Arabidopsis vascular complexity and connectivity controls PIN-FORMED1 dynamics and lateral vein patterning during embryogenesis

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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 BenchPress and 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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 manuscript by Yanagisawa is interesting and promising but troubled by serious conceptual misunderstandings and experimental limitations.

Comments for the author

Major Issues
1. I have many problems with the Authors' use of terms and expressions. In some cases, the problems are minor. For example, the use of "provascular", which means different things to different people: for some (e.g., Esau), it is a synonym of procambial; for others (e.g., Nelson), it refers to poorly defined preprocambial stages. To avoid confusion, I would suggest that the Authors stick to procambium/procambial and preprocambial.

In other cases, the terms used are internally inconsistent, but the problem is still minor. For example, the Authors do not use distal and proximal, or apical and basal, but distal and basal, incorrectly unifying the two types of positional classifications.

The problem becomes more serious with the Authors' use of the term/expression "convergence point" to indicate the point where the second loop connects to the first loop in the cotyledon. For the past 15+ years the term "convergence point" has been used to indicate a point of converging PIN1 polarity in the epidermis of shoot apex and leaf primordia — incidentally, I find it perplexing that the Authors have not noticed this while reading the literature in the field.

Finally, the problem is most serious with the Authors' use of the terms complexity and connectivity.

In graph theory and network science, complexity is a — no pun intended — a complex feature of networks: it takes into account at the very least the number of edges (i.e. the number of veins in our case), the relative connectedness of the network (i.e. the connectedness of the network in question relative to a fully connected network with the same number of edges), and the relative continuity of the network (i.e. how discontinuous is the network in question relative to a fully continuous network with the same number of edges). Much work has been done in the field by experimental, theoretical, and computational biologists to derive indices to measure complexity of natural and artificial networks, but most Authors now refrain from using those complexity indices because they are not as informative as the use of separate indices that describe the number of edges, the relative connectedness, and the relative continuity of the network. Indeed, two networks may have the same complexity even if they have different number of edges, relative connectedness and relative continuity. And when two networks have different complexity, it is impossible to say whether that's because they have different number of edges, different relative connectedness, or different relative continuity. For all these reasons, when comparing two networks, it's more informative to directly compare the separate indices that are use to measure complexity.

It seems to me that what the Authors refer to as "vein complexity" — incidentally, it should be "vein network complexity", as complexity is a feature of vein networks not of veins — is the *absolute* number of closed and open loops. I write it seems because in the Discussion (l. 385 and following) complexity is defined as only "the number of closed distal areoles and the presence of basal veins forming either closed or open basal areoles". But when two networks differ in such "vein complexity", it's unclear whether they would differ in the number of veins formed or in their ability to connect to other veins. This of course is a problem because saying that a gene controls vein complexity does not inform us on the function of the gene. Instead, saying that a gene controls vein formation, vein connection, or both informs us on exactly the developmental process the gene is controlling. Incidentally, the complexity of different networks is never statistically compared in this manuscript (e.g., in Fig. 1B, Fig. 3B, Fig. 3G, and Figure 4).

Sometimes instead, the Authors use the expression "vein pattern complexity"" (e.g., ll. 110, 138, and 141–143) — do they mean vein network complexity? How can a pattern be more or less complex than another pattern?

Besides, I do not see any evidence in this manuscript of differences in the position where veins form in vcc mutant cotyledons.

In the Discussion (l. 386 and following), the Authors also claim to have measured continuity, but I don't see any evidence of that: did the Author confounded continuity with connectedness? It seems so because in the following lines they apparently use "disconnection" and "fragmentation" and synonyms.

As to all the other features of vein networks the Authors measure (e.g., the relative positions of "middle junction" and "convergence point"), it's unclear what we are supposed to learn from that, as they are not used to infer gene function.

And the whole point of quantifying phenotypes in multiple mutants is that they can be used to infer relative position and relation of the pathways in which genes act, but the Authors fail to do so. For example, is the relation between pin1 and vcc additive, synergistic, or epistatic? And what do we learn from that? And if the two single mutants defects are indistinguishable, as it seems to be — but we don't know for sure because statistical analysis is missing — then the authors need to find characters that are unique to the two mutants and leverage them in the analysis; otherwise, how can we interpret the phenotype of a double mutant that seems to be no different from that of either single mutants: which of the two genes would be the one acting upstream of the other?

2. The data on PIN1 expression during cotyledon lateral vein formation are new but incomplete. For example there is no reference to — or images of — whether those veins form in association with epidermal convergence points of PIN1 polarity, whether loops are of composite origin, and whether bipolar cells are observed in cotyledons too. This is the first time PIN1 expression is analyzed in cotyledon development, so those data are essential.

Most important is the fourth drawing from the left in Fig. 2D, which is supposed to depict the appearance of an isolated island of PIN1 expression, presumably imaged in the second-last image in Fig. 2C; however, in that image the PIN1 expression domain is continuous with at least the first loop and the epidermis, and perhaps with the midvein too. In leaves, islands of PIN1 expression have never been observed; instead, several labs (e.g., Berleth, Kuhlemeier, Fukuda, Mattsson, Nelson, Scarpella, and Schultz, to name just a few) have independently observed appearance of PIN1 expression domains in continuity with other PIN1 expression domains in both WT and mutants. Therefore, the claim the Authors make that in both WT and vcc PIN1 expression domains form instead in isolation from other PIN1 expression domains needs to be substantiated by better images and quantification of the reproducibility of those images.

Furthermore, to my knowledge, PIN1 has never been reported in vacuoles of live-imaged or dissected and undamaged leaves, but the Authors wish to suggest that PIN1 normally accumulates in vacuoles of, for example, the midvein of the cotyledon in WT (Fig. 7B). The image in that panel is saturated and the signal indicated with the yellow arrowhead is "blooming", so it's difficult to tell if the Authors' interpretation is correct. Quantification of co-localization between PIN1 and vacuole markers at high resolution should resolve that issue; controls done with plasma membrane proteins known not to accumulate in the vacuole should obviously be included.

Finally, it's very confusing to learn in Figure 7 for the first time that PIN1 localization may be altered in vcc which could explain why PIN1 did not even look like it was localized to the plasma membrane in the vcc cotyledon in Fig. 3F. Defects in PIN1 localization in vcc cotyledons should be presented first to avoid confusing the reader and raising questions about artifactual imaging.

Minor Points
- ll. 65, 66.
>Polar localization of PIN1 determines the direction of auxin flow (Friml et al., 2003)
I think the correct reference for this is Wisniewska et al. 2006.
- ll. 84–88.
>lack of function of the transcription factors LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS5/TMO5-LIKE1, which regulate the proper expression of ARF5/MP, PIN1, and auxin and cytokinin biosynthetic genes, results in vascular differentiation defects in embryos and roots (De Rybel et al.,

2013; Ohashi-Ito et al., 2013; DeRybel et al., 2014; Ohashi-Ito et al., 2019; Smet et al., 2019).
I may be mistaken, but I do not recall evidence that LHW, TMO5, and TML1 control expression of MP or PIN1.

Reviewer 2

Advance summary and potential significance to field

Through a careful study combining cell biology (imaging) and genetics, the authors describe and suggest a new aspect of the role of the VCC protein in the regulation of the polarity of PIN1. They demonstrate a strong correlation between PIN1-GFP degradation, partial lost of polarity of PIN1-GFP and the localization of VCC.

In view of the potential function of VCC in membrane structuring, which unfortunately has not been addressed. This study could potentially constitute a new axis in the field of PIN1 polarity regulation.

Comments for the author

Overall this paper is well written. However, it seems to me that the overall results presented in this paper do not support the conclusions brought to the reader's attention. It is therefore necessary to deepen the questions raised and to answer them, as well as to improve the iconography, the definition of which is too weak to clearly establish conclusions. I encourage revision providing that authors addressed the major comments

Major comments
Paragraph 1 In this paragraph, the authors describe the phenotype of the vcc mutant in the cotyledons. This study has already been carried out in the previous article (Roschzttardtz et al., 2014).

However, the authors make a tedious and more detailed study of the positioning of the pattern. The reason why this study is being carried out does not seem to me to be clearly explicit and would deserve a better justification for the rest. On the other hand, even if the measurements are standardised with respect to the size of the cotyledon, I think it is important to carry out a study on the geometry of the cotyledons to analyse a possible phenotype (length, width and sphericity for example). This would reinforce the idea that the vcc phenotype is specific to the vascular network and not to the size or shape of the cotyledons.

Paragraph 2 The pattern of pPIN1::PIN1-GFP (nomenclature to be respected) is not very clear on the globular, it seems to be rather expressed in the ground meristem and little in the future vascular cells. Overall, the images are not always of good quality for evaluating phenotypes or other descriptions. For this type of study, I advise you to follow the procedure described by (Ursache et al., 2018) on fixed embryos, which does not alter the XFP patterns.

The distinction between preprocambial and procambial cells is based on identity markers. In the absence of these markers, how can these two cell types be distinguished?

Paragraph 3 The pattern of pPIN1::PIN1-GFP is similar in vcc and WT cotyledons of 120 to 160um (fig3C), this assertion requires showing images. In particular, it seems that in figure 3A we can consider two middle junction points in WT coming from both distal loops, whereas only one in vcc. How do you score these middle junction points coming from the first two areoles?
In studies involving PIN1 expression, immuno-localization is regularly used to unambiguously follow the expression and the polarity of this protein. Why not using this complementary strategy?
The state of homozygosity vs heterozygosity is not an irrefutable proof of the quantitative gene expression (for example gene silencing), so it would be important to support this assertion with a quantification measure (RT-QPCR, western blot with anti-PIN1, relative fluorescence quantification).

