The genetic basis of natural variation in the timing of vegetative phase change in *Arabidopsis thaliana*

Erin Doody, Yuqi Zha, Jia He and Scott Poethig
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MS TITLE: The genetic basis of natural variation in the timing of vegetative phase change in Arabidopsis thaliana

AUTHORS: Erin E Doody, Yuqi Zha, Jia He, and Scott Poethig

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

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

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

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

Reviewer 1

Advance summary and potential significance to field

Doody and coworkers have taken advantage of a large collection of accessions of Arabidopsis from diverse climates to address the question of whether traits associated with vegetative phase change, some of which might be adaptive reflect variation in expression of the miR156/SPL cassette. The traits they monitored were leaf shape and first appearance of abaxial trichomes. The latter has been used reliably in the Poethig lab, where this work was done, for decades in their screens for the mutants that have provided the key that unlocked our understanding of VPC. As vegetative growth progresses, each new leaf is less round and more oval than those that preceded it, in a pattern suggesting that shape, a qualitative trait, is also phase-specific. They compared the appearance of those traits with expression of miR156 and its targets, in both the panel of wild accessions and in a RIL generated from a cross between Col-O and Shakdara an accession with both early abaxial trichomes and early oval blades. They found that there was no tight correlation among timing of the morphological traits, gene expression, or onset of flowering, concluding that VPC is under multiple levels of regulation.

Comments for the author

Since leaf shape is known to influence physiology, such as heat dissipation in sun leaves of Taraxacum, it was reasonable to tether comparisons of shape among accessions from more or less challenging geographical locations. They developed a quantitative proxy for shape by measuring how flat (early leaves) or tapered (later leaves) the proximal end of each blade was and calculating a so-called leaf base angle. Since leaf angle is a term already employed in maize for the insertion of a leaf at the stem, this was confusing: a term that is more obviously related to the shape might be preferred-lamina base taper? Even without a new term, it would help the reader to articulate that leaf base angle is a proxy for shape, and a supplemental figure that compares leaf silhouettes to LBA for Col-O would help convey this. It would also help the reader to state when leaf 4 or leaf 3 shape is being compared.
Since, as it turned out, abaxial trichome appearance and shape were not linked tightly, I think it has hurt the ms. to have focused on shape. Contrary to their claim (line 68) that Telfer et al. looked at trichomes, shape, and margin serrations to identify juvenile traits, it was trichomes alone that have marked VPC there and in many other publications, and was reported here (line 161) as the marker for juvenility. I would have ordered figures 1 and 2 around trichomes: it would still have conveyed that shape varied independently. Figure 1D shows that VPC timing is independent of flowering, but the measurement was in number of days until the first flower opening (anthesis). The standard is not days but leaf number. While I suspect that leaf number wouldn't have given a different result, it would have been good to acknowledge (and justify) use of a nonstandard measure. Notably, Telfer et al found that growth conditions that delayed flowering (SD) also delayed appearance of abaxial trichomes, in Col-O Ler, and Ws. Since that correlation didn't show up in this report, it seems worthy of discussion.
Figure 1 B, C, D. Is there a more intuitive way to show this data that doesn't make the reader look through and compare values. Would like to also see a comparison between trichomes and days to first flower.
Figure 2D. Why not show leaf 4 when so much of the analysis uses its shape?
Figure2EF/ Figure 3AtoF – would be helpful to see this data compared in the same order to get a better understanding of the variation between observable leaf traits and gene expression patterns with leaf shape included.
Figure 3A – range of miR156 abundance in Sij-4. You have 2 fold difference across 1 accession and only 3 data points. If you are using this metric to order your data for analysis, this should include more data points to get a more meaningful average.
Figure 3C – the range of values from kldr-1 needs to be address for primiR156C abundance.
Figure 4D – can you put this data in a graph so it is easier to see a difference in first leaf with serrated edges (imageJ analysis of change of shape from round to more oval using all leaves, not just the odd ones) and how it relates to leaf angle. I see there is natural variation of first leaf with serrated edges/change in shape but I would like to see how that correlates to first leaf with abaxial trichomes and miR156 abundance.

Minor suggestion: rotate images in Fig 4 A so that leaf 4 is at the top in both.
SPL transcript abundance. If miR156s are present and active, do we know how mRNA abundance of the miR targets relate to actual protein being made?
There has to be a better way to show relationship between the different markers other than putting them in numerical order in one graph, and showing they don't follow that numerical order in other graphs. Do both miRs have exactly the same targets?
If you are proposing that VPC is influenced by something other than this miR156/SPL pathway, can you identify genes or processes in the QTL groups that are novel instead of looking for genes already known to be part of this process like the TOEs and SPLs?

Minor points:
L32: has miR157 been examined in VPC of many different plant species? Maybe just write 156.
L86: trees are not at the opposite end of the evolutionary spectrum of land plants from bryophytes.
L110: precocious what? VPC or flowering?
L147: needs a verb L282: production of abaxial…

Reviewer 2

Advance summary and potential significance to field

This manuscript reports on the analyses of 70 Arabidopsis accessions regarding their natural variation in VPC. As expected miR156 was among the loci this trait was attributable to, but the study also identified loci that indicate a miR156-independent regulation. This included the miR156 targets, the SPLs.
Importantly though not surprisingly, no relationship between the variation in flowering time and VPC was observed, indicating that both developmental transitions are controlled by separate mechanisms (though in part employing similar or the same candidates). Lastly, the authors discuss that dissociation of the traits might be associated to annual variation in temperature.
The manuscript is very well written and a pleasure to read and review. I do however have some comments, which largely deal with the interpretation of the results, and textual changes summarized at the end.

Comments for the author

Major comments
1. Recording flowering time: Why was floral anthesis (Days to first open flower) instead of the more commonly used parameters 'days to bolting' or 'total leaf number' (rosette leaf number plus cauline leaf number)? I suggest replacing the term flowering (time) with floral anthesis and adjust the discussion.
2. Appearance of traits (angle, trichomes, floral anthesis), abundance of mature miR156, expression of pre-miR156A and C, and three target genes (SPL3, SPL9, SPL15) do not correlate in a group of early accessions. However, none of the other potential target SPLs were quantified. This leaves the reader wondering whether any of the others might correlate better with the abundance of miR156 or the appearance of traits. Did the authors assess the variance of cleavage/target site sequences of the SPLs? This might explain, why they do not observe the expected correlation between miRNA abundance and target expression.
3. The study suggests that vernalization responsive floral induction is regulated independently of VPC. This assumption is based on findings in Sha, which when non-vernalized, still initiates VPC earlier than Col-0 and remains in the adult phase until vernalized. Can this be generalized? I am missing a discussion of this essential result.
4. The authors explain the differences between Col-0 and Sha with an accelerated decline in the levels of MIR156A and MIR156C transcript. Making such a statement would require measurements in a time or leaf series, which the authors do not have. The differential expression between the samples L1&2 and L5&6 is similar for Col-0 and Sha, although the levels are generally reduced in Sha when compared to Col-0.
5. Both Sha and Col-0 have similar miR156 levels in L5&6. However, transcript levels are close to 0. How reliable are assumptions drawn from this data?
6. What does lower sensitivity mean (line 350)? What senses miR156? Its output is the cleavage of its targets. This has to be rephrased and/or properly discussed.

