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Short Communication

The structural and molecular analysis of endoreduplicated nuclei in tomato (*Solanum lycopersicum*) fruit provides evidence for a ploidy-dependent increase in transcriptional activity

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Abstract Endopolyploidy, i.e. the amplification of genomic DNA without mitosis, is a widespread process in plants. Cells from the tomato fruit pericarp are characterized by a wide range of ploidy levels (from 2C to 256C). Although various functional hypotheses have been attributed to endoreduplication according to the literature, evidence for a specific role of endoreduplication in transcription and metabolism control is still lacking. We have developed a new method based on bacterial artificial chromosome fluorescent in situ hybridization (BAC-FISH) that allows the in situ determination of DNA ploidy levels of individual nuclei. The advantage of this method is illustrated by the analysis of ploidy levels and cell sizes within the pericarp tissue from mature green tomato fruits. Using this cellular approach we established the ploidy map of the pericarp tissue. Based on this map, we performed a structural analysis of endoreduplicated nuclei at the level of chromatin organization, nuclear shape and relationship with mitochondria. We demonstrated a link between the ploidy level of nuclei, the complexity of their shape and the number of mitochondria at the vicinity of polyploid nuclei. The use of the DNA FISH method demonstrated that endopolyploidy leads to the formation of polytene chromosomes, whereas the use of a RNA FISH method demonstrated that the rDNA transcription was increased during polyploidization. Performing quantitative PCR (qPCR) and RT-qPCR on sorted nuclei respectively, we confirmed that endoreduplication did amplified exponentially loci for a set of specific genes allowing us to demonstrade that endoreduplication results in an increasing transcriptional activity.

Key words: BAC-FISH, endoreduplication, karyoplasmic homeostasis, tomato fruit, transcription.

In the model fleshy fruit of tomato (*Solanum lycopersicum* Mill.), the early fruit development can be divided into three distinct phases (Gillaspy et al. 1993). Phase I corresponds to the development of the ovary, and the decision to set fruit. In Phase II the ovary develops through an intense activity of cell divisions. In phase III fruit growth is mainly sustained by cell expansion leading to a fruit which exhibits its almost final size and is able to ripen. Then fruit ripening is characterized by dramatic changes in color, texture and taste, which contribute to the build-up of the fruit sensory quality (for a review, see Pirrello et al. 2009). Cell size in tomato fruit can reach spectacular levels such as hundreds of times its initial size (e.g. >0.5 mm in diameter) (Cheniclet et al. 2005). These huge cells are found within the pericarp (fleshy part) and the locular (gel) tissue which are characterized by the arrest of mitotic activity. This spectacular cell expansion triggering the tomato final size is intimately associated to endopolyploidy (Cheniclet et al. 2005; Joubès and Chevalier 2000; Joubès et al. 1999) resulting in the appearance of very high ploidy levels (256–512C) at the end of fruit development (Bourdon et al. 2010). This increase in ploidy levels induces a hypertrophy of the nucleus which ultimately influences the final size of cells. Indeed there is a clear correlation between the mean cell size in the pericarp of various tomato genotypes and the mean ploidy level of developing fruit which can explain this observed gradation in cell size in tomato fruit (Cheniclet et al. 2005; Chevalier et al. 2011). Taking advantage of the extraordinary extent of

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the endoreduplication process occurring in tomato fruit, we contributed to provide new and important insights on the molecular mechanisms triggering the exit of the mitotic cycle towards the onset of the endoreduplication cycle associated to cell expansion (for a review, see Chevalier et al. 2011). We demonstrated very clearly that modifying endoreduplication during fruit development impacts on fruit growth and final fruit size (Gonzalez et al. 2007; Mathieu-Rivet et al. 2010).

Recently, it was demonstrated that endoreduplication is crucial for cell fate acquisition and maintenance, thus contributing to the formation of specialized cell type patterns, as an important determinant of cell identity (Bramsiepe et al. 2010; Roeder et al. 2010).

