

INVESTIGATING EARLY SIGNS OF ENDOREDUPLICATION IN *ARABIDOPSIS* SHOOT: AN UNKNOWN FACT

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Abstract: Endoreduplication is alternative form of cell cycle that involves the replication of DNA without mitosis. It commonly occurs in various tissues of plants like pavement cells of leaf, trichomes and sepals of flower and led to increase in size of the cell. Shoot apical meristem (SAM) is a region from where the aboveground organs of the plant arise. The cells in the meristem remain in meristematic state (mitotic) and get displaced from there to form the differentiated tissues. But how the nuclear DNA synthesis vary from the meristematic cells to the differentiated tissues is not very well studied. It has been observed through the present study that the cells in shoot tip of *Arabidopsis* are diploid in nature and starts to endoreduplicate at 1cm away from the shoot, down the stem which was justified by the flow cytometer-based DNA analysis of different *Arabidopsis* tissues. Petal is known to be the most variable part of the flower with different color, shape, size and fragrance but little is known about its characterization. The cell identities in petal are diverse with different cell types. One is small sized distal cells and the other is large sized proximal cells. The present study has addressed that the large size of proximal cells of petal is not merely a cell expansion but is something related to endoreduplication. This was evidenced by the DNA estimation of FACS (Florescent activated cell sorter) sorted petal cells of *Arabidopsis*. This is the first study in which SAM, stem, leaf and petal cells are taken into account for ploidy analysis by flow cytometry. In shoot apex we did not see endoreduplication however as the cells descend towards the stem or leaf they enter into differentiation pathways and undergo endoreduplication. On the other hand, different ploidies in petal cells shows the signs of endoreduplication which can be a way towards differentiation.

Keywords: ploidy, endoreduplication, petal, florescent activated cell sorter, *Arabidopsis*.

Introduction

In plants, organs are formed during embryogenesis and continuous the development through the activity of specialized tissue called shoot and root meristem. The cells in the meristem remain in the proliferating state and maintains a balance between the cell proliferation and differentiation throughout the development. In response to various signals, the cells displaced from meristem and form differentiated tissues (stem or lateral organs) [STEEVES & SUSSEX, 1989]. Most of the differentiated tissues have a tendency to undergo endoreduplication which is an alternate form of cell cycle [BARLOW & al. 1978]. During this phenomenon, nuclear DNA is replicated without mitosis due to which amount of DNA becomes greater than 2C. The indirect effect of ploidy increase is the change in gene expression, cell size, nuclear size and organ size [DEL POZO & RAMIREZ-PARRA, 2015; SLABODNICK & al. 2017; ZHAO & al. 2017]. Endoreduplication helps in gene amplification, radiation resistance and cell differentiation [BARLOW, 1978; GALBRAITH & al. 1991]. Endoreduplication is quite common in various tissues of plants like pavement cells of leaf, trichomes, sepals of flower. In fact, degree of endoreduplication is developmentally regulated in most somatic tissues in *Arabidopsis* depending on age and tissue types [GALBRAITH & al. 1991]. The cells in the

Arabidopsis sepals exhibit a characteristic pattern, with diverse sizes ranging from giant cells of 360 μm to the smallest cells of 10 μm in length. These giant cells of flower are the markers for sepal organ identity [ROEDER & al. 2010]. Similarly, petals are of great interest to horticulturalists to enhance the ornamental value of plants [NISHIHARA & NAKATSUKA, 2011]. The petal in *Arabidopsis* seems to be simple but has a more complex and diverse cell types. It generally arises from L2 and L3 layer of SAM from where the other aerial organs arise [JENIK & IRISH, 2000]. Petal growth and size is very important for attracting the pollinators. It is composed of diverse cell types which serve as a model for morphogenesis [IRISH, 2008]. The earlier studies have shown that petal has elongated proximal cells and small distal cells. But how these cell types are different in terms of its ploidy characterization is not known. Like a leaf, petal growth also involves initial cell proliferation followed by cell expansion, thus favors the interaction between cell number and size control during petal growth [MIZUKAMI & FISCHER, 2000; SZÉCSI & al. 2006]. Is endoreduplication, a part of normal petal growth? This question has not been addressed so far. In *Arabidopsis* mutants like *frll*, petal cells displayed little endoreduplication at the tip [HASE & al. 2005] but endoploidy in wild type petal is not known so far. Generally, endoreduplication is mostly associated with cells that become enlarged and is an important factor for controlling the cell size [MIZUKAMI, 2001; MELARAGNO & al. 1993]. The question which has been addressed in the present study is to know the endoreduplication signals in various tissues of *Arabidopsis* and whether it is related to cell expansion or not.

