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Relationship of nuclear genome size, cell volume and nuclei volume in endosperm of *Sorghum bicolor*

Razmerje velikosti jedrnega genoma, prostornine celic in prostornine jeder v endospermu sirka (*Sorghum bicolor*)

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Abstract: Endosperm cells of *Sorghum bicolor* undergo several rounds of endoreplication during seed development, resulting in somatic endopolyploidy with cells containing 3 C to 96 C nuclei (1 C represents the amount of DNA in an unreplicated haploid genome). Cells with higher DNA content are larger and contain larger nuclei. The function of large endosperm cells in *Sorghum bicolor* is storage of starch that will be used in germination. We analysed the ratios of nuclear genome size and volume of nuclei and cells to determine if karyoplasmic ratio is constant in cells of different endopolyploidy levels. Interestingly, the volume of cells and nuclei increases more than can be expected from the increase in genome size alone. Instead, a constant ratio was observed between genome size and surface of cells and nuclei. However, an isometric relationship was found between volume of nuclei and volume of cells, indicating that karyoplasmic ratio is constant in sense of dimensions of cellular compartments, rather than with nuclear genome size alone.

Keywords: cell volume, endopolyploidy, endoreplication, endosperm, nuclear genome size

Izvleček: Tekom razvoja semena sirka (*Sorghum bicolor*) v celicah endosperma poteče več ciklov endoreplikacije, kar se kaže v somatski endopoliploidiji tkiva, ki vsebuje celice s 3 C do 96 C jedri (1 C predstavlja količino DNA v nepodvojenem haploidnem genomu). Celice z večjo količino DNA so večje in vsebujejo večja jedra. Vloga velikih celic v endospermu sirka je shranjevanje založnega škroba, ki bo porabljen med kalitvijo. Analizirali smo razmerje med velikostjo jedrnega genoma in prostornino jeder ter celic, da bi preverili konstantnost karioplazemskega razmerja v celicah z različno stopnjo endopoliploidije. Zanimivo, prostornina celic in jeder se povečuje bolj kot bi pričakovali samo zaradi povečevanja velikosti genoma. Namesto tega smo opazili konstantno razmerje med velikostjo genoma in površino celic in jeder. Poleg tega smo pokazali, da obstaja izometrično razmerje med prostornino jeder in prostornino celic, kar kaže na to, da je karioplazemsko razmerje konstantno v smislu dimenzij celičnih sestavnih delov, ne pa glede na velikost jedrnega genoma.

Ključne besede: endopoliploidija, endoreplikacija, endosperm, prostornina celic, velikost jedrnega genoma

Introduction

Polyploidization in cell differentiation to produce large cells is a widespread developmental strategy throughout the animal and plant kingdoms (Orr-Weaver 2015). Endoreplication is a variation of a cell cycle, where DNA replication in the S-phase is not followed by mitotic division and sister chromatids are not separated into daughter cells. Repeated endoreplication cycles generate chromosomes with an exponentially growing number of chromatids (Joubès and Chevalier 2000, Sugimoto-Shirasu and Roberts 2003). Occurrence of endoreplication is common in plants, but is not related to the initial genome size, it is more correlated to the life strategy and phylogeny of plant groups (Barow and Meister 2003). The significance of endoreplication in organisms may not be due to any specialized function that is supported by endopolyploidy, but rather in the consequences of the endopolyploidy state that makes the polyploid cells different from diploid cells, such as the tissue growth with absence of mitosis, changed surface to volume ratio and differences in gene expression or growth factor gradients (Barlow 1978, Joubès and Chevalier 2000). However, the precise functions of polyploidization is still elusive today (Orr-Weaver 2015). Recently, Bourdon et al. (2012) provided the direct evidence that endopolyploidy increased transcription of rRNA and mRNA on a per-nucleus basis. The positive correlation between ploidy levels and cell size indicates that endopolyploid nuclei might be required for the formation of large cells (Kondorosi et al. 2000), but under certain conditions, the final size of cells and organs can be uncoupled from endoreplication (Cookson et al. 2006). The positive relationship between size of nuclei and size of cells was shown widely throughout the eukaryotes, for example the size of nuclei was correlated to cell volume in yeast (Jorgensen et al. 2007). The constant ratio of nucleus and cell volume was observed already a century ago and led to the hypothesis of a "karyoplasmic ratio" (Wilson 1925). Closely related observations, but should not be considered equivalent to the volume of nuclei, is that variations in genome size is related to cell size (Cavalier-Smith 2005).

