CHIQUITA1 maintains temporal transition between proliferation and differentiation in *Arabidopsis thaliana*

Flavia Bossi, Benjamin Jin, Elena Lazarus, Heather Cartwright, Yanniv Dorone and Sue Y. Rhee

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Original submission

First decision letter

MS ID#: DEVELOP/2022/200565

MS TITLE: CHIQUITA1 maintains temporal transition between proliferation and differentiation in Arabidopsis thaliana

AUTHORS: Flavia Bossi, Benjamin Jin, Elena Lazarus, Heather Cartwright, Yanniv Dorone, and Sue Y. Rhee

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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

Previously the authors identified CHIQUITA transcription factor from Arabidopsis as that interacts with paralogs and PRC2. Recently another research group found that the same gene family is involved in suppression of autophagy that is triggered by a response to drought stress. Here in this

study the authors found that the stunted statue of the chiq1 and chiq1,4,5,6 mutant is due to early cessation of cell cycling. Not only the mitotic cycle but also endocycle are decreased in the mutant. Interestingly, mutants show the “ephemerally” larger leaves that could be caused by early entry to cell expansion. Defect in the endocycle in the chiq mutant is apparent and significant, while some past, similar reports overestimate defects in the endoreduplication. The authors also measured the cell cycle length in mutants and wt from tracking imaging, and showed that tee cell cycle length is not affected in the mutant indicating that CHIQ affect cell population size for mitosis and endocycling.

Comments for the author

The story shown here is a little bit too simple, but data is clear and concise. I have some minor comments on this manuscript as shown below.
1) Introduction. “Differences in organ size among species are largely controlled by cell number rather than cell size (7, 8)”. This is an overstatement. If you compare size of petal cells among species of Brassicaceae, you will soon find that both, cell size and numbers are different.
2) Results “CHIQ1 encodes a protein of unknown function”. Is this correct? The authors previously revealed some molecular functions; Bao et al. 2020 also.
3) Results “siliques”. This term is recently not widely used, because this term is too specialized term for a particular type of fruits. Here we do not deal with any morphological characters of fruit structure, thus simply “fruits” is appropriate term.
4) Results “pavement cells display the typical jigsaw puzzle shape in mature leaves, which suggests that terminal differentiation is not compromised in chiq1-1.” Here the authors showed that chiq1 mutant is severely defective in endoreduplication. How about the trichome morphology? Similarly defective in the endocycling also in trichomes? If so how many branches?
5) Results “chiq-quad, which lacks CHIQ1, CHIQL4, CHIQL5 and CHIQL6,” why the authors chose these specific four members from paralogs?
6) Discussion “we show that CHIQ1 maintains the proliferative capacity of cells and delays the timing of cell cycle exit during organ development by combining cell population”: Here “delay” is not appropriate term, because this sentence describes the role of CHIQ1 in a normal condition. Keeps the timing?

Reviewer 2

Advance summary and potential significance to field

This paper shows that a group of plant-specific, novel transcriptional regulators are important for maintaining the division potential of cells during organ development. The function of CHIQ1 was most prominently examined in this manuscript and the authors nicely show that the mature organ phenotype of reduced size is due to cells exiting the proliferative phase early resulting in fewer smaller cells and, therefore, smaller organs.
These studies were done over time during organ development and, interestingly, show that initially chiq1 organs are the same size or larger than wild type organs. However, at maturity the chiq1 organs are smaller.
The authors eliminate longer cell cycle duration as the driver of the reduced cell proliferation in chiq1 and instead, find that cell proliferation rate at the organ level is reduced because fewer cells are dividing in chiq1.
Additionally, CHIQ1 interacts with other CHIQ-like proteins and the authors find that CHIQ4, 5, and 6 also function to modulate how long cells proliferate prior to beginning differentiation.
Understanding how organisms and organs modulate size is a fundamental biological question that also has important ramifications in crop improvement.

Comments for the author

Thank you so much for submitting such a high quality, clearly written, and carefully edited manuscript for review! It was straightforward to read with interesting results that are nicely placed into a broader context. Congratulations on a very nice paper!
Primary concern:

- My main quibble with the authors is their use of the term "Fold change" in their graphs when showing percent change relative to wild type. I believe that using Fold change is inaccurate and just plain confusing. For example, in figure 1 A, the y-axis is labeled primary root length (fold change). To me, fold change is used as a measure of how much something changes between one measurement and another, therefore just looking at the graph this implies that the roots of chiq1 are actually shrinking over time. Of course, this is not the case and it is just that they are smaller relative to WT. In the legend the authors do state that Figure 1A is "Scatter plot of normalized primary root length against the wild type..." so that helps clarify, but still can leave readers with questions about what is being shown. I very very strongly encourage the authors to simply put "relative primary root length" or "primary root length relative to WT" as the y-axis label. I believe that this will just be more straightforward for readers and is a more accurate reflection of the data shown. I strongly believe that this should be done throughout the manuscript wherever fold change is used.