Since endopolyploidy is involved in organ and cell differentiation and in many developmental and physiological processes (Lee et al. 2009), addressing the DNA content of individual nuclei in situ would be of great help in a developmental perspective of the endoreduplication function. We here investigated the temporal and spatial distribution of endoplolyploidy in the pericarp fruit tissue with the aim to provide an important step towards understanding its physiological role. In addition, we provided the first direct evidence that endoreduplication plays a role in increased transcription of rRNA and mRNA on a per-nucleus basis, and demonstrated that endoreduplication contributes in the establishment of a highly structured cellular system integrating multiple physiological.

Fluorescent In situ hybridization: a method to quantify ploidy levels in planta

DNA contents of individual nuclei can be determined essentially according to two kinds of methodology: densitometry and fluorometry. These two methods are based on DNA staining: densitometry is performed according to the Feulgen reaction using the Schiff's reagent (Greilhuber 2008), and fluorometry makes use of a fluorochrome such as 4′,6′-diamino-2-phenylindole (DAPI) or propidium iodide (Dolezel and Bartos 2005; Johnston et al. 1999). In both cases the quantification of the signal is monitored either by a photometer equipped with diaphragms suited to the nuclear size (photometric cytometry) or by analysis of images generally acquired with a charge-coupled device (CCD) camera (image cytometry). Although the use of these methodologies was proven to be efficient for the in situ measurement of ploidy levels in young tissues with small cells and nuclei, in monolayer tissues (epidermis) or in paraffin sections (Dermastia et al. 2009; Gendreau et al. 1998; Melaragno et al. 1993), they appear largely limited to be applied to a so three-dimensionally complex organ such as tomato fruit. Nevertheless, we attempted to measure the size of nuclei after DAPI staining as a quantitative marker of endopolyploidy in tomato pericarp, since the nuclear size is often reported to increase according to ploidy level (for a review, see Bourdon et al. 2010). Nuclei from the pericarp tissue were sorted by flow cytometry according to ploidy levels (ranging from 2C to 128C DNA content) and collected on microscope slides. We then measured their respective area. A clear correlation between nuclear area and ploidy level was indeed observed, but the large standard deviations led to partial overlapping of surface values corresponding to close ploidy levels. As a consequence, the area measurements of nuclei did not allow the determination of the precise ploidy level of a nucleus (Bourdon et al. 2011). When performed in situ on thin pericarp slices stained with DAPI, the determination of nuclear sizes and the subsequent estimation of ploidy levels from two-dimensional (2-D) images were obviously not robust, especially as the in situ orientation of the nuclei is much different from that of isolated nuclei. In conclusion, the very high ploidy levels (ca. 256C) and large cells, that are reached and generated respectively at the end of fruit development (Bourdon et al. 2010) together with the 3-D structure of the fruit pericarp, renders the use of this DNA-staining method totally inappropriate to determine cell ploidy level in situ.

We thus looked for a new technique aimed at measuring in situ the DNA ploidy level of individual endopolyploid nuclei in tomato fruit pericarp tissue. We developed a method based on the detection of a given chromosomal locus by Fluorescent In Situ Hybridization (FISH) whose amplification during endopolyploidy is expected to be directly correlated to DNA ploidy levels. The principle of this method relies on the direct visualization of the copy number of a locus, as revealed by fluorescent dots in the nuclear volume, and the determination of dot number to estimate the corresponding C DNA content. The main advantage of this method relies on object counting and is therefore not dependent upon fluorescence intensities. Using Bacterial Artificial Chromosome (BAC) clones, chosen for their tomato chromosome specificity, we tested this method on nuclei sorted by flow cytometry according to their ploidy levels as described in Bourdon et al. (2011). A strong, linear correlation ($R^2=0.92$) was found between ploidy levels and dot numbers, with a low and highly satisfactory standard deviation for dot number counts. Conversion of the dot number counts into DNA ploidy levels matched with the known ploidy levels of flow-cytometry sorted nuclei, thus demonstrating the relevance of this FISH method to assign a ploidy level to individual isolated nuclei.

