nature portfolio

Peer Review File

JULGI coordinates vascular development and leaf patterning through non-cell-autonomous regulation of miR165/166

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Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)
The manuscript title and abstract promised an interesting and creative study, combining mechanistic RNA biology with developmental biology. However, while the study may have promise within the RNA field (although JUL had already been described previously), the authors clearly are not developmental biologists. The study is in this regard flawed in many ways. This applies to the introduction, experimental design, as well as data interpretation.

A few examples of errors and misstatements in the introduction:
Line 40-44: “This pattern suggests that vascular tissues, beyond resource transport, acted as structural and developmental axes, potentially serving as organizing centers for the establishment of leaf polarity and patterning. Developmental evidence supports this link: showing that the asymmetric distribution of specific molecular components establishes polarity, providing positional cues that guide pattern formation during organogenesis 5.”
The reference cited reviews the role of asymmetric cell divisions in generating distinct cell identities through cell division. There is no evidence for the vasculature as an organizing center for adaxial-abaxial leaf polarity.

Line 45: In early leaf development, primary adaxial–abaxial polarity is first established6–9.
In fact, the cited work shows that adaxial-abaxial polarity is established before the formation of a vascular strand in the new primordium, contradicting the above premise.

Line 50-51: Subsequently, surrounding tissues align with this vascular axis, specifying and differentiating to mesophyll and epidermis10,15.
The epidermis is specified during embryogenesis, and is maintained as a separate lineage throughout plant development.

The authors also confuse pri-miRNA and pre-miRNA. Pri-miRNA is the long primary transcript generated, in this case, by PolII. The shorter hairpin RNA, as shown in Fig. 1c, is the pre-miRNA.

The qRT-PCR analysis to monitor expression levels of different MIR165/166 genes and other polarity markers was performed on 4 week-old leaves. After 4 weeks, leaves are fully grown and patterned, any transcript differences observed are non-informative for developmental patterning. These experiments should be done on immature leaves, or vegetative apices. Given that expression is shown relative to WT, it is not clear whether any relevant transcript levels are detected.

The method section does not state how expression was normalized. In line 124, the authors state “We used pri-MIR166c and pri-MIR166d as internal negative controls that lack the putative minimal JUL1-binding sequence”. This would be the wrong choice. If expressed in 4 week-old leaves, these genes would be abaxial markers, and thus their expression is expected to change in the mutant, if not directly in an indirect manner via its effect on adaxial-abaxial polarity. A housekeeping gene that is unaffected in jul1jul2 mutants is needed to normalize expression across samples. The same applies for all other qRT analyses.

Line 129-130: “To further assess the functional connection between JUL and miR165/166 in establishing the leaf adaxial–abaxial axis”. See the above point, the data presented does NOT address the establishment of adaxial-abaxial polarity. The

authors make statements like this throughout the manuscript, but analysis of the establishment of leaf polarity requires studying the shoot apical meristem. Moreover, the phenotypes reported for jul1 and jul1 jul2 are far too mild to be caused by defects in leaf polarity establishment. The authors confuse “a minor effect on leaf polarity” with the far more exciting term “leaf polarity establishment”.

The phenotypes of the proRPS5A:STTM165/166 lines are surprisingly weak. Short Tandem Target Mimics, when constructed correctly, cause a dramatic decrease in the level of the targeted miRNA. As a result, STTM lines effectively targeting miR165/166 (when viable) show severe developmental defects, and changes in the expression of polarity related genes (e.g. Yan et al., 2012 doi: 10.1105/tpc.111.094144). The explanation provided by the authors on lines 146-148 does not make sense at all. This would indicate that miR165/166 has no function in leaf development. This raises questions about expression or effectiveness of the proRPS5A:STTM165/166 transgene in the lines selected.

Regarding the vascular phenotypes shown in Extended data Fig. 3, the authors previously showed that JUL1 suppresses phloem development through its negative effects on SMXL4 and SMXL5. This complicates data interpretation. Are the observed defects due to effects on adaxial-abaxial polarity, lost regulation of SMXL genes, both, or even not yet discovered targets.

Line 174: the term “emerging” is wrongly used

Line 177: The authors conclude that JUL1-GFP accumulates throughout the leaf primordium. The quality of the data shown is not good enough to conclude this. The optical sections show many cells without GFP. Imaging actual leaf sections would generate a much greater resolution. Also, how many lines were analyzed? In other words, how reproducible is the data?

The tissue- or region-specific complementation of jul1jul2 is nice idea. However, how do the authors explain that expression of JUL1 on the adaxial side (BOP1 promoter) rescues the leaf curling defect of jul1 jul2? The latter is proposed to be caused by elevated miR165/166 levels in the mutant due to increased miRNA processing. However, the miR165/166 precursors are not expressed adaxially. This would point to an entirely different basis for the leaf curling phenotype.

Line 199: see above. The data presented does not support a role for JUL1 in adaxial-abaxial pattern formation. The mutants show a mild leaf curling, potentially consistent with an effect on polarity maintenance.

Evaluation of the RNA binding experiments are outside my expertise. However, two thoughts: 1) the on-bead binding assays are not reflective of what happens in the cell. The pri-miRNA transcript is able to form a hairpin structure, such that JUL1 has to compete with secondary structure formation. How realistic is that? Is single stranded pri-miRNA present, or can JUL compete with RNA folding? 2) The colocalization of JUL1 with MS2-tagged miR165a precursor is again not at all convincing. The resolution of this experiment is insufficient. MS2-GFP protein on its own is nuclear localized and, at the resolution provided, co-localizes with mRFP-JUL1 (left panel Fig 3e). The authors need to look at the sub-nuclear level. The statement that mRFP-JUL1(RA) does not co-localize with MS2-GFP is also not supported by the data. It is just that expression of mRFP-JUL1(RA) is comparatively low.

As is evident, I looked at the manuscript carefully up to the middle of page 9. However, I stopped reading at that point, as it was overwhelmingly clear that the current manuscript is not suitable for publication. The authors would be advised to consult a developmental biologist, and to greatly improve the quality of the data. Moreover, given that the authors identified over 65,000 regions capable of forming RG4s, they would need to think of additional experiments to convincingly make the point that increased miR165/166 levels are the cause, rather than an outcome of the jul1 jul2 phenotype. As it stands the study falls far short of its promise, there is no evidence for the model proposed.

