Ploidy and Size at Multiple Scales in the Arabidopsis Sepal

Dana O. Robinson, Jeremy E. Coate, Abhyudai Singh, Lilan Hong, Max Bush, Jeff J. Doyle, Adrienne H.K. Roeder

Plant Cell. Advance Publication Aug. 24, 2018; doi: 10.1105/tpc.18.00344

Corresponding author: Adrienne H.K. Roeder, ahr75@cornell.edu.

Review timeline:

TPC2018-00344-RA	Submission received:	May 27, 2018
	1st Decision:	June 10, 2018 *accept with minor revisions*
TPC2018-00344-RAR1	1st Revision received:	Aug. 9, 2018
	2nd Decision:	Aug. 10, 2018 *acceptance pending, sent to science editor*
	Final acceptance:	Aug. 22, 2018
	Advance publication:	Aug. 24, 2018

REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00344-RA 1st Editorial decision – *accept with minor revision* June 10, 2018

We have received reviews of your manuscript entitled "Ploidy and size in the Arabidopsis sepal." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in *The Plant Cell*. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following: The reviewers have some suggestions for clarifying a few points in the manuscript. Some additional analyses are suggested; these are left to your discretion but would bolster the impact of the manuscript.

The figures are generally unacceptable with respect to legibility. Please see the attached file for comments on the figures, which you can correct in the revision. You can download the attachment, open in acrobat and view in comment mode. please apply the comments to all figures in the paper. We recommend that you hire a professional illustrator.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00344-RAR1 1st Revision received Aug. 9, 2018

Reviewer comments and **author responses**:

Reviewer #1:

The manuscript from Robinson *et al.* addresses the relationship between ploidy levels and cell size to determine the final size of a plant organ. The authors used the model of the Arabidopsis sepal, where ploidy is naturally increased via endopolyploidy according to the endoreduplication process. They created polyploid plants (tetraploid and octoploid) using a colchicine treatment and subsequent selection of polyploid plants where they induced whole-genome multiplication (WGM). In this manuscript, they investigated scaling relationships between these two modes of ploidy increase (endopolyploidy and true polypoidy) and cell, nucleus and organ (petal) size. The main findings of the manuscript showed that pavement cell size and associated transcriptome increases linearly with whole-genome ploidy, but due a compensation mechanism affecting cell number, the size of the sepal organ increases more modestly. According to the karyoplasmic ratio theory, the ratio of nuclear size to cell size is constant in diploid and tetraploid, but slightly lower in octoploid plants.

This manuscript provides an important and original contribution relative to the determination of organ size in plants, and the contribution of cell ploidy, nuclear size, cell size, cell number.

The manuscript is well written, clear and concise. The experiments are well designed, and clearly described for reproducibility. The biological material used in this study is adequate, and fairly characterized with very elegant imaging and molecular techniques. All hypotheses are well presented and discussed."

We thank the reviewer for the positive comments.

"Major revisions
1- Title: this title sounds very rough. "Ploidy and size" refer to cell, and this manuscript studies how it contributes to "petal size determination". At least this latter expression could be part of the title."

We agree that the context of size should be broadened in the title. With size, we refer not only to cell size, but also organ size and nuclear size, which starts to become a long list. We have tried a number of options and the best compromise we have found is to add "multiple scales" so that the title now reads: "Ploidy and size at multiple scales in the Arabidopsis sepal."

"2- Introduction: page 2; I urge the authors to clearly describe the difference in Whole-Genome-Multiplication (WGM) polyploidy versus Endopolyploidy (here precisely Endoreduplication): the former results in the doubling of the number of chromosomes, while in the latter the chromosome number is unchanged but the number of chromatids per chromosome is doubled. This is indeed introduced page 5, line 132, but needs to be mentioned as early as in page 2 (paragraph starting line 52). As a consequence, Figure 1C should clearly illustrate this: in its present state, it gives the impression that the number of chromosomes is exponentially increased according to ploidy, but not following the endoreduplication process (unchanged number of chromosomes) but rather in an endomitosis process (which is another endopolyploid situation identical to WGM, except it is not present in the zygote and all descendant somatic cells, but induced -though rarely in plants- in differentiated cells). Please represent clearly chromosomes with duplicated chromatids to make a definitive difference with Figure 1D and illustrated WGM. Hence a better explanation of the difference between induced polyploidy by colchicine treatment (leading to whole genome multiplication, by doubling the number of chromosomes) and natural endopolyploidy by endoreduplication (maintaining the number of chromosomes, but doubling the number of chromatids) is required in the introduction."

