury in panel D to the figure legend.
We believe that the increase in CUC2-3X VENUS expression at 24 hours is likely due to the difference in developmental stage of the leaf between 0 hour and 24hour. In RT-qPCR we have taken samples from seedling after 12 hour of injury. Also, while collecting the samples for RT-qPCR we do not rule out the possibility of inclusion of adjacent shoot tissues besides the damaged ones. The difference in S6L (new S9L) and Figure4 can be attributed to these technical and temporal variations. We have added this information in revised methods. Also, turn-over of 3X VENUS YFP can contribute to differences between transcript levels measured by RT-qPCR and reporter activity by fluorescent signals from 3XYFP.For clarity we have added the details of duration after injury to the figure legend for S6L (new S9L) and Fig.4D.

15. Figure 5:ATHB8-YFP signals are indistinguishable from PIN1-GFP. Although the authors claimed that ATHB8-YFP can be detected in developing procambium, there are only GFP fluorescence in A and A'.
We agree with you that the YFP signal was not clear due to the predominant expression of PIN1-GFP. Therefore, in the revised version of the manuscript we have provided the GFP channel (representing PIN1-GFP), YFP channel (representing ATHB8-YFP) and merged images of all the panels for better clarity of the data (Fig5 A- F).In addition, we have replaced and included new panels showing expression of ATHB8-YFP and PIN1-GFP at 0h of incision in both wildtype and *plt3;plt5-2;plt7* for better clarity (Fig5 A,B,F(for 24h)). Thank you for pointing it out.

16. Figure 6:Perform the statistical test in panel A. In panel N, PLT before Step1 and Step2 should be PLT3, 5, 7 and PLT1, 2.There is no evidence on "YUC4→CUC2" in panel O.
We have now performed the statistical analysis for panel A in figure6.As suggested we have edited PLT to PLT3, 5, 7 and PLT1, 2 in step1 and step 2 respectively in the revised version of the schematic (Fig 6N). We apologize for the mistake in the schematic. Indeed, we do not have evidence for YUC4 regulation of CUC2.Currently we have genetic and molecular evidence only for the coherent feed forward loop, and we have corrected the schematic accordingly (Fig6.O)

17. line 500-501.There is no data of CUC2 in vascular regeneration of stems.

Thank you for encouraging us to provide functional data for the role of CUC2 in vascular regeneration of stem. Both *cuc2-3* and *cuc2-1D* mutants are severely impaired in vascular regeneration. We have now added new data on inflorescence vascular regeneration (Fig4. H; lines 310-311).

18. line 504:The authors should add PLT7 to "Activation of YUC4 by PLT5''.
Thank you for the suggestion. We have now added RT-qPCR data showing rapid transcriptional activation of YUC4 by PLT7 as early as 4hours even in the presence of translational inhibitor, cycloheximide (S12 J). We have added the new results in the text (line 407-408).

19. Supplementary Figure 6L.Perform the statistical test. Add the time information after the injury.
We have now performed the statistical analysis in S6L (new S9L) and we have added the information about the time of injury to the figure legend. Thank you for pointing it out.

## RESPONSE TO COMMENTS OF REVIEWER2

1. The authors state that PLT3,5,7 is necessary for leaf vein regeneration, and mutants fail to form regenerative veins (lines 41, 182-186, 248, 255). This in only partially true since regeneration decreases from 80% to 40% (Figure 2) but still occurs. These statements should be rephrased to indicate that PLT3,5,7 contributes to regeneration, but is not absolutely required.
Thank you for the suggestion. We have rephrased these sentences to reflect the data that PLT3,5,7 contributes to vascular regeneration in leaf (lines 46,171-176,824,832-833)

2. The rice PLT experiments are fine, but too much is interpreted from these data. Simply because rice PLTs can rescue the Arabidopsis phenotype doesn't mean their function is conserved in rice. Instead, similar experiments would need to be conducted in rice to show function in rice (are PLTs induced by wounding in rice? Are the PLT mutants in rice unable to heal wounds?). The abstract (line 47-48) and this section (line 286-288) need rephrasing. The discussion also (593-598).

Thank you very much for pointing this out. We agree with you that at present our data do not lead to the conclusion that the function of PLT genes is conserved across plant species. As you have stated, to make such conclusion one needs to carry out the analysis of function of rice PLT gene in rice too. At present we do not have any plt mutant in rice. Such studies will not be trivial as rice has a large PLT gene family and there is likelihood of redundancy among rice PLT genes. At present we are unable to carry out any analysis of wound healing in rice. I hope you would agree with us that such studies can take several years and is not in the scope of present work as the main focus of current manuscript is the regulatory mechanisms of wound repair and vascular regeneration in Arabidopsis.

In light of your comments, we have removed the sentence related to "conservation of PLT function across the plant species" from the abstract and edited the results and discussion accordingly (lines 255-257,468-470).