This is the first study in which SAM, stem, leaf and petal cell types are taken into account for ploidy analysis by flow cytometry. In shoot apex we did not see endoreduplication however as the cells descend towards the stem or leaf, they enter into differentiation pathways and undergo endoreduplication. Also, we are able to separate the two different cell types of *Arabidopsis* petal on the basis of their cell size through florescent activated cell sorter and have shown for the first time the presence of different ploidies in them. The cells towards the distal end of petal were found to be diploid whereas elongated/elliptical cells at the proximal end of the petal have undergone endoreduplication to approx. 128C. A relationship was also proposed between ploidy levels and cell size during petal development.

Material and methods

Protoplasting and florescent activated cell sorting (FACS) of SAM cell types

For protoplast isolation, 1 cm, 4 cm and 10 cm stem from shoot tip of *WT Col* and *Ler* was cut down and chopped into small pieces into 6 ml of protoplasting cocktail. On the other hand, approximately 50 petals of 28 days old mature *Arabidopsis* (*Ler*) plants were harvested and placed in falcon tube containing 6 ml of protoplasting cocktail. This cocktail was prepared by dissolving 1.25% w/v Cellulase (Yakult), 0.3% w/v Macerozyme (Yakult), Hemicellulase (Sigma), 0.4 M D-mannitol, 20 mM MES and 20 mM KCl (from a 1 M stock) in demineralized water and adjust the pH to 5.7 with 1 M Tris/HCl pH 7.5. Further the solution was heated to 55 °C for 10 minutes (to make it clear) and cooled down to room temperature before adding 0.1% w/v BSA (bovine serum albumin), 10 mM CaCl_2 , and 5 mM β -mercaptoethanol. The tubes were allowed to shake for about 1 hour and 15 min at 120 rpm. After shaking, the protoplast solution containing tissue was filtered through 40 μm cell strainer (BD Falcon) and the filtrate was centrifuged at 4 °C for 20 minutes at 500 g. The pellet was resuspended in 1 ml of the incubation solution (154 mM NaCl, 125 mM CaCl_2 , 5 mM KCl, 5 mM MES, adjust pH to 5.7 with KOH) and was used for flow cytometry for sorting of cells on the basis of size.

Cells were sorted on the basis of size by fluorescence activated cell sorter (BD, FACS Aria Fusion) with a 100 μ m nozzle at a rate of 2,000 to 5,000 events per second and sheath fluid pressure of 20 psi. Protoplasts sorting were established based on the following cell properties: a) a cluster of live protoplasts with intact membranes was selected based on a forward to side scatter ratio. b) Doublet exclusion was performed by plotting width versus area for forward and side scatter and finally gate was applied to identify cells small and big cells and sorting was performed. About 10,000 protoplasts were collected from each sample. Cells were sorted directly in LB01 isolation buffer (15 mM Tris, 2 mM Na₂EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100; pH was adjusted to 7.5 before adding β -mercaptoethanol to 15 mM) for ploidy and cell cycle analysis.

DNA Ploidy and cell cycle analysis

For DNA ploidy and cell cycle analysis, flow cytometry using DNA-selective fluorochromes method was used for the measurement of nuclear DNA content in the protoplast cells. For nuclei isolation, protoplasts sorted above in LB01 isolation buffer was incubated at dark for 15 min on ice with occasional shaking. After that homogenate was filtrated through 42 μ m nylon mesh and the DNA fluorochrome, propidium iodide (PI) was added to the filtrate at the concentration of 50 μ g/ ml simultaneously with 50 μ g/ ml of RNase in order to prevent the binding of PI to dsRNA. This solution was incubated in ice for 15 min-1 hr before analysis of nuclear DNA content through flow cytometer (BD Aria Fusion).