The effect of endoreduplication on chromatin structure is not simple in plants (Leitch and Dodsworth, 2017). In Drosophila cells, it is clear that endoreduplication produces polytene chromosomes. Most of the evidence in plants cells supports partial but incomplete polyteny that is also cell type dependent. For example, Fang and Spector (2005) imaged a fluorescent centromere marker in living Arabidopsis cells and found that endoreduplicated root nuclei had 10 centromeres consistent with complete polyteny, whereas endoreduplicated leaf epidermal nuclei had 20-80 small spots consistent with dissociation of centromeres. Furthermore, we examined Arabidopsis transgenic plants with a fluorescent dot on one chromosome. These plants contained a multimerized lac operator that has been integrated into a single locus on the chromosome which was bound by a fluorescently labeled Lac repressor (lines generated in Matzke et al., 2005). In Arabidopsis sepal epidermal cells, we found substantially more spots than would be expected for completely polytene chromosomes and fewer spots than would be expected for completely dissociated chromosomes, again indicating the chromosomes are likely to be partially polytene (unpublished). We have added: "The degree of polyteny can also be cell-type-specific: analysis of fluorescent centromere markers suggested that endoreduplicated Arabidopsis root cells were completely polytene at the centromere, whereas chromosomes in endoreduplicated leaf pavement cells were more dissociated (Fang, 2005)." (Lines 161-165)

Therefore, we wish to be cautious in the figure and only convey the number of copies, not the structure. We have changed the figure legend to indicate that the drawings represent only number, not structure of the chromosomes. We have added to the figure legend: "Note that this panel depicts only chromosome or chromatid number (red lines), not chromosome structure: endoreduplicated chromosomes may be partially or completely polytene."

We have also addressed this point earlier in the introduction as requested by the reviewer. First, we have defined C and N in this context in lines 42-46: "Two terms are used to denote ploidy: N refers to the number of separate chromosomes in a cell, while C refers to the copy number of each chromosome (Edgar and Orr-Weaver, 2001). The usage of these terms is complicated by total or partial polyteny (synapsis of endoreduplicated chromosomes), as is discussed below."

In lines 121-123, we have added "Endoreduplication multiplies the number of chromatids per chromosome (C); whether it also modifies N, the total number of chromosomes, depends on whether chromosomes remain polytene. (Polyteny is discussed further below)."

"3- The authors should make it very clear according to classical definitions: the DNA amount in the unreplicated haploid or gametic nucleus of an organism is referred to as its C-value, and the normal haploid chromosome number is referred to as N. As reported from the introduction of the highly cited paper from Edgar and Orr-Weaver (2011), "sperm nuclei are 1N, 1C, diploid cells in G1 are 2N, 2C, and diploid cells in G2 are 2N, 4C". Endoreduplicated cells in which the sister chromatids remain closely associated are thus 2N, 4C, or 2N, 8C, or 2N, 16C, and so on... Whenever the authors refer to WGM, they should express it as 2N, 4N and 8N for diploid, tetraploid and octoploid plants, to make a clear difference with the ploidy levels of endoreduplicated cells. This has to be corrected throughout the text, and specifically in Figure 5C,D,E where fold change in gene expression are compared between WGM and endoreduplication."

As stated above, in lines 42-46: "Two terms are used to denote ploidy: N refers to the number of separate chromosomes in a cell, while C refers to the copy number of each chromosome (Edgar and Orr-Weaver, 2001). The usage of these terms is complicated by total or partial polyteny (synapsis of endoreduplicated chromosomes), as is discussed below."

