

Reticulate leaf venation in *Pilea peperomioides* is a Voronoi diagram

Corresponding Author: Dr Saket Navlakha

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear Authors,

This paper presents extremely interesting results showing that the vein patterns of the Chinese money plant (*Pilea peperomioides*) closely resemble the patterns generated by Voronoi partitioning of hydathodes. It demonstrates that if we assume vascular bundles are formed by the flux of auxin with hydathodes as the auxin source, a pattern similar to Voronoi diagram can be generated. The results indicating that the vein pattern and the relationship with hydathodes up to a certain order closely resemble the Voronoi diagram are very precise. While the first half of the paper seems generally unproblematic, several questions remain regarding the mathematical model presented in the latter half.

[Major Points]

If vein formation is not simultaneous with hydathodes as the auxin source, a Voronoi-like pattern will not be formed. However, at least some veins are not formed simultaneously up to a certain order but rather sequentially. This contradicts the core assumption of this model, suggesting that a different mechanism may need to be considered. For example, it might be more consistent to assume that hydathodes exert a supplementary repulsive force up to a certain stage, avoiding this issue and allowing new vascular bundles to form.

Regarding hydathodes and early vascular development, there are several SEM photos in the supplementary information, but they need to be shown in a way that indicates the chronological sequence. While knowledge on the development of leaf margin hydathodes is accumulating in the model plant *Arabidopsis thaliana*, these hydathodes connect to the vascular bundles and are thus generally unrelated to vein patterns. Therefore, insights from *Pilea* are crucial for verification.

[Minor Points]

In the model description, it appears that color allocation is assigned on a logarithmic scale and then converted to the actual scale for display in the figures, making the correspondence with the heat map unclear. The legend should be shown on a logarithmic scale, as in Fig. S6A, with the scale ticks indicated in Fig. 4, Fig. 5, and all related supplementary figures.

Fig. 2C vertical axis "distance erro (Δd)" contains a typo.

In Fig. 3A, it is assumed that the conditions indicated by the four colors are represented in Fig. 3B for "5 weeks," but the colors are mixed and hard to see. Please make the lines for each color a bit thicker and space them out a little more.

(Remarks on code availability)
The data in this study were reproducible from the code and notebook(.ipynb).

Reviewer #2

(Remarks to the Author)

Article by Zheng et al. explores a potential mechanism underlying the formation of high-order leaf venation structures, such as loops. The authors focus on the Chinese money plant, which exhibits a beautiful arrangement of leaf vasculature that,

according to the authors, resembles Voronoi diagrams. In general, the technical details and descriptions of models/algorithms seem appropriate and technically sound. However, I have several points to address before recommending this article for publication:

1) Bias Focus on Flux-Based Models.

The authors devote significant attention to so-called flux-based models as the "paradigm" for explaining leaf venation patterning. While these models are indeed elegant, they rely on the concept of a "flux sensor," which has yet to be identified. It remains unclear how cells would sense fluxes across other cells. There are alternative models that could potentially explain looped patterns (e.g., Stoma et al., 2008; Wabnick et al., 2011), assuming specific source/sink arrangements and predicting bipolar PIN polarizations, which have been experimentally observed (Linh NM & Scarpella E., 2022). I wonder if the authors have considered other models or concepts beyond canalization/flux sensors, which might have identifiable molecular components.

2) Simultaneous Emergence of patterns.

From the simulations, it appears that loops and higher-order patterns emerge simultaneously on the leaf fabric. Although the convergence with Voronoi diagrams is impressive, this simultaneous emergence of veins does not occur in reality. Typically, based on the model plant *Arabidopsis*, there is a sequence of events leading to the formation of vasculature (the works of Scarpella lab provide a good indication of the temporal and spatial dynamics). First, there is the establishment of the midvein, followed by the formation of first-order veins and secondary veins. I would like the authors to address this discrepancy between their predictions and real observations. Can the model replicate the sequential vein patterning events?

3) Applicability to Other Species

Is the proposed model applicable to other species? Do similar Voronoi diagrams appear in the leaves of other plants?

4) What is the main novelty of the Manuscript.

I am having difficulty identifying the main novelty of this manuscript. Is it the ability to predict higher-order veins? The modeling approach (which seems unlikely), or the application of Voronoi diagrams to developmental biology?

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

Reticulate leaf venation patterns have been a conceptual challenge in theoretical biology for ages, not least because closed (reticulate) networks seem to defy a prominent explanation model, the auxin flow concept, as pioneered by Mitchison and Sachs. According to several variants of the auxin flow hypothesis veins form along preferred routes of auxin transport, a drainage concept that is more easily reconcilable with an open than with a closed venation system. Over the past twenty years, increased experimental and genetic access, the availability of new molecular markers and improved computational biology have triggered a fair number of new leaf venation models implicating also plant hormones beyond auxin, further hypothetical substances and mechanical features in the generation of leaf vein networks.