Minor comments:
- I found the leaf+GUS images in Figure 4 to be of quite low quality as they are somewhat blurry. Perhaps this is an artifact of the image quality in the manuscript for review, but if the authors have higher quality images I suggest adding them. That said the point the authors are trying to make is generally visible, even if the images appear a bit blurry.
- There is a repetitive bit in the methods that could be made more concise. I suggest unifying these two paragraphs into one as shown below.
"To construct a binary vector that expresses CHIQ1's wild type allele, 642 bp of the promoter region (including the 5' UTR) plus the coding region of AT2G45260 lacking the stop codon was amplified by PCR from Col-0 genomic DNA, cloned into the entry vector pDONR221 (Life Technologies), and transferred to the binary vector pGWB640 (41) using Gateway cloning (Life Technologies) to create the vector CHIQ1pro:CHIQ1-YFP.
To construct a binary vector that overexpresses CHIQ1's wild type allele, the AT2G45260 (CHIQ1) protein-coding sequence was amplified by PCR from Col-0 genomic DNA, cloned into the entry vector pDONR221 (Life Technologies), and transferred to the binary vector pB7HFC3_0 (42), using Gateway cloning (Life Technologies) to create the vector 35Spro:CHIQ1-FLAG."
Revise to: To construct a binary vector that expresses CHIQ1's wild type allele, 642 bp of the promoter region (including the 5' UTR), plus the coding region of AT2G45260 lacking the stop codon was amplified by PCR from Col-0 genomic DNA, cloned into the entry vector pDONR221 (Life Technologies). Then using Gateway cloning (Life Technologies), this fragment was then transferred to the binary vector pGWB640 (41) to create the vector CHIQ1pro:CHIQ1-YFP and to the binary vector pB7HFC3_0 (42) to create the vector 35Spro:CHIQ1-FLAG.
- I found a just single typo in the last paragraph of the discussion change "studie" to "studies". :)

Reviewer 3

Advance summary and potential significance to field

This topic is of sufficient interest for researchers follow trends and important development in understanding of the temporal transitioning between proliferation and differentiation.

Comments for the author

The organ size is determined by the rate and duration of cell divisions and expansions, but how the timing of temporal transitioning between proliferation and differentiation still remains unclear. In this manuscript, the authors reported that CHIQUITA1 is a positive regulator of vegetative and reproductive organ size which plays an important role in regulating the timing of the transition from proliferation to differentiation. Most of my comments are relatively minor, and there are several areas that can be improved before acceptance for publication:
1, in Figure 2A and 2C, the root length and leaf blade area of chiq1-1 mutants are slightly bigger than that of wild-type plants, which are significantly different from the observations of mature organs or aged plants. How to explain it?
2, in the Abstract, the authors mentioned that "CHIQ1 belongs to a plant-specific gene family of unknown molecular function and physically and genetically interacts with three close members of its family…". The authors should provide the experimental evidences.

3, serval genes have been shown to be involved in determining the size of organs (or cell proliferation and endoreduplication) in Arabidopsis, does CHIQUITA1 affect these gene expression?

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author...

1) Introduction. “Differences in organ size among species are largely controlled by cell number rather than cell size (7, 8)”. This is an overstatement. If you compare size of petal cells among species of Brassicaceae, you will soon find that both, cell size and numbers are different.

The statement was rephrased as “The final number of cells in an organ plays an important role in determining final organ size” (page 2).

2) Results “CHIQ1 encodes a protein of unknown function”. Is this correct? The authors previously revealed some molecular functions; Bao et al. 2020 also.

We still consider CHIQ1 and CHIQ proteins as proteins of unknown molecular function since the transcriptional regulator function proposed for CHIQ1 (Bossi et al 2017) have not been proven and Bao et al did not propose any molecular function for CHIQ1 (Bao et al 2020).
Our previous work suggested that CHIQ1 is a transcriptional regulator. However, we have not been able to prove if this is the case. We showed physical interaction between CHIQL6 with EMF2 (Bossi *et al.*, 2017), but we do not know if CHIQ proteins alter PRC2 function. Moreover, we were not able to detect transactivation activity *in planta* (Bossi *et al.*, 2017).
Bao et al indicated that CHIQ1 works in the autophagy pathway, localizes to autophagosomes and interacts with autophagy-related proteins, but failed to specify any particular molecular function (Bao *et al.*, 2020). Moreover, the authors indicated that CHIQ1’s autophagy-related role does not control plant growth (Bao and Bassham, 2020; Bao *et al.*, 2020). Therefore, CHIQ1’s molecular function during stress, growth, and development remains unknown.