Given the complexity of the partially polytene chromosomes, we have been cautious about using N in the results. Instead we prefer to write out diploid, tetraploid, or octoploid to convey the whole genome multiplication state. We can measure C, so we can report that accurately. In Figure 5C, D, and E we agree that these are a mixture of ploidies due to endoreduplication on top of whole genome multiplication, so our use of 2C, 4C and 8C was inaccurate. We have changed these to the words diploid, tetraploid and octoploid to be consistent with the rest of the text and figures.

"4- Result section, page 13, line 405; Figures 5C, D, E aim at comparing whether sepals from WGM-polyploid plants express the same genes as sepals with induced endoreduplication. Therefore, the authors compared the expression responses induced by changes in WGD (4C/2C, 8C/2C, 8C/4C respectively) versus changes in endoreduplicated expression (LGOox/WT). I am wondering about the relevance of these data for the comparison of expression responses displayed in Figures 5C,D,E.

The WGD data are based on the effects of the polyploid levels on gene expression; the preparation of the libraries took this effect into account, with the use of the RNA to DNA ratio, the SPIKE addition, and the normalization process using DSEQ2. However there is a mixture of nuclei originating from the "true" polyploid state (WGD) plus nuclei from endoreduplicated cells. The transcriptome data related to endopolyploidy are based on the comparison of the transcriptome of the LGO overexpressor line (LGOox) versus the wild-type (WT): in this case, the transcriptome is also the result of a mixture of ploidy levels from various endoreduplicated nuclei (natural or induced by the construct). It appears that the transcriptomic differences are very poorly significant and that no clear tendencies can be seen. Everything is so mixed that the whole transcriptomic response is buffered. Definitely I need to be convinced that comparing the fold changes of these two sets of data displayed in Figure 5C,D,E is relevant, and even if so, whether it is of interest to be shown in a figure. I would rather add it in a Supplemental figure. At least these data need a better justification or a better thorough discussion."

We agree that there are no clear tendencies or correlations in the scatter plots comparing genes differentially expressed between WGD (tetraploid/diploid, octoploid/diploid, and octoploid/tetraploid) and endoployploidy (LGOox/WT). That is the point we are making with those scatter plots and we believe it is an important point that these two different ways of changing ploidy do not cause differential expression of similar genes. Reviewer 2 also mentions this result is of interest. *The Plant Cell* has restrictions on supplemental data, so we would like to leave these scatterplots in the main figure.

In our previous version of the manuscript, we pointed to a weak correlation between the 8N/4N and the LGO OX/WT, but we agree that this is very weak and have removed it.

To make the data normalization comparable between WGM and endopolyploidy samples for the scatterplots in Figure 5 D-F (formerly 5C-E), we used TPM data for both and did not use ERCC spike in control normalization for WGM data. We note that when we do compare the data with the ERCC spike in control, we get similar results.

"5- Results section, page 13 line 379: data related with the increase in mRNA transcriptome are "not shown". At least please provide these data in a Supplemental file, so that the reader can understand the values of 2.1-fold larger for tetraploid versus dipoid, and 1.6-fold larger for octoploid versus tetrapoid sepals"

We have added the graph of mRNA transcriptome size, which is now Figure 5B.

"Minor revisions
6- Introduction page 4: a recent paper on transcriptome analysis of tomato fruit endoreduplicated nuclei, (Pirrello et al., 2018; Plant Journal 93; 387) showed that endoreduplication induces a global shift in gene expression that has an impact on how data should be normalized and interpreted. In addition, the changes in gene expression related to ploidy levels seemed to be associated to cell differentiation or cell specification."

Thanks for the reference. We have added a sentence in lines 135-136: "Global transcription increases with endopolyploidy in tomato fruit (Bourdon et al. 2012, Pirrello et al. 2018)."

"7- Introduction: page 4, paragraph starting line 119; the first sentence of this paragraph is improper. The commitment to endoreduplication (here mentioned as the entry into endocycles) is regulated by the loss of activity of CDKs via the binding of CDK inhibitors such as KRPs or SMRs. "Regulated by the activity of CDK inhibitors" is awkward."