The key finding in the manuscript by Zheng et al. is an impressive match between the observed locations of major veins relative to hydathodes in the *Urticaceae* species *Pilea peperomioides* and the geometry of a Voronoi diagram with hydathodes being in the positions of Voronoi polygon centers. The authors diligently determined the major vein and hydathode locations by direct measurements in mature leaves under normal and stressed growth conditions and provide a mathematically sound quantitative measure for deviations of the actual versus the predicted positions of hydathodes in a given vein geometry and vice versa. The manuscript is not limited to the mere discovery of these geometrical regularities, but goes on to provide an auxin-based model for the generation of a Voronoi pattern. Inspirational to this model is the prediction by Feugier et al. according to which no 'auxin drainage' veins are formed, if the allocation of auxin carriers in feedback to auxin flux is linear or less than linear. In this case veins may form as lines dividing the area between major auxin sources, positioned by colliding auxin concentration waves. In support of their hypothesis, the authors cite the fact that hydathodes have been identified as major sources of auxin.

The manuscript contains sufficient detail for recalculating the reported correlations and offers the raw data upon request. Beyond this firm evidence, the manuscript provides high-quality illustrations and informative videos for readers, who would like to get the message without scrutinizing the evidence in quantitative detail. In summary, these new observations in conjunction with a mathematically backed-up hypothetical basis is an important contribution to the ongoing discussion on the origins of reticulate leaf vein networks. It will be interesting to see whether the described correlations can also be observed in other reticulate leaves, especially the closed networks in monocotyledonous leaves.

As the authors are, justifiably, optimistic to have found an actual molecular mechanism to explain the phenomenon of vein reticularization in leaves, it is surprising that they did not use the opportunity to test their hypothesis more rigorously. Instead the manuscript is relatively vague with regard to the relevance of the postulated mechanism, even considers that this

mechanism might be confined to Urticaceae, and even there only to positions ‘major’ veins. This is surprising, because in most species, there is reticularization also observed within the minor vein networks. Not least, it is not even mentioned that in other species patterns of closed vein networks are already set up in amazingly young leaf primordia, i.e. long before mature hydathodes as major auxin sources are established. For the average reader, the question how applicable the postulated mechanism is in angiosperm leaves, is important, because reticularization is the rule not the exception. It might therefore have been desirable to have reticulate leaves of at least one other species being subjected to a similar assessment. This could dramatically increase the significance of the work.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

[Major Points]

The authors provide a clear and well-organized presentation of the auxin–PIN1-based model, and convincingly explain how a Voronoi-like pattern can emerge from it. In particular, the idea that secondary veins arise as ridges formed at the collision fronts of propagating auxin waves is articulated clearly, and this mechanism appears to be the most essential ingredient underlying the Voronoi-like geometry observed in the leaf.

The addition of experimental validation of PIN localization in *Pilea* substantially strengthens the manuscript and is very welcome. This direction significantly improves the biological plausibility of the proposed model.

However, in the validation based on PIN localization patterns, an important conceptual point remains insufficiently constrained. The emergence of a Voronoi partition fundamentally relies on the approximate simultaneity of auxin wave propagation from multiple hydathodes. Without such temporal synchrony, ridge formation at equidistant positions would not necessarily imply a Voronoi-like construction. Therefore, demonstrating or at least bounding the degree of temporal synchrony is crucial for the interpretation.

Direct observation of propagating auxin waves may be technically challenging. Nevertheless, a comparative analysis of the spatial extent and spread of PIN expression around multiple hydathodes within the same leaf could potentially provide indirect evidence for such synchrony. Even a qualitative comparison across several hydathodes might help support the assumption that the relevant patterning events occur within a narrow temporal window.

The authors also show that the initial venation pattern is robust under stress conditions. It remains unclear, however, whether leaves that expand under stress—while experiencing altered cell division and growth dynamics—ultimately deviate from an ideal Voronoi geometry. A brief discussion of whether growth under stress might distort the Voronoi-like pattern, even if the initial pattern is conserved, would be informative.

Finally, it is striking that the Voronoi construction based on hydathode positions aligns not only with secondary veins, but also with primary veins, in particular the midvein, despite the fact that hydathodes are unlikely to play a causal role in primary vein positioning. This suggests that hydathodes may be positioned at approximately equal distances from the primary venation framework. While a full explanation may be beyond the scope of the present study, a short discussion acknowledging this unexpected correspondence and its possible interpretations would be valuable.

[Minor Points]

Terminology consistency

The terms “Voronoi diagram”, “Voronoi tessellation”, and “Voronoi partition” are used somewhat interchangeably. For clarity, please define the terminology once and use it consistently throughout the manuscript.

Time-scale separation

A short comment on the relative time scales of auxin-wave-mediated patterning versus tissue growth would help justify the separation of patterning and growth in the model.

Spatial correlation and statistics

Please clarify how spatial correlations among hydathodes within the same leaf were considered when assessing statistical significance, as this may affect the effective number of independent samples.

Interpretation of stress experiments

It would be useful to clarify whether the stress experiments are intended to probe robustness of the initial patterning mechanism or potential long-term deviations from Voronoi geometry during growth.