Results and discussion

Early signs of endoreduplication observed just below shoot apical meristem in *Arabidopsis*

To explore the early sign of differentiation in search of genetic signature, we analyzed the ploidy in shoot and stem of *WT Col* and *Ler* ecotypes of *Arabidopsis* by flow cytometry. This study has revealed the specific endoreduplication pattern in stem and shoot. The shoot as a meristematic tissue found to consist of diploid 2C and 4C cells only (Figure 1a). Interestingly, the signs of endoreduplication were observed as we go down towards the stem and leaf of *Arabidopsis*. For stem, we have analyzed the ploidy in the nuclei isolated from the cells taken 1 cm, 4 cm and 10 cm from shoot tip of *WT Col* and *Ler*. The signs of endoreduplication with small peak of 8C was observed in the cells isolated from 1cm below the shoot tip (Figure 1d & 1g) in both ecotypes of *Arabidopsis*. Excited from this result, we extended the distance and analyzed the ploidy in the cells that were harvested 4 cm and 10 cm from shoot tip, respectively. 8C nuclei were found to be more abundant as distance increases down the stem (Figure 1e & 1h). Similarly, the cells with 16C were found to appear when we got down to 4cm and 10 cm in *WT Col*, *Ler* (Figure 1f & 1i). The pavement cells analyzed from leaf tissue showed endoreduplication from 4C, 8C, 16C and occasionally up to 32C and beyond (Figure 1b). The above findings suggest that indeed, ploidy is an essential mechanism that leads to stem elongation and cell growth in most plant species. The proportion of polyploid cells increases in stem as the distance increase from shoot tip. Thus, suggested that cells near the meristem maintains their genomic content closer to 2C, however, when these cells undergo differentiation in stem and leaf they start increasing their size by increasing the genomic content. The nuclear DNA content in various tissues has been depicted by histogram (Figure 1c). The results suggest that the nuclei harvested from 1 cm below the shoot tip have early signs of endoreduplication.