Reviewer #2

(Remarks to the Author)
In this manuscript, Hwang and colleagues revealed new molecular and physiological functions of JUL1 in addition to regulating phloem development (Nature plants, 2018). They identify pri-MIR165/166 as new targets of JUL1 and uncover the regulatory role of JUL1 in miRNA processing, therefore provide new insights into the understanding of JUL1’s molecular functions. They also provided solid genetic and physiological proof that JUL1regulates leaf development dependent on MIR165/166 processing, therefore coordinates vascular development and leaf patterning, and update our understanding of coordinated development of plant tissues. Overall, this manuscript provides new insights into leaf development from RNA regulatory’s side with high quality data.
Major points:
1) The authors have found JUL1 is differentially distributed in different cells, and this distribution seems to be very important for JUL1’s function and subsequently its regulatory role in leaf development, however the driving force of JUL1’s distribution is not clear. The authors may make some efforts to explore the factors that affect JUL1’s distribution, and the evolutionary selection of such factors across different species with different leaf shapes.
2) Considering the RG4 formation, canonical RG4 sequence should include at least 4 G-blocks. However, in the pri-MIR165/166 sequence, UUGAGGGGAAUGUUGUCUGGAUCGAGGAU, only 3 G-blocks are found. Although there are 4G in the first block, it still cannot form two separate G-blocks. This sequence doesn’t satisfy the sequence requirement for RG4 formation. Given the complexity of RNA folding, there may be possibility of RG4 formation as indicated in Extended Data Fig 8 through circular dichroism (CD) spectra. However, to confirm RG4 formation, other methods should be included.
Minor points:

1) P should be always italicized and capitalized, such as in figure legend Figure 3 f.
2) In reference, “Elife” should be in “eLife”.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Thanks for the authors' effort answering all questions, no more questions are raised.

Reviewer #3

(Remarks to the Author)
Overview

This is an interesting study exploring a new contributor to the adaxial-abaxial polarity network: the RNA binding protein JULGI. I was invited to evaluate the author’s response to Reviewer 1 who had numerous critiques of the paper. These critiques were conceptual as well as experimental in nature. Conceptually, the authors did not clearly delineate ‘establishment’ from ‘maintenance’ phases of leaf polarity. Experimentally, the reviewer made several concrete suggestions to improve their experiments. These included better tissue choices, imaging, and in planta experiments. I believe these critiques have merit.

For instance, establishment vs maintenance is an extremely important distinction, as a lot of effort has gone into carefully defining the relative contributions of each polarity determinant to each phase in the process. Reading their revision, the authors have taken this critique to heart and changed most of the language to better reflect the ‘maintenance’ role of JULGI. However, a few lines still need to be modified:

Line 62 states miR166 ‘establishes’ adaxial identity. However, Burian et al 2022 shows that AS2 and KAN are polarized across the ad-ab axis well before leaf emergence. The role of 166 is to limit HDZIPIIIs to the adaxial side; this is important because HDZIPIII factors are sufficient to convert abaxial identity into adaxial identity. However, HDZIPIII accumulation appears to be a response to – rather than the cause of – a polarized ad-ab axis. Line 363 has the same issue.

Line 71 states vascular development ‘coordinates leaf patterning’. It is more accurate to say it ‘contributes to leaf shape’. Or that it ‘mediates/interprets underlying ad-ab polarity to impact leaf shape’. However they choose to write it, the text must make clear that patterning itself is not set up by JULGI or the vasculature.

The legend of Fig 5 still says ‘establishes’. I believe this is the last instance, but the text must be fully checked for removal/clarification of this issue.

Regarding the experimental critiques, I did not see the original manuscript. However, the justifications laid out by the reviewer all seemed reasonable to me. Importantly, almost all these critiques have been addressed by the authors (see Weaknesses for two exceptions).

Strengths

My own assessment of this version of the manuscript is that it is experimentally strong and conceptually interesting. Factors that aid in interpreting the underlying ad-ab axis are important and understudied. Given the importance of ncRNAs to ad-ab polarity, taking an RNA biology approach is also appropriate and interesting.

The experimental rigor in this version of the manuscript is outstanding. Indeed, multiple orthogonal assays are used to test each claim. I believe it is worth laying them out to clearly illustrate the number of independent assays performed:

• To support a link between JUL1/2 and miR166 accumulation/activity: qRT for polarity determinants, STTM and qRT/phenotypic analyses
• To support non-cell-autonomy: single cell data mining, transcriptional reporter, multiple translational reporters
• To support binding of JUL1 to G-rich region of pri-MIR165a: REMSAs, bead-surface binding, circular dichroism, bacteriophage M2, in planta RIP
• To support the notion that JUL1 prevents formation of dsRNA: bead surface binding, FRET
• To support the notion that JUL1 prevents DCL1 from interacting with pri-MIR165A to process it into mir165: in planta RIP, pri-miRNA processing assay (see Weaknesses).
• To support the notion that JUL1/2 act in the immediate term to suppress miR165a accumulation: protoplast co-transfection (see Weaknesses), short term induced JUL1 O/E and KO lines, MIR165amut phenotypic/qRT analyses in Wt and jul1/2 mutants

I have concerns about a couple of these assays (see Weaknesses) but not enough to question their overall conclusions.

Weaknesses

The pri-miRNA processing assay does not convincingly show a difference in mature 165 accumulation with JUL1 vs JUL1(RA), i.e. Fig 4d. Thus, conclusions that JUL1 works by inhibiting DCL1 processing are not supported. However, statements that JUL1 inhibits DCL1 interaction with pri-miR165A remain supported.

The RT-PCR for pri and mature 165A in Fig 5A does not show increases in either as claimed in text. JUL1 (and mir166 binding site)-dependent effects on GFP signal (via confocal) and protein levels (via Western) are supported, however. Thus, the only claims that can be made for Fig 5A are: 1) the presence of an intact miR165a binding site reduces accumulation of GFP protein, and 2) this reduction can be blocked via increased dosage of JUL1. The statement “These results suggest that JUL1 binding to pri-MIR165a suppresses the accumulation of mature miR165/166, leading to higher levels of transcripts for HD-ZIP III genes, the targets of miR165/166.” should thus be revised as these assays do not support this claim.

Minor Comments and Suggestions

Lines 210, 214, 232 reference the wrong Extended Fig.

The authors should mention AGO10/PINHEAD/ZWILLE in the Intro or Discussion. AGO10 is a non-catalytic, adaxially-localized soak for miR166 (Zhu et al., 2011 Cell). As JULGI is an orthogonal post-transcriptional mechanism to limit 166 activity, a discussion of AGO10 in this context would be of interest.

In addition, AS2 should mentioned. There are many reasons to do this, including its involvement in the ad-ab pre-patterning (Burian et al) and the fact that it directly represses MIR166A (Husbands et al., 2015 Plant Cell).

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Dear editor,

We sincerely appreciate the reviewers' time and thoughtful efforts in evaluating our manuscript. Their comments have helped us substantially improve the clarity and rigor of the work, and we have addressed the scientific concerns raised as carefully and thoroughly as possible in the revised version.

However, we would like to respectfully raise a concern regarding the tone and balance of one of the reviewer's comments. While we fully welcome critical and constructive feedback, certain remarks appear to reflect a degree of bias and a lack of objectivity in assessing the manuscript.

For instance, the reviewer states that "*the authors clearly are not developmental biologists*" and refers to the study as being "*flawed in many ways*." We would like to clarify that, although some of the authors are trained primarily in RNA biology, others are developmental biologists with specific expertise in leaf polarity and vascular development. The overarching goal of this study was to integrate these complementary areas to explore the non-cell-autonomous role of JUL1 in modulating miR165/166 activity, as a coordinator between vascular development and leaf patterning, an interdisciplinary question that we believe is scientifically meaningful.