Cereal endosperm is a storage tissue that is comprised of cells with different endopolyploidy levels. The function of endosperm cells is accumulation of storage compounds, mainly starch (Kowles et al. 1992). Maize endosperm contains endoreplicated nuclei with at least 192 C DNA content (Vilhar et al. 2002). The endopolyploid state evolved before domestication, since the wild relative of maize, teosinte, contains nuclei with up to 96 C (Dermastia et al. 2009). The initial genome size of maize endosperm cells is 3 C, therefore endoreplication generates 6 C, 12 C, 24 C, etc. cells. The extent of endopolyploidy correlates with the yield of maize grain (Kowles et al. 1992).

The aim of this study was to re-evaluate the data measured by Kladnik et al. (2006) to examine the increase in volume of cells and nuclei of *Sorghum bicolor* with increase in nuclear genome size due to endoreplication and test whether the increase in volume deviates from isometry. Moreover, we examined the ratios of volume and surface of cells and nuclei with nuclear genome size. At last, but not least, we analysed the relationship of nuclear volume with cell volume to test the classical karyoplasmic ratio hypothesis.

Materials and methods

Tissue sections and staining

Developing caryopses of sorghum (*Sorghum bicolor* (L.) Moench) were sampled 5 to 16 days after pollination, fixed in FAA (3.7 % formaldehyde, 5 % acetic acid, 50 % ethanol), embedded in Paraplast Plus (Sherwood Medical Co., USA) and sectioned longitudinally to 12-20 μm thick sections as described in detail by Kladnik et al. (2006). Sections were stained for starch using an aqueous solution of 2 % iodine and 3 % potassium iodide (I_2/KI). Nuclear DNA was stained with Feulgen reagent according to Dolenc Koce et al. (2003) and Kladnik et al. (2006). The Feulgen reaction is quantitative for DNA if the only aldehydes remaining in the cell are those produced from the hydrolysis of DNA (Feulgen and Rossenbeck 1924). Sections were observed on an Axioskop 2 MOT microscope (Carl Zeiss, Germany) and images were acquired with an AxioCam MRc digital camera (Carl Zeiss Vision, Germany).

Measurement of genome size, cell volume and nuclei volume

Nuclear genome size was measured on Feulgen stained median longitudinal sections of caryopses at 5, 8, 10, 12 and 16 days after pollination (DAP) in three replicates. The nuclear DNA amount was measured by image densitometry using the interphase-peak method adapted for use with tissue sections (Vilhar et al. 2002, Dermastia et al. 2009). Integrated optical density (a measure of relative DNA amount), size and positions of the nuclei were measured in the whole endosperm transect. The amount of nuclear DNA was expressed in C-value units, with 1 C representing the nuclear DNA content of a non-replicated haploid genome. The volume and surface area of a nucleus was estimated as a sphere based on the area of the nucleus section in the acquired image. The size of cells was measured by outlining the cell walls, visible due to their autofluorescence in UV. Volume and surface of cells was estimated as a sphere based on the area of the cell transects in the image. In total, we measured nuclear DNA content and size of cells and nuclei for 995, 1651, 2711, 2470 and 2587 cells in 5, 8, 10, 12 and 16 DAP samples, respectively.

Data analysis

Data was analysed using R version 3.1.2 (R Core Team 2014) in RStudio version 0.98.1102 and GraphPad Prism version 6.01 for Windows (GraphPad Software, USA). The ratios of nuclear genome size with different cell size parameters were calculated by dividing measured relative DNA amount, normalized to C-units, with respective cell size parameter, measured in µm. Variation of volumes and ratios with respect to nuclear genome size was analysed using linear model on logarithmically transformed data in R and calculating R^2 value to express the portion of the variance explained by variation in nuclear genome size. The relationship between cell volume, nuclei volume and different ratios with genome size was analysed by calculating medians in all endopolyploidy classes (3 C to 96 C) and performing linear regression in Prism, thus obtaining the slope of the log-log relationship.

