Vein patterning by tissue-specific auxin transport

Priyanka Govindaraju, Carla Verna, Tongbo Zhu and Enrico Scarpella
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MS TITLE: Vein Patterning by Tissue-Specific Auxin Transport

AUTHORS: Priyanka Govindaraju, Carla Verna, Tongbo Zhu, and Enrico Scarpella

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.

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

Govindaraju et al describe a genetic analysis of the spatial requirements for PIN protein expression in leaf vein patterning in Arabidopsis. Previously, it was suggested (including work from the same author) that PIN convergence points in the epidermis of the leaf contribute to vein patterning, and this finding has been used in computational models of auxin-dependent vein patterning.
In the current manuscipt, the authors carefully examined localization of PIN1,3,4 and 7 in the developing leaf and in addition expressed PIN1-GFP in various cell types to determine where the protein is required for leaf vein development. The conclusion is that expression in the vascular domain (not the epidermis or ground tissue) is necessary and sufficient for its role in leaf vein

development. This is an intersting finding, that is well-supported in the pin1;3;4;7 mutant (less so in the pin1 mutant). While the quality of the work is outstanding (as always with this group) and while I see the value of correcting the prevailing idea that epidermal PIN1 matters for vein development, I also feel that the scope is rather limited and the substance is somewhat minimal.

Comments for the author

If the authors want to make a strong point, I would suggest either of the following courses:

1. Explore the impact of these findings on the existing computational models of vein development. Likely, a relatively minor effort would help define the imapct of this new insight, which is otherwise left to the community to solve.

2. Strengthen the evidence for (non-) relevance of epidermal PIN1 in a wild-type background. There is an efficient tissue-specific KO system (Decaestecker et al., Plant Cell 2019), that would allow the authors to knock out PIN1 only in the L1 layer in an otherwise wild-type background. Hopefully this experiment will suffer less from incomplete pentrance of complemetation transgenes.

Reviewer 2

Advance summary and potential significance to field

The detailed genetic work by Govindaraj et al examines the role of PIN1-mediated polar auxin transport in young leaf margin epidermis and vascular tissue in vein patterning. To test this, the authors expressed the PIN1-GFP under various tissue specific promoters, and observed whether they were able to complement pin mutants. Based on the results, the authors concluded that epidermal PIN1 expression is neither required nor sufficient for vein patterning, while vascular PIN1 expression is both required and sufficient.
The work is logically executed and illustrated, and the research question is important for plant development biology.

Comments for the author

I have one major concern:
The finding that ubiquitously in epidermis expressed PIN1-GFP (under pAtML1) does not complement pin mutants is a not a big surprise. Auxin canalization supposedly should involve complex feed-forward regulation between auxin and the transporters both in transcriptional and post-transcriptional level.
If PIN1-GFP is expressed ubiquitously in epidermis, its transcriptional feed-forward regulation with auxin will most likely not take place. Also, as the authors point out, the convergence point of PIN1 in the marginal epidermis consists only of few cells. So, if I understood this right, AtML1 domain and endogenous PIN1 expression overlap only in these few cells, as opposed to almost identical expression domain between endogenous PIN1 and SHR. Thus, I am afraid, the outcome of this study is quite expected. The authors point was to address the current hypothesis on the role of PIN1 convergence point in leaf margin epidermis, however, I am not sure whether author behind this hypothesis assumed that the major, inner vascular PIN expression does not have contribution – I think not? Or did I misunderstood something? I am happy to change my mind if the logic is better explained.

Minor points:
- plant signal auxin --> plant hormone auxin?
- Top of page 4: "….epidermal expression became restricted to the basalmost cells, and inner-tissue expression became restricted to developing veins (Fig. 1E–H)." Here and throughout the paper:

Does the "epidermal expression" refer to the leaf edges only? There is also epidermis in the central part of the flat leaf (in the abaxial and adaxial side). Please, clarify this. A cartoon with PIN1 expression in leaf transverse section would be informative.

First revision

Author response to reviewers' comments

Dear Reviewers,

Please find attached our revised manuscript “Vein Patterning by Tissue-Specific Auxin Transport”, and below our point-by-point response to your comments. Because the Development online submission system does not preserve the formatting of this document, we have also uploaded it as supplemental material.

To satisfy the word-limit requirement for Short Reports after the changes we made in response to your comments, we have moved details of the expression of PIN3, PIN4, and PIN7 to a supplemental table (Table S1).

To facilitate the identification of the changes we made to the original manuscript, we have uploaded as supplemental material a version of the revised manuscript in which changes are tracked.

We believe our new text and documentation have taken all your remarks into full account, and would like to thank you for your help, time, interest, and patience.