We acknowledge that a few developmental terms (e.g., "establishment" or "emerging") were used imprecisely in the original version. These instances have been revised to align with conventional developmental biology usage. However, we respectfully submit that these semantic issues do not compromise the main findings or interpretations. Throughout the manuscript, we clearly present JUL1 as a post-patterning regulator that contributes to the refinement or recalibration of polarity, not its initial establishment in leaf development. This distinction is made explicit in both the revised Introduction and Discussion sections.

We were especially concerned by the reviewer's final comment: "*I stopped reading at that point, as it was overwhelmingly clear that the current manuscript is not suitable for publication*." While we recognize that not all reviewers may agree with the conclusions or approach of a manuscript, we feel that discontinuing the review midway and issuing a blanket rejection without a full evaluation of the data limits the opportunity for fair and balanced assessment.

We appreciate the reviewer's suggestion to consult a developmental biologist, and we emphasize that developmental expertise, specifically in leaf polarity and vascular development, was integral to both the design and interpretation of this study. We believe this perspective strengthens the interdisciplinary nature of our work, even if it was not fully recognized in the review. We believe interdisciplinary efforts, especially at the interface of RNA biology and development, deserve rigorous but open-minded evaluation.

We hope that the revised manuscript, which incorporates extensive clarifications and new supporting data, will be considered on its scientific merits. For your reference, please find our point-by-point responses to the reviewers' comments below.

Best regards,

Ildoo

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript title and abstract promised an interesting and creative study, combining mechanistic RNA biology with developmental biology. However, while the study may have promise within the RNA field (although JUL had already been described previously), the authors

clearly are not developmental biologists. The study is in this regard flawed in many ways. This applies to the introduction, experimental design, as well as data interpretation.

A few examples of errors and misstatements in the introduction:
Line 40-44: "This pattern suggests that vascular tissues, beyond resource transport, acted as structural and developmental axes, potentially serving as organizing centers for the establishment of leaf polarity and patterning. Developmental evidence supports this link: showing that the asymmetric distribution of specific molecular components establishes polarity, providing positional cues that guide pattern formation during organogenesis 5."
The reference cited reviews the role of asymmetric cell divisions in generating distinct cell identities through cell division. There is no evidence for the vasculature as an organizing center for adaxial-abaxial leaf polarity.

Author response

We thank the reviewer for this insightful comment. We acknowledge that the cited reference (Ref. 5) primarily discusses the role of asymmetric cell division in establishing distinct cell identities through specific molecular mechanisms at the cellular and tissue levels, and does not directly support the notion that vascular tissues act as organizing centers for adaxial–abaxial polarity.

Our intention was not to imply that vascular strands initiate polarity establishment. Rather, we aimed to propose that vascular development may contribute to the spatial refinement or coordination of polarity-related patterning during leaf development, based on currently identified molecular mechanisms mediating intercellular and intertissue communication.

To support this hypothesis, we refer not solely to Ref. 5, but to a growing body of genetic and phenotypic evidence demonstrating functional interplay between leaf polarity regulators and vascular patterning, including:

- Gain-of-function of the adaxial factor *REVOLUTA* (*REV*) disrupts miR165/166-mediated cleavage, leading to ectopic xylem arrangement surrounding the phloem (Emery *et al.*, 2003, *Curr. Biol.*).
- Loss-of-function of *KANADI1*, an abaxial regulator, results in improper vascular bundle formation (Izhaki and Bowman, 2007, *Plant Cell*).
- Mutants of *ASYMMETRIC LEAVES2* (*AS2*) and *RH10*, involved in adaxial identity and RNA regulation, exhibit abaxialized leaves with xylem dissipation (Matsumura *et al*., 2016, *Biol. Open*).
- The *hca2* mutant shows enhanced cambial activity and vascular proliferation along with curly leaf formation (Guo *et al*., 2009, *Plant Cell*).
- Overexpression of *BREVIS RADIX* (*BRX*), a regulator of phloem differentiation, leads to adaxial–abaxial patterning defects (Zhang *et al*., 2021, *Hortic. Res.*).

These findings collectively suggest that leaf polarity and vascular development are tightly coordinated, likely via shared regulatory modules or non-cell-autonomous signaling pathways. We regret that this intended context may not have been sufficiently clear in the original text, and we have revised the relevant passage to more accurately reflect this interpretation and avoid misrepresentation.

Line 45: In early leaf development, primary adaxial–abaxial polarity is first established6–9.
In fact, the cited work shows that adaxial-abaxial polarity is established before the formation of a vascular strand in the new primordium, contradicting the above premise.

Author response

We appreciate the reviewer's insightful comment. We agree that our use of the term "establishment" may have been imprecise and potentially misleading in the context of developmental biology, where it specifically refers to the initial specification of adaxial–abaxial polarity.

It is well established that leaf polarity is first initiated at the shoot apical meristem, prior to vascular strand formation, and that vascular development is subsequently guided by this pre-established polarity. In fact, we describe this polarity-driven patterning process in the following sections of the manuscript (Lines 45-52) and in the discussion (Lines 353-362).

Our data do not suggest that vascular tissues serve as primary organizers of polarity establishment, but rather to propose that vascular development may reinforce or spatially coordinate polarity cues during later stages of leaf development. In our study, we interpret the role of JUL1 as that of a non-cell-autonomous coordinator, expressed in the vasculature, that contributes to the "refinement" or "recalibration" of leaf polarity as development progresses.

To avoid confusion and to better reflect our intended meaning, we have revised the relevant passage to replace "establishment" with a more appropriate term (Line 37-39).

Line 50-51: Subsequently, surrounding tissues align with this vascular axis, specifying and differentiating to mesophyll and epidermis10,15.
The epidermis is specified during embryogenesis, and is maintained as a separate lineage throughout plant development.

Author response

We appreciate the reviewer's correction and recognize the inaccuracy in our original statement. In our original wording, we unintentionally conflated the specification of the epidermal layer itself with the specification and differentiation of epidermal cell types such as pavement cells, trichomes and guard cells, which acquire their identities during leaf development after initiation of vascular development.

To avoid this confusion, we have revised the sentence as follows: "Subsequently, surrounding tissues align with this vascular axis, undergoing spatial organization and differentiation." (Lines 48–50)

The authors also confuse pri-miRNA and pre-miRNA. Pri-miRNA is the long primary transcript generated, in this case, by PolII. The shorter hairpin RNA, as shown in Fig. 1c, is the pre-miRNA.

Author response

We thank the reviewer for this comment. We would like to clarify that the structure shown in Fig. 1c corresponds to the predicted secondary structure of the full-length pri-MIR165a, not the pre-miRNA. To generate this structure, we used the published pri-MIR165a sequence (Zielezinski *et al*., 2015, *BMC Plant Biology*) and predicted its RNA folding using the RNAfold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). We hope this clarifies that pri-miRNA and pre-miRNA were not conflated in our analysis.