7. The initial focus on leaf angle lets the reader assume that “leaf angle” as the more robust trait associated to VPC will be one of the main outcome of this manuscript, however, the authors then decide to focus on QTLs associated to trichome production instead of leaf angle later on. Why was this decision made? Similar QTLs identified in both LD and SD suggest that trichome production is the more robust trait correlating with VPC. I wonder how a LD/SD trichome PCA would look like (not included in Figure S1).
8. I don’t understand how sensitivity to photoperiod or light quantity was linked to the data described in the paragraph above line 406/7.
9. Why were only nonsynonymous SNPs taken into account (paragraph line 418)? Synonymous SNPs might have led to causal mutations e.g. when affecting a miR156 recognition sites in the SPLs.
10. Further, the authors argue that the decreased expression of TOE1 might explain the early Sha VPC. However, they also write that TOE1 “promotes” earliness (line 460), which is not correct and definitely does not fit in the argument.
11. The authors find that lower pri-miR156A correlates with earliness phenotype of Sha. However, they argued in a previous paragraph that pri-miR156 expression does not correlate with the earlier phenotypes of a set of accessions. This leaves the reader puzzled and should be properly discussed.
12. The manuscript lacks biological data demonstrating that both miR156A and TOE1 are responsible for the earlier VPC of Sha. Such data would have supported the story.
12. The supplementary file Table S4 contains information on the peaks found on chromosome 2 and 5, but not on 1. The region covers supposedly covers FT (At1g65480, line 402), but also TPS1 (At1g78580), which has been associated with sugar-dependent miR156 and a partially miR156-independent regulation of SPLs. A potential link should be discussed.
13. The authors argue that since over-expressing miR156 in Col-0 has only a minor effect on flowering, VPC to the adult vegetative stage is unlikely to be essential for plants to undergo floral transition. While this might be true, the statement is very strong and lacks sufficient support. The mild flowering phenotype (which is laboratory and therefore likely growth condition-dependent) might also suggest that other pathways bypass miR156-dependent signals (likely in part converging on the SPLs) ensuring progression to a shortened adult phase and a timely floral transition. That this might at least be possible is nicely demonstrated by the data in this manuscript.
Minor comments Line 92: Typo, replace “it” with “its”
Line 292: Typo, remove one “in”
Figure 2: In the first part of the study the angle of leaf four was measured, but the leaf is not among the imprints shown in Fig. 2D.
Typo Figures 1 / 2: compare “Stepn” and “Steph”
Figures 2/3: data points (transparent grey circles) vanish on the background of black boxes. Consider arranging non-transparent circles to the front of the boxes. Some of the circles are elliptic, e.g. Figure 3D.
Figure 4: align “Leaf #” with numbers in 4B (adjust font size), tick missing on graph of 4D or remove “150”
Line 308: word missing in sentence Line 334: Figure 8 appears prematurely Line 341: wrong figure reference Line 359: replace “6A.C” with “with “6A-C”
Line 394: delete space between “Col-“ and “0”.
Line 404: replace “vrn1” with “VRN1”
Line 452: word missing or rephrase Line 487: word missing Line 505: replace “these” with “that”

Reviewer 3

Advance summary and potential significance to field

This manuscript studies vegetative phase change and flowering time in a collection of 70 accessions of Arabidopsis thaliana. The authors describe variation in vegetative phase change by scoring abaxial trichomes and leaf angle in long days and short days, and flowering time. Vegetative phase change traits vary independently among the accessions and no relationship to flowering is detected suggesting that the traits are regulated at least partially by different mechanisms. They then focus on 9 accessions that transitioned to adult phase early in development. Among these 9 accessions the levels of miR156 RNA or SPL gene mRNA did not explain the variation in phenotype among all accessions suggesting vegetative phase change is controlled by unknown genetic loci. They then focus on the Sha accession, which shows an extreme early transition to adult phase. By analyzing previously described RILs of a cross between Sha and Col they identify QTLs for vegetative phase

change based on abaxial trichomes and other traits, and for flowering time. They conclude that most QTL controlling vegetative phase change do not correspond to known genes affecting these traits, although one QTL on chromosome 2 correlates with TOE1 and MIR156A, and the RNAs encoded by these genes were reduced in abundance in Sha, suggesting that they are "promising candidates" for this QTL.
This manuscript describes a very large amount of phenotypic and genetic analysis that demonstrates the complexity of natural genetic variation in vegetative phase change and its relationship, if any, to flowering. The work also identifies MIR156A and TOE1, two known genes in the process, as possibly contributing to phenotypic variation in the Sha accession. This work will likely form the basis of many more detailed future studies. Some of the conclusions and some links to previous work could perhaps be explained in more detail, and some essentially negative results were explained in great detail.

Comments for the author

1. One conclusion of this work is that among natural accessions of Arabidopsis there is little relationship between flowering and vegetative phase change. More than 50% of the natural variation in flowering time has been described by several authors to be conferred by variations in the activity of the FLC/FRI system. The same lab did publish previously in Development (Willmann and Poethig, 2011) an analysis of the role of FLC in regulating vegetative phase change and found that FLC affects leaf shape and distribution of abaxial trichomes independently of its effect on flowering. This paper is not cited in the current paper, and I missed a full discussion of the relevance of the previous results for the current conclusions.
2. The conclusion of several experiments is that there is no correlation between different traits scored in these accessions. However, the data are complex with extreme quantitative variation across up to 70 accessions. I missed any formal statistical analysis of whether there are correlations among traits, and therefore an objective statistical demonstration that there is no correlation. For example, line 265 "no correlation between the angle of the leaf base and the first leaf with abaxial trichomes" and then the citation is the figure. So, by visual inspection of the Figure the reader should decide whether there is a correlation or not. Such conclusions should be formally drawn from statistical analyses and objective criteria, and this follows for others such as the relationship to flowering time.
3. The expression analyses of SPL genes and miR156 is based on RNA levels. However, is this enough to detect a correlation with phenotype because miR156 is also likely to affect the translation of SPL mRNAs? This issue becomes a problem for example on lines 309 to 311: "There was no clear relationship between the abundance of these transcripts and the expression of miR156 or their phase change phenotype". If miR156 is mainly affecting the protein abundance of SPLs could a "clear relationship" be hidden?
4. I found pages 15-21 very long as they explore possible candidate genes for QTLs and most of the conclusions are negative, perhaps most of these results could be summarized more briefly.
5. Sha accession has been used for QTL mapping of flowering time loci before, although in different crosses. For example, Salomé et al (2011) Genetics, and El-Lithy et al (2004) Plant Physiol. Perhaps these results should be discussed and integrated with the current results, especially as all analyses seem to detect loci on the bottom of chromosome 5.
6. Several previous papers (for example Wang et al 2009 Cell; Hyun et al, 2016 Developmental Cell) argued that the miR156/SPL system is much more important in controlling flowering in non-inductive SDs than in inductive LDs. It was not completely clear how much this conditional effect was considered in examining possible correlations between flowering time and vegetative phase change because different environmental conditions were used in different experiments.