PIN signal assessment

In the immunolocalization analysis, please clarify whether comparisons of PIN signal intensity are purely qualitative or semi-quantitative.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

In the substantially revised version, the authors present a compelling case that further supports the novelty and potential impact of this work on the field. I strongly support this study for publication and have no further comments.

(Remarks on code availability)

Yes, the code is properly annotated and accessible to use

Reviewer #3

(Remarks to the Author)

With the addition and excellent presentation of the immunolocalization data at stages of leaf development the technical and conceptional shortcomings of the previous submission have been addressed to the degree that the chosen experimental system allows. Also the limitations of this system have been openly and adequately discussed. This includes a very sharp definition of the vein classes in a *Pilea* leaf, to which the model applies. Of course, with this very clear and sharp definition of the study's scope, the manuscript gains accuracy, while it also limits its explanatory power. But in any case, it is interesting to see how high quality experimental and modelling input clearly outlines another possibility for closed vein network formation.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for their careful and detailed responses. The clarification regarding the importance of local rather than global synchrony, supported by additional simulations and qualitative PIN localization data, satisfactorily addresses my major concern. The added discussion appropriately constrains the interpretation of the model.

I am satisfied with the revisions and have no further major concerns.

(Remarks on code availability)

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Reply to the Reviewers

We thank the reviewers for appreciating our paper, and for the thoughtful and constructive comments. Below we include a point-by-point response to the all comments (the comments are cited in black; our responses are shown in blue).

Reviewer #1 (Remarks to the Author):

Dear Authors,

This paper presents extremely interesting results showing that the vein patterns of the Chinese money plant (*Pilea peperomioides*) closely resemble the patterns generated by Voronoi partitioning of hydathodes. It demonstrates that if we assume vascular bundles are formed by the flux of auxin with hydathodes as the auxin source, a pattern similar to Voronoi diagram can be generated. The results indicating that the vein pattern and the relationship with hydathodes up to a certain order closely resemble the Voronoi diagram are very precise. While the first half of the paper seems generally unproblematic, several questions remain regarding the mathematical model presented in the latter half.

We thank the reviewer for their interest in our results and for identifying pertinent questions.

[Major Points]

If vein formation is not simultaneous with hydathodes as the auxin source, a Voronoi-like pattern will not be formed. However, at least some veins are not formed simultaneously up to a certain order but rather sequentially. This contradicts the core assumption of this model, suggesting that a different mechanism may need to be considered. For example, it might be more consistent to assume that hydathodes exert a supplementary repulsive force up to a certain stage, avoiding this issue and allowing new vascular bundles to form.

We thank the reviewer for the opportunity to clarify this important point. We addressed the reviewer's concerns by:

(1) introducing a clear classification of *Pilea* veins, and clarifying that our model is restricted to a specific class of major veins, called secondary major veins (new Fig. 1C and lines 74-85);

(2) obtaining experimental data capturing the patterning process at the molecular level, which included evidence that the formation of hydathodes precedes that of the secondary veins we modeled and that hydathodes form near-simultaneously across the leaf in a specific, time-limited phase of leaf development (new Fig. 6 and Fig. S13; new section “Model validation: Experimental results”);

(3) showing that our model, adjusted for the new data, is consistent with these experimental results (new Fig. 7 and Fig. S14; new section “Model validation: Model review and improvements”); and

(4) demonstrating, through additional simulations, that the model is relatively insensitive to desynchronization of hydathode activation, especially when temporal shifts exhibit a long-distance gradient, in which local offsets between nearby hydathodes are limited (new Fig. S12 and lines 309-317).

While the action of a hypothetical repulsive force cannot be eliminated, in light of our results it does not seem to be necessary.

Regarding hydathodes and early vascular development, there are several SEM photos in the supplementary information, but they need to be shown in a way that indicates the chronological sequence. While knowledge on the development of leaf margin hydathodes is accumulating in the model plant *Arabidopsis thaliana*, these hydathodes connect to the vascular bundles and are thus generally unrelated to vein patterns. Therefore, insights from *Pilea* are crucial for verification.

We thank the reviewer for the opportunity to elaborate on this point. We obtained new experimental results, described in the new section “Model validation: Experimental results”, which capture the chronological developmental sequence of different structures. These results improve upon those originally presented in the SEM photos, the (old) Supplementary Text Section 2, and the (old) Table S5, which we therefore have now deleted.

[Minor Points]

In the model description, it appears that color allocation is assigned on a logarithmic scale and then converted to the actual scale for display in the figures, making the correspondence with the heat map unclear. The legend should be shown on a logarithmic scale, as in Fig. S6A, with the scale ticks indicated in Fig. 4, Fig. 5, and all related supplementary figures.

We thank the reviewer for their careful eye. We used a logarithmic scale to optimally visualize the large range of concentration levels emerging in the simulations shown in Fig. S6A. In the remaining simulations, we used a linear scale. We made this distinction explicit by indicating in Supplementary Tables S4-S5 the type of mapping and the minimum and maximum concentration values represented in the color bars. We hope that information will suffice to interpret our models, given that — in the absence of quantitative data — they are qualitative in nature.