3. It's not clear how the PLT7-GR overexpression experiments (Figure 3) are examples of vascular regeneration or wound healing. They appear more like de novo organ formation (Kareem et al 2015). Phenotypes associated with callus formation at the wound site, or an increase in vascular regeneration, could be measured instead.

Thank you for this very important and valuable advice. As per your suggestions, in the revised manuscript, we have replaced the de novo organogenesis data with phenotypes associated with leaf vascular regeneration and callus formation at the wound site upon inducible over expression of PLT5 and PLT7 (Fig3 A-D' and S8 D-F'). Consistent with our data on loss of function of PLT genes, we find that inducible over expression of PLT5 as well as of PLT7 promotes the wound repair and leads to increase in vascular regeneration. We have added the new data and revised the results section (lines 237-248).

4. The authors state that "The PLT-CUC2 module is required for vascular regeneration, but is dispensable for vascular tissue development per se" (line 48) but they don't measure vascular

development in the PLT, CUC2 quadruple mutants. Measurements could be made to test their statement. The vascular measurements used in the manuscript are measuring the number of loops in early leaf development. Other examples of vascular development or vascular anatomy changes are not tested so it's tough to say whether their statement is accurate. A more specific statement would be more appropriate.

Thank you very much for the suggestion. As per your suggestion we have now rephrased the statement in the abstract as "The PLT-CUC2 module is required for vascular regeneration, but is dispensable for midvein formation in leaf during normal development" (lines 51-52). We have also edited rest of the text accordingly. Because of the severe developmental phenotypes in PLT/CUC2 quadruple mutant, we analysed only the leaves of heterozygous plants for the midvein regeneration assay in supplementary table1. As you will notice, midvein regeneration is impaired in the genetic background heterozygous for *plt3;plt5-2;plt7* and *cuc2-3* (*plt3*$^{+}$/-;*plt5-2*$^{+}$/-;*plt7*$^{+}$/-;*cuc2-3*$^{+}$/-) but its formation during normal development is not compromised (S7L). The midvein in quadruple heterozygous mutant develops like wildtype and remains intact in all the samples tested (Fig S7L). We have now included the data for the number of vein loops of the PLT/CUC2 heterozygous leaves in Fig. S7F, S7G, S7L.

In our manuscript we have focussed on regeneration of midvein in response to local wounding in the leaf. Therefore, we restricted our analysis only to midvein formation during normal development. Because we had analysed vascular regeneration in leaves collected from 8dpg plants, we also analysed the normal development of vein loops during the same stage to ensure consistency in developmental stage. While studying loop formation during normal development, our main focuses were:

1> If there is any discontinuity in midvein formation during normal development.

2> We decided to score the number of loops because it is the closest lateral veins which get connected to the midvein to form the loop.

In all the mutants used for vascular regeneration assay, we never observed any discontinuity in the midvein in any developmental stage. Upon analysing the vein loop formation, we did not detect any significant difference in number of loops between the wildtype and mutants as we have already shown using leaves from 8dpg plants (S7F). Additionally, now we have also performed vein loop analysis using leaves collected from 10dpg old plants (S7G).

Minor points

5. PLT induction in Figure 1 and S1 is not always clear or obvious for intermediate time points. The green, blue and white circles and dotted lines are not always helpful and distract from the images. Blue and green arrows are too small to see. Incision or abrasion could be labelled on the relevant panels. Can panels 1H-K include zoom ins to better see the area of interest? Panels 1B and E are difficult to see signal – is there a clearer way to see the signal? PLT5 induction is not clear in S1.

Thank you for pointing out these corrections. We have now included separate panels for YFP channels corresponding to all images of PLT3,PLT5 and PLT7 showing abrasion and incision in inflorescence in both the main figure1 and supplementary figure1.We have removed all the green, blue and white markings denoting expression in the image panels and we have added the relevant labels in the revised figures. We have included zoomed in images of the wound site in Figure1 for panels I and K. Separate panels have now been added to show the expression in panels 1B,1E and S1.

6. Maximum intensity projections (ie Figure 1) for confocal stacks create an image where each pixel contains the maximum value over all images in the stack at the particular pixel location, and is not the best way to show data since it is not quantitative. Average intensity projections should be used instead and are more representative of the data in the stack.

In light of your suggestion, we have now included a version of figure1 using average intensity projection of the panels used in main figure1 (Fig S2). Due to the very weak intensity of signal we preferred the maximum intensity projection panels for main figure1. Additionally we have also included the raw data for both the projections in Mendeley (https://data.mendeley.com/datasets/mwyxw4v63h/draft?a=e64505aa-564b-4127-9d0c-afc900810544).

7. Figure 5 is tough to see. PIN1 and AtHB8 signal are not very clear – can some of the excessive arrows and markings be removed? Why does DR5 signal decrease so much in Figure

5G, 5G'.
We agree with you that the YFP signal is not clear due to the predominant expression of PIN-GFP. Therefore, in the revised version of the manuscript we have provided the GFP channel (representing PIN1-GFP), YFP channel (representing ATHB8-YFP) and merged images of all the panels for better clarity of the data ( Fig5A- F). Thank you for pointing it out. Also we have removed the extra arrows and markings. In Figure G and G' (now I and I') we have used 10x lens to show the expanded leaf at 24h post incision. However, images in panels (G,G',H and H') were acquired using 20x lens as the leaves were younger and not as big as in I and I'. Hence the difference in observed intensity can be attributed to the difference in the lenses used for image acquisition.