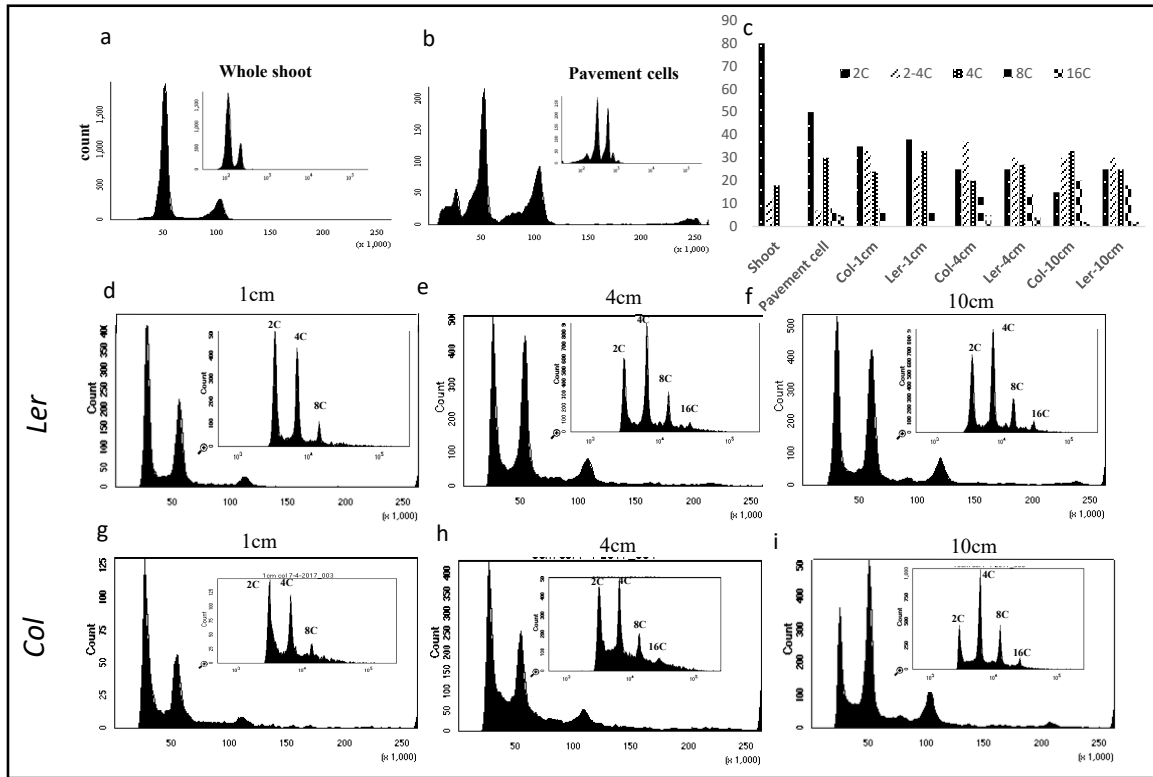


Figure 1. DNA ploidy in different tissues of *Arabidopsis*. a) Whole shoot; b) pavement cells; d) stem (1cm) down from shoot tip (*Ler* ecotype); e) stem (4 cm) down from shoot tip (*Ler* ecotype); f) stem (10 cm) down from shoot tip (*Ler* ecotype); g) stem (1cm) down from shoot tip (*Col* ecotype); h) stem (4 cm) down from shoot tip (*Col* ecotype); i) stem (10 cm) down from shoot tip (*Col* ecotype); c) Histogram showing DNA content in all the observed tissues

Endoreduplication was observed in petals

Further, ploidy was analyzed in floral petals. The protoplast was isolated from about 50 mature petals of *Arabidopsis* (Figure 2). They were subjected to FACS (BD Aria fusion) and were analyzed by taking forward scatter and side scatter parameters (area) into account. From the scatter plot, the two types of cells forming clusters were analyzed. One showing smaller cell size and the other with bigger cell size were observed. These both type of cells was sorted separately for cell cycle analysis (Figure 3). Cells sorted above on the basis of size were subjected to cell cycle analysis by flow cytometry. Nucleus was isolated and DNA content was estimated using propidium iodide. The smaller cells showed 2C and 4C peak accounting for 80% (2C) and 20% (4C) of total nuclei. Because *Arabidopsis* is a diploid species ($2n=10$), the 2C DNA level corresponds to the diploid state of the genome found in the G₁ phase, whereas the 4C DNA level results from the S-phase doubling of chromatids found in the G₂ phase (Figure 4a). It is thus an indicator of the capacity of cells to enter mitotic cycles. Therefore, the major 2C and 4C peaks suggest that the tissue is in a dividing state. Whereas, surprisingly, bigger cells showed a sharp peak corresponding to 128C was observed (Figure 4b). It means the cells in the proximal part of the petal during maturity have stop division and undergone endoreduplication, reaching to high ploidy level. The flow cytometric profiles displayed a reproducible 128C peak from nuclei in the proximal part of the petal at the mature stage. At this stage, in contrast, the number of nuclei with 2C, 4C, 8C, 16C, 32C and 64C DNA level decreased dramatically (Figure 4b & 4c). The study of *Arabidopsis* petals may help in providing information about the relationship between endoreduplication and cell growth during plant organ development. In the present study, one of the most striking features of petal cells observed was an uneven increase of their DNA content during development. This cytometric data showed that, in the proximal part of the petal, cells become more endopolyploid with up to a 128C ploidy level, whereas cells in the distal part of the petal maintained the diploid level throughout petal differentiation. The high proportion of nuclei at the 2C and 4C levels in distal part, indicate that the cells never endoreduplicate and their mitotic cell cycle is arrested either in the G₁ phase or in the G₂ phase of the diploid cell cycle. The study has shown that the epidermal cells in the proximal part of the petal are large and extremely elongated, but epidermal cells in the distal part of the petal are small and highly homogeneous. These findings, indicated that the formation of large differentiated cells is accompanied by an increased ploidy level.