The qRT-PCR analysis to monitor expression levels of different MIR165/166 genes and other polarity markers was performed on 4 week-old leaves. After 4 weeks, leaves are fully grown and patterned, any transcript differences observed are non-informative for developmental patterning. These experiments should be done on immature leaves, or vegetative apices. Given that expression is shown relative to WT, it is not clear whether any relevant transcript levels are detected.

Author response

We appreciate the reviewer's important comment and apologize for the lack of clarity in our original description. We agree that fully expanded 4-week-old leaves are not ideal for assessing transcriptional programs underlying developmental patterning.

Our intention in analyzing these samples was not to infer causal relationships in early patterning, but rather to validate the phenotypic outcomes by examining whether the altered adaxial–abaxial ratios in mature leaves correlate with changes in the expression of established polarity marker genes (Fig. 1 g and h and Fig. 2 f and g).

To specifically assess the role of JULGI in regulating primary and mature miR165/166 expression during early developmental stages, we performed qRT-PCR using 7-day-old seedlings, which contain the shoot apical meristem, leaf primordia, and developing leaves (Fig. 1 c).

We revised the Figure legends and Methods section accordingly to clarify the sampling stages and experimental rationale (Line 522-523).

The method section does not state how expression was normalized. In line 124, the authors state "We used pri-MIR166c and pri-MIR166d as internal negative controls that lack the putative minimal JUL1-binding sequence". This would be the wrong choice. If expressed in 4 week-old leaves, these genes would be abaxial markers, and thus their expression is expected to change in the mutant, if not directly in an indirect manner via its effect on adaxial-abaxial polarity. A housekeeping gene that is unaffected in jul1jul2 mutants is needed to normalize expression across samples. The same applies for all other qRT analyses.

Author response

We thank the reviewer for this important comment and fully acknowledge the concern regarding the use of pri-MIR166c and pri-MIR166d as internal negative controls. As pointed out, these genes are expressed in the abaxial domain and could be indirectly influenced in *jul1* or *jul1 jul2* mutants due to altered adaxial–abaxial polarity, especially in mature leaves.

To address this issue, we measured the expression of primary and mature miR165/166 transcripts in 7-day-old seedlings, which contain only immature leaves and leaf primordia. This developmental stage minimizes potential secondary effects on abaxial gene expression. For all qRT-PCR analyses, we normalized transcript levels to *UBQ1*, a housekeeping gene with stable expression that is unaffected in *jul1* and *jul1 jul2* mutants. Under these conditions, we observed no significant change in the expression of pri-MIR166c and pri-MIR166d, which lack the putative JUL1-binding sequence. In contrast, pri-MIR165a and pri-MIR166a, which contain this motif, showed marked reductions in transcript levels in both *jul1* and *jul1 jul2* mutants (Fig. 1d). These results support that pri-MIR166c and pri-MIR166d are not transcriptionally affected in these backgrounds under the tested conditions, thereby validating their use as non-target comparators. The differential expression patterns further support the specificity of JUL1-mediated transcriptional regulation.

We apologize for the confusion caused by the wording in line 124 and have revised the the Figure legends and Methods section to clearly describe the normalization strategy used in all qRT-PCR experiments (Line 522-523).

Line 129-130: "To further assess the functional connection between JUL and miR165/166 in establishing the leaf adaxial–abaxial axis". See the above point, the data presented does NOT address the establishment of adaxial-abaxial polarity. The authors make statements like this throughout the manuscript, but analysis of the establishment of leaf polarity requires studying the shoot apical meristem. Moreover, the phenotypes reported for jul1 and jul1 jul2 are far too

mild to be caused by defects in leaf polarity establishment. The authors confuse "a minor effect on leaf polarity" with the far more exciting term "leaf polarity establishment".

Author response

We appreciate the reviewer's comment and acknowledge that our original wording was imprecise. As noted above, we do not intend to suggest that JUL1 plays a role in the initial establishment of adaxial–abaxial polarity at the shoot apical meristem. Rather, our data support the view that JUL1 contributes to the modulation or refinement of polarity-related gene expression during later stages of leaf development, likely in coordination with vascular patterning (see Lines 353–362).

We recognize that the use of the term "establishment" may have overstated the functional implications and caused confusion. To better reflect the scope of our findings and avoid misinterpretation, we have revised the sentence to read:

"To further assess the functional connection between JUL and miR165/166 in leaf adaxial–abaxial patterning, …" (Lines 129–130).

The phenotypes of the proRPS5A:STTM165/166 lines are surprisingly weak. Short Tandem Target Mimics, when constructed correctly, cause a dramatic decrease in the level of the targeted miRNA. As a result, STTM lines effectively targeting miR165/166 (when viable) show severe developmental defects, and changes in the expression of polarity related genes (e.g. Yan et al., 2012 doi: 10.1105/tpc.111.094144). The explanation provided by the authors on lines 146-148 does not make sense at all. This would indicate that miR165/166 has no function in leaf development. This raises questions about expression or effectiveness of the proRPS5A:STTM165/166 transgene in the lines selected.

Author response

We thank the reviewer for this insightful comment. We agree that functional STTM constructs targeting miR165/166 can cause dramatic phenotypes when expressed under strong, constitutive promoters, as shown in the study by Yan *et al.* (2012, Plant Cell). In that work, STTM165/166 was driven by the 35S promoter, resulting in robust suppression of miR165/166 and severe developmental defects.

In our study, we used the same STTM165/166 sequence design as described by Yan et al. (Rebuttal Figure 1). To validate the functionality of our construct, we expressed STTM165/166 under the 35S promoter and observed phenotypes consistent with strong miR165/166 depletion, thereby confirming that the construct is effective when expressed at high levels.

For our experimental lines, we intentionally placed STTM165/166 under the control of the *RPS5A* promoter, which is active in the meristem and vascular initials but is less ubiquitous and potent than 35S. We hypothesize that this more spatially and temporally restricted expression, along with lower promoter strength, leads to partial not complete suppression of miR165/166, thereby producing milder but still detectable phenotypes.

[Figure Redacted]

Rebuttal Figure 1. Phenotype of STTM165/166 under the 35S promoter

To directly assess knockdown efficiency, we measured mature miR165/166 levels in the shoot apices of 7-day-old seedlings. These data are now included in Figure 1e–i and show a modest but reproducible reduction of miR165/166 (Fig. 1i), accompanied by moderate de-repression of target genes. Correspondingly, phenotypes were present but not as severe as those in 35S lines.

We would like to emphasize that these results do not imply miR165/166 is dispensable for leaf development. Rather, they reflect the limited knockdown achieved with *RPS5A*-driven expression of STTM165/166. Importantly, our genetic interaction analyses show that the leaf phenotypes of

jul1 jul2 mutants are dependent on miR165/166 function, reinforcing the biological relevance of this regulatory relationship.