First revision

Author response to reviewers' comments

We are grateful to the reviewers for their very careful consideration of this manuscript. We have done our best to address all of their concerns, and conducted additional experiments where they were requested. We re-wrote the manuscript extensively and also re-organized the figures. These changes are indicated below and highlighted in the manuscript. We hope the reviewers agree that the manuscript is much improved as a result of these changes.

Reviewer 1 Advance Summary and Potential Significance to Field:
Doody and coworkers have taken advantage of a large collection of accessions of Arabidopsis from diverse climates to address the question of whether traits associated with vegetative phase change, some of which might be adaptive, reflect variation in expression of the miR156/SPL cassette. The traits they monitored were leaf shape and first appearance of abaxial trichomes. The latter has been used reliably in the Poethig lab, where this work was done, for decades in their screens for the mutants that have provided the key that unlocked our understanding of VPC. As vegetative growth progresses, each new leaf is less round and more oval than those that preceded it, in a pattern suggesting that shape, a qualitative trait, is also phase-specific. They compared the appearance of those traits with expression of miR156 and its targets, in both the panel of wild accessions and in a RIL generated from a cross between Col-O and Shakdara, an accession with both early abaxial trichomes and early oval blades. They found that there was no tight correlation among timing of the morphological traits, gene expression, or onset of flowering, concluding that VPC is under multiple levels of regulation.

Reviewer 1 Comments for the Author:
Since leaf shape is known to influence physiology, such as heat dissipation in sun leaves of Taraxacum, it was reasonable to tether comparisons of shape among accessions from more or less challenging geographical locations. They developed a quantitative proxy for shape by measuring how flat (early leaves) or tapered (later leaves) the proximal end of each blade was and calculating a so-called leaf base angle. Since leaf angle is a term already employed in maize for the insertion of a leaf at the stem, this was confusing: a term that is more obviously related to the shape might be preferred-lamina base taper? Even without a new term, it would help the reader to articulate that leaf base angle is a proxy for shape, and a supplemental figure that compares leaf silhouettes to LBA for Col-O would help convey this. It would also help the reader to state when leaf 4 or leaf 3 shape is being compared.

This paper is not the first to use leaf base angle as a measure for vegetative phase change in Arabidopsis, (Fouracre and Poethig, 2019; He et al., 2018; Wang et al., 2021; Willmann and Poethig, 2011; Wu and Poethig, 2006; Xu et al., 2021) amongst others. However, it is true that some readers may be confused by the short hand term “leaf angle” to refer to leaf base angle. To avoid confusion, we changed to using the term leaf base angle, instead of leaf angle, where necessary and added a graphic to illustrate how leaf base angle was measured in Col-0 leaves 4 and 7 in supplemental figure 1A. We were also more explicit about when leaf four or three base angle was used in an analysis (leaf three was only use in RIL analysis)

Since, as it turned out, abaxial trichome appearance and shape were not linked tightly, I think it has hurt the ms. to have focused on shape. Contrary to their claim (line 68) that Telfer et al. looked at trichomes, shape, and margin serrations to identify juvenile traits, it was trichomes alone that have marked VPC there and in many other publications, and was reported here (line 161) as the marker for juvenility. I would have ordered figures 1 and 2 around trichomes: it would still have conveyed that shape varied independently.

Although abaxial trichomes were the first marker we used to study vegetative phase change (Telfer et al, 1998), subsequent studies have shown that several other traits also change in a phase specific fashion in Arabidopsis. Inspired by our discovery (Leichty and Poethig, 2019) that different phase-specific traits in swollen thorn acacias change at slightly different nodes in different genotypes, we decided to investigate the relationship between abaxial trichomes and leaf shape in Arabidopsis. We emphasize leaf shape in this paper because recent studies have shown that abaxial trichomes are regulated by flowering genes in the TOE1/miR172 pathway (Wang et al., 2019; Xu et al., 2019).

This discovery, along with our previous results (Wu and Poethig, 2006) suggest that leaf shape is a more reliable marker of vegetative phase change than abaxial trichomes. Additionally, our analysis of the Central Asian accessions revealed that leaf shape correlated with environmental conditions (trichome production did not), indicating this phenotype might have adaptive importance, and making it a logical path for us to follow to the order of this paper.

Figure 1D shows that VPC timing is independent of flowering, but the measurement was in number of days until the first flower opening (anthesis). The standard is not days but leaf number. While I suspect that leaf number wouldn't have given a different result, it would have been good to acknowledge (and justify) use of a nonstandard measure.

Now Figure 2E and Figure S2. Although leaf number is commonly used as a convenient way to measure flowering time in *Arabidopsis*, this measure depends on the assumption that the genotypes being compared do not vary in their rate of leaf initiation, or that the rate of leaf initiation varies in the same direction as flowering time. We and others have shown that genes involved in vegetative phase change have a major effect on the rate of leaf initiation, independent of their effect on flowering time (Wang et al., 2019; Xu et al., 2016), so leaf number is not a good measure of flowering time in studies of vegetative phase change. Furthermore, we found that the rate of leaf initiation in different accessions is not completely correlated with flowering time, meaning that leaf number cannot be used to compare flowering time between accessions. We added a Supplemental Figure S2 to illustrate this point. In short, we believe that the best measure of flowering time is when a plant actually produces flowers.

Notably, Telfer et al found that growth conditions that delayed flowering (SD) also delayed appearance of abaxial trichomes, in Col-O, Ler, and Ws. Since that correlation didn't show up in this report, it seems worthy of discussion.

We too found that conditions that delay flowering (specifically SD) also delay abaxial trichome production, possibility because miR172 is strongly up-regulated upon floral induction and represses the expression of TOE1, which has been shown to repress abaxial trichome production (Wang et al., 2019; Xu et al., 2019). In contrast, although leaf base angle is highly correlated between LD and SD, it maintains no relationship to flowering (Fig. 2B). Additionally, in Sha, where vernalization slightly delayed phase change based on abaxial trichomes, abaxial trichome production remained early compared to Col-0, which we think can be generalized to other accessions with active FRI/FLC. Thus, different phase-specific traits are differentially sensitive to environmental conditions. This is consistent with our previous results, (Wu and Poethig, 2006) which suggested that vegetative phase change is regulated by multiple interacting mechanisms. We have expanded on this in the Discussion.

Figure 1 B, C, D. Is there a more intuitive way to show this data that doesn't make the reader look through and compare values. Would like to also see a comparison between trichomes and days to first flower.

Now Figures 1, 2, and Figure S1. We divided this figure into two, (SD and LD data) moved regression analysis from supplemental material to main Figures. We also added a comparison of abaxial trichomes to flowering time to this figure. The text was adjusted accordingly.