The correlation between the cell size and the degree of endopolyploidy supports the idea that the nuclear DNA content might play a key role in controlling cell size. Endoreduplication in *Arabidopsis* petals might be a major driving force for cell differentiation. According to GALBRAITH & al. (1991), endoreduplication is not present in the floral organs of *Arabidopsis thaliana*, all cells are at the 2C level [GALBRAITH & al. 1991]. But Roeder et al. 2012 have shown for the first time that sepals of *Arabidopsis* have the giant cells that have a power to undergo endoreduplication. Our data is well supported by this report of giant cell formation in the early development of sepal [LEE & al. 2009; BREUER & al. 2010]. Endoreduplication has also been documented in different organs of the cabbage flower. Filament tissue showed ploidy level up to 64C, carpel and petal showed ploidy up to 8C and 32C respectively [KUDO & KIMURA, 2001].

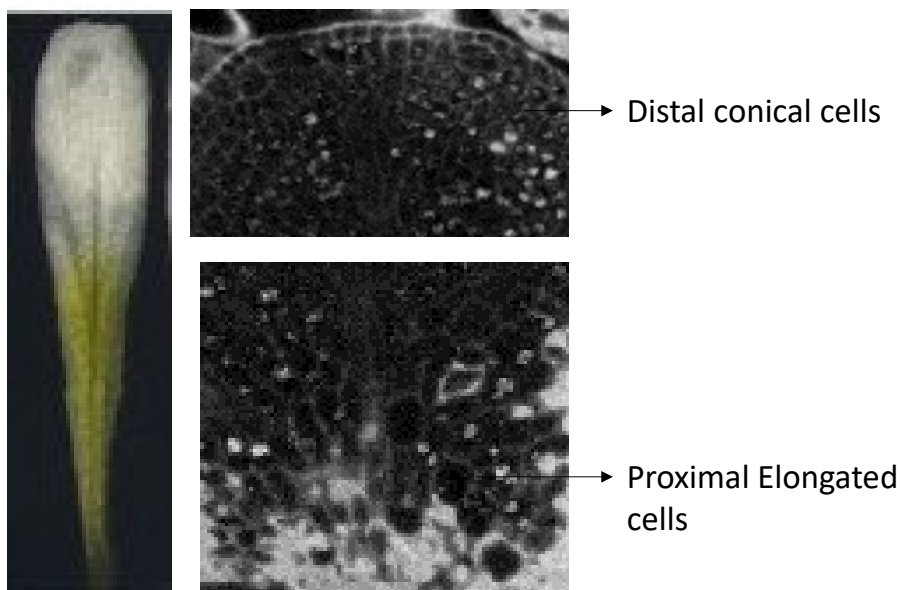


Figure 2. *Arabidopsis* flower: **A.** Morphological view of *Arabidopsis* petal taken through Nikon camera fitted to dissecting microscope. **B.** Confocal microscopic view of *Arabidopsis* petal showing the cells stained with propidium iodide.

A correlation between cell size and DNA content has also been reported in many eukaryotic organisms. Size of endoreduplicated cells is larger than normal cells [LARKINS & al. 2001]. *Arabidopsis* mutants with increased or reduced ploidy levels in trichomes invariably showed an increase or a reduction in the final cell size [FOLKERS & al. 1997] which supports an idea that endoreduplication is coupled with cell size. However, some reports have shown that uncoupling also occurs in endoreduplication and cell elongation. It was observed during germination of *Arabidopsis* seedlings where endoreduplication takes place before the elongation of hypocotyl cells [GENDREAU & al. 1997]. Endopolyploid nuclei can be advantageous for specialized functions. It is proposed to increase the metabolic activity, rRNA synthesis and transcriptional activity [BALUSKA & KUBICA, 1992] like in maize and pea [CAVALLINI & al. 1995; LIU & al. 1996]. The control of endoreduplication may allow cells to reach extraordinary sizes [CEBOLLA & al. 1999]. So, the process of endoreduplication is very important for manipulating the cell size in horticultural and agronomically important crops.