Results

The endosperm of *Sorghum bicolor* is composed of cells with variable sizes, containing nuclei

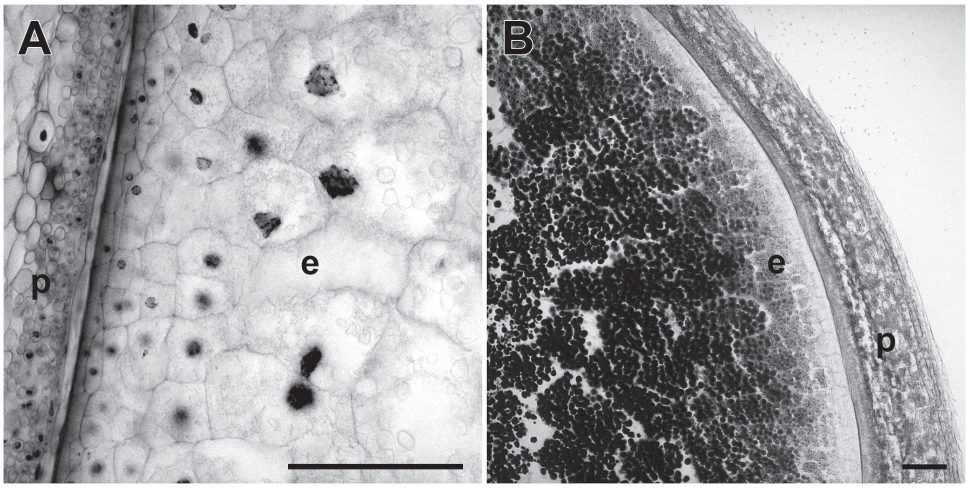

Figure 1: Endosperm of *Sorghum bicolor* 16 days after pollination. A - Feulgen stained tissue section, showing size of nuclei and cells of different endopolyploidy levels. B - Starch deposition in endosperm cells. Labels: e, endosperm; p, pericarp. Bar represents 100 µm.

Slika 1: Endosperm sirka (*Sorghum bicolor*) 16 dni po oprašitvi. A - Tkivna rezina barvana po Feulgen-u, ki prikazuje velikost jeder in celic različnih endopoliploidnih stopenj. B - Nalaganje škroba v celicah endosperma. Oznake: e, endosperm; p, perikarp. Merilo predstavlja 100 µm.

with different amounts of DNA that is replicated in multiple endocycles (Fig. 1A). The endosperm cells contain large amounts of starch in the form of starch grains, with exception of the smallest cells in the outermost layer of the endosperm (Fig. 1B). The cells in the sorghum endosperm undergo several rounds of endoreplication, resulting in nuclei with DNA content up to 96 C (Kladnik et al. 2006).

We have tested the effects of nuclear genome size variation on variations in volume of cells and nuclei. Duplication of DNA during endoreplication cycles and growth of cells and nuclei are exponential processes by nature, so all relationships were plotted on log-log graphs. Scatterplots (Fig. 2) represent all measured data for samples 10 to 16 days after pollination (DAP), where all possible endopolyploidy classes were present (3 C to 96 C). Five and eight DAP samples lacked the highest endopolyploidy levels (Kladnik et al. 2006). The volume of cells increases with increasing nuclear genome size (Fig. 2A), with 65 % of variation explained by variation in genome size ($R^2 = 0.65$). Volume of nuclei is tightly related to nuclear DNA amount (Fig. 2B), with 95 % of variation explained by genome size ($R^2 = 0.95$). Furthermore, we examined the ratio of nuclear genome size with cell dimensions. The ratio of genome size with cell volume (Fig. 2C), expressed as C-units per unit of cell volume, shows a slight negative correlation with genome size with only 13 % of its variation explained by genome size ($R^2 = 0.13$). The ratio of genome size with nuclei volume (Fig. 2D), expressed as C-units per unit of nuclei volume, shows a negative correlation, with 64 % of variation accounted for genome size ($R^2 = 0.64$). However, when we calculate a ratio of genome size versus cell surface (Fig. 2E) or nuclei surface (Fig. 2F), the variation in the ratio is no longer dependent on variation in nuclear genome size ($R^2 = 0.01$ and 0.02, respectively).