Tomato pericarp is characterized by an organized mosaic distribution of ploidy levels

Using flow cytometry and nuclei preparation from tomato pericarp, all ploidy levels ranging from 2C to 256C can be detected within this tissue, indicating

a strong heterogeneity. To specify the organization of cells according to their ploidy level across tomato pericarp, we then applied the developed FISH method to analyze endopolyploidy patterns in mature green tomato pericarp and establish a ploidy map of the pericarp (Figure 1). Our results revealed that pericarp is a highly organized tissue. Ploidy was found to increase gradually from the outer epidermis (2C) to the sixth cell layer (64C). Throughout mesocarp, the ploidy is elevated (64–128C), except in the eleventh cell layer that displays a slight decrease in ploidy levels (between 32C and 64C). In the last cell layer (no. 13) forming the inner epidermis, lower ploidy levels are reached (16C). Cells close to vascular bundles display intermediate ploidy levels (between 8C and 32C). Despite this high degree of tissular organization, the ploidy map confirmed that there is a significant heterogeneity of ploidy levels not only across the pericarp tissue but also inside each cell layers, each one displaying 2 to 5 different ploidy levels.

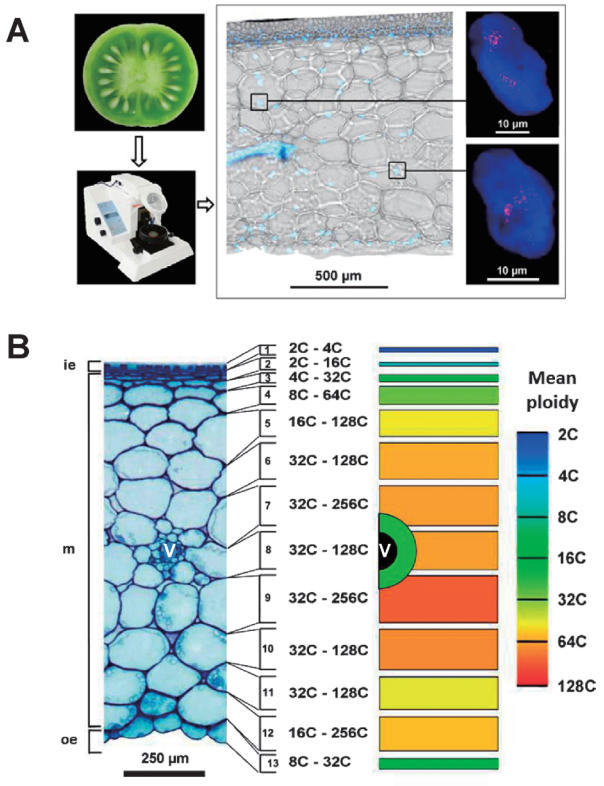

Figure 1. Application of a BAC-FISH method to establish the ploidy map of tomato fruit pericarp. A. Principle of the BAC-FISH method. Pericarp pieces from mature green fruit equatorial region were prepared and fixed in a 4% paraformaldehyde solution. 150 μm sections were obtained using a vibrating blade microtome prior to perform the BAC-FISH method. Using a BAC specific to chromosome 7 as a probe, the hybridizing signals visualized as red spots (Texas Red) were counted to assign to each observed nucleus the respective deduced ploidy level. B. Ploidy map of mature green tomato pericarp. Mean ploidy levels in the 13 composing cell layers of the mature green fruit pericarp are indicating according to the heat map. Oe: outer epidermis; m: mesocarp; ie: inner epidermis; V: vascular bundle.

Since the FISH technique allows the preservation of cell structures within a tissue, we took advantage of this convenient method to determine the relationship existing between cell size and ploidy levels in every cell layers of the fruit pericarp. The global distribution of cell area according to cell layer matched remarkably that of ploidy levels (as recorded by the Endoreduplication Index, EI). This observation is in total accordance with the frequently reported link between cell growth and endopolyploidisation (Kondorosi and Kondorosi 2004; Sugimoto-Shirasu and Roberts 2003). Although a positive correlation between ploidy level and cell size was observed at the level of individual cells, a high ploidy level could be observed for some small cells (for instance, within the external cell layers 2 and 3 of the pericarp), suggesting that endopolyploidy can take place in the quasi-absence of cell growth or before the onset of the cell growth phase. This deviation in the correlation may be attributed to a cell layer effect, thus indicating that cell size is not only dependent on ploidy levels but also on the position of the cell within the pericarp.