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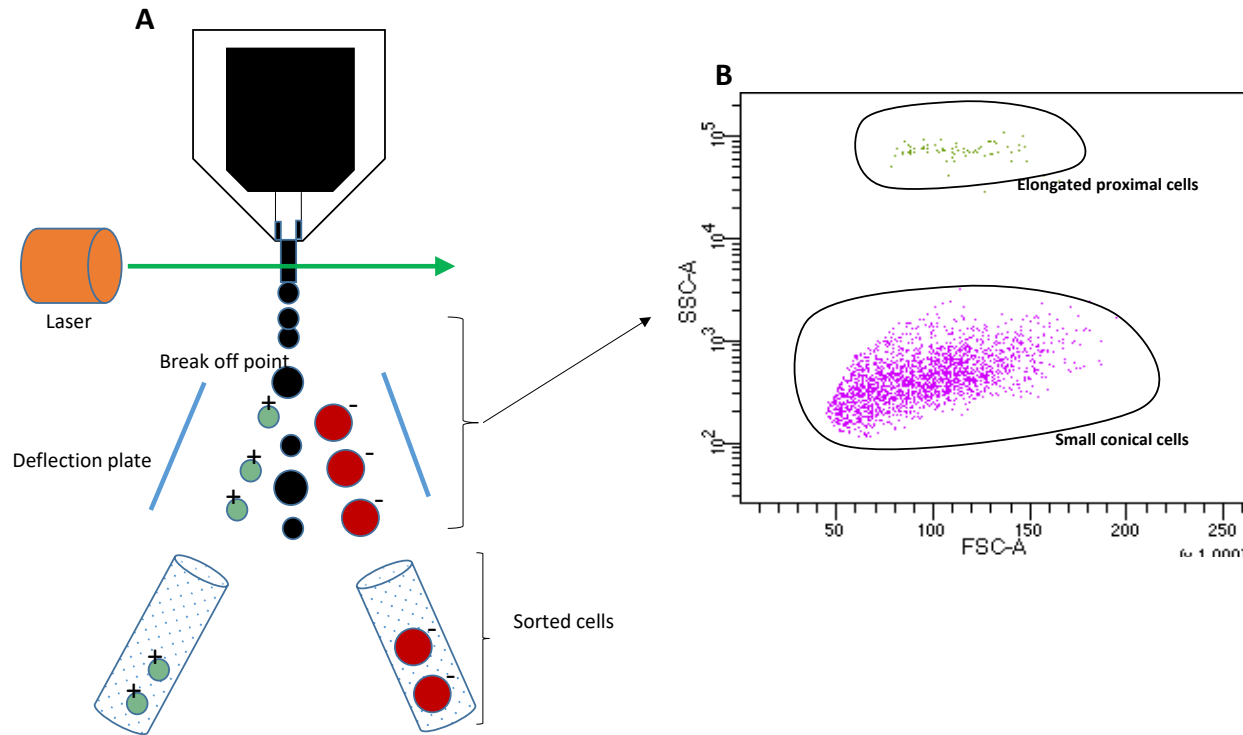


Figure 3 **A.** Diagrammatic representation of florescent activated cell sorter (FACS) showing the sorting of protoplast isolated from *Arabidopsis Ler* petal on the basis of their size. **B.** The scatter plot between SSC and FSC generated on the basis of size and complexity showing two types of nuclei in wild *Ler* petals. Gating was done to sort these two populations separately.