Figure 2D. Why not show leaf 4 when so much of the analysis uses its shape?

Done- Now figure 3D.

Figure2EF/ Figure 3AtoF – would be helpful to see this data compared in the same order to get a better understanding of the variation between observable leaf traits and gene expression patterns with leaf shape included.

Done- Now Figure 3E/F, Figure 4A-C and Figure 5A-F.

Figure 3A – range of miR156 abundance in Sij-4. You have 2 fold difference across 1 accession and only 3 data points. If you are using this metric to order your data for analysis, this should include more data points to get a more meaningful average.

Now Figures 4 and 5. We increased biological replicates to 5 and reordered these figures according to leaf base angle phenotype.

Figure 3C – the range of values from kldr-1 needs to be address for primiR156C abundance.

Now Figure 5. We increased the number of biological replicates to 5.

Figure 4D – can you put this data in a graph so it is easier to see a difference in first leaf with serrated edges (imageJ analysis of change of shape from round to more oval using all leaves, not just the odd ones) and how it relates to leaf angle. I see there is natural variation of first leaf with serrated edges/change in shape but I would like to see how that correlates to first leaf with abaxial trichomes and miR156 abundance.

Now Figure 6D. It is a computational heavy task to measure leaf serrations across leaf number (time consuming image processing for thousands of leaves), which is why we only counted number of serrations on leaf seven in RILs). We expect there is variation here, and that leaf serrations may be uncorrelated with abaxial trichome production and leaf base angle, but did that that it was worthwhile to conduct this very detailed analysis when the results of a single leaf conveyed the same point. It is worth noting that most studies of vegetative phase change only present the first leaf with abaxial trichomes rather than trichome density on multiple leaves, even though trichome density increases with leaf position (Willmann and Poethig, 2011), just as the number of leaf serrations does. We added leaf outlines where appropriate to help the reader assess differences in leaf shape phenotypes between Col-0 and Sha.

Minor suggestion: rotate images in Fig 4 A so that leaf 4 is at the top in both.

Done- Now Figure 6A.

SPL transcript abundance. If miR156s are present and active, do we know how mRNA abundance of the miR targets relate to actual protein being made?

This is a key point. We examined this question in the Col-0 ecotype using reporter genes (He et al., 2018) and found that very small differences in miR156 levels can have dramatic effects on SPL protein levels while having essentially no effect on *SPL* transcript levels. Consequently, it is possible that the relatively small differences in miR156 levels observed in this study could be biologically significant. Unfortunately, it is extremely difficult to measure SPL protein levels in a wide range of accessions because antibodies to *SPL* proteins do not exist. We have tried and failed to produce such antibodies multiple times. The only way to measure *SPL* protein levels is with reporter proteins and it was impractical to introduce such reporter constructs by crossing (which is necessary to guard against position effects) into the many accessions we investigated in this study. We added a section on this issue to the discussion in this revised version (Line 558)

There has to be a better way to show relationship between the different markers other than putting them in numerical order in one graph, and showing they don't follow that numerical order in other graphs. Do both miRs have exactly the same targets?

Yes.

If you are proposing that VPC is influenced by something other than this miR156/SPL pathway, can you identify genes or processes in the QTL groups that are novel instead of looking for genes already known to be part of this process like the TOEs and SPLs?

We tried to do this, but there are thousands of genes under each QTL peak, and it is not obvious which of these genes is responsible for the trait. This is a common problem in QTL analysis. The only solution is to map the QTL with high precision, which we are in the process of doing.

Minor points: All done.
L32: has miR157 been examined in VPC of many different plant species? Maybe just write 156.

It has shown to regulate VPC in Arabidopsis (He et al., 2018).

L86: trees are not at the opposite end of the evolutionary spectrum of land plants from bryophytes.

Now line 85. Wording here was changed.

L110: precocious what? VPC or flowering?
L147: needs a verb
L282: production of abaxial…

Reviewer 2 Advance Summary and Potential Significance to Field:
This manuscript reports on the analyses of 70 Arabidopsis accessions regarding their natural variation in VPC. As expected miR156 was among the loci this trait was attributable to, but the study also identified loci that indicate a miR156-independent regulation. This included the miR156 targets, the SPLs.
Importantly though not surprisingly, no relationship between the variation in flowering time and VPC was observed, indicating that both developmental transitions are controlled by separate mechanisms (though in part employing similar or the same candidates). Lastly, the authors discuss that dissociation of the traits might be associated to annual variation in temperature.
The manuscript is very well written and a pleasure to read and review. I do however have some comments, which largely deal with the interpretation of the results, and textual changes summarized at the end.

Reviewer 2 Comments for the Author:
Major comments
1. Recording flowering time: Why was floral anthesis (Days to first open flower) instead of the more commonly used parameters ‘days to bolting’ or ‘total leaf number’ (rosette leaf number plus cauline leaf number)? I suggest replacing the term flowering (time) with floral anthesis and adjust the discussion.

We have used days to the first open flower in previous papers because it is self-explanatory and describes exactly what we observed. “Anthesis” is not a correct substitute for “days to first open flower” because every flower undergoes anthesis (this term refers to the opening of a flower bud), not to the opening of the first flower bud on the shoot. In other words, the correctly substitute for “days to the first open flower” is “days to the first anthesis”, which is one word shorter but introduces the problem that many, if not most, *Arabidopsis* molecular geneticists have no idea what anthesis is. Our reason for using flowering time to measure flowering time instead of using leaf number to measure flowering time is described above.

2. Appearance of traits (angle, trichomes, floral anthesis), abundance of mature miR156, expression of pre-miR156A and C, and three target genes (SPL3, SPL9, SPL15) do not correlate in a group of early accessions. However, none of the other potential target SPLs were quantified. This leaves the reader wondering whether any of the others might correlate better with the abundance of miR156 or the appearance of traits. Did the authors assess the variance of cleavage/target site sequences of the SPLs? This might explain, why they do not observe the expected correlation between miRNA abundance and target expression.

We measured three additional *SPL*s, to now include at least two from each clade (*SPL5, SPL10, SPL11*). Although *SPL13* is a key regulator of VPC, we did not include this gene in our analysis because Col-0 contains a duplication of this gene, which is not present in other accessions. Similar results were found (i.e. some were elevated in accord with the phenotype of these accessions, but there was not a consistent pattern). All target site sequences are identical in these accessions. With the exception of *SPL2*, which is highly polymorphic in these accessions, the coding sequence of *SPL* genes is quite conserved in the accessions we examined. The high degree of polymorphism in *SPL2* suggests that this gene is not under strong selection and may not be very important for the regulation of vegetative phase change. Consequently, we did not include it in our analyses.

3. The study suggests that vernalization responsive floral induction is regulated independently of VPC. This assumption is based on findings in Sha, which when non-vernalized, still initiates VPC earlier than Col-0 and remains in the adult phase until vernalized. Can this be generalized? I am missing a discussion of this essential result.