We have revised the sentence to say "Cyclin-dependent Kinase Inhibitors (CKIs) promote commitment of cells to endoreduplication by binding to and inhibiting Cyclin-dependent kinase (CDK) complexes." (Lines 137-139)

"8- Introduction: page 5, line 131; to my knowledge, there is no report demonstrating that endoreduplicated cells can divide (even seldomly). Endoreduplication and the doubling of the chromatids is rather considered as a one-way process with no possible return."

Endoreduplicated cells do sometimes (if very rarely) divide. We have observed an instance in a wild-type Arabidopsis sepal ourselves, which was published in a supplemental movie of Roeder et al., 2010. We have also added references to Schoenfelder et al. (2014), which shows that endocycles are regularly followed by cell division in Drosophila rectal papillae, and Weinl et al. (2005), which shows cells neighboring trichomes initially endoreduplicate and subsequently resume division when a truncated KRP1 is expressed under a trichome specific promoter. We have changed the word seldom to "very rarely" and added the references to line 150.

"9- Results section, page 10, line 285: ML1::H2B-GFP is not an epidermis-specific nuclear marker per se, but due to the specificity of expression of ML1 "used as an epidermis-specific nuclear marker"."

We have modified the language in lines 318-319 in accord with this suggestion: "*ML1::H2B-GFP*, used as an epidermis-specific nuclear marker"

"10- Result section, Figure 3E: the name of the Y-axis is missing."

Thanks, we have added it.

"11- Discussion section, page 18, line 543: "Melaregno et al.""

We have fixed it.

"12- Discussion section, page 22, line 655: incomplete sentence."

We have changed the style of this list from "1." to "(1)" to avoid this ambiguity. (Line 700)

"13- Discussion section, page 22, line 673; Bourdon et al. (2012, Development 139: 3817) have shown that in tomato endoreduplicated nuclei, the nucleolus area increased proportionally to the nuclear area based on the increase of ploidy. Therefore, endoreduplication does exert an effect on the nucleolus volume, and its transcriptional activity for rRNA synthesis."

Yes, the size of the nucleolus does scale with ploidy. For the denominator, we envision a hypothetical mechanism in which the number of nucleoli is counted, which remains constant despite endoreduplication. We have added a sentence to clarify this point in lines 720-722: "Such a mechanism would have to count number instead of measure size, as nucleolar size has been shown to increase with ploidy (Bourdon et al., 2012)."

Reviewer #2:

"The authors re-examined correlation between ploidy and cell size in sepal epidermis of Arabidopsis, which has been shown to be one of the best model system to study roles of endoreduplication in cell expansion by the laboratory of the last author, Dr. Roeder.

Here they distinguished the effect of ploidy change between by whole genome duplication (WGD) and by endoreduplication. Moreover, they showed that nuclear size is not well correlated with cell size. Cell-type specificity on the relationship was also confirmed. While past studies simplified the roles of endoreduplication on regulation of cell size too much, these findings urge us to reconsider the past interpretations. Moreover, while past studies hypothesized that the effect/roles of polyploidization on cell size is the same between by WGD and by endoreduplication, this study clearly denied it. More specifically they revealed that WGD generates expression profiles differed from those caused by endopolyploidy.

Overall the experimental analyses were properly done, and thus if a few following points are fixed, this report will be welcomed by researchers who studies organ- and/or cell-size control and roles of polyploidy in plants."

We thank the reviewer for the positive comments.

"Major comments
(1) The authors wrote that "pavement cell area is used as a proxy for cell surface are because pavement cells are highly vacuolated, the volume of the cytoplasm may be better represented by surface area than total cell volume" (page 16). This seems to be reasonable in the thin-shaped pavement cells, but in a ball-shaped cells, cell area from a particular viewpoint should be [cell volume]^(2/3). This point must be clarified in the earlier part of this report. Indeed in the introduction, the authors cited past reports on the cell size difference (Line 65, Page 2 to Line 69, Page 3) as tetraploid cells are 1.5- to 1.76-fold larger in diploid cells. Because these values are an area-based data, these values are not 2-fold but 1.5- to a.76-fold. The authors are requested to clarify it in the Introduction."