Paragraph 4 It is not very clear how the authors identified the pin1-1 plants. In fact, this identification can only be possible in the progeny of a heterozygous, it is important to know the segregation of the pin1-1 plants to be sure that all the mutants have been taken into account. Moreover, if it seems clear that the distinction between plants with 1 single cotyledon, 3 cotyledons or 2 fused cotyledons can be established, there remains an important category of plants with cotyledons whose phyllotaxis is not perfectly opposed "staggered" cotyledons.

Concerning the of pid mutants, it seems that the authors have selected two independent mutants expressing the same type of mutation. These cases are rare, how many plants have been selected to see this phenotype?

Similar question for the offspring of pid mutants, in the absence of genotyping, what is segregation of the pid mutants (based only on 3 cotyledons phenotypes?).

This paragraph lacks a clear conclusion on the epistasis relationships between and pid pin1, vcc.

Paragraph 6 :
Why studying the root meristematic zone (justification)?
Figure 6B : The images do not allow a clear view of the different cell layers. A more median optical section would really allow distinguishing the different cell layers (see protocols Ursache et al., 2017)

Figure 6D: This is not a co-localization study, which requires statistical processing by dedicated software but just an indication that fluorescence is close to the membrane; the same study with the PI would have given the same results.

Overall the images are not of sufficient quality to convince the readers

Paragraph 7 The VCC expression in the ground meristem in fig7A is not very clear; it seems to be confined to the vascular cells (globular stage)
Fig7B needs close-up; images seem over-exposed and thus very difficult to distinguish cytoplasmic puncta and vacuoles signal.
figure 8A and C are of poor quality)
How was this quantification carried out? on optical sections? on projections?
I don't see anything in the material and methods
Figure 8F how do you quantify the more pronounced polarity in the plasma membrane of procambial cells? (2D, 3D, projection?)
It is difficult to evaluate the polarity on cells because the resolution does not allow dissociating the two membranes coming from the interface. For example, the strong polarity observed on procambial cells expressing pPIN1::PIN1-GFP may be the result of the fluorescence of two contiguous membranes, this is all the more true as procambial cells expressing PIN1:GFP are isolated and restricted.

It is difficult to study the cotyledons at this stage in dynamics and to act on them. However, embryo culture is possible from the torpedo stage. To demonstrate the importance of VCC in the localization and stability of PIN1 at the plasma membrane It would be important to manipulate endocytic pathway (genetic or pharmacology) in order to modify vacuole trafficking and observe the change in the vascular network.

Discussion In two different studies Mahravy et al.,(2011 and 2014) point out the role of cytokinin in both the endocytic trafficking and the polarity of PIN1-dependent auxin transport. They provided evidences that cytokinin directs PIN1 to lytic vacuoles for degradation. Unfortunately, I have not seen any experiences or discussion to link these two studies with your data.

Reviewer 3

Advance summary and potential significance to field

In this work, Yanagisawa and co-authors describe the role of VCC in regulating the localization and abundance of PIN1 during lateral vein formation of embryonic cotyledons. By thorough examination of PIN1 localization during embryogenesis, the authors spatio-temporally define two PIN1 fields that correlate with the origin of apical and basal secondary veins. Moreover, they claim that an auxin-mediated VCC induction is required to promote PIN1 internalization into the vacuole and therefore, promote the transition between pre-procambial to procambial cell fate. While the characterization of this process expands our knowledge about the mechanisms by which plants increase the complexity of their vascular networks, further evidence is required to support the authorÂ's claims about the subcellular PIN1 localization and cell fate transitions.

Comments for the author

Major comments
1-The authors nicely show that a PIN1 field is established perpendicular to the middle vein in late torpedo stage as the initial point to originate the lateral veins. This process is followed by the generation of a second PIN1 domain coinciding with basal strand initiation at early bent cotyledon stages. Yet, it is not clear to this reviewer how can the authors explain the branching process only based on the presence/absence of a correct PIN1 localization excluding other important developmental process such as periclinal divisions. Is a phase of periclinal divisions involved in the generation of branching? In these lines, it seems that the authors define pre-procambial cell identity to procambial cell identity only based on PIN1 expression and cell shape. Can they use other marker identity gene to better define the transition between pre-procambial and procambial cells? Indeed, how are PIN1 expressing ground cells different from PIN1 expressing pre-procambial cells?

2- The authors claim that an increase in PIN1 dosage is partially sufficient to restore a normal vein pattern in vcc cotyledons. However, these assumptions are only based on hemizygous or homozygous seedlings. Since it seems that VCC activity is not required in the initiation of the first PIN1 domain, only in the establishment of the second PIN1 field, further experiments involving a precise induction of PIN1 expression in those cells at this particular stage are necessary to sustain such a claim.

3- Figure 4A. "Representative images of distorted distal areoles (red arrows) in pin1-1 and vcc pin1-1 cotyledons are shown". How can the authors be certain that these are indeed the distal areoles formed first, could they be smaller loops formed later during vein patterning as a compensatory mechanism for low vein complexity? It would be interesting to also show representative images of pid and pidvcc cotyledons and not only the quantifications (Figure 4B).

4-Figure 6E. Could the authors provide better or at least bigger images to show the localization of VCC in the vacuolar lumen?

5-Figure 8E. It is not clear to this reviewer that VCC internalization into the vacuole occurs mostly in PIN1-GFP expressing cells based on their quantification. Which would be the significance of VCC internalization into the vacuole in other cells such as endodermal cells. Moreover, in figure 8F the authors show a "Representative image of polarized PIN1-GFP in elongating pre-procambial cells". In M&M the authors explain that "PIN1 polarity was analyzed by quantifying PIN1-GFP Fluorescent signal at the plasma membrane of at least two consecutive cells". Considering that in Figure 8F this reviewer can distinguish just two consecutive cells where PIN1 seems to be polarized, and in the first of the two, there is signal on the lateral side of this cell, are two consecutive cells enough to assess polarity? Furthermore, if comparing this image to Figure 8I the vcc mutant shows a very similar localization pattern, where PIN1 is polarly localized in two consecutive cells and in the first of the two, PIN1 is also localized on the lateral side of that cell. So how is polarity or incomplete polarity truly identified and further quantified?

6-The authors claim that the failure of the second PIN1 domain in vcc cotyledons is due to an enhanced internalization of PIN1 into the vacuoles and therefore, a deficient PIN1 polarization required to promote the transition between pre-procambial to procambial cells. Yet, a high vacuolar localization of PIN1-GFP could be observed in elongated procambial cells of the middle vein strand. Could only PIN1 internalization explain the defective transition between pre-procambial into procambial cells in vcc mutants? Moreover, it seems that the authors have based

their interpretation of cell fate not only into PIN1 polar distribution but also the shape of the cells. However, in Figure 8I, it is hard to say that some procambial cells showing PIN1 polar localization are elongated or are different shape-wise to the ones assigned as pre-procambial cells in vcc images. Could the authors use another identity marker or trait to clarify this?

7-While in the discussion VCC function is mainly discussed in terms of regulating PIN1 activity in an independent path of PINOID, very little is mentioned about the importance of the interaction between VCC and OPS. Can the authors discuss a bit further about the potential role of VCC in terms of regulating the activity of OPS? Is OPS also internalized and requires VCC activity to display its correct localization? Are other brassinosteroid-related mutants involved in cotyledon vascular development during embryogenesis?

8-Also in the discussion, the authors suggest that VCC expression is subjected to an auxin-mediated feedback loop involving most likely MONOPTEROS. Yet, Smith et al; Development 2020 demonstrated the existence of a MP-independent pathway in modulating vein identity. Could the authors comment on the role of VCC in establishing vascular identity at least based on its expression pattern during embryogenesis?

Minor comments
9-The authors frequently use indistinguishably differentiation and specification. Since both are very different process in a developmental context, I would strongly recommend the authors to be more precise in the use of these terms for reader's clarity. In several cases the authors use "differentiated/differentiation" to describe the transition from pre-procambial to procambial strands or procambial to vascular strands. In either case, I believe this terminology to be incorrect as vascular differentiation occurs between 2-3 days after germination (James S. Busse and Ray F. Evert 1999). One example is on Line 72 "These PIN1-positive cell files differentiate into procambium".

Likewise, the authors frequently refer to PIN1 expression when they mean localization. This terminology can be confusing and considering that PIN1 has a transitional expression pattern and a very dynamic localization during vein patterning, I think the authors should be more precise when referring to PIN1 subcellular dynamics.

10-Lines 310-312. Based on the graphs depicted in Figure S3C it seems inaccurate to claim that both VCC YFP tagged versions largely restore vcc phenotypes. To talk about a partial rescue seems more appropriate. Additionally, do the authors observe the correct polar localization of PIN1 in tdTomato-VCC vcc plants?

11-Figure 6. VCC YFP is shown in the root meristem, is there a root vascular phenotype or root meristem phenotype in vcc? It would be helpful for the reader to indicate that to better demonstrate the role of VCC in vascular development in general.

12-Throughout the entire manuscript, there are some references missing after statements that should be referenced for example page 3 line 45 "….most of the transport occurs from and to the leaves."
Missing Reference Line 69….of ground cells and localizes to the plasma membrane with no detectable polarity"
Missing Reference Line 75
Missing Reference Line 82
Missing Reference Line 95
13-Line 53 ..by the ---of procambial cells. In this sentence do the authors mean that new veins form in denovo organs like leaves?
14-Figure 1A. Are the images of cotyledons shown here of WT background or vcc or a mix?
15-Figure 8I. Figure states red asterisks image displays red arrowheads.

First revision

Author response to reviewers' comments

We would like to thank the reviewers for the thoughtful comments on our manuscript. We have now revised the manuscript extensively. We have addressed the concerns raised by the reviewers by providing new data and/or better explanations. The main changes in the manuscript are:

1) Better microscopy images (Figures 2C, new 4F, 7B, 7E, 8B, 9C)
2) Analysis of expression of the early vascular reporter ATHB8 during the formation of basal strands in embryonic cotyledons.
3) Analysis of PIN1 polarity during formation of lateral strands (new Figure 3)
4) Analysis of shape and size of cotyledons in vcc and wild type seedlings (new Supplemental Fig S1)
5) Statistical analysis of vein network patterns in wild type and mutants (Supplemental Table 1).
6) New imaging data on the origin of the second PIN1 domain (new Supplemental Figure 3)

All changes have been highlighted in yellow in the revised manuscript. Below, we answer the comments raised by the reviewers one by one.