To avoid misinterpretation, we explicitly mention that, currently, we have no evidence that supports or contradicts the hypothesis that CHIQ1 is a transcriptional regulator in pages 3 and 9.

3) Results “siliques”. This term is recently not widely used, because this term is too specialized term for a particular type of fruits. Here we do not deal with any morphological characters of fruit structure, thus simply “fruits” is appropriate term.

The word “silique/s” was replaced with “fruit/s” in the text, Figure 1 and its legend.

4) Results “pavement cells display the typical jigsaw puzzle shape in mature leaves, which suggests that terminal differentiation is not compromised in chiq1-1.” Here the authors showed that chiq1 mutant is severely defective in endoreduplication. How about the trichome morphology? Similarly defective in the endocycling also in trichomes? If so how many branches?

Trichome morphology, including the number and size of branches, was not affected in *chiq1-1* leaves. However, *chiq1-1* seventh leaf had fewer trichomes. This is consistent with the reduced number in the other epidermal cell types and further corroborates that *CHIQ1* does not alter the process of differentiation itself, but its timing.

The data on trichome morphology and number is shown in Figure S3 and briefly described on pages 4-5.

5) Results “chiq-quad, which lacks CHIQ1, CHIQL4, CHIQL5 and CHIQL6,” why the authors chose these specific four members from paralogs?

We chose CHIQL4, CHIQL5 and CHIQL6 based on the CHIQ's family phylogeny, the domain composition and length of CHIQ proteins, and proteomic work that identified CHIQ1 protein interactors. These results were described in (Bossi *et al.*, 2017).

Briefly, in (Bossi *et al.*, 2017) we showed that CHIQ1 co-immunoprecipitated with CHIQL4, CHIQL5 and CHIQL6 *in planta*. Further studies corroborated that CHIQL5 and CHIQL6 proteins interacted with CHIQ1 protein in yeast-two-hybrid studies, pull-downs in tobacco leaves, and bimolecular fluorescence complementation in Arabidopsis protoplasts (Bossi *et al.*, 2017).
Based on the CHIQ family phylogenetic tree and protein length and domain composition, we hypothesized that CHIQL4 and CHIQL5 might be redundant. Therefore, we generated the quadruple mutant *chiq1-1*, *chiql4*, *chiql5*, *chiql6*. Analysis on the quadruple mutant showed that removing four proteins from the CHIQ family further enhances the cell proliferation phenotype.

This clarification was added on page 7.

6) Discussion "we show that CHIQ1 maintains the proliferative capacity of cells and delays the timing of cell cycle exit during organ development by combining cell population": Here "delay" is not appropriate term, because this sentence describes the role of CHIQ1 in a normal condition. Keeps the timing?

We agree that in describing the role of CHIQ1 under normal conditions, the word "delay" is not appropriate. The word "delay" was replaced by "keep" on pages 6 and 7.

Reviewer 2 Comments for the Author...
Thank you so much for submitting such a high quality, clearly written, and carefully edited manuscript for review! It was straightforward to read with interesting results that are nicely placed into a broader context. Congratulations on a very nice paper!

Primary concern:
- My main quibble with the authors is their use of the term "Fold change" in their graphs when showing percent change relative to wild type. I believe that using Fold change is inaccurate and just plain confusing. For example, in figure 1 A, the y-axis is labeled primary root length (fold change). To me, fold change is used as a measure of how much something changes between one measurement and another, therefore just looking at the graph this implies that the roots of chiq1 are actually shrinking over time. Of course, this is not the case and it is just that they are smaller relative to WT. In the legend the authors do state that Figure 1A is "Scatter plot of normalized primary root length against the wild type..." so that helps clarify, but still can leave readers with questions about what is being shown. I very very strongly encourage the authors to simply put "relative primary root length" or "primary root length relative to WT" as the y-axis label. I believe that this will just be more straightforward for readers and is a more accurate reflection of the data shown. I strongly believe that this should be done throughout the manuscript wherever fold change is used.

Thank you for your feedback. We replaced the "fold change" y-axis labels with "[trait being measured] relative to Col-0" in Figures 2, 3, S1, S2 and S5.