Regarding the vascular phenotypes shown in Extended data Fig. 3, the authors previously showed that JUL1 suppresses phloem development through its negative effects on SMXL4 and SMXL5. This complicates data interpretation. Are the observed defects due to effects on adaxial-abaxial polarity, lost regulation of SMXL genes, both, or even not yet discovered targets.

Author response

We appreciate the reviewer's important point and fully agree that multiple regulatory layers, both known and potentially unidentified targets of JUL1, may contribute to the leaf phenotypes observed in *jul1 jul2* mutants.

Among the known targets, *SMXL4* and *SMXL5* are expressed specifically in phloem lineage cells, and their protein accumulation is similarly restricted to the phloem (Cho *et al.*, 2018, *Nature Plants*; Wallner *et al.*, 2017, *Current Biology*). Given this spatial restriction, their direct involvement in establishing or modifying adaxial–abaxial leaf polarity is unlikely. Consistent with this, *smxl4 smxl5* double mutants do not exhibit any defects in leaf polarity (Wallner *et al.*, 2017, *Current Biology*).

To further evaluate this relationship, we examined plants carrying a targeted deletion of the JUL1-binding site in the 5′ UTR of *SMXL5* (Park *et al.*, 2025, *PNAS*). These lines displayed an increase in phloem cell number, supporting the role of JUL1 in repressing *SMXL5*-mediated phloem development. However, they did not exhibit the leaf curling phenotypes observed in *jul1 jul2* mutants. Together, these findings suggest that the leaf patterning phenotypes are not mediated by *SMXL4/5*, and instead implicate a distinct regulatory mechanism.

In support of this, our biochemical data show that JUL1 directly binds to pri-miR165/166 and modulates their processing, and that JUL1 protein is broadly distributed beyond the phloem. These observations support a model in which JUL1 functions as a coordinator of both vascular development and leaf patterning, likely through independent pathways rather than solely via repression of *SMXL* genes.

Line 174: the term "emerging" is wrongly used

Author response

We appreciate the reviewer's comment and acknowledge that our original use of the term "emerging" was imprecise. Our intent was not to suggest that the leaf was physically emerging from the shoot apical meristem, but rather to refer to an early stage of leaf development. We agree that the term "emerging" may be misleading in the context of developmental biology. To clarify and avoid overstatement, we have revised the sentence as follows:

"We detected GFP fluorescence indicative of *MIR165a* transcriptional activity in the abaxial domain of young leaf primordia in *proMIR165a:GUS-GFP* transgenic seedlings." (Lines 174–175)

Line 177: The authors conclude that JUL1-GFP accumulates throughout the leaf primordium. The quality of the data shown is not good enough to conclude this. The optical sections show many cells without GFP. Imaging actual leaf sections would generate a much greater resolution. Also, how many lines were analyzed? In other words, how reproducible is the data?

Author response

We appreciate the reviewer's comment regarding the JUL1-GFP localization data. To assess reproducibility, we analyzed two independent *proJUL1:JUL1-GFP* transgenic lines and examined

approximately 15 individual seedlings per line. In all cases, we observed a consistent pattern of JUL1-GFP distribution that was broader than that of the corresponding transcriptional reporter.

That said, we fully acknowledge that the resolution of the current confocal optical sections may not be sufficient to support the phrasing "throughout the leaf primordium." In particular, we recognize that GFP signal may appear uneven due to optical interference from cellular structures such as chloroplasts, which can obscure fluorescence detection. In response, we have revised the manuscript text to avoid overstatement and more accurately describe the observed expression pattern.

Importantly, we note that JUL1-GFP signal is detectable even in abaxial epidermal cells of the leaf primordium, where pri-MIR165a is expressed (Fig. 2a, b), supporting our conclusion that JUL1 may influence miR165/166 biogenesis in a non-cell-autonomous manner.

The tissue- or region-specific complementation of jul1jul2 is nice idea. However, how do the authors explain that expression of JUL1 on the adaxial side (BOP1 promoter) rescues the leaf curling defect of jul1 jul2? The latter is proposed to be caused by elevated miR165/166 levels in the mutant due to increased miRNA processing. However, the miR165/166 precursors are not expressed adaxially. This would point to an entirely different basis for the leaf curling phenotype.

Author response

We thank the reviewer for raising this important point. We acknowledge that pri-MIR165/166 transcripts are primarily expressed in the abaxial domain of the leaf. However, in our study, we observed that JUL1-GFP protein is distributed beyond its transcriptional origin, suggesting that JUL1 may function in a non-cell-autonomous manner.

To test this possibility, we performed tissue-specific complementation of the *jul1 jul2* mutant using the adaxial-specific *BOP1* promoter. If JUL1 acted strictly in a cell-autonomous fashion, adaxial expression would not be expected to affect miR165/166 processing in the abaxial domain or restore the mutant phenotype. However, the observed rescue of the leaf curling phenotype by *BOP1*-driven JUL1 expression suggests that JUL1 can function non-cell-autonomously, potentially by influencing downstream processes such as the movement or stability of pri-miRNAs, or through indirect regulatory effects across tissue layers.

These findings support the interpretation that JUL1, although expressed in specific tissues, can influence leaf development through non-cell-autonomous mechanisms. This is consistent with the broader distribution of JUL1-GFP protein observed in our localization assays (Fig. 2a).

Line 199: see above. The data presented does not support a role for JUL1 in adaxial-abaxial pattern formation. The mutants show a mild leaf curling, potentially consistent with an effect on polarity maintenance.

Author response

We respectfully note that our manuscript does not propose a role for JUL1 in the establishment of adaxial–abaxial polarity.

As described in the discussion part (line 358-364), JUL1 is expressed following the onset of phloem development and acts to modulate the polar distribution of the miR165/166–HD-ZIP III module.

We specifically use terms such as "recalibrate" and "feedback" to reflect a function in refinement or maintenance, rather than in the initial establishment of polarity. We apologize if the phrasing elsewhere in the manuscript caused any confusion, and we have made minor revisions to the introduction and results to clarify this distinction (Line 405–409).

Evaluation of the RNA binding experiments are outside my expertise. However, two thoughts: 1) the on-bead binding assays are not reflective of what happens in the cell. The pri-miRNA transcript is able to form a hairpin structure, such that JUL1 has to compete with secondary structure formation. How realistic is that? Is single stranded pri-miRNA present, or can JUL compete with RNA folding?

Author response

We thank the reviewer for raising this important point. We fully agree that *in vitro* on-bead binding assays do not fully recapitulate the structural and dynamic context of pri-miRNA folding *in vivo*, where RNA secondary structures may limit access by RNA-binding proteins.

To address this limitation, we performed RNA immunoprecipitation (RIP) using *jul1 proJUL1:JUL1-HA* complementation lines to evaluate the association between JUL1 and pri-MIR165a under native cellular conditions (Fig. 3f). While RIP cannot directly determine whether JUL1 competes with pri-miRNA secondary structures or preferentially binds to single-stranded regions, the enrichment of pri-MIR165a in JUL1-IP samples supports that JUL1 interacts with the transcript in a physiological context.