On behalf of all the authors,

Sincerely,

Enrico Scarpella

Reviewer 1
Advance Summary and Potential Significance to Field:

Govindaraju et al describe a genetic analysis of the spatial requirements for PIN protein expression in leaf vein patterning in Arabidopsis. Previously, it was suggested (including work from the same author) that PIN convergence points in the epidermis of the leaf contribute to vein patterning, and this finding has been used in computational models of auxin-dependent vein patterning. In the current manuscipt, the authors carefully examined localization of PIN1,3,4 and 7 in the developing leaf, and in addition expressed PIN1-GFP in various cell types to determine where the protein is required for leaf vein development. The conclusion is that expression in the vascular domain (not the epidermis or ground tissue) is necessary and sufficient for its role in leaf vein development. This is an intersting finding, that is well-supported in the pin1;3;4;7 mutant (less so in the pin1 mutant). While the quality of the work is outstanding (as always with this group) and while I see the value of correcting the prevailing idea that epidermal PIN1 matters for vein development, I also feel that the scope is rather limited and the substance is somewhat minimal.

We thank Reviewer 1 for their encouraging evaluation of our work. That evaluation made us realize that the broad implications of our findings for plant developmental biology were not sufficiently clear in our original manuscript; we therefore welcome the opportunity to clarify this important point.

For the past >15 years, a set of developmental processes has been thought to depend on PIN1 expression in the shoot epidermis: the patterned positioning, growth, and differentiation of flower primordia; the patterned positioning of leaf primordia; the formation of dissected leaves; the formation of leaf serrations; and leaf vein patterning (e.g., (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Hay et al., 2006; Scarpella et al., 2006; Wenzel et al., 2007; Barkoulas et al., 2008)). Consistent with that thought, PIN1 expression in the shoot epidermis has been shown to be required and sufficient for all those processes, except — until our manuscript — leaf vein patterning (e.g., (Bilsborough et al., 2011; Kierzkowski et al., 2013; Kierzkowski et al., 2019; Li et al., 2019)). Therefore, for the past >15 years the same mechanism has been inductively inferred to be underlying all those processes, including leaf vein patterning. That — as we show in our manuscript — PIN1 expression in the shoot epidermis is neither sufficient nor required for leaf vein patterning is thus a most unexpected finding, which points to a fundamental difference between, on the one

hand, leaf vein patterning and, on the other hand, the patterned positioning, growth, and differentiation of flower primordia; the patterned positioning of leaf primordia; the formation of dissected leaves; and the formation of leaf serrations. All these considerations are important but were not in our original manuscript; we have now included them in our revised manuscript.

Reviewer 1 Comments for the Author:

If the authors want to make a strong point, I would suggest either of the following courses:

1. Explore the impact of these findings on the existing computational models of vein development. Likely, a relatively minor effort would help define the imapct of this new insight, which is otherwise left to the community to solve.

We thank Reviewer 1 for their valuable suggestion. We have been collaborating with computational scientists for several years now to develop models of vein patterning that are rooted in experimental evidence. What we have discovered, however, is that in addition to assuming that PIN1 expression in the epidermis is required for vein patterning, all existing models suffer from a fatal assumption: that auxin transport is sufficient for vein formation. We have recently shown that such an assumption is unjustified: veins are still formed in a reproducible, though abnormal, pattern in plants that lack the functions of all the *PIN* genes with vein patterning activity, and the residual patterning activity does not seem to depend on other auxin transporters; instead, it seems to depend on the movement of an unidentified auxin-dependent signal (Verna et al., 2019). Our work with computational scientists has made abundantly clear that until we learn more about the nature and behavior of that signal, all attempts at developing models of vein patterning that are rooted in experimental evidence are destined to be frustrated. Over the past few years, we have made significant progress toward the identification of such signal, but we still lack sufficient details to develop a biologically plausible model of vein patterning. Nevertheless, we would like to suggest that the nature and behavior of that signal and the derived model of vein patterning fall outside the scope of the current manuscript.

2. Strengthen the evidence for (non-) relevance of epidermal PIN1 in a wild-type background. There is an efficient tissue-specific KO system (Decaestecker et al., Plant Cell 2019), that would allow the authors to knock out PIN1 only in the L1 layer in an otherwise wild-type background. Hopefully this experiment will suffer less from incomplete pentrance of complemetation transgenes.