To test if increase in volume of cells and nuclei, related to increase in genome size, follows a power law ($y = bx^a$), that is if their relationship is allometric, we calculated median values of volumes of cells and nuclei belonging to different endopolyploidy classes, and plotted volume versus C-value on a log-log scale (Fig. 3A). If the relation between both is exponential, the data would be distributed on a straight line. The slope of the fitted line (exponent *a* in the above equation) indicates the type of allometry (Barow 2006). Both for cell volume and nuclei volume, the slope was larger than 1, indicating positive allometry, 1.44 and 1.34, respectively (Tab. 1). The volume of cells and nuclei increases with a higher rate, than can be attributed to the increase in genome size alone.

Maintenance of karyoplasmic ratio can be examined by calculating ratio of nuclear genome size versus cell volume, thus obtaining the amount of genome (C-units) per unit of cell volume (Fig. 3B). The ratio is related to different endoploidy levels; on log-log scale their relationship is linear with a negative slope (Tab. 1), indicating that progressively less genome units are found in a unit of cell volume. Similarly, the ratio of genome size per volume of nuclei also shows a negative linear relationship with increasing ploidy level (Fig. 3B, Tab. 1). However, when calculating the ratio between genome size and surface area of cells or nuclei, and comparing it to ploidy level, the ratio is constant in the whole range of endoploidy levels (Fig. 3B). The slope of the linear relationship between genome size to surface area on a log scale is not significantly different from zero (Tab. 1). Finally, if using nuclear genome size only to separate cells into different endoploidy classes, the relationship between volume of nuclei and volume of cells is isometric, with a slope not significantly different from 1 (Fig. 3C, Tab. 1).