Minor comments:
- I found the leaf+GUS images in Figure 4 to be of quite low quality as they are somewhat blurry. Perhaps this is an artifact of the image quality in the manuscript for review, but if the authors have higher quality images I suggest adding them. That said the point the authors are trying to make is generally visible, even if the images appear a bit blurry.

We apologize that the image quality was low in the manuscript for review. The images in Figure 4D were replaced by higher quality versions of the same images.

- There is a repetitive bit in the methods that could be made more concise. I suggest unifying these two paragraphs into one as shown below.
"To construct a binary vector that expresses CHIQ1's wild type allele, 642 bp of the promoter region

(including the 5' UTR) plus the coding region of AT2G45260 lacking the stop codon was amplified by PCR from Col-0 genomic DNA, cloned into the entry vector pDONR221 (Life Technologies), and transferred to the binary vector pGWB640 (41) using Gateway cloning (Life Technologies) to create the vector CHIQ1pro:CHIQ1-YFP.
To construct a binary vector that overexpresses CHIQ1's wild type allele, the AT2G45260 (CHIQ1) protein-coding sequence was amplified by PCR from Col-0 genomic DNA, cloned into the entry vector pDONR221 (Life Technologies), and transferred to the binary vector pB7HFC3_0 (42), using Gateway cloning (Life Technologies) to create the vector 35Spro:CHIQ1-FLAG."
Revise to: To construct a binary vector that expresses CHIQ1's wild type allele, 642 bp of the promoter region (including the 5' UTR), plus the coding region of AT2G45260 lacking the stop codon was amplified by PCR from Col-0 genomic DNA, cloned into the entry vector pDONR221 (Life Technologies). Then using Gateway cloning (Life Technologies), this fragment was then transferred to the binary vector pGWB640 (41) to create the vector CHIQ1pro:CHIQ1-YFP and to the binary vector pB7HFC3_0 (42) to create the vector 35Spro:CHIQ1-FLAG.

Thank you for the suggestion. However, CHIQ1pro:CHIQ1-YFP and 35Spro:CHIQ1-FLAG were created using different entry vectors and this information is lost in the revised version suggested by the reviewer.
To minimize repetition, we consolidated binary vector construction as follows (pages 9-10): "642 bp of CHIQ1 (AT2G45260)'s promoter region (including the 5' UTR) plus its coding region lacking the stop codon and the CHIQ1 protein-coding sequence were amplified by PCR from Col-0 genomic DNA and cloned into the entry vector pDONR221 (Life Technologies) to create pDONR221-CHIQ1pro:CHIQ1 and pDONR221-CHIQ1 vectors, respectively. pDONR221- CHIQ1pro:CHIQ1 was transferred to the binary vector pGWB640 (41) using Gateway cloning (Life Technologies) to create the vector CHIQ1pro:CHIQ1-YFP, which expresses the CHIQ1- YFP protein fusion under CHIQ1's endogenous promoter. pDONR221-CHIQ1 was transferred to the binary vector pB7HFC3_0 (42), using Gateway cloning (Life Technologies) to create the vector 35Spro:CHIQ1-FLAG, which expresses CHIQ1's wild type allele under the constitutive promoter 35S."

- I found a just single typo in the last paragraph of the discussion change "studie" to "studies". :)

The typo has been corrected on page 9.

Reviewer 3 Comments for the Author...
The organ size is determined by the rate and duration of cell divisions and expansions, but how the timing of temporal transitioning between proliferation and differentiation still remains unclear. In this manuscript, the authors reported that CHIQUITA1 is a positive regulator of vegetative and reproductive organ size, which plays an important role in regulating the timing of the transition from proliferation to differentiation. Most of my comments are relatively minor, and there are several areas that can be improved before acceptance for publication: 1, in Figure 2A and 2C, the root length and leaf blade area of chiq1-1 mutants are slightly bigger than that of wild-type plants, which are significantly different from the observations of mature organs or aged plants. How to explain it?

We think that the early transition from proliferation to differentiation is causing a temporary increase in organ size in *chiq1-1* as explained on page 5.

Our data supports this hypothesis as follows:
1- Fig. 4 and S5 shows that average cell size at days 6 and 8 is greater in *chiq1-1* than in wild type; described on page 5.
2- Fig S6 shows that *chiq1-1* leaves have a larger number of big cells than the wild type. For example, the cell size population from *chiq1-1* 8 day-old leaves had 15% of cells bigger than 201 um^2, 30% more than the value observed in wild type (11% of cells bigger than 201 um^2) (Fig. S6); described on page 5.
3- Fig S6 shows that young *chiq1-1* leaves have fewer small cells; described on page 5. 4- Fig. 4 shows that *chiq1-1* leaves exit proliferation prematurely; described on page 6.