This conclusion is also supported by our analysis (Willmann and Poethig, 2011) of vegetative phase change in a vernalization-dependent genotype (*FRI FLC*) and a vernalization independent genotype (*FRI flc-3*) of Col-0. We are currently finalizing a detailed study of the relationship between VPC and the acquisition of reproductive competence in the Columbia ecotype of Arabidopsis. Consistent with the results presented in this paper, variation in the expression level of the genes most directly involved in vegetative phase change, specifically miR156/miR157 and their direct targets the *SPL* genes, has almost no effect on responsiveness of plants to a LD floral inductive signal. In contrast, variation in the expression of miR172 and its direct targets the *TOE* genes, has a very significant effect on the competence to respond to a LD stimulus. Our results indicate that in Arabidopsis reproductive competence (i.e. the ability to respond to a positive inductive signal) is regulated primarily by the expression level of *miR172*, not the miR156/SPL pathway.

4. The authors explain the differences between Col-0 and Sha with an accelerated decline in the levels of MIR156A and MIR156C transcript. Making such a statement would require measurements in a time or leaf series, which the authors do not have. The differential expression between the samples L1&2 and L5&6 is similar for Col-0 and Sha, although the levels are generally reduced in Sha when compared to Col-0.

In fact, as this reviewer notes, we did present a time series for the expression of miR156 in the original version of the paper, although it only involved three leaves (note that we consider variation in the amount of miR156 in different leaves a time series because these leaves are produced at different times). However, it is true that these data make it difficult to decide if the decline in miR156 is accelerated in Sha relative to Col-0 because Sha already had lower levels of miR156 by the time we sampled leaves 1 and 2. We concluded that the rate of decline for miR156 is greater in Sha than in Col-0 because Sha has lower levels of miR156 in leaves 1 and 2 than Col-0, but has essentially the same non-zero level of miR156 in leaves 5&6. This result implies that Sha reached this non-zero level faster than Col-0. But, given that we did not do a high resolution analysis of the expression pattern of miR156 in these accessions and the fact that this issue is not central to the major conclusions of this paper, we have revised the wording to indicate that our data do not allow us to resolve the question of whether Sha has overall less miR156, or has a faster rate of decline. (Line 392)

5. Both Sha and Col-0 have similar miR156 levels in L5&6. However, transcript levels are close to 0. How reliable are assumptions drawn from this data?

As we showed earlier, (He et al, 2018) the amount of miR156 in leaves 5&6 of Col-0 is significantly lower than the amount in leaves 1&2, but is not zero because it continues to decline up until at least leaf 15, which has about 1/3 of the amount of miR156 present in leaf 5. Given that we can reliably detect small differences in miR156 levels using RT-qPCR, and the observation that these very small differences are biologically significant—as demonstrated by the phenotype of miR156 mutants that have small differences in miR156 levels, as well as other experiments described in (He et al., 2018)—we think the conclusions are likely significant. Additionally, it should be emphasized that Sha transitions to the adult phase by leaf 4 (compared to leaf 6 in Col-0), suggesting that the difference in miR156 between Sha and Col-0 is biologically significant. But, as noted above, we have decided not to press this point in this revised version. (Line 390)

6. What does lower sensitivity mean (line 350)? What senses miR156? Its output is the cleavage of its targets. This has to be rephrased and/or properly discussed.

This is an excellent point, and we have rephrased this section. (Line 395-397)

7. The initial focus on leaf angle lets the reader assume that “leaf angle” as the more robust trait associated to VPC will be one of the main outcome of this manuscript, however, the authors then decide to focus on QTLs associated to trichome production instead of leaf angle later on. Why was

this decision made? Similar QTLs identified in both LD and SD suggest that trichome production is the more robust trait correlating with VPC.
We present QTL results for both abaxial trichomes and for leaf angle, However, it is the case that the QTL data for abaxial trichome production was less variable and the QTLs were more robust than for other traits. There is more variation within RIL genotypes for LA than for leaves lacking abaxial trichomes, making QTL mapping less statistically powerful. As a result we have more confidence in the QTL results for abaxial trichome production.

I wonder how a LD/SD trichome PCA would look like (not included in Figure S1).

These traits are highly correlated. Regression graphs showing this relationship are now added to Figure S1.

8. I don't understand how sensitivity to photoperiod or light quantity was linked to the data described in the paragraph above line 406/7.

Rephrased this section. (Now lines 429)

9. Why were only nonsynonymous SNPs taken into account (paragraph line 418)? Synonymous SNPs might have led to causal mutations e.g. when affecting a miR156 recognition sites in the SPLs.

We did look at all SNPs (including intergenic) not just nonsynonymous ones. This is indicated in the revised manuscript. (Now line 468)

10. Further, the authors argue that the decreased expression of TOE1 might explain the early Sha VPC. However, they also write that TOE1 "promotes" earliness (line 460), which is not correct and definitely does not fit in the argument.

Thanks for catching this. We have rephrased this part. (Now line 495)

11. The authors find that lower pri-miR156A correlates with earliness phenotype of Sha. However, they argued in a previous paragraph that pri-miR156 expression does not correlate with the earlier phenotypes of a set of accessions. This leaves the reader puzzled and should be properly discussed.

In the earlier sections of the text, we state that miR156 likely has a role in Sha but not the other Central Asian accessions. We edited to further emphasize this (Lines 345-348). The point is that miR156A was a viable option for regulation of phase change in Sha (and Leb-3, Kly-4) from the beginning, which is one of the reasons we chose to conduct a QTL analysis of this accession.

12. The manuscript lacks biological data demonstrating that both miR156A and TOE1 are responsible for the earlier VPC of Sha. Such data would have supported the story.

We are conducting a detailed molecular and genetic analysis of this region to assess the roles of these genes in the early phase change phenotype of Sha. The results of this study are years away, and we believe that they are outside the scope of this study.

12. The supplementary file Table S4 contains information on the peaks found on chromosome 2 and 5, but not on 1. The region covers supposedly covers FT (At1g65480, line 402), but also TPS1 (At1g78580), which has been associated with sugar-dependent miR156 and a partially miR156-independent regulation of SPLs. A potential link should be discussed.

The QTL on the top of chromosome 1 that contains FT and TPS1 accounts for <3% of the variance of abaxial trichome development and has no relationship to leaf shape. This makes it highly unlikely that it plays a role in vegetative phase change in Sha. We have now added it as a candidate for the flowering time QTL. (Line 447)

13. The authors argue that since over-expressing miR156 in Col-0 has only a minor effect on flowering, VPC to the adult vegetative stage is unlikely to be essential for plants to undergo floral transition. While this might be true, the statement is very strong and lacks sufficient support. The

mild flowering phenotype (which is laboratory and therefore likely growth condition-dependent) might also suggest that other pathways bypass miR156-dependent signals (likely in part converging on the SPLs) ensuring progression to a shortened adult phase and a timely floral transition. That this might at least be possible is nicely demonstrated by the data in this manuscript.