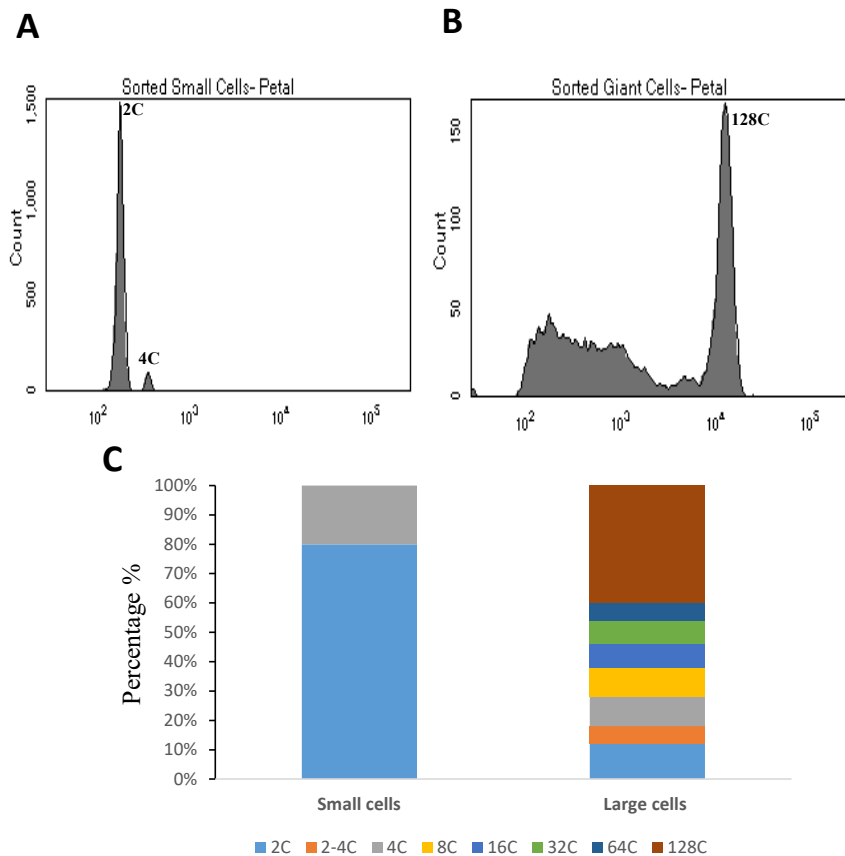


Figure 4. A. DNA ploidy in the sorted small nuclei is showing 2C and 4C peaks whereas B. the large nuclei is showing the higher ploidy (endoreduplication) upto 128C. C. Histogram showing DNA content in small and large cells of *Arabidopsis* petals.

References

- BALUŠKA F. & KUBICA. Š. 1992. Relationship between the content of the basic nuclear proteins, chromatin structure, rDNA transcription and cell size in different tissues of the maize root apex. *Journal of Experimental Botany*. **43**(7): 991-996. <https://doi.org/10.1093/jxb/43.7.991>
- BARLOW P. W. 1978. Endopolyploidy: towards an understanding of its biological significance. *Acta Biotheoretica*. **27**: 1-18. <https://doi.org/10.1007/BF00048400>
- BREUER C., ISHIDA T. & SUGIMOTO K. 2010. Developmental control of endocycles and cell growth in plants. *Current Opinion in Plant Biology*. **13**(6): 654-660. <https://doi.org/10.1016/j.pbi.2010.10.00>
- CAVALLINI A., NATALI L. & BALCONI C. 1995. Chromosome endoreduplication in endosperm cells of two maize genotypes and their progenies. *Protoplasma*. **189**: 156-162. <https://doi.org/10.1007/BF01280169>
- CEBOLLA A., VINARDELL J. M. & KISS E. 1999. The mitotic inhibitor *ccs52* is required for endoreduplication and ploidy dependent cell enlargement in plants. *The EMBO Journal*. **18**(16): 4476-4484. <https://doi.org/10.1093/emboj/18.16.4476>
- DEL POZO J. C. & RAMIREZ-PARRA E. 2015. Whole genome duplications in plants: an overview from *Arabidopsis*. *Journal of Experimental Botany*. **66**(22): 6991-7003. <https://doi.org/10.1093/jxb/erv432>