We agree that this issue should be brought up in the introduction before we use cell area as a measure. We have now added to the introduction in lines 79-85: "A naïve expectation might be that cell volume should double with duplication of the genome. For a spherical cell, a doubling of the volume would cause a 1.58-fold increase in in cell cross-section area, comparable to the 1.5- to 1.76-fold changes in area observed. However, Melaragno, et al. have proposed that the relevant volume is that of the cytoplasm, which in highly vacuolated cells (like plant epidermal cells) is best estimated by the surface area; thus, we might alternately expect a doubling of surface area (Melaragno et al, 1993)."

We have added Supplemental Figure 2 showing that the epidermal cells in the sepal are largely cylindrical, although tapered at the ends (see also major comment 9 below).

In the discussion, we have added: "We can estimate cell volume by modeling pavement cells as cylinders (Supplemental Figure 2) and further assuming that the radius and height increase proportionately. In this model, a doubling of cell volume would cause a 1.58-fold increase in cell area. The fold changes we observed, ca. 1.75-fold, are significantly different from both 2-fold and 1.58-fold (one-sample t-test, $p<0.01$), suggesting that the size scaling is more complex than doubling either parameter or that a more precise measure of the volume of the cytoplasm is required." (Lines 582-588)

"(2) Line 93 and Line 393. In addition to the cited reference, Nakamura et al. 2015 PLoS One reported that cell-wall-related gene expression is altered in tetraploid hypocotyls of Arabidopsis."

In lines 108-109, we have added a reference to Narukawa H, Yokoyama R, Komaki S, Sugimoto K, Nishitani K. Stimulation of Cell Elongation by Tetraploidy in Hypocotyls of Dark-Grown Arabidopsis Seedlings. Tsukaya H, ed. *PLoS ONE*. 2015;10(8):e0134547–13. doi:10.1371/journal.pone.0134547.

This paper shows an increase in cuticle biosynthesis (GO terms: lipid transport, lipid localization and lipid binding) gene expression through microarray analysis of tetraploid versus diploid hypocotyls. It specifically mentions that no GO terms associated with cell wall metabolism were associated with upregulated or downregulated genes.

(3) Lines 283-291 and Line 348. In tetraploids, both, mitosis and endocycle than in diploids. Please discuss about some possible mechanisms on the reduction of both cycles.

We think a compensation phenomenon accounts for the reduction in both mitosis and endocycles. Given our live imaging data, the most likely hypothesis is that a maturation process occurs somewhat earlier in tetraploid plants than diploid, terminating both mitosis and endocycles. In our Discussion (lines 600-611), we suggest, "Our live imaging data indicate that this cell number change arises late in development: actively growing sepals of all ploidy levels have the same number of cell files and grow at roughly the same rate (Figure 6). A relatively late-acting mechanism must affect proliferation to enact compensation. One possibility consistent with our results is that maturation and termination of cell division and growth occur earlier in sepals with a higher base ploidy level. (The same mechanism may also cause earlier termination of endocycles, which might account for the small reduction in endoreduplication observed in tetraploid and octoploid sepals.) The cause of this cell number reduction is so far unknown—is this intrinsic to the growth of polyploid cells, or might it be a response to a total organ size checkpoint (Krizek, 2009; Potter and Xu, 2001; Powell and Lenhard, 2012; Sugimoto-Shirasu and Roberts, 2003; Tsukaya, 2008)?"

(4) Lines 382-383. the authors discussed that "transcriptomes scale with ploidy in a way that closely reflects the scaling of sepal pavement cell area with ploidy". As for the above point (1), this should be carefully discussed considering the cell volume. Particularly, mRNA level should be kept in a concentration manner per volume.