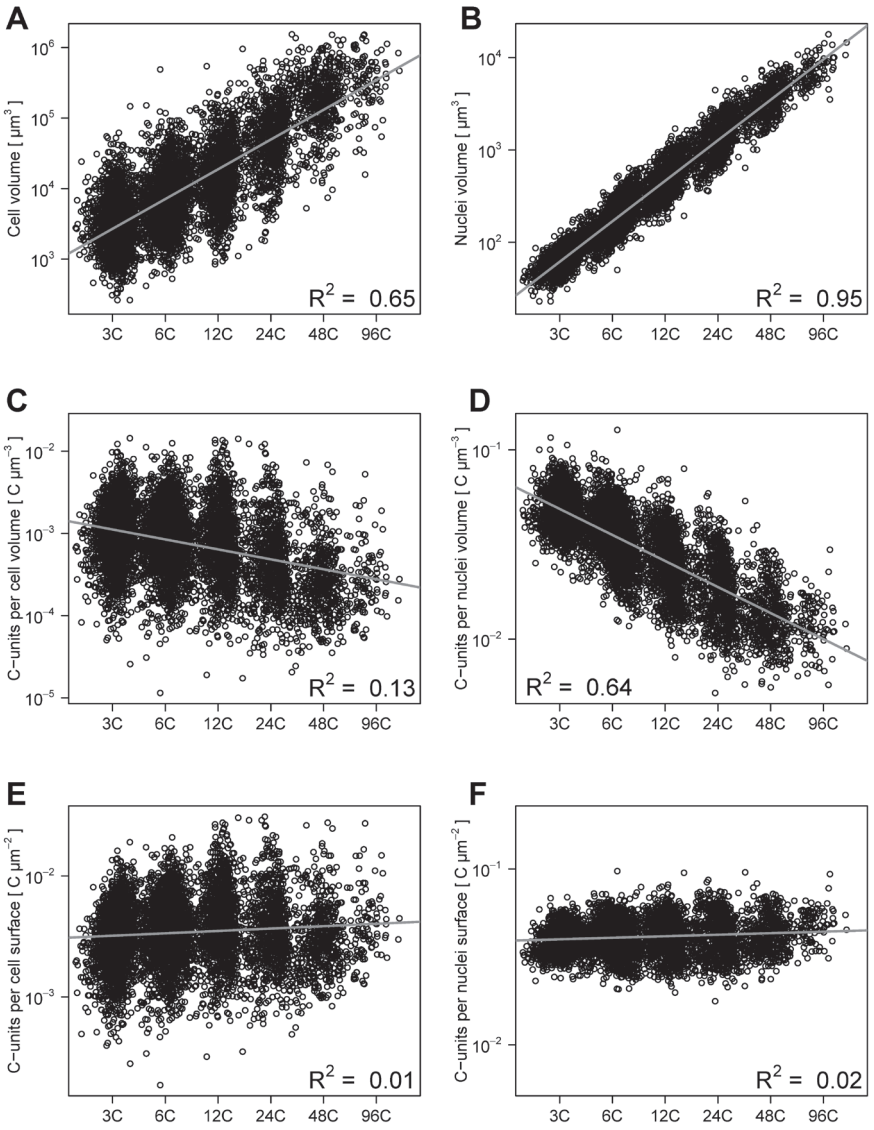

Figure 2: Volume of cells and nuclei of different endopolyploidy levels in endosperm of *Sorghum bicolor* and ratios of nuclear genome size to cell size parameters. A - cell volume, B - nuclei volume, C - ratio of nuclear genome size per cell volume, D - genome size per nuclei volume, E - genome size per cell surface area, F - genome size per nuclei surface area. The range of observed nuclear genome sizes was 3 C to 96 C. Grey line represents a linear fit applied to logarithmically transformed data, R^2 value represents the percentage of variation in the examined parameter explained by the variation in nuclear genome size.

Slika 2: Prostornina celic in jeder različnih stopenj endopoliploidnosti v endospermu sirka in razmerja med velikostjo genoma in parametri velikosti celic. A - prostornina celic, B - prostornina jeder, C - velikosti genoma na prostornino celic, D - velikost genoma na prostornino jeder, E - velikost genoma na površino celic, F - velikost genoma na površino jeder. Razpon velikosti jedrnega genoma je 3 C do 96 C. Sive črte predstavljajo linearni model na logaritmiranih podatkih, R^2 vrednost je delež variance v preučevanem parametru, pojasnjen z endopoliploidijo.

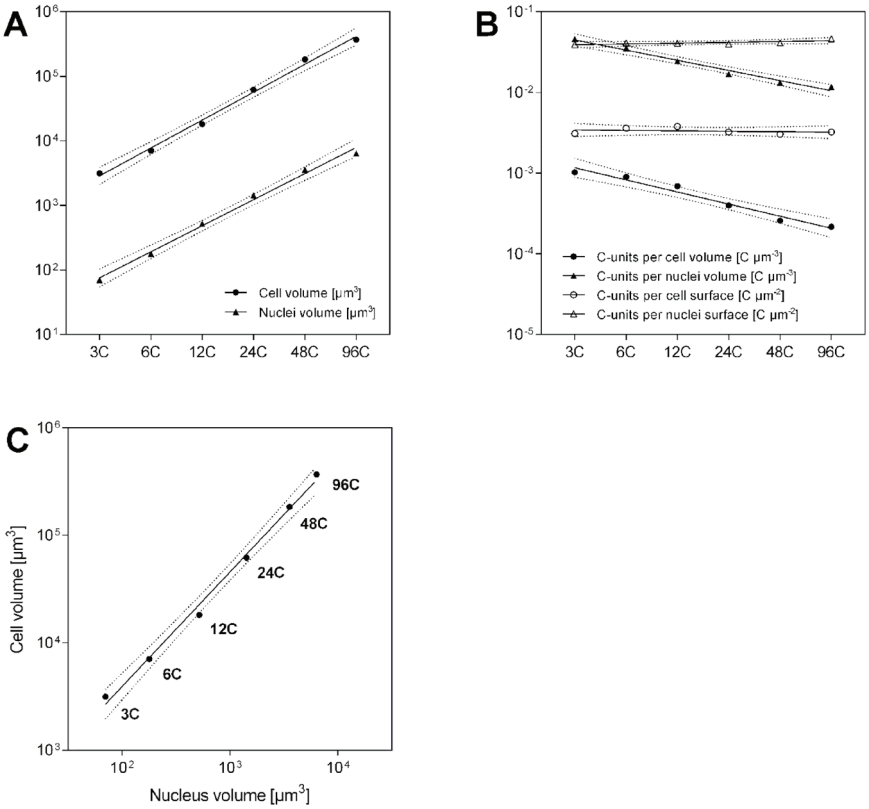

Figure 3: Relationship between endopolyploidy level and size of cells and nuclei. A - Volume of cells and nuclei of different endopolyploidy levels (3 C to 96 C). B - Ratios of nuclear genome size with cell size parameters (volume of cells and nuclei, surface of cells and nuclei). C - Relationship between volume of nuclei and volume of cells of different endoploidy levels. Data presented are median values calculated for individual endopolyploidy classes. Solid lines represent linear regressions with 95 % confidence intervals (dashed lines).