In addition, using a protoplast transient expression system, we observed that JUL1 reduced the levels of RNA bound to the double-stranded RNA-binding domain of DCL1 (Fig. 4c), suggesting that JUL1 can affect DCL1-mediated pri-miRNA processing.

Thus, although we acknowledge the structural complexity of pri-miRNAs, our combined *in vitro*, protoplast, and *in planta* RIP data collectively support JUL1 binding to pri-MIR165a *in vivo* and demonstrate its potential impact on DCL1-dependent miRNA biogenesis.

2) The colocalization of JUL1 with MS2-tagged miR165a precursor is again not at all convincing. The resolution of this experiment is insufficient. MS2-GFP protein on its own is nuclear localized and, at the resolution provided, co-localizes with mRFP-JUL1 (left panel Fig 3e). The authors need to look at the sub-nuclear level. The statement that mRFP-JUL1(RA) does not co-localize with MS2-GFP is also not supported by the data. It is just that expression of mRFP-JUL1(RA) is comparatively low.

Author response

We thank the reviewer for the critical feedback. We acknowledge that the imaging resolution in the current dataset is not sufficient to conclusively demonstrate subnuclear colocalization between mRFP-JUL1 and MS2-tagged pri-miR165a. We also agree that, given the broad nuclear localization of MS2-GFP, its apparent overlap with mRFP-JUL1 at the resolution presented may reflect co-occupancy of the nuclear compartment rather than true molecular colocalization.

Regarding the JUL1(RA) mutant, we agree with the reviewer that the apparent lack of colocalization with MS2-GFP may be confounded by lower expression levels of mRFP-JUL1(RA), rather than reflecting a biologically meaningful loss of interaction.

To address these concerns, using an Airyscan-equipped LSM800 microscope, we obtained higher-resolution images that revealed MS2-GFP colocalization with distinct cytosolic compartments associated with mRFP-JUL1 in an RNA probe–dependent manner. Quantitative fluorescence intensity measurements within these condensates further confirmed probe-dependent enrichment of MS2-GFP signals, providing stronger evidence of colocalization (Fig. 3e).

As is evident, I looked at the manuscript carefully up to the middle of page 9. However, I stopped reading at that point, as it was overwhelmingly clear that the current manuscript is not suitable for publication. The authors would be advised to consult a developmental biologist, and to greatly improve the quality of the data. Moreover, given that the authors identified over 65,000 regions capable of forming RG4s, they would need to think of additional experiments to convincingly

make the point that increased miR165/166 levels are the cause, rather than an outcome of the jul1 jul2 phenotype. As it stands the study falls far short of its promise, there is no evidence for the model proposed.

Reviewer #2 (Remarks to the Author):

In this manuscript, Hwang and colleagues revealed new molecular and physiological functions of JUL1 in addition to regulating phloem development (Nature plants, 2018). They identify pri-MIR165/166 as new targets of JUL1 and uncover the regulatory role of JUL1 in miRNA processing, therefore provide new insights into the understanding of JUL1's molecular functions. They also provided solid genetic and physiological proof that JUL1regulates leaf development dependent on MIR165/166 processing, therefore coordinates vascular development and leaf patterning, and update our understanding of coordinated development of plant tissues. Overall, this manuscript provides new insights into leaf development from RNA regulatory's side with high quality data.
Major points:
1) The authors have found JUL1 is differentially distributed in different cells, and this distribution seems to be very important for JUL1's function and subsequently its regulatory role in leaf development, however the driving force of JUL1's distribution is not clear. The authors may make some efforts to explore the factors that affect JUL1's distribution, and the evolutionary selection of such factors across different species with different leaf shapes.

Author response

We appreciate the reviewer's insightful comment. We agree that the cell type–specific distribution of JUL1 is likely to be critical for its regulatory function in leaf development. Our observations indicate that *JUL1* transcription is primarily confined to vascular tissues, while JUL1 protein forms a gradient-like pattern that extends into surrounding cells. Given the size of JUL1-GFP (~42 kDa), which falls within the range permissive for plasmodesmal transport, we consider that passive diffusion may contribute to establishing this distribution.

At the same time, we recognize that additional mechanisms—such as selective retention, degradation, or active transport—could also play important roles. While the present study was not designed to dissect the underlying drivers of JUL1 mobility, we agree this is an important direction for future research. In particular, identifying upstream factors that regulate JUL1 distribution will provide valuable molecular insights into how its non-cell-autonomous function is controlled.

With respect to the evolutionary context, *JULGI* homologs are broadly conserved across vascular plants and are also found in certain non-vascular ancestral species (Park et al., 2025, PNAS). These findings suggest that the functional establishment of *JULGI* is closely linked with the evolution of vascular tissues, since the vascular-derived, non-cell-autonomous feedback regulation we propose presupposes the presence of vasculature. Consistent with this idea, we observed defects in leaf patterning in other plant systems such as tomato and tobacco, supporting conservation of this mechanism across vascular plants to refine both vascular development and organ polarity. Ongoing studies in our group aim to elucidate the ancestral roles of *JULGI* prior to the emergence of vascular tissues, which we expect will provide more direct insights into the evolutionary trajectory raised by the reviewer.

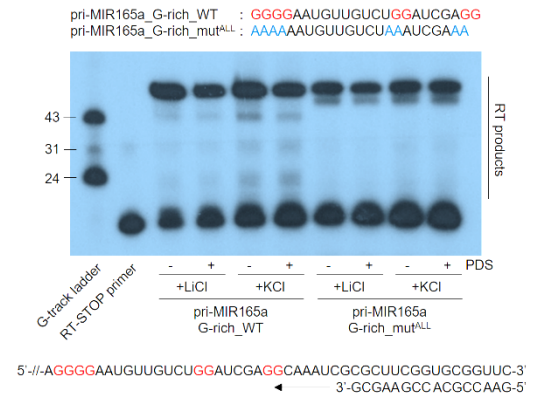
2) Considering the RG4 formation, canonical RG4 sequence should include at least 4 G-blocks. However, in the pri-MIR165/166 sequence, UUGAGGGGAAUGUUGUCUGGAUCGAGGAU, only 3

G-blocks are found. Although there are 4G in the first block, it still cannot form two separate G-blocks. This sequence doesn't satisfy the sequence requirement for RG4 formation. Given the complexity of RNA folding, there may be possibility of RG4 formation as indicated in Extended Data Fig 8 through circular dichroism (CD) spectra. However, to confirm RG4 formation, other methods should be included.

Author response

We appreciate this insightful comment. We were aware of the limitation that the pri-MIR165/166 sequence contains only three G-blocks and thus does not meet the canonical sequence requirement for RG4 formation, which typically includes at least four G-blocks (5′-G≥3N1-7G≥3N1-7G≥3N1-7G≥3-3′). Indeed, the prediction scores obtained from *in silico* analyses were also lower compared to canonical RG4 sequences. For this reason, we did not interpret the observed features as definitive evidence of RG4 formation or directly link them to the molecular mechanism. Rather, we used this prediction solely as a reference criterion to guide the identification of potential JULGI targets. In addition, we performed CD spectral analysis to confirm that the selected sequence, although based on RG4 prediction, behaved consistently during analysis, thereby validating the integrity of the experimental procedure.