Fig. 2C vertical axis "distance erro (Δd)" contains a typo.

We thank the reviewer for bringing to our attention that typo, which we've now fixed.

In Fig. 3A, it is assumed that the conditions indicated by the four colors are represented in Fig. 3B for "5 weeks," but the colors are mixed and hard to see. Please make the lines for each color a bit thicker and space them out a little more.

We thank the reviewer for the opportunity to make our manuscript more accessible, which we've now done according to the reviewer's suggestion.

Reviewer #1 (Remarks on code availability): The data in this study were reproducible from the code and notebook (.ipynb).

Reviewer #2 (Remarks to the Author):

Article by Zheng et al. explores a potential mechanism underlying the formation of high-order leaf venation structures, such as loops. The authors focus on the Chinese money plant, which exhibits a beautiful arrangement of leaf vasculature that, according to the authors, resembles Voronoi diagrams. In general, the technical details and descriptions of models/algorithms seem appropriate and technically sound. However, I have several points to address before recommending this article for publication:

We thank the reviewer for appreciating the soundness of our results. We address the reviewer's comments below.

1) Bias Focus on Flux-Based Models.

The authors devote significant attention to so-called flux-based models as the "paradigm" for explaining leaf venation patterning. While these models are indeed elegant, they rely on the concept of a "flux sensor," which has yet to be identified. It remains unclear how cells would sense fluxes across other cells. There are alternative models that could potentially explain looped patterns (e.g., Stoma et al., 2008; Wabnick et al., 2011), assuming specific source/sink arrangements and predicting bipolar PIN polarizations, which have been experimentally observed (Linh NM & Scarpella E., 2022). I wonder if the authors have considered other models or concepts beyond canalization/flux sensors, which might have identifiable molecular components.

The reviewer's comment revolves around the central question of auxin-related morphogenesis: What is the mechanism of PIN polarization in cells? This is a difficult, long-standing question, as witnessed by many topical and review papers, among them those cited by the reviewer.

The original theory is canalization, introduced in 1969 by Sachs to explain vascular patterning in plants, and first implemented as a computational model by Mitchison in 1980. Canalization relies on the assumption that cells can measure and respond to auxin fluxes through their membranes. Models operating under this assumption are collectively termed with-the-flux polarization models.

Another class of models was introduced in 2006 by Jönsson et al. and by Smith et al. to explain the patterning of convergence points of PIN polarity underlying phyllotaxis. Models in this class, collectively termed up-the-gradient models, operate under the assumption that cells can measure and respond to the distribution of auxin concentration in the neighboring cells.

Both classes of models raise fundamental questions: How can a cell sense auxin fluxes? How can it sense auxin concentration in neighboring cells? Neither question has yet been experimentally resolved. Some attempts — most notably the controversy surrounding the putative role of the ABP1 protein (an early perspective is described by Wabnik et al., 2011) — have an interesting history on their own, continuing to this day. Moreover, until now, none of these models has been shown to offer a credible path toward solving the notoriously open problem of the morphogenesis of vascular patterns with loops (the relatively most successful attempt by Mitchison (1980) relies on the questionable assumption of periodic switching of the strengths of auxin sources).

In this context, we selected the polarization model with directional fluxes (O'Connor et al., 2014; Cieslak et al., 2015) as the basis for our work for the following reasons:

- (1) This model introduces a simple molecular mechanism capable of sensing fluxes, which alleviates earlier concerns — echoed by the reviewer — regarding the biological plausibility of such a mechanism.
- (2) The proposed mechanism builds upon known processes of PIN trafficking between the cytoplasm and cell membranes through exo- and endo- cytos, which further increases its plausibility.
- (3) The model can polarize cells in both with-the-flux and up-the-gradient manners, depending on parameter values. In particular, it subsumes earlier models, including Stoma's weak polarization model mentioned by the reviewer, which partially inspired our work (line 229 and reference [59] in our paper). Incidentally, it also generates bipolar cells (see, for example, Fig. 7b in Cieslak et al., 2015). This generality was welcome, as it provided a convenient and flexible setting for experimenting with models.

Having carefully chosen the cell polarization model, we built our model of vascular pattern formation in *Pilea* leaves upon it. Moreover, in the course of revising our submission, we demonstrated the consistency of our model with molecular-level data in the new "Model validation" section. While confronting our model with alternative theoretical hypotheses would also be of interest, in the revised version

we chose to focus on comparing our model with experimental results, which we consider a particularly constructive direction.

2) Simultaneous Emergence of patterns.

From the simulations, it appears that loops and higher-order patterns emerge simultaneously on the leaf fabric. Although the convergence with Voronoi diagrams is impressive, this simultaneous emergence of veins does not occur in reality. Typically, based on the model plant *Arabidopsis*, there is a sequence of events leading to the formation of vasculature (the works of Scarpella lab provide a good indication of the temporal and spatial dynamics). First, there is the establishment of the midvein, followed by the formation of first-order veins and secondary veins. I would like the authors to address this discrepancy between their predictions and real observations. Can the model replicate the sequential vein patterning events?