Slika 3: Razmerje med stopnjo endopoliploidnosti in velikostjo celic in jeder. A - Prostornina celic in jeder različnih stopenj endopoliploidnosti (3 C do 96 C). B - Razmerja med velikostjo jedrnega genoma in parametrov velikosti celic (prostornina celic in jeder, površina celic in jeder). C - Razmerje med prostornino jeder in prostornino celic različnih stopenj endopoliploidnosti. Predstavljeni podatki so mediane vrednosti izračunane za posamezne razrede endopoliploidnosti. Polne črte predstavljajo linearno regresijo s 95 % intervalom zaupanja (črtkane črte).

Table 1: Relationship between endopolyploidy and cell size parameters. Slope of log-log relationship calculated from median values for individual endopolyploidy classes (Fig. 3A, B) or log-log relationship between cell volume and nucleus volume (Fig. 3C). Slope = 1 indicates isometry, slope > 1 indicates positive allometry (for volumes), slope = 0 indicates no relationship. Slope error based on 95% confidence intervals, * - deviation from zero is significant ($p < 0.001$), ns0, ns1 - deviation from zero or 1 not significant ($p > 0.05$); R^2 value is a portion of variance in the examined parameter explained by endopolyploidy (Fig. 2) or nucleus volume ($p < 0.001$).

Tabela 1: Razmerje med endopoliploidijo in parametri velikosti celic. Naklon log-log razmerja, izračunanega iz medianih vrednosti za posamezne razrede endopoliploidnih celic (Fig. 3A, B) ali log-log razmerja med prostornino celic in prostornino jeder (Fig. 3C). Naklon = 1 pomeni izometrijo, naklon > 1 pomeni pozitivno alometrijo (za prostornine), naklon = 0 pomeni, da ni odvisnosti. Napaka naklona izvira iz 95 % intervala zaupanja, ns0, ns1 - odklon od 0 oz. 1 ni statistično značilen ($p > 0.05$); R^2 vrednost je delež variance v preučevanem parametru, pojasnjen z endopoliploidijo (Fig. 2) ali prostornino jedra ($p < 0.001$).

	Slope	R^2 (linear model)
Cell volume [μm^3]	$1.44 \pm 0.05^{*}$	0.65
Nuclei volume [μm^3]	$1.34 \pm 0.05^{*}$	0.95
C-units per cell volume [C μm^{-3}]	$-0.50 \pm 0.05^{*}$	0.13
C-units per nuclei volume [C μm^{-3}]	$-0.42 \pm 0.03^{*}$	0.64
C-units per cell surface [C μm^{-2}]	-0.02 ± 0.03^{ns0}	0.01
C-units per nuclei surface [C μm^{-2}]	0.03 ± 0.01^{ns0}	0.02
Cell volume by nuclei volume	1.07 ± 0.04^{ns1}	0.68

Discussion

The early observations of a constant relationship between volume of nuclei and volume of cells led to the "karyoplasmic ratio" hypothesis (Wilson 1925). The exponential nature of this relationship was reported already by Sinnot and Trombetta (1936). Numerous studies reported on a tight relationship between volume of cells and nuclei and genome size (Cavalier-Smith 2005, Barow 2006). A clear relationship between nuclear genome size (endopolyploidy level) and volume of nuclei and cells was shown in *Sorghum bicolor* endosperm. The large endoreplicated endosperm cells are associated with deposition of starch (Kladnik et al. 2006). In the present study, the relationship was further characterized. The volume of cells is increasing by a higher rate than would be proportional with doubling of the ploidy level, since the slope of their log-log relationship is higher than 1, indicative of positive allometry (Barow 2006). The volume of cells more than doubles with each endocycle, and this in turn influences the ratio of genome size per cell volume that shows a negative slope in relation to ploidy level, although with a low R^2 value. The motivation for calculating a ratio of genome size with cell volume is to express the amount of genome that is in charge of a unit of tissue volume, or simply "genome concentration". Surprisingly, the volume of nuclei also shows a similar positive allometric relationship with nuclear genome size and a negative slope for the genome size to nuclei volume ratio. This indicates that the volume of nuclei increases with a higher rate as can be expected from the duplication of DNA alone. A similar positive allometry for nuclei has been observed in mesocarp cells of *Cucumis melo* (Kladnik, unpublished observation).