- FOLKERS U., BERGER J. & HÜLSKAMP M. 1997. Cell morphogenesis of trichomes in *Arabidopsis*: differential control of primary and secondary branching by branch initiation regulators and cell growth. *Development*. **124**(19): 3779-3786. <https://doi.org/10.1242/dev.124.19.3779>
- GALBRAITH D. W., HARKINS K. R. & KNAPP S. 1991. Systemic endopolyploidy in *Arabidopsis thaliana*. *Plant Physiology*. **96**(3): 985-989. <https://doi.org/10.1104/pp.96.3.985>
- GENDREAU E., TRAAS J. & DESNOS T. 1997. Cellular basis of hypocotyl growth in *Arabidopsis thaliana*. *Plant Physiology*. **114**(1): 295-305. <https://doi.org/10.1104/pp.114.1.295>
- HASE Y., FUJIOKA S. & YOSHIDA S. 2005. Ectopic endoreduplication caused by sterol alteration results in serrated petals in *Arabidopsis*. *Journal of Experimental Botany*. **56**(414): 1263-1268. <https://doi.org/10.1093/jxb/eri122>
- IRISH V. F. 2008. The *Arabidopsis* petal: a model for plant organogenesis. *Trends in Plant Science*. **13**(8): 430-436. <https://doi.org/10.1016/j.tplants.2008.05.006>
- KUDO N. & KIMURA Y. 2001. Patterns of endopolyploidy during seedling development in cabbage (*Brassica oleracea* L.). *Annals of Botany*. **87**(2): 275-281. <https://doi.org/10.1006/anbo.2000.1337>
- LARKINS B. A., DILKES B. P., DANTE R. A., COELHO C. M., WOO Y. & LIU Y. 2001. Investigating the hows and whys of DNA endoreduplication. *Journal of Experimental Botany*. **52**(355): 183-192. <https://doi.org/10.1093/jexbot/52.355.183>
- LEE H. O., DAVIDSON J. M. & DURONIO R. J. 2009. Endoreplication: polyploidy with purpose. *Genes Development*. **23**(21): 2461-2477. <https://doi.org/10.1101/gad.1829209>
- LIU C. M., JOHNSON S. & HEDLEY C. L. 1996. The generation of a legume embryo: morphological and cellular defects in pea mutants. In: WANG T. L. & CUMING A. (eds.). *Embryogenesis: the generation of a plant*. Oxford: Bioscience: 191-213.
- MELARAGNO J. E., MEHROTRA B. & COLEMAN A. W. 1993. Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. *The Plant Cell*. **5**(11): 1661-1668. <https://doi.org/10.1105/tpc.5.11.1661>
- MIZUKAMI Y. 2001. A matter of size: developmental control of organ size in plants. *Current Opinion of Plant Biology*. **4**(6): 533-539. [https://doi.org/10.1016/s1369-5266\(00\)00212-0](https://doi.org/10.1016/s1369-5266(00)00212-0)
- MIZUKAMI Y. & FISCHER R. L. 2000. Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proceedings National Academy of Sciences USA*. **97**(2): 942-947. <https://doi.org/10.1073/pnas.97.2.942>
- NISHIHARA M. & NAKATSUKA T. 2011. Genetic engineering of flavonoid pigments to modify flower color in floricultural plants. *Biotechnology Letters*. **33**(3): 433-441. <https://doi.org/10.1007/s10529-010-0461-z>
- ROEDER A. H. K., CHICKARMANE V., CUNHA A., OBARA B., MANIUNATH B. S. & MEYEROWITZ E. M. 2010. Variability in the control of cell division underlies sepal epidermal patterning in *Arabidopsis thaliana*. *PLoS Biology*. **8**(5): e1000367. <https://doi.org/10.1371/journal.pbio.1000367>
- ROEDER A. H. K., CUNHA A. & OHNO C. K. 2012. Cell cycle regulates cell type in the *Arabidopsis* sepal. *Development*. **139**(23): 4416-4427. <https://doi.org/10.1242/dev.082925>
- SLABODNICK R., RUBY J. G. & REIFF S. B. 2017. The macronuclear genome of *Stentor coerules* reveals tiny introns in a giant cell. *Current Biology*. **27**(4): 569-575. <https://doi.org/10.1016/j.cub.2016.12.057>
- STEEVES T. A. & SUSSEX I. M. 1989. *Patterns in plant development*. New York, NY: Cambridge University Press.
- SZÉCSI J., JOLYC. & BORDJI K. 2006. BIGPETALp, a bHLH transcription factor is involved in the control of *Arabidopsis* petal size. *The EMBO Journal*. **25**: 3912-3920. <https://doi.org/10.1038/sj.emboj.7601270>
- ZHAO Z., LI Y. & LIU H. 2017. Genome-wide expression analysis of salt-stressed diploid and autotetraploid *Paulownia tomentosa*. *PLoS One*. **12**(10): e0185455. <https://doi.org/10.1371/journal.pone.0185455>

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