For further confirmation of RG4 formation in this sequence, we performed RT-STOP assays using templates containing either the wild-type G-rich elements or mutated elements of pri-MIR165a (Rebuttal figure 2). We observed a stop pattern immediately upstream of the last G-block under potassium or potassium/PDS (an RG4 stabilizer) treatment, indicating intra-molecular RG4 formation. However, stops were predominantly detected near the beginning of the first and second G-blocks. Importantly, full-length transcripts remained the major products compared to the stalling products detected by RT-STOP. These results support the reviewer's point that pri-MIR165/166 likely has a reduced capacity to form stable intra-molecular RG4 structures, while also raising the possibility that inter-molecular RG4 formation might occur under certain conditions.



Rebuttal figure 2. RT-STOP assay of G-rich elements in pri-MIR165a

In line with the reviewer's suggestion, we have now explicitly stated in the revised manuscript that the G-rich elements in pri-MIR165a deviate from the canonical RG4 sequence and therefore likely have a lower probability of forming stable intra-molecular RG4 structures. The revised text reads as follows:

"We confirmed experimentally that pri-MIR165a potentially forms RG4 structures *by in vitro* circular dichroism (CD) analysis (Supplemental Figure 7). However, considering the canonical RG4 forming sequence, (5′-G≥3N1-7G≥3N1-7G≥3N1-7G≥3 -3′), the G-rich elements in pri-MIR165a seem to have a lower probability of forming an RG4 structure."

Minor points:

1) P should be always italicized and capitalized, such as in figure legend Figure 3 f.

Author response

We have revised the manuscript so that *P* is consistently italicized and capitalized throughout, including in Figure 3f and all other figure legends.

2) In reference, "Elife" should be in "eLife".

Author response

We have corrected the journal name "Elife" to the proper format "eLife" in the reference list.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author)
Thanks for the authors' effort answering all questions, no more questions are raised.

Reviewer #3 (Remarks to the Author)
Overview
This is an interesting study exploring a new contributor to the adaxial-abaxial polarity network: the RNA binding protein JULGI. I was invited to evaluate the author's response to Reviewer 1 who had numerous critiques of the paper. These critiques were conceptual as well as experimental in nature. Conceptually, the authors did not clearly delineate 'establishment' from 'maintenance' phases of leaf polarity. Experimentally, the reviewer made several concrete suggestions to improve their experiments. These included better tissue choices, imaging, and in planta experiments. I believe these critiques have merit.

For instance, establishment vs maintenance is an extremely important distinction, as a lot of effort has gone into carefully defining the relative contributions of each polarity determinant to each phase in the process. Reading their revision, the authors have taken this critique to heart and changed most of the language to better reflect the 'maintenance' role of JULGI. However, a few lines still need to be modified:

Line 62 states miR166 'establishes' adaxial identity. However, Burian et al 2022 shows that AS2 and KAN are polarized across the ad-ab axis well before leaf emergence. The role of 166 is to limit HDZIPIIIs to the adaxial side; this is important because HDZIPIII factors are sufficient to convert abaxial identity into adaxial identity. However, HDZIPIII accumulation appears to be a response to – rather than the cause of – a polarized ad-ab axis. Line 363 has the same issue.

Line 71 states vascular development 'coordinates leaf patterning'. It is more accurate to say it 'contributes to leaf shape'. Or that it 'mediates/interprets underlying ad-ab polarity to impact leaf shape'. However they choose to write it, the text must make clear that patterning itself is not set up by JULGI or the vasculature.

The legend of Fig 5 still says 'establishes'. I believe this is the last instance, but the text must be fully checked for removal/clarification of this issue.

Author response

We thank the reviewer for this insightful comment. We fully agree with the reviewer's point, and have revised the relevant sentences accordingly:

(Line 61) In leaf development, abaxially expressed microRNA165/166 (miR165/166) maintains abaxial identity by restricting the activity of class III homeodomain leucine zipper (HD-ZIP III) transcription factors.

(Line 364) In the abaxial domain, miR165/166 maintains abaxial identity by repressing HD-ZIP III, the key determinants of adaxial fate.

(Line 70) However, the spatiotemporal mechanisms by which vascular development mediates underlying adaxial-abaxial polarity to shape leaf development, particularly through ncRNA regulation to integrate vascular and adjacent tissues during leaf formation, remain largely unknown.

Figure 5. JUL1 refines leaf adaxial-abaxial patterning by suppressing miR165/166 levels in the abaxial domain.

Regarding the experimental critiques, I did not see the original manuscript. However, the justifications laid out by the reviewer all seemed reasonable to me. Importantly, almost all these critiques have been addressed by the authors (see Weaknesses for two exceptions).

Strengths

My own assessment of this version of the manuscript is that it is experimentally strong and conceptually interesting. Factors that aid in interpreting the underlying ad-ab axis are important and understudied. Given the importance of ncRNAs to ad-ab polarity, taking an RNA biology approach is also appropriate and interesting.

The experimental rigor in this version of the manuscript is outstanding. Indeed, multiple orthogonal assays are used to test each claim. I believe it is worth laying them out to clearly illustrate the number of independent assays performed:

- *To support a link between JUL1/2 and miR166 accumulation/activity: qRT for polarity determinants, STTM and qRT/phenotypic analyses*
- *To support non-cell-autonomy: single cell data mining, transcriptional reporter, multiple translational reporters*
- *To support binding of JUL1 to G-rich region of pri-MIR165a: REMSAs, bead-surface binding, circular dichroism, bacteriophage M2, in planta RIP*
- *To support the notion that JUL1 prevents formation of dsRNA: bead surface binding, FRET*
- *To support the notion that JUL1 prevents DCL1 from interacting with pri-MIR165A to process it into mir165: in planta RIP, pri-miRNA processing assay (see Weaknesses).*
- *To support the notion that JUL1/2 act in the immediate term to suppress miR165a accumulation: protoplast co-transfection (see Weaknesses), short term induced JUL1 O/E and KO lines, MIR165amut phenotypic/qRT analyses in Wt and jul1/2 mutants*

I have concerns about a couple of these assays (see Weaknesses) but not enough to question their overall conclusions.

Weaknesses

The pri-miRNA processing assay does not convincingly show a difference in mature 165

accumulation with JUL1 vs JUL1(RA), i.e. Fig 4d. Thus, conclusions that JUL1 works by inhibiting DCL1 processing are not supported. However, statements that JUL1 inhibits DCL1 interaction with pri-miR165A remain supported.