Reviewer 1

Reviewer 1:
Major Issues

1a. I have many problems with the Authors' use of terms and expressions. In some cases, the problems are minor. For example, the use of "provascular", which means different things to different people: for some (e.g., Esau), it is a synonym of procambial; for others (e.g., Nelson), it refers to poorly defined preprocambial stages. To avoid confusion, I would suggest that the Authors stick to procambium/procambial and preprocambial.

Answer: We have replaced the term “provascular” throughout the manuscript, according to the reviewer’s suggestion.

1b. In other cases, the terms used are internally inconsistent, but the problem is still minor. For example, the Authors do not use distal and proximal, or apical and basal, but distal and basal, incorrectly unifying the two types of positional classifications.

Answer: We agree with the reviewer that “distal and proximal” or “apical and basal” would be good term pairs to define veins. However, we already used “distal” and “basal” in our 2014 publication when we first reported the vein patterning defects in the *vcc* mutant cotyledons (Roschzttardtz et al 2014). We think that changing the terminology and the classification of vein patterns, making it inconsistent with our previous report, would lead to unnecessary confusion.

1c. The problem becomes more serious with the Authors' use of the term/expression "convergence point" to indicate the point where the second loop connects to the first loop in the cotyledon. For the past 15+ years, the term "convergence point" has been used to indicate a point of converging PIN1 polarity in the epidermis of shoot apex and leaf primordia — incidentally, I find it perplexing that the Authors have not noticed this while reading the literature in the field.

Answer: We have replaced the expression “convergence point” with “merging point” to avoid confusion with other region/features, as pointed out by the reviewer.

1d. Finally, the problem is most serious with the Authors' use of the terms complexity and connectivity. In graph theory and network science, complexity is a — no pun intended — a complex feature of networks: it takes into account at the very least the number of edges (i.e. the number of veins in our case), the relative connectedness of the network (i.e. the connectedness of the network in question relative to a fully connected network with the same number of edges), and the relative continuity of the network (i.e. how discontinuous is the network in question relative to a fully continuous network with the same number of edges). Much work has been done in the field by experimental, theoretical, and computational biologists to derive indices to

measure complexity of natural and artificial networks, but most Authors now refrain from using those complexity indices because they are not as informative as the use of separate indices that describe the number of edges, the relative connectedness, and the relative continuity of the network. Indeed, two networks may have the same complexity even if they have different number of edges, relative connectedness, and relative continuity. And when two networks have different complexity, it is impossible to say whether that's because they have different number of edges, different relative connectedness, or different relative continuity. For all these reasons, when comparing two networks, it's more informative to directly compare the separate indices that are use to measure complexity.
It seems to me that what the Authors refer to as "vein complexity" — incidentally, it should be "vein network complexity", as complexity is a feature of vein networks not of veins — is the *absolute* number of closed and open loops. I write it seems because in the Discussion (l. 385 and following) complexity is defined as only "the number of closed distal areoles and the presence of basal veins forming either closed or open basal areoles". But when two networks differ in such "vein complexity", it's unclear whether they would differ in the number of veins formed or in their ability to connect to other veins. This of course is a problem because saying that a gene controls vein complexity does not inform us on the function of the gene. Instead, saying that a gene controls vein formation, vein connection, or both informs us on exactly the developmental process the gene is controlling. Incidentally, the complexity of different networks is never statistically compared in this manuscript (e.g., in Fig. 1B, Fig. 3B, Fig. 3G, and Figure 4). Sometimes instead, the Authors use the expression "vein pattern complexity"" (e.g., ll. 110, 138, and 141–143) — do they mean vein network complexity? How can a pattern be more or less complex than another pattern? Besides, I do not see any evidence in this manuscript of differences in the position where veins form in vcc mutant cotyledons.

Answer: We replaced the expression “vein complexity” with “vein network complexity”, following the reviewer’s suggestion.
We have also performed Pearson’ Chi-squared analyses to determine the statistical significance of the differences in vein network complexity and the occurrence of vein discontinuities among different genotypes.

1e. In the Discussion (l. 386 and following), the Authors also claim to have measured continuity, but I don't see any evidence of that: did the Author confounded continuity with connectedness? It seems so because in the following lines they apparently use "disconnection" and "fragmentation" and synonyms.

Answer: We changed the term “continuity” to “connectivity” throughout the text.

1f. As to all the other features of vein networks the Authors measure (e.g., the relative positions of "middle junction" and "convergence point"), it's unclear what we are supposed to learn from that, as they are not used to infer gene function.

Answer: We analyzed these features to find possible explanations for the reduced formation of basal veins in the vcc mutants. For example, we found that the middle junction is located in a more basal position when basal veins either do not merge with the middle vein or are absent altogether. Based on this, one would expect that the *vcc* mutant cotyledons, which form fewer basal veins, would show an overall displacement of the middle junction to more basal positions compared to wild type. However, our analysis showed the opposite trend (Fig. 1D). This rules out the possibility that the reduced formation of basal veins in the *vcc* mutant is due to the early misplacement of the middle junction during cotyledon development. This is explained in the results section (lines 146- 151)
In the discussion, we further explained our conclusions regarding these observations:
Line 451: According to this observation, we hypothesize that the middle-junction and merging positions are located at significantly more distal positions in *vcc* cotyledons because basal strands originated from the second PIN1 domain frequently fail to specify procambial/vascular cells, and therefore, most of the basal veins in *vcc* mutant cotyledons derives from the first PIN1 domain (Fig. 10A).

1g. And the whole point of quantifying phenotypes in multiple mutants is that they can be used to infer relative position and relation of the pathways in which genes act, but the Authors fail to

do so. For example, is the relation between pin1 and vcc additive, synergistic, or epistatic? And what do we learn from that? And if the two single mutants defects are indistinguishable, as it eems to be — but we don't know for sure because statistical analysis is missing — then the authors need to find characters that are unique to the two mutants and leverage them in the analysis; otherwise, how can we interpret the phenotype of a double mutant that seems to be no different from that of either single mutants: which of the two genes would be the one acting upstream of the other?

Answer: As indicated above, we have now performed statistical analyses (Pearson's Chi-squared test of independence) to determine the significance of the differences seen in vein network complexity and vein discontinuities in cotyledons from different genotypes. With these new analyses, we have determined that there are statistically significant differences in the representation of vein network complexity patterns between wild type and *pin1-1* (p=0.006), wild type and *vcc* (p= 0.0001), and *pin1-1* and *vcc pin1-1* (p= 0.006); however, the differences between *pin1-1* and *vcc* single mutants was not significant (p = 0.87) whereas the difference between *vcc* and *vcc pin1-1* was barely significant (p= 0.04). In terms of vein disconnections, we found no statistically significant differences between either *vcc* and *pin1-1* (p= 0.86) or each of the single mutants and the *vcc pin1-1* double mutant (p=0.3 for both cases). Based on this new analysis, we have concluded that the phenotypic defects of the single *vcc* and *pin1-1* mutants are not statistically different from the double mutant and therefore, we cannot place one gene upstream of the other. However, based on the presence of abnormal apical areoles in *pin1-1* and *vcc pin1-1* but not in *vcc* single mutants, for this particular feature, PIN1 seems to be epistatic to VCC. Similarly, we found no statistically significance differences in vein network complexity between *pid* and *vcc* single mutants or between single and *vcc pid* double mutants. However, the occurrence of vein discontinuities in the double *vcc pid* mutant was significantly higher (p<0.0001) than in any of the single mutants, indicating a synergistic effect of the two mutations on vein disconnections.
New text has been added to the Result section to explain these results (lines 269-284 and 294-306).

2a. The data on PIN1 expression during cotyledon lateral vein formation are new but incomplete. For example, there is no reference to — or images of — whether those veins form in association with epidermal convergence points of PIN1 polarity, whether loops are of composite origin, and whether bipolar cells are observed in cotyledons too. This is the first time PIN1 expression is analyzed in cotyledon development, so those data are essential.

Answer: a new figure (Figure 3) has been added to show association of the PIN1 domains with the epidermis and PIN1 localization at the merging point. New text is now in the manuscript explaining these new results:

Line 182: Similar to what has been described in leaf primordia (Scarpella et al., 2006; Wenzel et al., 2007), this PIN1 domain originates underneath an epidermal cell in which PIN1-GFP is localized at opposite anticlinal sides (bipolar localization) as well as the side facing the subepidermal PIN1 domain (Fig 3A).

Line 199: In the cell located at the merging point, PIN1-GFP was localized at the sides oriented along the distal strand (bipolar localization) as well as at the side of contact with the basal strand (Fig. 3C). We confirmed that the isodiametric cells in developing basal strand were pre-procambial based on their ability to express the pre-procambial marker pATHB8::NLS-YFP (Sawchuk et al., 2007) (Fig 3D,E).

2b. Most important is the fourth drawing from the left in Fig. 2D, which is supposed to depict the appearance of an isolated island of PIN1 expression, presumably imaged in the second-last image in Fig. 2C; however, in that image the PIN1 expression domain is continuous with at least the first loop and the epidermis, and perhaps with the midvein too. In leaves, islands of PIN1 expression have never been observed; instead, several labs (e.g., Berleth, Kuhlemeier, Fukuda, Mattsson, Nelson, Scarpella, and Schultz, to name just a few) have independently observed appearance of PIN1 expression domains in continuity with other PIN1 expression domains in both WT and mutants. Therefore, the claim the Authors make that in both WT and vcc PIN1 expression domains form instead in isolation from other PIN1 expression domains needs to be substantiated by better

images and quantification of the reproducibility of those images.