We appreciate the reviewer's comment: Indeed, veins of different orders form sequentially in *Pilea*, too. The midvein and the two lateral veins — collectively termed primary veins— emanate from the point of leaf blade attachment to the petiole and emerge first. Insights from the studies of leaf morphogenesis in *Arabidopsis* and other plants suggest that in *Pilea* too the midvein is likely formed before the lateral veins, but our data did not allow us to discern that order directly. The highly visible secondary veins running between the hydathodes are patterned next, and our model captures only the morphogenesis of these veins. The fact that it also approximates the paths of the midvein and lateral veins is not entirely coincidental, given the overall Voronoi-like geometry of *Pilea* venation patterns, but our model generates the primary veins out of sequence. Finally, tertiary (minor) veins, which form networks emanating from the hydathodes, are patterned last; we have not simulated their morphogenesis either.

We have now clearly identified the different vein types present in *Pilea* leaves (new Fig. 1C) and explicitly stated that our model only captures the morphogenesis of secondary veins (lines 74-85). Focusing on these veins, we have supported the assumption of their approximately simultaneous emergence experimentally, including evidence that their formation succeeds that of hydathodes (new Fig. 6 and new section “Model validation: Experimental results”). We also tested via simulation the robustness of our model to variation in the appearance time of hydathodes across the leaf (new Fig. S12 and lines 309-317). For further details please see our reply to the first comment of Reviewer 1, who raised similar concerns. Extending our model to include primary and tertiary veins represents a logical next step for future work, which we now highlight in the Discussion (lines 431-450).

3) Applicability to Other Species.

Is the proposed model applicable to other species? Do similar Voronoi diagrams appear in the leaves of other plants?

We appreciate the reviewer's intriguing questions. In a nutshell, we have found no other species in which the relation of leaf venation to Voronoi patterns would be as striking as in *Pilea*. We hypothesize, however, that this may be due to secondary factors rather than a fundamental limitation of our wave-collision model.

For example, the round shape of *Pilea* leaves, with the petiole attached near the center of the blade (peltate morphology), may reflect relatively uniform expansion of the blade during growth, which in turn may be the reason why the Voronoi-like property is well-preserved despite leaf growth, and why abstracting growth was possible in our model. Such abstraction may be impossible in non-peltate leaves, in which the emerging venation pattern becomes distorted by leaf growth. Moreover, non-uniform leaf growth may be accompanied by a more significant temporal shift in the emergence of hydathodes, implying, again, a departure from a Voronoi pattern.

We have added a paragraph in the Discussion (lines 451-462) that highlights the open character of these questions, suggests several species with laminar hydathodes for further investigation, and includes references indicating a possible path for further extension to leaves without laminar hydathodes.

4. What is the main novelty of the Manuscript. I am having difficulty identifying the main novelty of this manuscript. Is it the ability to predict higher-order veins? The modeling approach (which seems unlikely), or the application of Voronoi diagrams to developmental biology?

We thank the reviewer for the opportunity to clarify this important point. We believe the novelty of our manuscript is four-fold.

(1) We demonstrated and quantitatively analyzed the relation of the *Pilea* vascular network to Voronoi diagrams. We consider this relation interesting because of its unexpected character bringing together disparate research areas, and because it offers an insightful geometric characterization of a complex natural pattern.

(2) We presented an original computational model that provides a plausible explanation of the emergence of a key component of this vascular network, namely the conspicuous pattern of secondary veins. In the revised version of our manuscript, we also provide original experimental data that support this model.

(3) We introduced a paradigm of auxin-based patterning — collision of auxin waves — which, to our knowledge, is different from all previously developed auxin-based models. In particular, it complements canalization as a vein-patterning mechanism.

(4) We provide an explanation for the formation of closed venation patterns, a long-standing open problem. Although we only demonstrate its applicability to a single

species — *Pilea peperomioides* — it may provide a stepping stone toward a more general understanding of the morphogenesis of closed venation patterns, as outlined in our response to Point 3 above.

These contributions are listed in the Abstract and in the last two paragraphs of the Introduction, which — prompted by the reviewer's comments — we have slightly reinforced (lines 49-63). Please note that we have intentionally tried to avoid explicit claims to novelty, in agreement with the style recommendations of many journals (which is also our preference): “Language such as ‘new’, ‘novel’, ‘for the first time’, ‘unprecedented’, etc., should be avoided [...]” (<https://www.nature.com/documents/commsj-life-style-formatting-guide-accept.pdf>). We hope that, in the revised manuscript, the novelty of our results is evident from the content, without being declared explicitly.

Reviewer #3 (Remarks to the Author):

Reticulate leaf venation patterns have been a conceptual challenge in theoretical biology for ages, not least because closed (reticulate) networks seem to defy a prominent explanation model, the auxin flow concept, as pioneered by Mitchison and Sachs. According to several variants of the auxin flow hypothesis veins form along preferred routes of auxin transport, a drainage concept that is more easily reconcilable with an open than with a closed venation system. Over the past twenty years, increased experimental and genetic access, the availability of new molecular markers and improved computational biology have triggered a fair number of new leaf venation models implicating also plant hormones beyond auxin, further hypothetical substances and mechanical features in the generation of leaf vein networks.