In addition, the literature (Andriankaja et al., 2012; Jones et al., 2017) suggests that small cells might represent actively dividing cells implying that big cells are expanding cells.

Based on our data and the current literature, we infer that some cells start expanding earlier in *chiq1-1* than in wild type, leading temporarily, to bigger organs. As larger cells cover more area, a small increase in their number has a great impact on overall organ size.

2, in the Abstract, the authors mentioned that "CHIQ1 belongs to a plant-specific gene family of unknown molecular function and physically and genetically interacts with three close members of its family…". The authors should provide the experimental evidences.

CHIQ1 was first described in (Bossi *et al.*, 2017), where it was identified as an uncharacterized gene with orthologs only in plant species.

The experimental evidence for physical interaction among CHIQ proteins was previously reported (Bossi *et al.*, 2017). Therefore, we removed "physically" from the Abstract as these results are not described in this paper.

The experimental evidence for genetic interaction is presented in Fig. 5 and S8 and described on page 7. Here, we show that removing additional *chiq* genes enhances the cell proliferation phenotype indicating that CHIQ proteins regulate cell proliferation using the same genetic pathway.

3, several genes have been shown to be involved in determining the size of organs (or cell proliferation and endoreduplication) in Arabidopsis, does CHIQUITA1 affect these gene expression?

Yes. *CHIQ1* affects the expression of genes involved in cell proliferation.

In this paper (Fig. 4), studies using cell proliferation markers showed that the domain of expression of the cell cycle genes *CYCLIN D3.3, CYCLIN B,* and *CYCLIN-DEPENDENT KINASE B1.1* is reduced in *chiq1-1* (described on page 6). We did not analyze the expression of endoreduplication-specific genes.

In order to capture early transcriptome changes in *chiq1-1*, we performed genome-wide gene expression analysis in leaves from 6 day-old seedlings from wild type and *chiq1-1* (not included in this paper). These samples were chosen because they are enriched for dividing cells. Cell proliferation genes such as cyclins and cyclin-dependent kinases were not found to be mis-regulated in these samples. These results suggest that *CHIQ1* either alters the expression of cell proliferation genes only later in development (as shown in Fig. 4) or in a spatially and temporally specific manner (on a few cells, for example) in young leaves that gets masked by whole tissue analysis. Alternatively, *CHIQ1* could affect the expression of still uncharacterized cell proliferation genes.

References:

Bao, Y. and Bassham, D. C. (2020). COST1 balances plant growth and stress tolerance via attenuation of autophagy. *Autophagy* 16, 1157–1158.

Bao, Y., Song, W.-M., Wang, P., Yu, X., Li, B., Jiang, C., Shiu, S.-H., Zhang, H. and Bassham, D. C. (2020). COST1 regulates autophagy to control plant drought tolerance. *Proc. Natl. Acad. Sci. U. S. A.* 117, 7482–7493.

Bossi, F., Fan, J., Xiao, J., Chandra, L., Shen, M., Dorone, Y., Wagner, D. and Rhee, S. Y. (2017). Systematic discovery of novel eukaryotic transcriptional regulators using sequence homology independent prediction. *BMC Genomics* 18, 480.

Second decision letter

MS ID#: DEVELOP/2022/200565

MS TITLE: CHIQUITA1 maintains temporal transition between proliferation and differentiation in Arabidopsis thaliana

AUTHORS: Flavia Bossi, Benjamin Jin, Elena Lazarus, Heather Cartwright, Yanniv Dorone, and Sue Y. Rhee
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

The authors fully answered to my previous comments/requests; now I am satisfied with the present revision. Good job!

Comments for the author

I approve this revision for the publication.

Reviewer 2

Advance summary and potential significance to field

This is a review of the revisions made to this manuscript after the first round of peer review. Please see the first round of comment I made to view this summary.

Comments for the author

I am satisfied with the revisions that the reviewers made to this manuscript following the first round of comments by the reviewers. All of my comments and suggestion have been addressed. Thanks!

Reviewer 3

Advance summary and potential significance to field

This paper indicates that the Arabidopsis CHIQUITA1 gene controls plant growth through modulating transition between proliferation and differentiation.

Comments for the author

In this revised version of the manuscript, the authors have added additional data and answered the questions which I addressed, it now is acceptable for publication.