Answer: A new figure (Figure 3) has been added to show association of the second PIN1 domais with the epidermis.
Fig 2D was fixed to better reflect the formation of the second PIN1 domain in association with the epidermis.
The second image from the right in Fig 2C was replaced with a clearer image.

2c. Furthermore, to my knowledge, PIN1 has never been reported in vacuoles of live-imaged or dissected and undamaged leaves, but the Authors wish to suggest that PIN1 normally accumulates in vacuoles of, for example, the midvein of the cotyledon in WT (Fig. 7B). The image in that panel is saturated and the signal indicated with the yellow arrowhead is "blooming", so it's difficult to tell if the Authors' interpretation is correct. Quantification of co- localization between PIN1 and vacuole markers at high resolution should resolve that issue; controls done with plasma membrane proteins known not to accumulate in the vacuole should obviously be included.

Answer: Degradation of plasma membrane proteins, including PIN1 and other PIN proteins, in vacuoles have been extensively reported by our labs and others (Spitzer et al 2009 Plant Cell; Buono et al 2016 Plant Physiology). Shirakawa et al (2009, Plant Cell Physiol) and Marhavy et al (2011, Dev Cell) reported PIN1 accumulation in vacuoles in dark-treated leaves and in cytokinin-treated roots, respectively. To better show the localization of PIN1-GFP in vacuoles, we included higher resolution, non-saturated images in Figure 8B. Localization of PIN1-GFP in relationship to a tonoplast marker (VAMP711-mCherry) is presented in Fig9 A and B

2d. Finally, it's very confusing to learn in Figure 7 for the first time that PIN1 localization may be altered in vcc, which could explain why PIN1 did not even look like it was localized to the plasma membrane in the vcc cotyledon in Fig. 3F. Defects in PIN1 localization in vcc cotyledons should be presented first to avoid confusing the reader and raising questions about artefactual imaging.

Answer: In this manuscript, we follow this logical sequence: First, we show that the vein network complexity defects in the *vcc* mutant cotyledons are related to reduced basal vein formation; second, we show that the formation/stability of the second PIN1 domain, which is responsible for the formation of basal strands is abnormal in the vcc mutant; finally, we show that the polar localization of PIN1 in the pre-procambial cells derived from the second PIN1 domains is compromised in the *vcc* mutant. It would be very hard to start the manuscript by showing the abnormal localization of PIN1 in pre-procambial cells derived from the second PIN1 domain without explaining how the vascular defects in this mutant are related to these specific group of cells.
We have replaced the original Figure 3F (New Figure 4F) with a better image, but still the fluorescence signal needs to be enhanced to visualize a weak signal from the second PIN1 domain. As a result, the fluorescence signal from the middle and distal strands (especially from vacuoles) is saturated. This explanation was added in the Figure 4F legend.

Minor Points

- ll. 65, 66.
>Polar localization of PIN1 determines the direction of auxin flow (Friml et al., 2003) I think the correct reference for this is Wisniewska et al. 2006.

Answer: The reference was changed.

- ll. 84–88.
>lack of function of the transcription factors LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS5/TMO5-LIKE1, which regulate the proper expression of ARF5/MP, PIN1, and auxin and cytokinin biosynthetic genes, results in vascular differentiation defects in embryos and roots (De Rybel et al., 2013; Ohashi-Ito et al., 2013; DeRybel et al., 2014; Ohashi-Ito et al., 2019; Smet et al., 2019).
I may be mistaken, but I do not recall evidence that LHW, TMO5, and TML1 control expression of MP or PIN1.
Answer: Ohashi-Ito et al (2013) described that PIN1 expression patterns in embryos (based on PIN1

imaging) and MP expression (based on qPCR) were altered in *lhw* mutants.

Reviewer 2 Advance Summary and Potential Significance to Field:
Through a careful study combining cell biology (imaging) and genetics, the authors describe and suggest a new aspect of the role of the VCC protein in the regulation of the polarity of PIN1. They demonstrate a strong correlation between PIN1-GFP degradation, partial lost of polarity of PIN1-GFP and the localization of VCC. In view of the potential function of VCC in membrane structuring, which unfortunately has not been addressed. This study could potentially constitute a new axis in the field of PIN1 polarity regulation.

Reviewer 2 Comments for the Author:
Overall this paper is well written. However, it seems to me that the overall results presented in this paper do not support the conclusions brought to the reader's attention. It is therefore necessary to deepen the questions raised and to answer them, as well as to improve the iconography, the definition of which is too weak to clearly establish conclusions. I encourage revision providing that authors addressed the major comments

Major comments

1. Paragraph 1
In this paragraph, the authors describe the phenotype of the vcc mutant in the cotyledons. This study has already been carried out in the previous article (Roschzttardtz et al., 2014). However, the authors make a tedious and more detailed study of the positioning of the pattern. The reason why this study is being carried out does not seem to me to be clearly explicit and would deserve a better justification for the rest. On the other hand, even if the measurements are standardised with respect to the size of the cotyledon, I think it is important to carry out a study on the geometry of the cotyledons to analyse a possible phenotype (length, width and sphericity for example). This would reinforce the idea that the vcc phenotype is specific to the vascular network and not to the size or shape of the cotyledons.

Answer: New measurements of cotyledon length, width, and shape (circularity and roundness) are now included in Figure S1. We found no statistically significant differences between wild type and *vcc* cotyledons in terms of size or shape.

2a. Paragraph 2
The pattern of pPIN1::PIN1-GFP (nomenclature to be respected) is not very clear on the globular, it seems to be rather expressed in the ground meristem and little in the future vascular cells. Overall, the images are not always of good quality for evaluating phenotypes or other descriptions. For this type of study, I advise you to follow the procedure described by (Ursache et al., 2018) on fixed embryos, which does not alter the XFP patterns.

Answer: "PIN1-GFP" was changed to "*pPIN1::PIN1-GFP*" throughout the manuscript, as suggested by the reviewer.
We have replaced the following figure panels with better micrographs: new Figure 4F, 7B, 7E, 8B, 9C

Also, to address the reviewer's concern about the expression of *pPIN1::PIN1-GFP* in the globular embryo, we have changed the description in the text to be more consistent with the Figures.
Line 160: Consistent with previous reports (Steinmann et al., 1999; Izhaki and Bowman, 2007; Ploense et al., 2009), PIN1 was detected in all cells of the globular embryo, except the hypophysis, and its expression became restricted to protodermal and pre-procambial cells at heart and torpedo stages (Fig. 2A).

We used the procedure suggested by the reviewer to image cells expressing the pre-procambial marker pATHB8-NLS-YFP (See new Fig 3)

2b. The distinction between preprocambial and procambial cells is based on identity markers. In the absence of these markers, how can these two cell types be distinguished?

Answer: The ground meristem cells from which procambial cells arise express PIN1. Procambial

cells are readily identifiable based on their elongated profiles (Scarpella et al 2004). As procambial cells form along strands, PIN1-expressing pre-procambial cells located in forming strands can also be readily identified based on their position. To confirm that the short cells expressing PIN1-GFP and located in the areas where basal strands form were indeed pre-procambial cells, we analyzed wild type embryos expressing a pre-procambial fluorescent marker (*pATHB8::NLS-YFP*) provided by Dr Enrico Scarpella. New data showing expression of this reporter in short (pre-procambial) and elongating cells in forming strands in Arabidopsis cotyledons is now included in Fig 3D and E.

3a. Paragraph 3
The pattern of pPIN1::PIN1-GFP is similar in vcc and WT cotyledons of 120 to 160um (fig3C), this assertion requires showing images.

Answer: The expression pattern of *pPIN1::PIN1-GFP* in wild-type and *vcc* cotyledons 140 µm in length is now shown in new Figure S3.

3b. In particular, it seems that in figure 3A we can consider two middle junction points in WT coming from both distal loops, whereas only one in vcc. How do you score these middle junction points coming from the first two areoles?
Answer: We measured the position of middle junction points in half cotyledons. Therefore, for cotyledons such as those depicted in Fig 4A (former Fig 3A), we scored independently the position of the two middle junction points, one at each side of the middle vein.

3c. In studies involving PIN1 expression, immuno-localization is regularly used to unambiguously follow the expression and the polarity of this protein. Why not using this complementary strategy?

Answer: We believe that whenever possible, the best way to appreciate protein localization is through in vivo imaging. We have now included better images to show PIN1 polarization and pseudo-colored images according fluorescence intensity to better illustrate PIN1 distribution (new Fig 3).

3d. The state of homozygosity vs heterozygosity is not an irrefutable proof of the quantitative gene expression (for example gene silencing), so it would be important to support this assertion with a quantification measure (RT-QPCR, western blot with anti-PIN1, relative fluorescence quantification).

Answer: We have quantified PIN1 transcript levels by RT-qPCR as shown in Fig. 4H.

4a. Paragraph 4
It is not very clear how the authors identified the pin1-1 plants. In fact, this identification can only be possible in the progeny of a heterozygous, it is important to know the segregation of the pin1-1 plants to be sure that all the mutants have been taken into account. Moreover, if it seems clear that the distinction between plants with 1 single cotyledon, 3 cotyledons or 2 fused cotyledons can be established, there remains an important category of plants with cotyledons whose phyllotaxis is not perfectly opposed "staggered" cotyledons.