We thank Reviewer 1 for the valuable suggestion, which — if we understand it correctly — is motivated by the reviewer's impression that our transgene-based experiments in the *pin1* mutant background suffer from incomplete penetrance. We believe, however, it is the penetrance of the vein pattern defects of *pin1* mutants that is incomplete (Sawchuk et al., 2013; Verna et al., 2019; this manuscript), not the phenotypic rescue achieved by means of transgenes in our manuscript. No additional experiment in a background that lacks *PIN1* function — whether in all the tissues or only in some of them — will ever change that.

To show conclusively that it is not the transgene-mediated rescue of the vein pattern phenotype of *pin1* that is incompletely penetrant, we have now chosen a *pin1* phenotype that, unlike the vein pattern, is completely penetrant: the pin-shaped inflorescence. We reasoned that if it was the transgene-mediated phenotypic rescue to be incompletely penetrant, attempts to rescue the *pin1* inflorescence phenotype with ATML1::cPIN1:GFP, which fails to rescue even partially the vein pattern phenotype of *pin1*, would at best lead to partial rescue of the *pin1* inflorescence phenotype. As we show in the new Figure S3, however, ATML1::cPIN1:GFP rescued completely the *pin1* inflorescence phenotype. By contrast, PIN1::cPIN1:GFP, which rescues the incompletely vein pattern phenotype of *pin1*, failed to rescue even partially the *pin1* inflorescence phenotype. We take this as evidence that our transgene-based approach has the potential to rescue *pin1* phenotypes completely, provided such defects are completely penetrant.

Because the vein pattern defects of *pin1* are incompletely penetrant, the only alternative is to perform transgene-mediated rescue in a mutant background in which vein pattern defects are completely penetrant. And that is precisely what we had done in our original manuscript by phenotypically rescuing by means of transgenes the completely penetrant vein pattern phenotype

of the *pin1,3;4;7* quadruple mutant (revised Figures 4 and S5). PIN1::cPIN1:GFP completely rescued the completely penetrant vein pattern phenotype of *pin1,3;4;7*, which we take as further evidence that our transgene-based approach has the potential to rescue phenotypes completely, provided such defects are completely penetrant.

Nevertheless, we believe the suggestion of Reviewer 1's is valuable and opens the door to additional experimental tests on the role of PIN-mediated auxin transport in vein patterning. That is why we contacted two groups and received, under collaborative terms, published and unpublished material. Unfortunately, however, on March 15 our university asked that all labs working on research unrelated to COVID-19, including my lab, be shut down indefinitely. We will only be glad to resume the experiments inspired by Reviewer 1's suggestion.

Reviewer 2

Advance Summary and Potential Significance to Field:

The detailed genetic work by Govindaraj et al examines the role of PIN1-mediated polar auxin transport in young leaf margin epidermis and vascular tissue in vein patterning. To test this, the authors expressed the PIN1-GFP under various tissue specific promoters, and observed whether they were able to complement pin mutants. Based on the results, the authors concluded that epidermal PIN1 expression is neither required nor sufficient for vein patterning, while vascular PIN1 expression is both required and sufficient.

The work is logically executed and illustrated, and the research question is important for plant development biology.

We thank Reviewer 2 for their positive evaluation of our work.

Reviewer 2 Comments for the Author:

I have one major concern:

The finding that ubiquitously in epidermis expressed PIN1-GFP (under pAtML1) does not complement pin mutants is a not a big surprise. Auxin canalization supposedly should involve complex feed-forward regulation between auxin and the transporters both in transcriptional and post-transcriptional level. If PIN1-GFP is expressed ubiquitously in epidermis, its transcriptional feed-forward regulation with auxin will most likely not take place. Also, as the authors point out, the convergence point of PIN1 in the marginal epidermis consists only of few cells. So, if I understood this right, AtML1 domain and endogenous PIN1 expression overlap only in these few cells, as opposed to almost identical expression domain between endogenous PIN1 and SHR. Thus, I am afraid, the outcome of this study is quite expected. The authors point was to address the current hypothesis on the role of PIN1 convergence point in leaf margin epidermis, however, I am not sure whether author behind this hypothesis assumed that the major, inner vascular PIN expression does not have contribution - I think not? Or did I misunderstood something? I am happy to change my mind if the logic is better explained.