8. YUC4-PLT ChIP or Dapseq data would be useful to show.
Thank you for the suggestion. Like PLT5 induction of YUC4, our new RT-qPCR data (S12 J) shows rapid transcriptional activation of YUC4 upon over expression of steroid inducible version of PLT7 as early as 4 hours even in the presence of translational inhibitor, cycloheximide suggesting the direct activation. We have added this new data in the revised manuscript (S12 J, line 407-408).

Earlier we had performed CHIP seq. However, because of the huge variations in independent experiments we are unable to arrive at any conclusive results for YUC4 using the CHIPseq assay. Upon analysing the DAPseq data we did not detect any binding site for PLT7 on YUC4 promoter and data for PLT5 binding to YUC4 promoter in not there in DAPseq (neomorph.salk.edu) (O'Malley et al., 2016). So at present our interpretation of transcriptional activation of YUC4 by PLT is largely dependent on our analysis based on steroid inducible over expression of PLT5 and PLT7 and other related molecular and genetic experiments described in the manuscript.

9. Quantifying vein loops in the yuc1yuc4 mutant would be a useful control.
Indeed yuc4;yuc1 homozygous double mutant is an apt negative control for vein loop quantification. It is important to note that while midvein formation in yuc4;yuc1 mutant remains normal like wildtype, the number of loops surrounding the midvein are significantly reduced as compared to wildtype. Thank you for the valuable suggestion. We have now added the data to the revised graph (Fig.S7F, S7G, S7N; lines 436-438).

10. Figure 6 model is drawn to make it appear that CUC2 is dependent on PLTs. The data in the paper suggest that CUC2 appears only partially dependent on PLTs. It would make more sense to have both CUC2 and PLT at the top of the diagram, and both regulating YUC4. A horizontal arrow could be drawn from PLT to CUC2 to indicate that CUC2 is partially regulated by PLTs.
Thank you for the suggestion. We have edited the schematic in light of your comment (Figure 6O).

11. What is "AND gate" (line 609)?
An "AND" gate is a digital logic gate which functions according to a truth table. A HIGH output results only if all the inputs to the AND gate are HIGH eg. 1+1→1.Here in the manuscript we are comparing the promoters of PLT and CUC2 to such inputs and YUC4 activation to the output. In our case, since a coherent feedforward loop is functional, YUC4 can be activated even if one of the gene product, either PLT or CUC2 is available as a functional protein. Therefore, we state that "the regulatory logic at the promoter is not strictly an 'AND gate'". Details regarding logic gates in biological context have been explained in the book-: An introduction to systems biology: design principles of biological circuits (Alon, U., 2006) which has been referred to in the discussion (line 483).

12. Figure S9 – R, S, T are not clear differences. Can these differences be quantified?
In light of your suggestion we have now quantified the area of callusing and added the data (S13 E).

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Second decision letter

MS ID#: DEVELOP/2019/185710

MS TITLE: A coherent feed forward loop drives vascular regeneration in damaged aerial organs growing in normal developmental-context

AUTHORS: Dhanya Radhakrishnan, Anju Pallipurath Shanmukhan, Abdul Kareem, Mohammed Aiyaz, Vijina Varapparambathu, Ashna Toms, Merijn Kerstens, Devisree Valsakumar, Amit N. Landge, Anil Shaji, M.K Mathew, Megan G. Sawchuk, Enrico Scarpella, Beth A. Krizek, Idan Efroni, Ari Pekka Mahonen, Viola Willemsen, Ben Scheres, and Kalika Prasad
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

*Advance summary and potential significance to field*

The authors found the PLT-CUC2 module is necessary for vascular regeneration after injuries in Arabidopsis thaliana. Their findings are quite interesting and give an impact on the research field of developmental biology.

*Comments for the author*

I am fully satisfied with their revision for our requests. There is no revised point in the present manuscript. I am looking forward to seeing this paper.

Reviewer 2

*Advance summary and potential significance to field*

The manuscript follows up on previous work by the Prasad group looking at the role of the plethora genes during regeneration (Kareem et al 2015). Here, they undercover a role for PLT3,5,7 during vascular reconnection and callus formation in wounded leaves and stems. They go onto to show that CUC2 also plays a role, and together, these genes upregulate auxin biosynthesis via YUC4. The manuscript provides a nice mechanistic overview for how increases in plethora levels lead to wound healing in aerial tissues.

*Comments for the author*

Thank you for addressing my comments. They have all been addressed well. I found a couple minor typos in the manuscript:

- line 52. should say " in leaves"
- line 181 should say "refer to the methods section"
- line 334. should say Fig. S11E'