Answer: The method used to identify *pin1-1* and *pid* homozygous plants as well as the segregation rates of these mutants were included in the “Vein pattern analysis” section under Materials and Methods.
Line 533: Homozygous *pin1-1* mutants were identified by the presence of 1 or 3 cotyledons, fused cotyledons or leaves, and abnormal phyllotaxis. As only 10% of the progeny from *PIN1/pin1-1* (*n*=635) and *vcc PIN1/pin1-1* (*n*=732) plants showed cotyledon or leaf mutant phenotypes, the remaining plants were grown longer to observe the pin-like inflorescent meristem typical of *pin1-1* mutants; the cotyledons of these older plants were analyzed. Homozygous *pid* mutants were identified as seedlings with 3 cotyledons, representing 23% and 26% of the progeny from *PID/pid-17* (*n*=448) and *vcc PID/pid-18* (*n*=363) plants, respectively.
4b. Concerning the of pid mutants, it seems that the authors have selected two independent mutants expressing the same type of mutation. These cases are rare, how many plants have been selected to see this phenotype? Similar question for the offspring of pid mutants, in the absence of genotyping, what is segregation of the pid mutants (based only on 3 cotyledons phenotypes?).

Answer: We regularly find similar mutations in different plants transformed with the same guide RNA using CRISPR/Cas9 technology. For the editing of *PID*, we identified four independent lines with edits confirmed by sequencing; all four lines showed seedlings with 3 cotyledons. For this study, we analyzed two of those lines, one in Col-0 background (*pid-17*) and another in the *vcc* background (*vcc pid-18*); both lines have a single base-pair insertion in the same location of the coding sequence and the predicted amino acid sequence was identical for both cases (Fig S4).

4c. This paragraph lacks a clear conclusion on the epistasis relationships between and pid pin1, vcc.

Answer: With the incorporation of the new statistical analyses comparing vein network patterns in wild-type, *pin1-1, pid, vcc*, and double mutants we have now been able to conclude:
Line 280: These results suggest that both PIN and VCC act on the same pathway controlling basal vein formation and vein connectivity in cotyledons and that combined mutations in VCC or PIN1 do not lead to enhanced vein defects. However, based on the presence of abnormally small areoles in *pin1-1* and *vcc pin1-1* but not in *vcc* single mutants, for this particular feature, PIN1 seems to be epistatic to VCC.

Line 302: These results are consistent with both VCC and PID acting with PIN1 through a common mechanism to determine basal strands in cotyledons; however, the drastic increase in distal vein gaps in the double mutants suggests that VCC and PID may control distal vein connectivity in a partially or completely independent manner.

5a. Paragraph 6 :
Why studying the root meristematic zone (justification)?

Answer: We decided to analyze localization in roots because *pVCC:3x-YFP-VCC* was more strongly expressed in the root meristematic zone than in embryos.
We added the following sentence (Lines 332-333) as justification: To study the subcellular localization of VCC, we first analyzed the root tip region of seedlings where 3x-YFP-VCC was strongly expressed.

5b. Figure 6B : The images do not allow a clear view of the different cell layers. A more median optical section would really allow distinguishing the different cell layers (see protocols Ursache et al., 2017)
Answer: Figure 6B (now Fig 7B) was replaced with a more median optical section.

5c. Figure 6D: This is not a co-localization study, which requires statistical processing by dedicated software but just an indication that fluorescence is close to the membrane; the same study with the PI would have given the same results.

Answer: The corresponding sentence was modified.
Line 340: “Signal intensity profiles revealed that 3x-YFP-VCC and FM4-64 signals overlap at the plasma membrane of cortex, endodermal, and vascular cells whereas the internal signal seen in meristematic endodermal and vascular cells correspond to vacuolar lumen (Fig. 7D,F).”

5d. Overall the images are not of sufficient quality to convince the readers

Answer: We have replaced and added additional images to better show the localization of VCC at the plasma membrane and in the vacuolar lumen where is likely degraded as reported for many other plasma membrane proteins in plants and other eukaryotes.

6a. Paragraph 7
The VCC expression in the ground meristem in fig7A is not very clear; it seems to be confined to the vascular cells (globular stage)

Answer: We better described the expression pattern of VCC in developing embryos as follows:
Line 348: VCC was expressed strongly in vascular cells and weakly in ground cells at the globular stage (Fig. 8A). From heart to torpedo stages VCC was detected in pre-procambial and

procambial cells and surrounding ground cells (Fig. 8A).

6b. Fig7B needs close-up; images seem over-exposed and thus very difficult to distinguish cytoplasmic puncta and vacuoles signal.
Answer: Imaging settings were adjusted to capture signal from the plasma membrane. Under these conditions, the strong signal from vacuoles becomes saturated. We have now added non-saturated close-up images in Figure 8B (former Figure 7B).

7. figure 8A and C are of poor quality. How was this quantification carried out? on optical sections? on projections? I don't see anything in the material and methods

Answer: We have replaced Fig 9C (former Fig 8C) with a better image. We have also explained in detail how the fluorescence intensity plot was calculated in “Fluoresce signal intensity profiles” section under Materials and Methods:
Line 572: Single optical sections showing plasma membrane or mid-planes of vacuoles were selected for the analysis.

8. Figure 8F how do you quantify the more pronounced polarity in the plasma membrane of procambial cells? (2D, 3D, projection?). It is difficult to evaluate the polarity on cells because the resolution does not allow dissociating the two membranes coming from the interface. For example, the strong polarity observed on procambial cells expressing pPIN1::PIN1-GFP may be the result of the fluorescence of two contiguous membranes, this is all the more true as procambial cells expressing PIN1:GFP are isolated and restricted.

Answer: We agree with the reviewer that even though we can clearly detect polarized PIN1 localization, it is very difficult to discern at which side of cells PIN1-GFP is located in sub-epidermal embryo cells. Therefore, when it was not possible to tell from which side of the cell the signal was originating from, we just referred to these cells as having polarized PIN1 distribution.

9. It is difficult to study the cotyledons at this stage in dynamics and to act on them. However, embryo culture is possible from the torpedo stage. To demonstrate the importance of VCC in the localization and stability of PIN1 at the plasma membrane, It would be important to manipulate endocytic pathway (genetic or pharmacology) in order to modify vacuole trafficking and observe the change in the vascular network.

Answer: Although Arabidopsis embryos can be grown in culture, we are not sure whether VCC and PIN1 would behave exactly is the same way as in their counterparts developed in planta. Adding drugs to perform pharmacological manipulations in cultured embryos would only add more concerns about artifactual defects. Since this is the first study on the dynamics of PIN1 and VCC during the formation of procambial strands in embryo cotyledons, we think it is more valuable to use embryos developed in planta.

10. Discussion
In two different studies Mahravy et al.,(2011 and 2014) point out the role of cytokinin in both the endocytic trafficking and the polarity of PIN1-dependent auxin transport. They provided evidences that cytokinin directs PIN1 to lytic vacuoles for degradation. Unfortunately, I have not seen any experiences or discussion to link these two studies with your data.

Answer: We have now discussed the role of cytokinin in PIN1 trafficking as suggested by the reviewer:
Line 430: Cytokinin also controls PIN1 polarization and dynamics. Cytokinin enhances differential internalization of PIN1 from specific cell sides and stimulates its degradation in vacuoles (Marhavý et al., 2011; Marhavy et al., 2014). Whether VCC is involved in cytokinin signaling to regulate PIN1 dynamics is unknown; however, polarity defects and premature degradation of PIN1 in *vcc* mutants is consistent with that possibility.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this work, Yanagisawa and co-authors describe the role of VCC in regulating the localization and abundance of PIN1 during lateral vein formation of embryonic cotyledons. By thorough

examination of PIN1 localization during embryogenesis, the authors spatio-temporally define two PIN1 fields that correlate with the origin of apical and basal secondary veins. Moreover, they claim that an auxin-mediated VCC induction is required to promote PIN1 internalization into the vacuole and therefore, promote the transition between pre-procambial to procambial cell fate. While the characterization of this process expands our knowledge about the mechanisms by which plants increase the complexity of their vascular networks, further evidence is required to support the author's claims about the subcellular PIN1 localization and cell fate transitions.

Reviewer 3 Comments for the Author:
Major comments
1a-The authors nicely show that a PIN1 field is established perpendicular to the middle vein in late torpedo stage as the initial point to originate the lateral veins. This process is followed by the generation of a second PIN1 domain coinciding with basal strand initiation at early bent cotyledon stages. Yet, it is not clear to this reviewer how can the authors explain the branching process only based on the presence/absence of a correct PIN1 localization excluding other important developmental process such as periclinal divisions. Is a phase of periclinal divisions involved in the generation of branching?

Answer: Unfortunately, we cannot image the same embryos for long period of times to analyze cell division activity in these PIN1-expressing regions. Typically, periclinal divisions occur after the elongation of the pre-procambial cells and determine the width of the future vascular bundle. In the *vcc* mutant, we observed the early establishment of the second PIN1 domain but pre-procambial cells fail to elongate and prematurely degrade PIN1, which makes us conclude that VCC is acting upstream the proliferation of procambial cells by periclinal divisions.

1b. In these lines, it seems that the authors define pre-procambial cell identity to procambial cell identity only based on PIN1 expression and cell shape. Can they use other marker identity gene to better define the transition between pre-procambial and procambial cells? Indeed, how are PIN1 expressing ground cells different from PIN1 expressing pre-procambial cells?

Answer: The ground meristem cells from which procambial cells arise express PIN1. Procambial cells are readily identifiable based on their elongated profiles (Scarpella et al 2004). As procambial cells form along strands, PIN1-expressing pre-procambial cells located in forming strands can also be readily identified based on their position. To confirm that the short cells expressing PIN1-GFP and located in the areas where basal strands form were indeed pre- procambial cells, we analyzed wild type embryos expressing a pre-procambial fluorescent marker (*pATHB8::NLS-YFP*) provided by Dr Enrico Scarpella. New data showing expression of this reporter in short (pre-procambial) and elongating cells in forming strands in Arabidopsis cotyledons is now included in Fig 3D and E.

2- The authors claim that an increase in PIN1 dosage is partially sufficient to restore a normal vein pattern in vcc cotyledons. However, these assumptions are only based on hemizygous or homozygous seedlings. Since it seems that VCC activity is not required in the initiation of the first PIN1 domain, only in the establishment of the second PIN1 field, further experiments involving a precise induction of PIN1 expression in those cells at this particular stage are necessary to sustain such a claim.