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high-quality illustrations and informative videos for readers, who would like to get the message without scrutinizing the evidence in quantitative detail. In summary, these new observations in conjunction with a mathematically backed-up hypothetical basis is an important contribution to the ongoing discussion on the origins of reticulate leaf vein networks. It will be interesting to see whether the described correlations can also be observed in other reticulate leaves, especially the closed networks in monocotyledonous leaves.

As the authors are, justifiably, optimistic to have found an actual molecular mechanism to explain the phenomenon of vein reticularization in leaves, it is surprising that they did not use the opportunity to test their hypothesis more rigorously. Instead the manuscript is relatively vague with regard to the relevance of the postulated mechanism, even considers that this mechanism might be confined to Urticaceae, and even there only to positions 'major' veins. This is surprising, because in most species, there is reticularization also observed within the minor vein networks. Not least, it is not even mentioned that in other species patterns of closed vein networks are already set up in amazingly young leaf primordia, i.e. long before mature hydathodes as major auxin sources are established. For the average reader, the question how applicable the postulated mechanism is in angiosperm leaves, is important, because reticularization is the rule not the exception. It might therefore have been desirable to have reticulate leaves of at least one other species being subjected to a similar assessment. This could dramatically increase the significance of the work.

We are very thankful for the reviewer's comments, which both appreciate our work and highlight the shortcomings of our original submission.

(1) We shared the reviewer's and editor's unease with the lack of validation of our model beyond the agreement between generated and observed patterns. Consequently, we tested our model as rigorously as possible, with the constraint that *Pilea* is not a model plant and the only technique allowing us to address patterning at the molecular level – without embarking on an entirely new project involving genetic transformation of *Pilea* – was immunolocalization (new Fig. 6 and Fig. S13; new section “Model validation: Experimental results”). We found it encouraging that the experimental results were consistent with the basic tenet of our model: the collision of waves of high auxin concentration emanating from the hydathodes. We were also gratified that the discrepancies between our original model and experimental results – most significantly, the high concentration of membrane-localized PIN in the model versus the low concentration observed experimentally – could be addressed by introducing plausible adjustments to the model (new Fig. 7 and Fig. S14; new section “Model validation: Model review and improvement”).

(2) Interestingly, the experimental results also suggest that the patterning of *Pilea* secondary veins may be qualitatively different from that of primary and tertiary veins, in which the observed concentration of membrane-localized PIN was high. Our present model does not extend to these veins, but it is likely that the patterning

of primary veins can be explained by standard canalization. The patterning of tertiary (minor) veins presents a more difficult problem: while some tertiary veins link hydathodes to secondary or primary veins — an arrangement that can also be attributed to canalization — the overall pattern of tertiary veins is reticulate, and neither canalization nor wave collision provides a ready explanation for this. In our opinion, the wave-collision model credibly explains some, but not all, aspects of vein reticulation. We added a paragraph highlighting other aspects of leaf development, including primary and tertiary veins, that would need to be added to build a comprehensive model of the *Pilea* vein network (lines 429-450). Given the long-standing open character of the question of reticulation, we believe that even our partial progress represents a substantial step forward.

(3) While our experimental results validate the model to the extent afforded by immunolocalization, the question of the model's generality remains. As mentioned in our replies to Reviewers 1 and 2, we leave this as an open problem because the hypothesis we consider most likely — namely, that the macroscopically observed vascular patterns in leaves with laminar hydathodes represent Voronoi patterns distorted by growth — would require a quantitative analysis of leaf growth, and the incorporation of growth into the model (lines 448-450). These would be a very interesting but substantial endeavor, which we were not able to incorporate into this paper. We also suggest other species with laminar hydathodes for further investigation, as well as possible paths to extend our work to leaves without laminar hydathodes, in the Discussion (lines 451-462).

Reviewer #1 (Remarks to the Author)

[Major Points]

The authors provide a clear and well-organized presentation of the auxin–PIN1-based model, and convincingly explain how a Voronoi-like pattern can emerge from it. In particular, the idea that secondary veins arise as ridges formed at the collision fronts of propagating auxin waves is articulated clearly, and this mechanism appears to be the most essential ingredient underlying the Voronoi-like geometry observed in the leaf.

The addition of experimental validation of PIN localization in *Pilea* substantially strengthens the manuscript and is very welcome. This direction significantly improves the biological plausibility of the proposed model.

We thank the reviewer for appreciating our proposed model and its now closer connection to the underlying biology.

However, in the validation based on PIN localization patterns, an important conceptual point remains insufficiently constrained. The emergence of a Voronoi partition fundamentally relies on the approximate simultaneity of auxin wave propagation from multiple hydathodes. Without such temporal synchrony, ridge formation at equidistant positions would not necessarily imply a Voronoi-like construction. Therefore, demonstrating or at least bounding the degree of temporal synchrony is crucial for the interpretation.