Author response

We appreciate the reviewer's thoughtful comment. The assay in Fig. 4d was not intended to compare JUL1 and JUL1(RA), but rather to test whether differences in pri-miRNA binding affinity affect DCL1 processing efficiency. Specifically, pri-MIR165a_WT and pri-MIR165a_mut$^{\#3}$ differ in their binding affinity to JUL1, while retaining identical mature miR165a sequence. Across all assay conditions, JUL1 addition consistently reduced mature miR165a accumulation relative to reactions without JUL1, and pri-MIR165a_mut$^{\#3}$, the weaker binder, produced higher mature miR165a levels, supporting a binding-dependent inhibitory effect on DCL1-mediated processing.

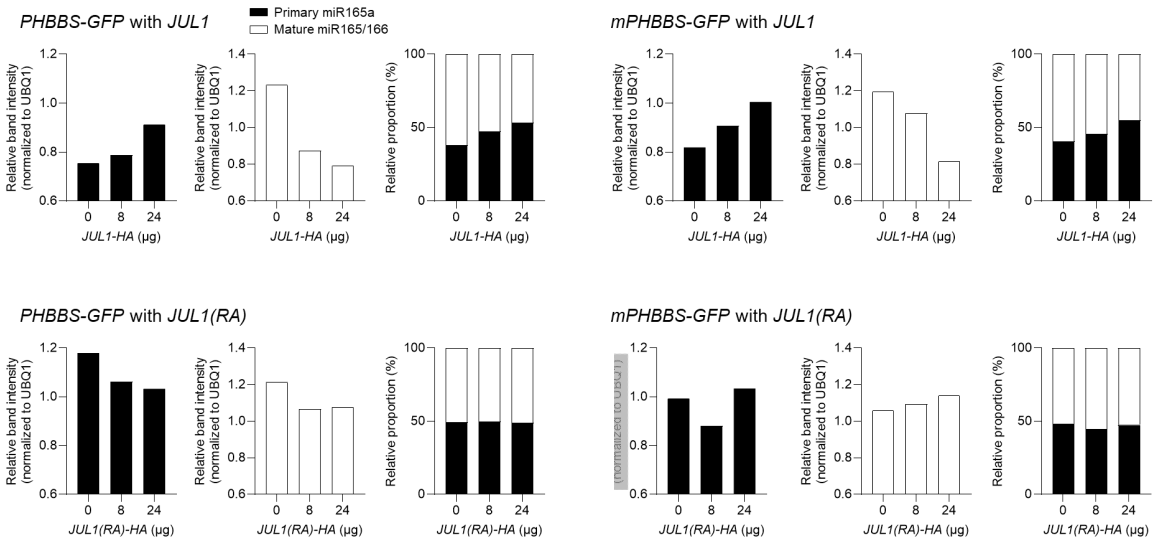
We agree, however, that our original wording could be misread as implying direct inhibition of DCL1's catalytic activity. We have revised the text to clarify that JUL1 interferes with pri-miRNA incorporation into the DCL1 dsRNA-binding domain rather than inhibiting DCL1 catalysis:

(Line 294) "…indicating that JUL1 reduces the efficiency of DCL1-mediated processing of pri-MIR165a into mature miR165. Collectively, these results suggest that JUL1 interferes with the incorporation of pri-MIR165a into the dsRNA-binding domain of DCL1, possibly through competitive binding or steric hindrance, thereby suppressing miR165a biogenesis."

The RT-PCR for pri and mature 165A in Fig 5A does not show increases in either as claimed in text. JUL1 (and mir166 binding site)-dependent effects on GFP signal (via confocal) and protein levels (via Western) are supported, however. Thus, the only claims that can be made for Fig 5A are: 1) the presence of an intact miR165a binding site reduces accumulation of GFP protein, and 2) this reduction can be blocked via increased dosage of JUL1. The statement "These results suggest that JUL1 binding to pri-MIR165a suppresses the accumulation of mature miR165/166, leading to higher levels of transcripts for HD-ZIP III genes, the targets of miR165/166." should thus be revised as these assays do not support this claim.

Author response

We thank the reviewer for this valuable observation. We agree that the visual differences in the RT-PCR bands in Fig. 5A appear subtle, although changes in band intensity are evident in a JUL1 dosage-dependent manner. To clarify this, we performed densitometric quantification of the pri- and mature miRNA bands (Revision Figure 1, below). This analysis revealed an approximately 20% increase in pri-miR165a and ~30% decrease in mature miR165/166 upon *JUL1* expression, whereas no such changes were detected with JUL1(RA). These quantitative results indicate that JUL1 modestly, but specifically, affects pri-miR165a processing efficiency in this assay. However, as the reviewer noted, this conclusion is not essential to support the main finding without the *in planta* data, and we have therefore removed the conclusive statement from the text.



Revision Figure 1. Quantification of RT-PCR bands in Fig. 5A

Importantly, these data serve as supportive evidence consistent with the *in planta* results shown in Fig. 1d and Fig. 5b,c, where reciprocal and statistically robust changes in pri- and mature miR165/166 were observed in *jul1*/*jul1 jul2* mutants, inducible *JUL1* overexpression, and *JUL1* knockdown lines.

Minor Comments and Suggestions

Lines 210, 214, 232 reference the wrong Extended Fig.

Author response

We thank the reviewer for carefully noting this error. The references in lines 210, 214, and 232 were indeed incorrect and should refer to Extended Data Fig. 8 rather than Extended Data Fig. 7. These have been corrected in the revised manuscript.

The authors should mention AGO10/PINHEAD/ZWILLE in the Intro or Discussion. AGO10 is a non-catalytic, adaxially-localized soak for miR166 (Zhu et al., 2011 Cell). As JULGI is an orthogonal post-transcriptional mechanism to limit 166 activity, a discussion of AGO10 in this context would be of interest.

In addition, AS2 should mentioned. There are many reasons to do this, including its involvement in the ad-ab pre-patterning (Burian et al) and the fact that it directly represses MIR166A (Husbands et al., 2015 Plant Cell).

Author response

We thank the reviewer for this insightful suggestion. We fully agree that AGO10/PINHEAD/ZWILLE and ASYMMETRIC LEAVES2 (AS2) are key components of the adaxial–abaxial regulatory framework and highly relevant for contextualizing the role of JUL1. In the revised manuscript, we have added a discussion highlighting how AGO10, AS1/AS2, and HD-ZIP II maintain adaxial identity by limiting miR165/166 expression or activity in the adaxial domain, and how JUL1 operates through a spatially distinct mechanism by modulating miR165/166 biogenesis in the abaxial domain.

This addition clarifies that JUL1 represents an orthogonal post-transcriptional layer that refines adaxial–abaxial balance rather than reinforcing adaxial suppression. We added this part as follows in Discussion,

(Line 369) Mechanistically, this process parallels the action of ARGONAUTE10 (AGO10), as both limit miR165/166 activity through post-transcriptional regulation[58,59]. Whereas AGO10, ASYMMETRIC LEAVES1/2[60], and HD-ZIP II[28] act in the adaxial domain to restrict miR165/166 accumulation, JUL1 acts in the abaxial domain to modulate miR165/166 biogenesis. Thus, JUL1 fine-tunes the abaxial source strength of miR165/166, adding a distinct layer of spatial regulation to the polarity network.