Answer: The experiment suggested by the reviewer would be indeed very informative but unfortunately, we don't have the molecular tools to induce PIN1 expression specifically in the cells that form the second PIN1 domain.

3a- Figure 4A. "Representative images of distorted distal areoles (red arrows) in pin1-1 and vcc pin1-1 cotyledons are shown". How can the authors be certain that these are indeed the distal areoles formed first, could they be smaller loops formed later during vein patterning as a compensatory mechanism for low vein complexity?

Answer: We have now modified the text to acknowledge we have not determined the origin of the abnormally small areoles:
Line 264: Although we did not determine the origin of these abnormal areoles, for the purpose of our analysis, we considered them as distal areoles based on their position.

3b. It would be interesting to also show representative images of pid and pid vcc cotyledons and not only the quantifications (Figure 4B).

Answer: We have now included representative images of *pid* and *vcc pid* cotyledons in Figure 5B (former Fig 4B).

4-Figure 6E. Could the authors provide better or at least bigger images to show the localization of VCC in the vacuolar lumen?

Answer: We have now included a better image in Figure 7E (former Fig 6E) to better document YFP-VCC localization at the vacuolar lumen.

5a-Figure 8E. It is not clear to this reviewer that VCC internalization into the vacuole occurs mostly in PIN1-GFP expressing cells based on their quantification. Which would be the significance of VCC internalization into the vacuole in other cells such as endodermal cells.

Answer: According to our quantifications, the ratio vacuolar to plasma membrane localization of VCC is higher in procambial cells expressing PIN1. This suggest that the vacuolar degradation of VCC is enhanced in these cells. However, although to a lesser extent, VCC is also found in the vacuole of ground cells that do not express PIN1-GFP. As most plasma membrane proteins are degraded inside vacuoles, the localization of a fraction of VCC in the vacuoles of ground cells is not surprising. However, we do not know what the function of VCC is in ground cells.
This analysis only allows to conclude that the degradation of VCC is enhanced in procambial cells compared to ground cells in developing embryos.

5b. Moreover, in figure 8F the authors show a "Representative image of polarized PIN1-GFP in elongating pre-procambial cells". In M&M the authors explain that "PIN1 polarity was analyzed by quantifying PIN1-GFP Fluorescent signal at the plasma membrane of at least two consecutive cells". Considering that in Figure 8F this reviewer can distinguish just two consecutive cells where PIN1 seems to be polarized, and in the first of the two, there is signal on the lateral side of this cell, are two consecutive cells enough to assess polarity? Furthermore, if comparing this image to Figure 8I the vcc mutant shows a very similar localization pattern, where PIN1 is polarly localized in two consecutive cells and in the first of the two, PIN1 is also localized on the lateral side of that cell. So how is polarity or incomplete polarity truly identified and further quantified?

Answer: We apologize for the confusion. An extra arrowhead was mistakenly added in Figure 8F, and it has now been removed. When we find stronger GFP signals at the opposite sides of a cell (even if we cannot discern at which side of cells PIN1-GFP is located), we have referred to those cases as polarized PIN1 localization.

6a-The authors claim that the failure of the second PIN1 domain in vcc cotyledons is due to an enhanced internalization of PIN1 into the vacuoles and therefore, a deficient PIN1 polarization required to promote the transition between pre-procambial to procambial cells. Yet, a high vacuolar localization of PIN1-GFP could be observed in elongated procambial cells of the middle vein strand. Could only PIN1 internalization explain the defective transition between pre-procambial into procambial cells in vcc mutants?

Answer: PIN1 is internalized in both wild-type and vcc elongated procambial cells. However, in *vcc* cotyledons, PIN1 is internalized prematurely in pre-procambial cells prior to elongation, only at the second PIN1 domain. Based on this observation, we concluded that VCC is important for the transition from preprocambial to procambial cells at the second PIN1 domain.

6b. Moreover, it seems that the authors have based their interpretation of cell fate not only into PIN1 polar distribution but also the shape of the cells. However, in Figure 8I, it is hard to say that some procambial cells showing PIN1 polar localization are elongated or are different shape- wise to the ones assigned as pre-procambial cells in vcc images. Could the authors use another identity marker or trait to clarify this?

Answer: The ground meristem cells from which procambial cells arise express PIN1. Procambial

cells are readily identifiable based on their elongated profiles (Scarpella et al 2004). As procambial cells form along strands, PIN1-expressing pre-procambial cells located in forming strands can also be readily identified based on their position. To confirm that the short cells expressing PIN1-GFP and located in the areas where basal strands form were indeed pre-procambial cells, we analyzed wild type embryos expressing a pre-procambial fluorescent marker (*pATHB8::NLS-YFP*) provided by Dr Enrico Scarpella. New data showing expression of this reporter in short (pre-procambial) and elongating cells in forming strands in Arabidopsis cotyledons is now included in Fig 3D and E.

7- While in the discussion VCC function is mainly discussed in terms of regulating PIN1 activity in an independent path of PINOID, very little is mentioned about the importance of the interaction between VCC and OPS. Can the authors discuss a bit further about the potential role of VCC in terms of regulating the activity of OPS? Is OPS also internalized and requires VCC activity to display its correct localization? Are other brassinosteroid-related mutants involved in cotyledon vascular development during embryogenesis?

Answer: We have not analyzed OPS localization in the *vcc* mutant. Some brassinosteroid-related mutants do show vasculature defects in cotyledons. However, we do not present any data about a direct connection between VCC and brassinosteroids and therefore, to keep the discussion focused and within the manuscript length limits, we have decided not to extend our discussion on VCC and brassinosteroids.

8- Also in the discussion, the authors suggest that VCC expression is subjected to an auxin-mediated feedback loop involving most likely MONOPTEROS. Yet, Smith et al; Development 2020 demonstrated the existence of a MP-independent pathway in modulating vein identity.
Could the authors comment on the role of VCC in establishing vascular identity at least based on its expression pattern during embryogenesis?

As the reviewer mentioned, Smit et al (2020) showed that MONOPTEROS/ARF5 and auxin signaling is required but not sufficient for specification of vascular identity in Arabidopsis embryo. However, MONOPTEROS/ARF5 has been proposed to regulate the expression of VCC (Möller et al 2017) and consistently, we show that the expression of VCC is upregulated in response to application of exogenous auxin. Although we have no evidence to discard the regulation of VCC independently of auxin and MP, both published data and our current results suggest that VCC is part of the auxin-mediated vascular development pathways. The similar expression of VCC and MONOPTEROS/ARF5 further support this notion.

Minor comments
9- The authors frequently use indistinguishably differentiation and specification. Since both are very different process in a developmental context, I would strongly recommend the authors to be more precise in the use of these terms for reader's clarity. In several cases the authors use "differentiated/differentiation" to describe the transition from pre-procambial to procambial strands or procambial to vascular strands. In either case, I believe this terminology to be incorrect as vascular differentiation occurs between 2-3 days after germination (James S. Busse and Ray F. Evert 1999). One example is on Line 72 "These PIN1-positive cell files differentiate into procambium".

Answer: We have changed "differentiation" to "specification".

Likewise, the authors frequently refer to PIN1 expression when they mean localization. This terminology can be confusing and considering that PIN1 has a transitional expression pattern and a very dynamic localization during vein patterning, I think the authors should be more precise when referring to PIN1 subcellular dynamics.

Answer: We have revised the terminology used to describe expressions patterns and localization.

10- Lines 310-312. Based on the graphs depicted in Figure S3C it seems inaccurate to claim that both VCC YFP tagged versions largely restore vcc phenotypes. To talk about a partial rescue seems more appropriate.

Answer: The word “largely” was replaced with “partially”, as suggested by the reviewer.

Additionally, do the authors observe the correct polar localization of PIN1 in tdTomato-VCC vcc plants?
Answer: Yes, we observed normal PIN1 localization in tdTomato-VCC *vcc* PIN1-GFP plants.

11- Figure 6. VCC YFP is shown in the root meristem, is there a root vascular phenotype or root meristem phenotype in vcc? It would be helpful for the reader to indicate that to better demonstrate the role of VCC in vascular development in general.
Answer: Although VCC is strongly expressed in roots, we have not found any defect in *vcc* root vasculature. We suspect that, as there are 15 genes in Arabidopsis encoding DUF1218 proteins Roschzttardtz et al 2014), there is functional redundancy within the family.

12- Throughout the entire manuscript, there are some references missing after statements that should be referenced
for example page 3 line 45 “….most of the transport occurs from and to the leaves.”

Answer: New references were added throughout. This specific sentence was removed.

Missing Reference Line 69….of ground cells and localizes to the plasma membrane with no detectable polarity”

Answer: Scarpella et al., 2006 was added to now line 68.

Missing Reference Line 75

Answer: The sentence was modified. Missing

Reference Line 82

Answer: Placing of citations was changed.

Missing Reference Line 95

Answer: Roschzttardtz et al., 2014 was added.

13- Line 53 ..by the ---of procambial cells. In this sentence do the authors mean that new veins form in denovo organs like leaves?

Answer: This sentence was modified as follows:
Line 47: The basic pattern of vein architecture is first established during embryo development and after seed germination, xylem and phloem differentiate from procambial cells (Sieburth, 1999).

14- Figure 1A. Are the images of cotyledons shown here of WT background or vcc or a mix?

Answer: These images illustrate examples of different vascular features and come from both wild-type and *vcc* cotyledons.

15- Figure 8I. Figure states red asterisks image displays red arrowheads.

Answer: Red asterisks were replaced with red arrowheads. This is now Figure 9I.

Second decision letter

MS ID#: DEVELOP/2020/197210

MS TITLE: Arabidopsis vascular complexity and connectivity controls PIN-FORMED1 dynamics and lateral vein patterning during embryogenesis

AUTHORS: Makoto Yanagisawa, Arthur Poitout, and Marisa Otegui
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 BenchPress and 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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 manuscript by Yanagisawa et al. is a revision of a previously submitted manuscript. I thank the authors for taking some of my comments into account while preparing the revised manuscript.