To clarify the relationship with transcriptome, we have added the sentence: "Because cytoplasmic volume scales linearly with cell area rather than cell volume in highly vacuolated cells, this suggests that transcription scales with cytoplasmic volume." (Lines 419-421)

"(5) Line 395. The reference "[32]" should be clarified."

This reference is Wu, C.Y., et al., Control of transcription by cell size. PLoS Biol, 2010. We have made sure it is imported correctly into the resubmitted manuscript. (Lines 433-434)

"(6) Line 423. "mitotic cell cycle duration is strongly correlated with genome size": this is not the case in the report by Storchova et al. 2006 Nature."

We have modified this line to read "mitotic cell cycle duration is generally strongly correlated with genome size (Francis et al., 2008; Serrano-Mislata et al., 2015; Simova and Herben, 2012) (though Storchova et al. did not observe a correlation in yeast (Storchova et al., 2006))". (Line 459)

"(7) On the relationship between polyploidy and nuclear or cell size, there are several reports in 1970s. Please examine them, too."

In lines 174-181, we have added: "That polyploids typically have larger cells than diploids has been known since the earliest investigations of polyploidy (Muntzing, 1936). In the 1960s and 70s, numerous observations of correlations between DNA content and cellular parameters, including nuclear size, cell size, and cell cycle duration (Bennett, 1971; Price, 1973; Van't Hof, 1963) provided data for the "nucleotype" theory of Bennett (Bennett, 1972, 1996), which stated that some elements of phenotype are affected directly by bulk DNA amount irrespective of gene content. A somewhat similar, nucleo-skeletal theory was also propounded by Cavalier Smith (1978)."

"(8) *crwn1* mutant story. Does transcriptomes scale reduced in the mutant?"

This would be an interesting experiment which would provide information about the relationship of nuclear size (independent of ploidy) to transcription; however, it is beyond the scope of our study, which focuses on the effects of differences in ploidy.

(9) Lines 539-542. In case of octoploid, if cell thickness is changed by octoploidization, cell-area-based measurements may result in underestimation. Isn't it possible that this underestimation affected the interpretation on the data for octoploids?

We have added Supplemental Figure 2 showing cross sections of the sepal epidermal cells. Using the MorphoGraphX software, we have created digital cross sections from high resolution confocal stack images. All cells are essentially cylindrical, and the cross sections appear circular. However, we note that the cylinders taper at the ends. As expected, we do find the cross-section area is significantly larger in octoploids than diploids and tetraploids.

"Minor comments
(1) Italicized "Arabidopsis" (title and in the text). This style had been used in Arabidopsis community for a long time,

but recent publications usually use roman-styled "Arabidopsis", because italicized "Arabidopsis" means genus *Arabidopsis*, including *A. suecica*, *A. camtchatica*, and *A. lyrata*."

We have made this change throughout the text.

"(2) The authors often use "--'s" style. This is better to be changed, because this is a spoken style."

We defer to the *Plant Cell* editors on this issue.

"(3) Arabidopsis octoploids often show aneuploidy, that might be a reason why octoploids show retarded growth. Did you check the possibility by some ways? (Flow cytometry is not enough for detecting one chromosome loss in 8C. Chromosome number counting or confirmation of the same phenotypes among several independently isolated octoploid lines might be good for the test)."

We are aware that aneuploidy might be an issue; we tried to account for this by representing at least two independently synthesized octoploid lines in each experiment. We observed consistent phenotypes among all octoploid lines generated, which includes octoploids generated in multiple different fluorescent marker lines. We also used at least two independently generated tetraploids for each experiment. We've added the sentence "Because flow cytometry may not always detect aneuploidy, we report results from at least two independently synthesized tetraploid and octoploid lines in all experiments." (Lines 302-304)

"(4) What protein is encoded by the *CRWN1* gene?"

We have added the sentence "*CRWN1* encodes a coiled-coil protein in the Nuclear Matrix Constituent Protein (NMCP) family." (Lines 554-555)

(5) Line 655. "DNA its associated proteins". Is this "DNA with associated proteins"?