There are many examples of species that flower in the juvenile phase including at least 70 species of *Acacia*, several species of *Eucalyptus*, *Juniperus communis* and others. Furthermore, we have shown that in *Acacia*, juvenilized species that express constitutive high levels of miR156 nevertheless flower at about the same time as species that have low levels of miR156. The widely held assumption that reproductive competence is tightly linked to the juvenile-to-adult vegetative transition under natural conditions has minimal experimental support (one species) and is not supported by our results. We appreciate that Coupland's lab has found that *SPL15* is important for flowering under non-inductive conditions (although it is not essential under our conditions), (Hyun et al., 2016) but this is only means that *SPL15* is a fail-safe mechanism—when all else fails, call in the *SPL* genes. Even the Coupland lab acknowledges that the miR156/SPL pathway plays little to no role in flowering in Arabidopsis under floral inductive conditions because plants that constitutively over-expression miR156 flower only a few days later than wild type (Fornara and Coupland, 2009; Hyun et al., 2017) . This is important because the concept of a developmental clock for flowering implies that developmental age controls flowering under conditions that are otherwise conducive to flowering, not under conditions that are not conducive to flowering. This is how the vernalization requirement is defined: under conditions that are otherwise conducive to flowering (LD, 22^{o}C etc) plants that possess functional alleles of FRI and FLC will flower late unless they have been exposed to prolonged cold.

Minor comments: All have been corrected.
Line 92: Typo, replace "it" with "its"
Line 292: Typo, remove one "in"
Figure 2: In the first part of the study the angle of leaf four was measured, but the leaf is not among the imprints shown in Fig. 2D.
Typo Figures 1 / 2: compare "Stepn" and "Steph"
Figures 2/3: data points (transparent grey circles) vanish on the background of black boxes. Consider arranging non-transparent circles to the front of the boxes. Some of the circles are elliptic, e.g. Figure 3D.
Figure 4: align "Leaf #" with numbers in 4B (adjust font size), tick missing on graph of 4D or remove "150"
Line 308: word missing in sentence
Line 334: Figure 8 appears prematurely, (now Figure 10) Moved data to Figure 6.
Line 341: wrong figure reference
Line 359: replace "6A.C" with "with "6A-C"
Line 394: delete space between "Col-" and "0".
Line 404: replace "vrn1" with "VRN1"
Line 452: word missing or rephrase
Line 487: word missing
Line 505: replace "these" with "that"

Reviewer 3 Advance Summary and Potential Significance to Field:
This manuscript studies vegetative phase change and flowering time in a collection of 70 accessions of Arabidopsis thaliana. The authors describe variation in vegetative phase change by scoring abaxial trichomes and leaf angle in long days and short days, and flowering time. Vegetative phase change traits vary independently among the accessions and no relationship to flowering is detected suggesting that the traits are regulated at least partially by different mechanisms. They then focus on 9 accessions that transitioned to adult phase early in development. Among these 9 accessions the levels of miR156 RNA or SPL gene mRNA did not explain the variation in phenotype among all accessions suggesting vegetative phase change is controlled by unknown genetic loci. They then focus on the Sha accession, which shows an extreme early transition to adult phase. By analyzing previously described RILs of a cross between Sha and Col they identify QTLs for vegetative phase change based on abaxial trichomes and other traits, and for flowering time. They conclude that most QTL controlling vegetative phase change do not correspond to known genes affecting these traits, although one QTL on chromosome 2 correlates with TOE1 and MIR156A, and the RNAs encoded by these genes were reduced in abundance in Sha, suggesting that they are "promising

candidates" for this QTL.
This manuscript describes a very large amount of phenotypic and genetic analysis that demonstrates the complexity of natural genetic variation in vegetative phase change and its relationship, if any, to flowering. The work also identifies MIR156A and TOE1, two known genes in the process, as possibly contributing to phenotypic variation in the Sha accession. This work will likely form the basis of many more detailed future studies. Some of the conclusions and some links to previous work could perhaps be explained in more detail, and some essentially negative results were explained in great detail.

Reviewer 3 Comments for the Author:

1.One conclusion of this work is that among natural accessions of Arabidopsis there is little relationship between flowering and vegetative phase change. More than 50% of the natural variation in flowering time has been described by several authors to be conferred by variations in the activity of the FLC/FRI system. The same lab did publish previously in Development (Willmann and Poethig, 2011) an analysis of the role of FLC in regulating vegetative phase change and found that FLC affects leaf shape and distribution of abaxial trichomes independently of its effect on flowering. This paper is not cited in the current paper, and I missed a full discussion of the relevance of the previous results for the current conclusions.

Because the majority of the experiments in this paper were performed with vernalized plants, we don't expect FRI/FLC to have a large effect on the phenotypes we observed. We now mention this in the discission. (Lines 581-583).

2.The conclusion of several experiments is that there is no correlation between different traits scored in these accessions. However, the data are complex with extreme quantitative variation across up to 70 accessions. I missed any formal statistical analysis of whether there are correlations among traits, and therefore an objective statistical demonstration that there is no correlation. For example, line 265 "no correlation between the angle of the leaf base and the first leaf with abaxial trichomes" and then the citation is the figure. So, by visual inspection of the Figure the reader should decide whether there is a correlation or not. Such conclusions should be formally drawn from statistical analyses and objective criteria, and this follows for others such as the relationship to flowering time.

We have now included regression analyses in the Figs. 1 and 2 to support our conclusions.

3.The expression analyses of SPL genes and miR156 is based on RNA levels. However, is this enough to detect a correlation with phenotype because miR156 is also likely to affect the translation of SPL mRNAs? This issue becomes a problem for example on lines 309 to 311: "There was no clear relationship between the abundance of these transcripts and the expression of miR156 or their phase change phenotype". If miR156 is mainly affecting the protein abundance of SPLs could a "clear relationship" be hidden?

This is an important point, and we completely agree with this reviewer. Our results (He et al., 2018) demonstrate that SPL genes are regulated primarily at a translational level, and that small differences in miR156 expression do not lead to major changes in SPL transcripts, but can have major effects on SPL proteins. We now address this issue in the discussion. (Lines 558-565)

4. I found pages 15-21 very long as they explore possible candidate genes for QTLs and most of the conclusions are negative, perhaps most of these results could be summarized more briefly.

We have shortened these sections a bit.

5. Sha accession has been used for QTL mapping of flowering time loci before, although in different crosses. For example, Salomé et al (2011) Genetics, and El-Lithy et al (2004) Plant Physiol. Perhaps these results should be discussed and integrated with the current results, especially as all analyses seem to detect loci on the bottom of chromosome 5

These papers and a few others were added to the discussion of flowering time analysis in Sha (Lines 450-452).

6.Several previous papers (for example Wang et al 2009 Cell; Hyun et al, 2016 Developmental Cell) argued that the miR156/SPL system is much more important in controlling flowering in non-inductive SDs than in inductive LDs. It was not completely clear how much this conditional effect was considered in examining possible correlations between flowering time and vegetative phase change because different environmental conditions were used in different experiments.

We only examined flowering time under inductive LD conditions because photoperiod has minimal effect on the timing of VPC compared to its effect on flowering (Xu et al., 2016).