Direct observation of propagating auxin waves may be technically challenging. Nevertheless, a comparative analysis of the spatial extent and spread of PIN expression around multiple hydathodes within the same leaf could potentially provide indirect evidence for such synchrony. Even a qualitative comparison across several hydathodes might help support the assumption that the relevant patterning events occur within a narrow temporal window.

Of primary importance is local rather than global synchrony of hydathodes. In Fig. S12 (lines 311–319), we compared three spatio-temporal patterns of hydathode activation and found that as long as nearby hydathodes are activated at approximately the same time, a near-exact Voronoi diagram robustly forms. This substantially relaxes the degree of synchrony required by the model.

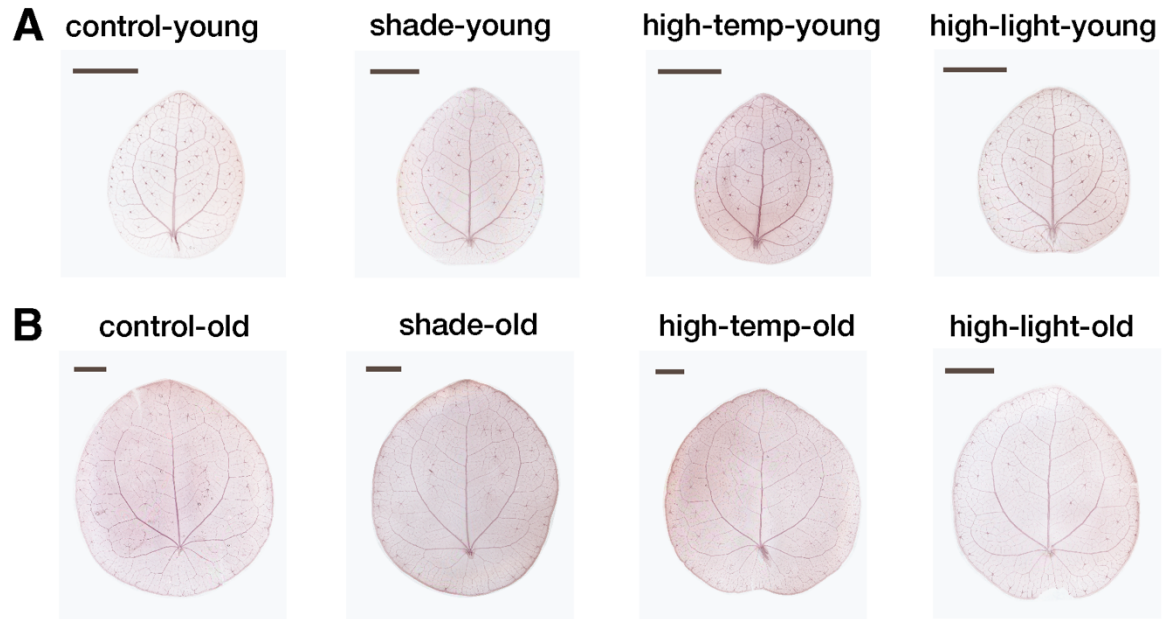
In addition, Fig. 6 contains three examples of pairs of adjacent hydathodes and surrounding PIN expression and localization. Fig. 6E,F shows similar spatial patterns and levels of PIN expression around the two hydathodes and in a developing vein that almost perfectly bisects the space between the hydathodes. This is consistent with concurrent activation of auxin sources associated with hydathodes. Fig. 6H shows three late-stage hydathodes with relatively lower

levels of surrounding PIN expression; the vein separating the bottom-most hydathode from the top two hydathodes lies slightly closer to the periphery. This would suggest that the wave of propagation from the bottom-most hydathode started earlier than that from the top two hydathodes. Thus, the data includes cases where nearby hydathodes appear to have been activated at the same time, as well as instances with some local asynchrony, which contributes to the approximate nature of the Voronoi diagram formed on the leaf. However, we have not observed any significant differences in spatial PIN distributions around nearby hydathodes that would invalidate the model. We included a discussion of these observations and interpretations on lines 353–357 and 382–387, respectively.

Thus, while direct observation of propagating waves using immunolocalization is indeed challenging, these qualitative results suggest that the window during which nearby hydathodes are activated is sufficiently narrow to allow approximate Voronoi diagrams to form.

The authors also show that the initial venation pattern is robust under stress conditions. It remains unclear, however, whether leaves that expand under stress, while experiencing altered cell division and growth dynamics, ultimately deviate from an ideal Voronoi geometry. A brief discussion of whether growth under stress might distort the Voronoi-like pattern, even if the initial pattern is conserved, would be informative.