Comments for the author

1. In response to one of my previous comments, the authors wrote: "We have also performed Pearson’ Chi-squared analyses to determine the statistical significance of the differences in vein network complexity and the occurrence of vein discontinuities among different genotypes."

However, Pearson’s chi-squared test requires knowledge of the expected frequency of phenotypes, which is only possible if such phenotypes have complete penetrance and expressivity because levels of incomplete penetrance and expressivity are not reproducible and depend on varied parameters, including the environment. That the phenotypes of WT, vcc pin1, or vcc;pin1 are at the very least incompletely penetrant prevents the calculation of reliable expected phenotype frequencies to be used in a chi-squared test. Moreover, by reducing the vein pattern defects of vcc, pin1, and vcc;pin1 to two classes — simple and complex — the authors reduce significantly the power of their analysis, thereby reducing the possibility to detect differences between the three genotypes. The gold standard in clinical studies for non-parametric distributions such as those of classes of discrete phenotypes are the Kruskal-Wallis and Mann-Whitney tests (with Bonferroni correction if necessary to correct for multiple comparisons).

2. ll. 189–192 and 202,203. The inner PIN1 expression domain that is associated with the formation of leaf midvein and lateral veins does not form underneath a bipolar epidermal cell, but underneath an epidermal cell toward which polarities of neighboring cells converge.

3. The original pin1-1 stock from the stock center contains a ttg mutation, which should be taken into account in Figure 5.

4. ll. 293, 294. I don't think strength of defects can be used as a means to determine which of two genes acts upstream of the other in the same pathway: that the defects of vcc are weaker than those of pin1 may simply reflect more widespread functional redundancy among VCC-related genes. Moreover, the observation in this manuscript that overexpression of PIN1 in PIN1::PIN1:GFP rescues vcc defects is consistent with the opposite interpretation: that VCC and PIN1 act in the same pathway and PIN1 acts downstream of it — an example of epistatic suppression.

5. vcc;pid-17 cannot be compared with pid-18. Double mutants must be compared with the same single mutant alleles used to generate the double mutants. Alternatively, the authors should show that the defects of pid-17 are no different from those of pid-18.

6. It's unclear why the authors switch at first to roots to study VCC expression and localization. This seems to make no sense in the context of the story — there are no defects in vcc root development presented in this study. The authors should justify this experiment better — in the text of the manuscript — or move it to the supplemental material.

7. In response to one of my previous comments ("PIN1 has never been reported in vacuoles of live-imaged or dissected and undamaged leaves"), the authors wrote that it had been reported in Spitzer et al. 2009 and in Buono et al. 2016; however, I was unable to find evidence in those papers that PIN1 accumulates in vacuoles of WT cells, especially in leaves or cotyledons. That PIN1 may accumulate in vacuoles in dark-treated leaves and in cytokinin-treated roots (Shirakawa et al. 2009; Marhavy et al. 2011) seems irrelevant to the authors' study, or is it? I maintain that PIN1 — detected either through FP fusions or anti-PIN1 antibodies — has never been reported in vacuoles of live-imaged or dissected and undamaged leaves, so that the authors observed such accumulation in cotyledons needs to be explained; could it be because cotyledons develop in the dark — i.e. inside seed coats and siliques — while leaves develop in the light?

8. Figure 3 or its legend. Please indicate stages of embryos from which the details in panels A–C are derived.

9. Figure 9 or its legend. Please indicate stages of embryos from which the details in panels A,C,F,G,I are derived.

10. In Fig. 9A, the PIN1 expression domain associated with the bottom left loop is disconnected from both epidermis, midvein, and top-left loop, which never happens. Please correct.

11. In response to one of my comments ("I do not recall evidence that LHW, TMO5, and TML1 control expression of MP or PIN1"), the authors correctly indicate that Ohashi-Iro et al. 2013 provide evidence that expression of MP and PIN1 is controlled by LHW, but failed to provide evidence in support of the claim that expression of TMO5 and TML1 controls expression of MP and PIN1.

Reviewer 3

Advance Summary and Potential Significance to Field

This study improves our understanding about how the timing of PIN1 degradation in pre-procambial cells is modulated by VCC and impacts the vein network complexity of embryonic cotyledons.

Comments for the author

The current manuscript has improved in comparison with the previous version. Yet, a deeper re-phrasing of some parts of the texts or more accurate interpretation of the results is needed. It would be desirable to obtain plants simultaneously expressing AthB8 and PIN1-GFP to better correlate the developmental stage of these cells and the levels of PIN1. Moreover, I do believe that PIN1 dosage in vcc mutants has not been satisfactorily addressed, especially given the little transcriptional differences observed among the genotypes analyzed

Second revision

Author response to reviewers' comments

We would like to thank the reviewers for the thoughtful comments on our manuscript. In this revised version, we have addressed their comments and highlighted changes in yellow. Below, we answer the comments raised by the reviewers one by one.

Reviewer 1 Advance summary and potential significance to field
The manuscript by Yanagisawa et al. is a revision of a previously submitted manuscript. I thank the authors for taking some of my comments into account while preparing the revised manuscript.

Reviewer 1 Comments for the author
1. In response to one of my previous comments, the authors wrote: "We have also performed Pearson' Chi- squared analyses to determine the statistical significance of the differences in vein network complexity and the occurrence of vein discontinuities among different genotypes." However, Pearson's chi-squared test requires knowledge of the expected frequency of phenotypes, which is only possible if such phenotypes have complete penetrance and expressivity because levels of incomplete penetrance and expressivity are not reproducible and depend on varied parameters, including the environment. That the phenotypes of WT, vcc, pin1, or vcc;pin1 are at the very least incompletely penetrant prevents the calculation of reliable expected phenotype frequencies to be used in a chi-squared test. Moreover, by reducing the vein pattern defects of vcc, pin1, and vcc;pin1 to two classes — simple and complex — the authors reduce significantly the power of their analysis, thereby reducing the possibility to detect differences between the three genotypes. The gold standard in clinical studies for non-parametric distributions such as those of classes of discrete phenotypes are the Kruskal-Wallis and Mann-Whitney tests (with Bonferroni correction if necessary to correct for multiple comparisons).

Answer: We apologize for the misunderstanding: we did not perform chi-squared tests of goodness-of-fit, which indeed require knowledge of the expected frequency of phenotypes. We instead performed chi-squared tests of independence, to compare the frequencies of vein patterns across genotypes. (to see if the pattern frequencies are dependent on the genotype or independent -equal across genotypes). These tests do not require knowledge of the exact frequency of phenotypes in each genotype. The frequencies are estimated from the data, and the degree-of-freedom for the chi-square distribution is adjusted accordingly.

Reducing the data to "simple" and "complex" categories increases the power to detect differences between genotypes with regards to the frequency of these global patterns, because the sample size in each category is increased, compared to smaller sample sizes in a larger number of categories. If also focuses the question being asked, thereby increasing interpretability. The very small p-values also show that our study has enough power to detect differences between genotypes.

Kruskal-Wallis and Mann-Whitney tests are appropriate for numerical data, when the distribution of the numerical values do not fit a normal distribution, like the reviewer points out. In our experiments, our data are not numerical but categorical (e.g. 2-2, 3-0, oda, bfe, …). Categorical data are summarized with the count of each category. The Kruskal-Wallis and Mann-Whitney tests are not appropriate for count data. Count data correspond to binomial distributions (for 2 categories) or multinomial distributions more generally (2 or more categories). Chi-square tests are specifically designed to model these distributions.

For the non-disconnected patterns, these patterns could potentially be given numerical values, if a downstream analysis using a Kruskal-Wallis or Mann-Whitney test:

category/	numerical value
4-0/	5
3-1/	4
2-2/	3

3-0/ 2??
2-1/ 1??
2-0/ 0??

but we feel that mapping categories to a single numerical axis is arbitrary, and that it is not necessary given that our approach (which is appropriate for categorical data) has the power the detect differences between genotypes.

Therefore, we continue to believe that our chi-square methodology is the most appropriate.

2. ll. 189–192 and 202,203. The inner PIN1 expression domain that is associated with the formation of leaf midvein and lateral veins does not form underneath a bipolar epidermal cell, but underneath an epidermal cell toward which polarities of neighboring cells converge.

Answer: Addressing the reviewer's comment, we have now re-written the following sentence: - Line 185 "Similar to what has been described in leaf primordia (Scarpella et al., 2006; Wenzel et al., 2007), this PIN1 domain originates underneath an epidermal cell in which PIN1-GFP is localized at the anticlinal sides facing the neighboring epidermal cells and the periclinal side towards a subepidermal cell (Fig 3A)."

3. The original pin1-1 stock from the stock center contains a ttg mutation, which should be taken into account in Figure 5.

Answer: As we have crossed the pin1-1 mutant into the vcc background, we have not observed any vein defects in the segregating progeny with pale seed coats except for those seedlings homozygous for pin1-1 or vcc, suggesting that the ttg mutation does not alter vein patterns in cotyledons.

4. ll. 293, 294. I don't think strength of defects can be used as a means to determine which of two genes acts upstream of the other in the same pathway: that the defects of vcc are weaker than those of pin1 may simply reflect more widespread functional redundancy among VCC-related genes. Moreover, the observation in this manuscript that overexpression of PIN1 in PIN1::PIN1:GFP rescues vcc defects is consistent with the opposite interpretation: that VCC and PIN1 act in the same pathway and PIN1 acts downstream of it — an example of epistatic suppression.

Answer: We agree with the reviewer. We have removed the sentence (page 10) where we proposed epistasis on PIN1 over VCC as more than one interpretation is possible based on our results.

5. vcc;pid-17 cannot be compared with pid-18. Double mutants must be compared with the same single mutant alleles used to generate the double mutants. Alternatively, the authors should show that the defects of pid-17 are no different from those of pid-18.