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Willmann, M. R. and Poethig, R. S. (2011). The effect of the floral repressor FLC on the timing and progression of vegetative phase change in Arabidopsis. *Development* **138**, 677–685.

Wu, G. and Poethig, R. S. (2006). Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. *Development* **133**, 3539–3547.

Xu, M., Hu, T., Zhao, J., Park, M.-Y., Earley, K. W., Wu, G., Yang, L. and Poethig, R. S. (2016). Developmental Functions of miR156-Regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) Genes in Arabidopsis thaliana. *PLOS Genetics* **12**, e1006263.

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Xu, M., Hu, T. and Poethig, R. S. (2021). Low light intensity delays vegetative phase change. *Plant Physiology* **00**, 12.

Second decision letter

MS ID#: DEVELOP/2021/200321

MS TITLE: The genetic basis of natural variation in the timing of vegetative phase change in Arabidopsis thaliana

AUTHORS: Erin E Doody, Yuqi Zha, Jia He, and Scott Poethig

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

Doody and coworkers have taken advantage of a large collection of accessions of Arabidopsis from diverse climates to address the question of whether traits associated with vegetative phase change, some of which might be adaptive reflect variation in expression of the miR156/SPL cassette. The traits they monitored were leaf shape and first appearance of abaxial trichomes. The latter has been used reliably in the Poethig lab, where this work was done, for decades in their screens for the mutants that have provided the key that unlocked our understanding of VPC. As vegetative growth progresses, each new leaf is less round and more oval than those that preceded it, in a pattern suggesting that shape, a qualitative trait, is also phase-specific. They compared the appearance of those traits with expression of miR156 and its targets, in both the panel of wild accessions and in a RIL generated from a cross between Col-O and Shakdara an accession with both early abaxial trichomes and early oval blades. They found that there was no tight correlation among timing of the morphological traits, gene expression, or onset of flowering, concluding that VPC is under multiple levels of regulation.

Comments for the author

I find the revised ms. much improved and look forward to seeing it published in Development.

Reviewer 2

Advance summary and potential significance to field

The manuscript has been improved much.
I however still have a few concerns that largely deal with the way flowering time analyses were performed and interpreted. As I see that this was also criticized by another reviewer and in my opinion was not sufficiently addressed by the authors, I believe it is of importance to be mentioned here again.

Comments for the author

Major concerns
I really don't understand the argument for using 'days to first (open) flower' instead of using the commonly used 'rosette leaf numbers' or 'days to bolting'.
That 'rosette leaf numbers' and 'days to bolting' do correlate well with 'days to first (open) flower' is nicely shown in Figure S2. Small differences in the leaf initiation rate of the accessions do not

seem to be an issue here. So why reinventing the wheel and not stick to what is commonly used in the field to help the reader to independently evaluate the results?
The authors explain the observed differences regarding the correlation between the appearance of trichomes and “flowering time” in LD versus SD by the photoperiod having an influence on phase-specific traits and AP2-like transcription factors regulating both trichome production and flowering time but not necessarily VPC.
First, the comparisons made are ‘LD flowering’ with ‘LD trichomes’ (correlates), and ‘LD flowering’ with ‘SD trichomes’ (does not correlate!), but no comparison of ‘SD flowering’ with ‘SD trichomes’ is provided. It cannot be excluded that the latter would significantly correlate as well.
The authors should here pay attention to the fact that the photoperiod pathway of the flowering network only operates in LD and not in SD. Importantly, SD conditions are also conductive conditions, as Arabidopsis is a facultative LD plants – meaning it also induces flowering in SD, just not via the photoperiod pathway. The beauty of this fact is that the impact of other pathways overridden in LD (e.g. the age pathway) can be accessed in SD. I am entirely missing this fact in the interpretation of the data.
In Sha flowering and VPC are independently regulated. However, I am still not convinced that an Arabidopsis plant would be able to flower without passing through VPC first. Therefore, the arguments developed from this result are way too strong and should at least be softened.
In addition, the differential regulation of Sha flowering time might be explained by the differences observed in the expression of SPL15 (and SPL9) and presumably a different way to respond to/sense/read out miR156 levels. However, I would have loved to see expression values of the other SPLs, especially SPL13, which has the most prominent impact on VPC.
This leads me to a very general observation, that though the authors provide a complete list of SPL transcripts they always (?) measure (SPL3, SPL5, SPL9, SPL10, SPL11, SPL15 - see line 352), they rarely present the results of this list. This is a pity, as I would have loved to specifically see the expression patterns of SPL13 (only presented in Figure 11A, not part of the list), as this is presumably the one most associated to the regulation of VPC.
Lastly, Figure S1A illustrates how leaf base angle was measured. Can the authors please explain why the lines generating the angle depicted in the leaf 7 (adult) are aligned with the second serration instead of with the first like it was done for leaf 4 (juvenile)? If this is how the data were obtained, the angles would have been forced to be smaller for adult leaves… Wouldn't make any sense to me. But maybe I'm missing a point here?

Minor concerns
Title of Figure S1 indicates a correlation of leaf base angle with abaxial trichome production. This is however not shown on the panel.
Line 65 “gemination” to “germination”
Lines 447/8 TPS1 encodes an enzyme involved in sugar metabolism producing T6P (references missing), a signal which impacts both flowering (references missing) and VPC (Ponnu et al., 2020)
Line 529 repetition

Second revision

Author response to reviewers' comments

We are grateful to the reviewers for their consideration of this manuscript. We have addressed their concerns and have made some changes that are indicated below and highlighted in the manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript has been improved much.
I however still have a few concerns that largely deal with the way flowering time analyses were performed and interpreted. As I see that this was also criticized by another reviewer and in my opinion was not sufficiently addressed by the authors, I believe it is of importance to be mentioned here again.

Major concerns:

1. I really don't understand the argument for using 'days to first (open) flower' instead of using the commonly used 'rosette leaf numbers' or 'days to bolting'.
That 'rosette leaf numbers' and 'days to bolting' do correlate well with 'days to first (open) flower' is nicely shown in Figure S2. Small differences in the leaf initiation rate of the accessions do not seem to be an issue here. So why reinventing the wheel and not stick to what is commonly used in the field to help the reader to independently evaluate the results?

It is unclear why this reviewer is so adamant about this issue. As we pointed out in our original response, leaf number is an indirect measure of flowering time, and relies on the unstated assumption that the genotypes being examined do not vary in their rate of leaf initiation. As we showed in the revised version of this manuscript, these ecotypes clearly vary in their rate of leaf initiation, with more than a six-leaf variation in leaf number between genotypes after 25 days. This becomes a much larger difference once these accessions flower (some more than 50 days later). Additionally, it has been previously shown that while leaf number and days to flower are often correlated, these two traits can be genetically de-coupled.

Salomé., et al. (2011). Genetic Architecture of Flowering-Time Variation in *Arabidopsis thaliana*, *Genetics*.