We've amended this line (Line 702) to "DNA and its associated proteins".

"(6) Figure 6G. This panel is too small in relative to the other panels in this Figure 6."

We have increased the size of this panel.

Reviewer #3:

"The manuscript by Robinson et al addresses the link between nuclear DNA content and cell size and number, using the epidermal cells of the sepals of Arabidopsis thaliana as a model system. They provide a comprehensive analysis of DNA content, nuclear volume, cell area, cellular RNA content and cell number of wild type (having endoreduplication to induce differences in ploidy between cells), a range of lines with altered dose of LGO (loss of giant cells), tetra and octoploid lines and the *crown1* mutant that has a smaller nuclear volume. They meticulously test possible correlation between these parameters, clearly linking cell area to nuclear DNA content (not nuclear volume) albeit not linearly and show a compensation mechanism where larger cells formed by polyploid plants. Overall, I think this is an elegant study that provides a clear dissection of this longstanding issue, that would be well suited for publication in a high-profile journal as the *Plant Cell*."

We thank the reviewer for the positive comments on our manuscript.

"However, there are a number of aspects that are weak, that could to my mind can easily be solved/improved:
- The authors analysed the effect of whole genome duplications on guard cell size. In doing so they confirmed earlier findings that stomatal density decreased with increased ploidy levels. This could of course be purely a consequence of pavement cells getting larger. It would be crucial to know if stomatal index (fraction of cells that are guard cells) is constant or decreased. This could be easily calculated from the data already collected."

We find that stomatal index is not affected by ploidy; we have added a panel illustrating this to Supplemental Figure 3 (Panel C), and have also added a sentence describing this figure to the main text (Lines 362-364): "Stomatal index, the ratio of pavement cell to guard cell number, is also fairly constant across the ploidy series (Supplementary Figure 3C)."

- After studying polyploid lines, the authors conclude that differences in cell number at mature stage, cannot be observed at an early stage of development. In lines 457 - 459 this leads to the conclusion that this discrepancy may be explained by sepals at high ploidy levels terminating cell division earlier. To my mind this could be tested by quantifying cell number evolution in function of sepal development. It's a bit of work, but the authors have all the tools in hand.

We would also really like to know more precisely where and when the difference in cell number occurs. For diploid, tetraploid and octoploid inflorescences, we imaged a sepal from each flower in the inflorescence to create a developmental series. Then we segmented and counted nuclei. Unfortunately, we could not detect a clear trend in when the difference in cell numbers occurs.

"- After determining at the population level the theoretical minimum nuclear size at each ploidy level, the authors discuss in line 507 - 509 that some cells are observed that are below this value. The simple explanation that the accuracy of the measurements may be the limiting factor to draw a hard conclusion should be addressed."

We have edited: "we observe that some nuclei in the dataset are smaller than this average minimum value (possibly due to segmentation errors)." (Line 543)

- In the first paragraph of the discussion the authors address the discrepancy that doubling of DNA content does not coincide with a doubling in cell area. They recognise that area may not translate into volume directly. Indeed, cells may also become "deeper", and not much of this would be needed to allow for a doubling. This could easily be tested by making cross sections.

We have added cross sections in Supplemental Figure 2. The cells are similar to tapered cylinders and the radius does increase with ploidy. We add further theoretical calculations about how the volume should increase as the radius and height increase for a cylinder in the discussion: "We can estimate cell volume by modeling pavement cells as cylinders (Supplemental Figure 2) and further assuming that the radius and height increase proportionately. In this model, a doubling of cell volume would cause a 1.58-fold increase in cell area. The fold changes we observed, ca. 1.75-fold, are significantly different from both 2-fold and 1.58-fold (one-sample t-test, $p<0.01$), suggesting that the size scaling is more complex than doubling either parameter or that a more precise measure of the volume of the cytoplasm is required." (Lines 582-588)

"- Figure 2B. Why not include the full distribution, including 2 and 4C, particularly given in C and D we are also looking at the total cell population."