Answer: PINOID (At2g34650) and VCC (At2g32280) loci are both located in chromosome 2, very close to each other. We have failed to generate double mutants between existing pid and vcc lines because these loci are linked. Therefore, we decided to use CRISPR/CAS9 editing in Col-0 and vcc backgrounds independently. The resulting mutations in each background (pid-17 in WT and pid-18 in vcc) are both a single nucleotide insertion in the same position but whereas an extra G was inserted in pid-17, and extra T was inserted in pid-18. However, the predicted translated proteins (PID17 and PID18) are identical to each other (as shown in Fig S4). We have screened hundreds of plants and have been unable to identify identical mutations in both backgrounds. Since the pid-18 to pid-17 alleles encode proteins of identical amino acid sequence, we decided to use for this analysis vcc pid-18 and pid-17. We do agree with Reviewer 2 that is important to be completely clear that the two alleles are not identical, so we have modified the text accordingly:

Lines 287-296: To test whether there is a genetic interaction between VCC and PID, we decided to generate vcc pid double mutants. However, as PINOID (At2g34650) and VCC (At2g32280) are located fairly close from each other in chromosome 2, we were unable to generate double mutants by crossing vcc with existing pid mutants. Instead, using CRISPR/Cas9 technology (Wang et al., 2015), we generated pid mutations in wild type Col-0 and vcc plants. Although we were unable to isolate identical pid mutations in both genetic backgrounds, we found in both lines a single nucleotide insertion at position 233 (G in the Col-0 and T in vcc) after the translation start site that causes a

codon reading frame shift after amino acid 78 of the PID protein. Although the two resulting pid alleles are not identical, their deduced translated protein products are (Fig. S4). We named these new alleles pid-17 and vcc pid-18 (Fig. S4).

6. It's unclear why the authors switch at first to roots to study VCC expression and localization. This seems to make no sense in the context of the story — there are no defects in vcc root development presented in this study. The authors should justify this experiment better — in the text of the manuscript — or move it to the supplemental material.

Answer: As the reviewer points out, the vcc mutant does not show root developmental defects. However, VCC expression in the root tip is relatively high, and consistently, we detected a strong 3x-YFP-VCC signal in the root meristematic region. Previously, VCC was reported to localize to the endoplasmic reticulum in root cells (Wilson-Sanchez et al 2018), challenging its functional connection to PIN1 at the plasma membrane. This is the first report of VCC localization based on a functional VCC-tagged protein under the control of the endogenous VCC promoter region. As roots are ideal for confocal imaging of deep tissues, where VCC is mostly expressed, we think it is important to keep this information in the manuscript and as a figure. We have explained the reasoning for presenting root imaging data in the Results section:

Lines 336-340: Although the vcc mutant does not have detectable root developmental defects, VCC expression in the root tip is relatively high, and consistently, we detected a strong 3x-YFP-VCC signal in the root meristematic region. As roots are ideal for confocal imaging of deep tissues, where VCC is mostly expressed, we decided to analyze the subcellular localization of 3x-YFP-VCC in root cells.

7. In response to one of my previous comments ("PIN1 has never been reported in vacuoles of live-imaged or dissected and undamaged leaves"), the authors wrote that it had been reported in Spitzer et al. 2009 and in Buono et al. 2016; however, I was unable to find evidence in those papers that PIN1 accumulates in vacuoles of WT cells, especially in leaves or cotyledons. That PIN1 may accumulate in vacuoles in dark-treated leaves and in cytokinin-treated roots (Shirakawa et al. 2009; Marhavy et al. 2011) seems irrelevant to the authors' study, or is it? I maintain that PIN1 — detected either through FP fusions or anti-PIN1 antibodies — has never been reported in vacuoles of live-imaged or dissected and undamaged leaves, so that the authors observed such accumulation in cotyledons needs to be explained; could it be because cotyledons develop in the dark — i.e. inside seed coats and siliques — while leaves develop in the light?

Answer: The detection of GFP from PIN1-GFP or other tagged proteins depends on the amount of GFP transported into the vacuole, a proposed light-induced change in conformation of vacuolar GFP that would make it more susceptible to degradation (Tamura et al 2003 Plant Journal 35:545-555), as well as the vacuolar lytic activity and pH. In previous papers, we measured the vacuolar pH of embryo cells at torpedo stage and mesophyll cells and found that in both cases is close to 6 (Otegui et al 2006 Plant Cell 18: 2567-2581; Otegui et al 2005, Plant Journal 41:831-844), therefore, pH itself does not seem to be the reason for the difference in GFP stability. We do not know whether there are differences in vacuolar proteolytic activity between torpedo embryos and mesophyll cells but as the reviewers points out, light exposure could be a factor as dark-treated leaves expressing PIN1-GFP do show GFP signal in their vacuoles (Shirakawa et al. 2009 Plant Cell Physiology 50:1319-1328). We think, though, it is important to mention that even though PIN1-GFP is not readily visualized in vacuoles of leaf cells, it does not mean that it is not been sorted to the vacuole for degradation just like in root cells.

8. Figure 3 or its legend. Please indicate stages of embryos from which the details in panels A–C are derived.

Answer: We have added the embryo stages for panels A to C in the figure legend.

9. Figure 9 or its legend. Please indicate stages of embryos from which the details in panels A,C,F,G,I are derived.

Answer: We have added the embryo stages for panels A, C, F, G, and I in the figure legend.

10. In Fig. 9A, the PIN1 expression domain associated with the bottom left loop is disconnected from both epidermis, midvein, and top-left loop, which never happens. Please correct.

Answer: Thank you for catching this mistake. The diagram in Figure 10A have been corrected

11. In response to one of my comments ("I do not recall evidence that LHW, TMO5, and TML1 control expression of MP or PIN1"), the authors correctly indicate that Ohashi-Iro et al. 2013 provide evidence that expression of MP and PIN1 is controlled by LHW, but failed to provide evidence in support of the claim that expression of TMO5 and TML1 controls expression of MP and PIN1.

Answer: The reviewer is correct. We have changed the text to:

Lines 78-83: Mutations in ARF5/MP result in severe defects in vascular formation in cotyledons (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998) and consistently, lack of function of the transcription factor LONESOME HIGHWAY (LHW), which regulates the proper expression of ARF5/MP, PIN1, and auxin and cytokinin biosynthetic genes, results in vascular development defects in embryos and roots (De Rybel et al., 2013; Ohashi-Ito et al., 2013; De Rybel et al., 2014; Ohashi-Ito et al., 2019; Smet et al., 2019).

Reviewer 3 Advance summary and potential significance to field
This study improves our understanding about how the timing of PIN1 degradation in pre-procambial cells is modulated by VCC and impacts the vein network complexity of embryonic cotyledons.

Reviewer 3 Comments for the author
The current manuscript has improved in comparison with the previous version. Yet, a deeper re-phrasing of some parts of the texts or more accurate interpretation of the results is needed. It would be desirable to obtain plants simultaneously expressing AthB8 and PIN1-GFP to better correlate the developmental stage of these cells and the levels of PIN1.

Answer: We agree with the reviewer it would be desirable to image both AtHB8 and PIN1 but this would take several months to achieve and we think, with limited new insights.

Moreover, I do believe that PIN1 dosage in vcc mutants has not been satisfactorily addressed, especially given the little transcriptional differences observed among the genotypes analyzed.

Answer: We can only measure PIN1 transcripts by qRT-PCR and their levels correlate nicely with the extent of vein patterning rescue in the vcc mutant lines, which would indicate that the stability of the second PIN1 domain is very sensitive to small changes in PIN1 transcript accumulation.

Third decision letter

MS ID#: DEVELOP/2020/197210

MS TITLE: Arabidopsis vascular complexity and connectivity controls PIN-FORMED1 dynamics and lateral vein patterning during embryogenesis

AUTHORS: Makoto Yanagisawa, Arthur Poitout, and Marisa Otegui

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 manuscript by Yanagisawa et al. is a revised version of a previously submitted study.

Comments for the author

1. Statistical analysis. I thank the authors for the clarification. It is true that the Kruskal Wallis / Mann Whitney test requires assigning a numerical value to each phenotype class, and this is usually done by assigning the lowest value to the WT phenotype class and increasingly higher values to increasingly stronger classes. This approach is the gold standard in clinical studies and seems to be applicable to the authors' study, so I urge the authors to reconsider their claim that their test is *the most* appropriate. Nevertheless, I note that the authors may not feel comfortable with defining which of any two phenotype classes deviates more from the WT, in which case a more "neutral" test like the one they have used is perfectly acceptable.

The authors are right that by reducing the number of phenotype classes the number of samples in each class increases, thereby increasing the power of their statistical analysis. However, I did not intend the term
"power" in a statistical sense — I apologize if I misled the authors. Instead, I meant to say that the smaller the number of phenotype classes, the more difficult it is to detect differences between genotypes. By keeping the original number of phenotype classes — and, as the authors add, by increasing the number of samples — the authors might have been able to tease out the genetic interaction between vcc and pin1.

2. To a comment of mine, the authors replied the following:
>As we have crossed the pin1-1 mutant into the vcc background, we have not observed any vein defects in the segregating progeny with pale seed coats except for those seedlings homozygous for pin1-1 or vcc suggesting that the ttg mutation does not alter vein patterns in cotyledons.

I think this is a very important piece of information that goes to the authors' credit and that should be included in the manuscript — perhaps in the figure legend or in the Materials & Methods.

3. PIN1 localization in vacuoles. I agree with the authors that this is an important observation that should be included in this manuscript, but I also think that precisely because it has never been reported before in normally grown WT embryos, shoots, or roots — whether PIN1 had been detected by anti-PIN1 antibodies or by tagging it with fluorescence proteins — it should be discussed. After all, a balanced Discussion section typically includes discussion of findings that, based on previous literature, are unexpected.