We thank Reviewer 2 for the opportunity to clarify this important point. Starting from 2003, a set of developmental processes has been thought to depend on PIN1 expression in the epidermis of the shoot: the patterned positioning, growth, and differentiation of flower primordia; the patterned positioning of leaf primordia; the formation of dissected leaves; the formation of leaf serrations; and leaf vein patterning (e.g., (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Hay et al., 2006; Scarpella et al., 2006; Wenzel et al., 2007; Barkoulas et al., 2008)). All those processes have been thought to depend on positive feedback between PIN1 polar localization and, depending on the model, auxin concentration, auxin transport, or a mechanical signal (e.g., (Jonsson et al., 2006; Smith et al., 2006; Bayer et al., 2009; Smith and Bayer, 2009; Heisler et al., 2010; Bilsborough et al., 2011)). And for all those processes, except — until our manuscript — leaf vein patterning, it has been shown that PIN1 expression in the epidermis of the shoot by the *ATML1* promoter is required and sufficient (e.g., (Bilsborough et al., 2011; Kierzkowski et al., 2013; Kierzkowski et al., 2019; Li et al., 2019)). Therefore, that — as we show in our manuscript — PIN1 expression in the epidermis of the shoot by the *ATML1* promoter is neither sufficient nor required for leaf vein patterning is in fact most surprising. In fact, so surprising that we have now tested

whether our ATML1::cPIN1:GFP construct normalized, as previously reported ATML1::gPIN1:GFP constructs did (Bilsborough et al., 2011; Kierzkowski et al., 2013; Kierzkowski et al., 2019), the pin-shaped inflorescence phenotype of *pin1*. We have found that it does (new Figure S3), suggesting that the inability of our ATML1::cPIN1:GFP construct to rescue the leaf vein pattern defects of *pin1* is not an experimental artifact. Instead, our findings point to a mechanistic difference between, on the one hand, leaf vein patterning and, on the other hand, the patterned positioning, growth, and differentiation of flower primordia; the patterned positioning of leaf primordia; the formation of dissected leaves; and the formation of leaf serrations. All these observations are important but missing in our original manuscript; we thank Reviewer 2 for bringing this logical gap to our attention. We have now revised the text of the Introduction and the Results & Discussion to bridge that gap.

As to whether the authors, including ourselves, who assumed that PIN1 expression in the epidermis of the shoot would be sufficient for leaf vein patterning also thought that PIN1 expression in the inner tissues of the leaf contributed (or not) to leaf vein patterning, we believe that was never a point of contention in our manuscript. Should Reviewer 2 instead think that it was, we would be grateful if they could point out where exactly in our manuscript we gave that impression, so that we could remediate. Nevertheless, we would like to respectfully suggest that what those authors, including ourselves, thought is irrelevant: it is one thing to *think* that PIN1 expression in the inner tissues of the leaf contributes (or not) to leaf vein patterning; it is an entirely different thing to show *experimentally*, as we did in our manuscript, that such inner expression is both required and sufficient.

Minor points:

-plant signal auxin -> plant hormone auxin?

We thank Reviewer 2 for the suggestion; we have now changed both instances of “plant signal auxin” to “plant hormone auxin”.

-Top of page 4: “….epidermal expression became restricted to the basalmost cells, and inner-tissue expression became restricted to developing veins (Fig. 1E-H).” Here and throughout the paper:

Does the “epidermal expression” refer to the leaf edges only? There is also epidermis in the central part of the flat leaf (in the abaxial and adaxial side). Please, clarify this. A cartoon with PIN1 expression in leaf transverse section would be informative.

We thank Reviewer 2 for the opportunity to clarify this point: Throughout our manuscript, unless otherwise stated, “epidermal expression” refers to expression in the whole epidermis of the leaf, including the adaxial, marginal, and abaxial epidermis, and not to expression in the sole marginal epidermis. To visualize that, we have added a map of PIN1::gPIN1:GFP expression in both a front view, median plane and a transverse section of a developing leaf (Fig. 1E).

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Second decision letter

MS ID#: DEVELOP/2019/187666

MS TITLE: Vein Patterning by Tissue-Specific Auxin Transport

AUTHORS: Priyanka Govindaraju, Carla Verna, Tongbo Zhu, and Enrico Scarpella
ARTICLE TYPE: Research Report

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

Reviewer 1

Advance summary and potential significance to field

The authors have clarified some issues that were not very clear in the initial submission, and make a clear case for their conclusions. I agree that it would be unreasonable to ask for more experimental work, given that the access to the labs is essential and uncertain, and also considering what additional support this would bring. I enthusiastically recommend acceptance of this version.

Comments for the author

No specific suggestions

Reviewer 2

Advance summary and potential significance to field

It has been long thought that PIN1-mediated polar auxin transport through epidermis of a developing leaf is essential for leaf venation. This paper shows for the first time that this process is not required nor sufficient for vein patterning, unlike it is in the other shoot patterning processes.

Comments for the author

By better explaining the literature behind the importance of epidermal expression of PIN1 in various shoot patterning processes, the authors have now addressed my main concern. Also, other, minor concerns have now been addressed.
Thus, I have no further comments.