We have added a version of this chart including 2C and 4C data as Supplementary Figure 1; we excluded these data in the main figure because (1) we specifically want to quantify the proportion of definitively endoreduplicated (8C and above cells here), and (2) the relatively small values here (0.01-4%) are hard to visualize on an axis shared with 2C and 4C values in the 30-70% range. As noted in the next point 4C cells are ambiguous because they could be endoreduplicated or could be in G2 phase of the cell cycle.

"- Figure 4C One has to be cautious with the 4C (for diploid plants), as indicated in the main text did these cells really endoreduplicate or exit the mitotic cycle while in G2? Consider presenting the endoreduplication index (average nr of rounds of DNA duplication):
IE = 0 x 2C + 1 x 4C + 2 x 8C + 3 x 16C + 4 x 32 C
Where I would replace 2C by the base ploidy level for each line."

Good point. We added the phrase "(or in G2)" below the axis label "1 endocycle" to address this ambiguity.

"Globally I find the text rather long. Personally, I like this, but I'm not sure if it fits in the limits imposed by the journal."

We defer to the *Plant Cell* editors on this issue.

Editor's Comments:

"-Supplemental materials should be restricted to large datasets and tables, presentation of replicates, and validation of reagents, methods, or genotypes. Any data that are used to support claims must be in the main manuscript.

Supplemental figure legends must indicate what figure in the main manuscript is supported by the supplemental data presented."

Supplemental Files 1 and 2 are large datasets, which support Figure 5.

Supplemental Figure 1 supports Figure 2. It is the same as panel 2B with the addition of 2C and 4C data that the reviewer requested.

Supplemental Figure 2 supports Figure 3, showing the cross sections of the cells as a control.

Supplemental Figure 3 supports Figure 3, essentially replicating the scaling measurements in another cell type.

Supplemental Figure 4 supports Figure 7, shows further details of the scatter plots in Figure 7E.

The support labels have been added to the figure legends.

"-Use freeze panes option in the excel pages as appropriate. Also, please edit the text to subscript items in the header labels or use symbols or italics as appropriate. Please define E."

Panes were frozen where appropriate in supplemental files 1 and 2 to keep header rows and/or columns visible when you scroll through the data.

The "2" in log2(FC) was changed to subscript in all instances in supplemental file 1.

The header to column E in supplemental file 1 was revised for clarity to "Max length", and is defined in the fourth tab ("Header definitions")

"-Sampling methods and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc.), along with a clear description of and rationale for any statistical analyses conducted. The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted."

We have added details on the biological replicates for transcriptomic analysis: "Each biological replicate consisted of a total of 100-200 sepals collected and pooled from 2-4 individual plants." (Lines 849-850)

Response to figure comments on pdf:

- **For all figures, we have enlarged and unified the text size throughout. Now the text within all figures is Calibri 8 point and the panel labels are Calibri 12 bold.**
- **Text has been centered around the graphs.**
- **We have used µ instead of u.**
- **We have made sure graphs have the same width bars, with a few important exceptions where the width of the bars conveys meeting. For example, in Figure 4, we have purposely made the bars in C different width from A and B. In A and B, each bar represents a single replicate. In C, the data is pooled and the bar is widened to indicate that it is averages across samples. We think it is important to continue to make this distinction using bar thickness. Likewise, in Figure 7J, the bar widths are narrower than the rest of the figure to convey that these bars represent subsets of the data.**
- **In Figure 1, we have made the arrows consistent as requested.**
- **In Figure 2, the color scheme is the same for all panels throughout the figure. It would disrupt the whole figure to change the color scheme only for panel C. Instead we have made the legend larger and clearer.**
- **In Figure 2, we have removed the lines at the top of panel A.**
- **In Figure 6, we have added a label to the y-axis of A and scaled G to fit with the figure.**

TPC2018-00344-RAR1 2nd Editorial decision – *acceptance pending* Aug. 10, 2018

We are pleased to inform you that your paper entitled "Ploidy and size at multiple scales in the Arabidopsis sepal"

has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor	**Aug. 22, 2018**