Non-uniform and/or anisotropic leaf expansion (growth) following vein patterning will indeed distort an initial Voronoi pattern. While we observed many changes in plant architecture under stress conditions (e.g., hydathode pore size in Fig. 3B; leaf size, number of leaves, number of hydathodes per leaf in Fig. S4), we did not observe any additional departure from the Voronoi geometry in stress conditions compared to in control conditions (Fig. S5). This is likely because these stress conditions do not alter the relatively uniform character of leaf growth, reflected in the round shape of *Pilea* leaves irrespective of their size. Fig. S1 shows five examples leaves from control conditions, and below, we include additional examples of both young and old leaves from all four conditions, which has been added to the Supplement as Fig. S15. We also added discussion of this observation on lines 197–201.



We suspect, however, that some reticulate venation patterns observed in nature, though not recognized as Voronoi, may in fact originate as Voronoi patterns that are subsequently distorted by non-uniform and/or anisotropic expansion (see Discussion, lines 470–472). A quantitative investigation of this phenomenon is an interesting topic for future work, which would require different analysis methods and incorporation of leaf growth into the model.

Finally, it is striking that the Voronoi construction based on hydathode positions aligns not only with secondary veins, but also with primary veins, in particular the midvein, despite the fact that hydathodes are unlikely to play a causal role in primary vein positioning. This suggests that hydathodes may be positioned at approximately equal distances from the primary venation framework. While a full explanation may be beyond the scope of the present study, a short discussion acknowledging this unexpected correspondence and its possible interpretations would be valuable.

We agree with the reviewer that hydathodes are unlikely to play a causal role in patterning primary veins. In fact, the opposite is more likely to be true, i.e., that the positioning of primary veins affects the patterning of hydathodes. The coincidental patterning of a “mid-vein” by our model suggests that hydathodes immediately to the left and right of the mid-vein lie at similar distances to the mid-vein (although they are not symmetrically distributed, resulting in a zig-zag pattern). Propagating waves from these hydathodes then form a “mid-vein” look-alike. We now added discussion of this on lines 446–448.

[Minor Points]

Terminology consistency

The terms Voronoi diagram, Voronoi tessellation, and Voronoi partition, are used somewhat interchangeably. For clarity, please define the terminology once and use it consistently throughout the manuscript.

We thank the reviewer for catching this inconsistency. We have changed all instances to “Voronoi diagram”.

Time-scale separation

A short comment on the relative time scales of auxin-wave-mediated patterning versus tissue growth would help justify the separation of patterning and growth in the model.

This is indeed an intriguing question, but unfortunately, our data do not allow us to precisely tease apart the time scales of wave propagation and tissue growth. However, separating patterning from growth is justified in our model because we did not observe noticeable changes in leaf shape from P4 to P5 leaves, despite the leaves almost doubling in size (P4: ~1.5–2mm in length from tip to base versus P5: ~3–4 mm). Uniform growth would not distort patterning predicted by the model.

Spatial correlation and statistics

Please clarify how spatial correlations among hydathodes within the same leaf were considered when assessing statistical significance, as this may affect the effective number of independent samples.

A Bayesian linear mixed-effects model was used to account for possible statistical dependencies (see Methods, lines 547–560).

Interpretation of stress experiments

It would be useful to clarify whether the stress experiments are intended to probe robustness of the initial patterning mechanism or potential long-term deviations from Voronoi geometry during growth.

We appreciate the reviewer's observation and have now clarified that the stress experiments were designed to test whether deviations from Voronoi geometry are induced by stress (lines 180–181).

PIN signal assessment

In the immunolocalization analysis, please clarify whether comparisons of PIN signal intensity are purely qualitative or semi-quantitative.

Because the human eye can distinguish only a limited number of shades of gray, we used a lookup-table to facilitate comparisons of signal intensities within and across images. The lookup-table assigns different colors to different pixel gray

values, corresponding to different fluorescence signal intensities. Thus, the colors accurately reflect differences in signal intensity and are not merely qualitative. Consistent signal quantification across all image regions and across all images was not possible because it would require the signal to remain below saturation in all pixels being compared. Since signal intensity varies greatly within and across images, avoiding saturation would cause the weakest signals to fall below the detection threshold, which would be detrimental to our analysis.

Reviewer #2 (Remarks to the Author)

In the substantially revised version, the authors present a compelling case that further supports the novelty and potential impact of this work on the field. I strongly support this study for publication and have no further comments.

We thank the reviewer for appreciating our revised manuscript and for supporting publication.

Reviewer #2 (Remarks on code availability):

Yes, the code is properly annotated and accessible to use

Reviewer #3 (Remarks to the Author)

With the addition and excellent presentation of the immunolocalization data at stages of leaf development the technical and conceptional shortcomings of the previous submission have been addressed to the degree that the chosen experimental system allows. Also the limitations of this system have been openly and adequately discussed. This includes a very sharp definition of the vein classes in a *Pilea* leaf, to which the model applies. Of course, with this very clear and sharp definition of the study's scope, the manuscript gains accuracy, while it also limits its explanatory power. But in any case, it is interesting to see how high quality experimental and modelling input clearly outlines another possibility for closed vein network formation.

We thank the reviewer for appreciating our results and for having prompted us, in the previous review, to perform the immunolocalization experiments.