Of great interest is an observation that genome size to surface ratio is constant both for cells and nuclei. The possible explanation for both is however different. Nucleus volume increases with increasing endopolyploidy level at a rate higher than 1, but the genome to surface ratio remains constant throughout the endopolyploidy range. This indicates that the surface area of the nucleus (nuclear envelope) is crucial for the function of nucleus. Indeed, increased nucleus surface

in endopolyploid cells was observed in tomato pericarp, where large endopolyploidy nuclei show deep invaginations (Bourdon et al. 2012). The authors hypothesize that nuclear exchange ability is maintained by keeping the ratio between nuclear envelope area with nuclear volume constant. Another possibility for maintenance of nuclear exchange ability is peripheral distribution of DNA in nuclei of higher endopolyploidy levels, observed in mesocarp of *Cucumis melo* (Kladnik, unpublished observation). In the present analysis, a higher rate of nuclear volume increase in endopolyploid nuclei of sorghum endosperm as can be expected from duplication of DNA, compared to a constant ratio of genome size to nuclear surface, indicates that the nuclear exchange ability is most likely the important factor that needs to be maintained with increase of nuclear genome size in endoreplication. However, the explanation for a higher rate of cell volume growth is not so straightforward. The positive allometric relationship between cell volume and nuclear genome size is seemingly not in accordance with a hypothesis of a constant karyoplasmic ratio. As noted by Edgar et al. (2014), expansion of plant cells is often driven by increasing the size of the fluid-filled vacuole and it is not necessarily accompanied by increased mass of proteinaceous cytoplasm. Here, the endopolyploid endosperm cells are filled with starch grains and we could speculate that the volume of cytoplasm is related more to the surface area of the cell than with the volume of the whole cell. On the other hand, we have shown an ideal karyoplasmic ratio if we compare cell volume to the volume of the nucleus, not to the size of the genome it contains. Therefore, we can conclude that in this case, the main factor influencing endosperm cell size is not its DNA content, but rather the nuclear exchange ability that is limiting gene expression and cell metabolism.

Summary

The data on sorghum endosperm cell volume, nuclei volume and nuclear genome size (Kladnik et al. 2006) was re-evaluated to examine the relationship between cell parameters. Endosperm cells contain endoreplicated nuclei with DNA content from 3 C to 96 C and the volume of cells and nuclei is positively correlated with nuclear genome size. The larger cells with higher DNA content are located in the central part of endosperm and accumulate starch. The relationship of cell and nuclei volume with genome size is positively allometric, ie. the volume increases by a higher rate than can be expected from DNA duplication alone. Instead, a constant ratio was observed between genome size and surface of cells and nuclei. Finally, an isometric relationship was shown between the volume of nuclei and volume of cells, indicating that a constant karyoplasmic ratio is related to the volume of nucleus, not to its DNA content.

Povzetek

Podatke o prostornini celic in jeder, ter velikosti jedrnega genoma v endospermu sirka (Kladnik et al. 2006) smo dodatno analizirali, da bi ovrednotili razmerja med celičnimi parametri. Celice endosperma vsebujejo endoreplicirana jedra, ki vsebujejo 3 C do 96 C DNA. Prostornina celic in jeder je v pozitivni korelaciji z velikostjo jedrnega genoma. Večje celice z višjo vsebnostjo DNA so v osrednjem delu endosperma in kopičijo založni škrob. Razmerje prostornine celic in jeder z velikostjo genoma je pozitivno alometrično, kar pomeni, da se prostornina povečuje hitreje, kot bi pričakovali samo zaradi samega podvajanja DNA. Po drugi strani smo opazili konstantno razmerje med velikostjo genoma in površino celic in jeder. Pokazali smo tudi izometrično razmerje med prostornino jeder in prostornino celic, kar kaže na to, da je konstantno karioplazemsko razmerje povezano s prostornino jedra, ne pa s količino DNA, ki jo le-ta vsebuje.

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