Most importantly, this reviewer fails to explain why leaf number is a better measure of flowering time than flowering time, other than this is the measure that people normally use. The reason people normally use leaf number is that it is easier to measure because you don't have to check your plants daily, not because it is a better measure. We are unwilling to use an indirect measure of flowering time simply because this measure is widely used, and would also like to point out some recent papers that have used days to first flower as a marker for flowering time.

Alonso-Blanco, C., et al. (2016). 1,135 genomes reveal the global pattern of polymorphism in *Arabidopsis thaliana*. *Cell* **166**, 481–491.

Atwell, S., Huang, Y., Vilhjálmsson, B. et al. Genome-wide association study of 107 phenotypes in Arabidopsis thaliana inbred lines. Nature 465, 627–631 (2010).

Additionally, we direct the editor to the following papers, which show that genes involved in phase change (mir156, SPL genes) have a significant effect on the rate of leaf initiation, and that flowering time is very poorly correlated with leaf number in plants with varying levels of SPL activity. For example, many *spl* genotypes flower at exactly the same time as wild type, but with significantly more rosette leaves because they have a more rapid rate of leaf initiation. This is relevant because we are specifically interested in natural variation in the timing of vegetative phase change, which is likely controlled by SPL genes.

Wang et al. (2008) Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in Arabidopsis thaliana. Plant Cel 20:1231-1243.

Xu et al. (2016) Developmental functions of miR156-regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes in Arabidopsis thaliana. PLoS Genet. 12(8):e1006263

2. The authors explain the observed differences regarding the correlation between the appearance of trichomes and "flowering time" in LD versus SD by the photoperiod having an influence on phase-specific traits and AP2-like transcription factors regulating both trichome production and flowering time but not necessarily VPC.
First, the comparisons made are 'LD flowering' with 'LD trichomes' (correlates), and 'LD flowering' with 'SD trichomes' (does not correlate!), but no comparison of 'SD flowering' with 'SD trichomes' is provided. It cannot be excluded that the latter would significantly correlate as well. The authors should here pay attention to the fact that the photoperiod pathway of the flowering network only operates in LD and not in SD. Importantly, SD conditions are also conductive conditions, as Arabidopsis is a facultative LD plants – meaning it also induces flowering in SD, just not via the photoperiod pathway. The beauty of this fact is that the impact of other pathways overridden in LD

(e.g. the age pathway) can be accessed in SD. I am entirely missing this fact in the interpretation of the data.

We previously showed (Wilmann and Poethig, 2011; Xu et al.. 2016) that under short day conditions abaxial trichome production is delayed by about 1-2 leaves (equivalent to about 2 days), while flowering time is delayed by greater than 30 days. The very large discrepancy between these numbers supports our conclusion that there is little or no correlation between VPC and flowering time. We now mention this supporting information in the discussion (Line 601-603).

We are unclear what point this reviewer is trying to make in noting that the photoperiod pathway only operates in LD. We are of course aware of this fact, but its relevance to this study is not obvious. For example, it is unclear why this reviewer thinks that "SD conditions also induce flowering in SD, just not via the photoperiod pathway". What is the evidence that SD have an inductive effect on flowering, and what relevance does this have to our study? Coupland's lab showed that SPL15 promotes flowering in SD, and GA and the TOE genes also play a role in flowering in SD, but these signals are not dependent on SD, because they also affect flowering in LD.

We do acknowledge that we do not have flowering time data from SD because the majority of these accessions require many months to flower in these conditions. We do, however, believe that because appearance of abaxial trichomes is highly correlated between SD and LD, it is reasonable to compare these traits.

3. In Sha flowering and VPC are independently regulated. However, I am still not convinced that an Arabidopsis plant would be able to flower without passing through VPC first. Therefore, the arguments developed from this result are way too strong and should at least be softened.

This reviewer does not offer any arguments for why he/she is not convinced that an Arabidopsis plant can flower without passing through vegetative phase change, so we cannot respond to this criticism. As we noted in the revised version of the manuscript, the requirement for VPC may vary between species, and we believe this statement addresses this reviewer's concern.

4. In addition, the differential regulation of Sha flowering time might be explained by the differences observed in the expression of SPL15 (and SPL9) and presumably a different way to respond to/sense/read out miR156 levels. However, I would have loved to see expression values of the other SPLs, especially SPL13, which has the most prominent impact on VPC. This leads me to a very general observation, that though the authors provide a complete list of SPL transcripts they always (?) measure (SPL3, SPL5, SPL9, SPL10, SPL11, SPL15 - see line 352), they rarely present the results of this list. This is a pity, as I would have loved to specifically see the expression patterns of SPL13 (only presented in Figure 11A, not part of the list), as this is presumably the one most associated to the regulation of VPC.

We examined the expression level of all the SPL genes that have been shown, or proposed, to have an effect on flowering except for SPL2 and SPL13. We described why we excluded these two genes in the our last response to reviewers, but the reviewer seems to have missed this. As we pointed out, we did not include SPL13 expression because this locus is duplicated in Col-0, but not in other ecotypes (Xu et al., 2016). We did examine SPL13 expression, and predictably the Central Asian accessions had roughly 50% abundance compared to Col-0. Consequently—in the absence of any additional factors--the amount of SPL13 transcript is not contributing to timing of vegetative phase change in these accessions, and we did not include this data in the manuscript because it seemed irrelevant to compare expression of SPL13 between these accessions. We did not examine SPL2 because it is highly polymorphic in these accessions and therefore unlikely to play a significant role in vegetative phase change. We have added this explanation to the manuscript to prevent further confusion (Lines 354-360).

5. Lastly, Figure S1A illustrates how leaf base angle was measured. Can the authors please explain why the lines generating the angle depicted in the leaf 7 (adult) are aligned with the second serration instead of with the first like it was done for leaf 4 (juvenile)? If this is how the data were obtained, the angles would have been forced to be smaller for adult leaves… Wouldn't make any sense to me. But maybe I'm missing a point here?

Measuring the base of the lamina to incorporate serrations does not depict the actual shape of the leaf margin, because it would artificially force leaf angles wider in accessions with more serrations than others. As a result, we try more accurately to measure the actual boundary of the lamina in our measurements by excluding the contributions of serrations to base angle.

Minor concerns

Title of Figure S1 indicates a correlation of leaf base angle with abaxial trichome production. This is however not shown on the panel. We have changed the title of this figure.

Line 65 “gemination” to “germination” Fixed.

Lines 447/8 TPS1 encodes an enzyme involved in sugar metabolism producing T6P (references missing), a signal which impacts both flowering (references missing) and VPC (Ponnu et al., 2020) We have added some additional references that cite TPS1 as a candidate but maintain the argument that the QTL on the bottom of Chr1 is not strongly associated with vegetative traits in Sha, but is associated with flowering time (Line 456-459).

Line 529 repetition: Fixed.

Third decision letter

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MS TITLE: The genetic basis of natural variation in the timing of vegetative phase change in Arabidopsis thaliana

AUTHORS: Erin E Doody, Yuqi Zha, Jia He, and Scott Poethig
ARTICLE TYPE: Research Article

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