Tomato chromosomes display a polytenic structure

Several cellular mechanisms may lead to endopolyploidy, such as the generation of multinucleate cells originating from acytokinetic mitosis, nuclear fusion, endomitosis or endoreduplication. The last two mechanisms are the most frequently encountered modes of polyploidization in eukaryotes (Lee et al. 2010). During endomitosis, the sister chromatids separate after chromosome doubling and condensation and return to the interphase state within an intact nuclear membrane as in the mitotic cycle. Thus endomitosis differs essentially from mitosis by the absence of a mitotic spindle and leads to a doubling of chromosome number during each endomitotic cycle. Endomitosis is barely encountered in the plant kingdom (D'Amato 1984), but may arise as a consequence of a mutation of genes essential for the progression into the cell cycle, such as *GIGAS CELL1*, a negative regulator of the Anaphase-Promoting Complex/Cyclosome that is required for proper mitotic progression (Iwata et al. 2011). The second mechanism to generate endopolyploid nuclei corresponds to endoreduplication. This mechanism is characterized by a duplication of DNA without separation of chromatids. Chromatids thus remain attached to the centromere; chromosomes with multivalent (2, 4, 8, 16, etc...) chromatids are thus produced and consequently the chromosome number stays unchanged (Joubès and Chevalier 2000). The application of the FISH technique on sorted nuclei or on pericarp tissue in situ, using chromosome-specific BAC probes, resulted in 2 clusters of hybridization signals inside nuclei, whatever the ploidy level is. When a DNA probe encompassing the

45S rDNA locus located on chromosome 2 within the Nucleolar Organising Regions (NORs) of the tomato genome (Arumuganathan et al. 1994; Chang et al. 2008; Jo et al. 2009) was used, we observed two large hybridisation signals whose relative size correlated with the extent of ploidy. Finally, the use of Chromomycin A3, a DNA-specific dye that preferentially stains GC-rich regions such as in the NORs, stained two large chromosomal regions similar to the 45S rDNA hybridization signal. Altogether these experiments demonstrated that chromatids stay linked together within polyploid chromosomes, that the chromosome number is unchanged during endopolyploidisation in tomato fruit tissues. As a result we could ascertain that endopolyploidisation in tomato originates from the endoreduplication process, leading to the production of polytenic chromosomes. The significance of the occurrence of polytenic chromosomes with linked sister chromatids might represent biological and functional advantages for a cell. By restricting gene duplication within a nuclear region, the same transcriptional machinery, involving transcription factors and RNA polymerases, can be used for transcriptional activity.

Karyoplasmic exchanges are maintained in endoreduplicated nuclei

The consequence of endopolyploidization on nuclear morphology within the pericarp tissue was analyzed by fluorescent microscopy and Transmission Electronic Microscopy (TEM). We observed the shape of nuclei within the three most contrasted region in terms of ploidy levels, ca. the Outer Epidermis (OE, where cells display low ploidy levels), the Internal Epidermis (IE, where ploidy levels reach medium values), and the Mesocarp (M, characterized by high ploidy levels). The use of the lipophilic fluorescent dye $DiOC_6(3)$ (for labeling intracellular membranes; Terasaki and Reese 1992) and DAPI (for DNA) to stain the fruit pericarp sections, highlighted a strong correlation between ploidy level and the complexity of the nuclear shape (Bourdon et al. 2012). Indeed small and less polyploid nuclei from OE are nearly perfectly round. Medium sized nuclei from IE are slightly grooved. Large and highly polyploid nuclei from M display a complex shape with numerous invaginations. Interestingly, the nuclear-cytoplasm exchange ability estimated by measuring the nuclear area (A) to the perimeter (P) (P/A) ratio on TEM images of sections of nuclei all across the pericarp tissue was found to be maintained during endopolyploidisation despite the increase in nuclear volume. Hence this suggests that the invaginated morphology of endoreduplicated nuclei enables the maintenance of an efficient nucleus-to-cytoplasm communications, by minimizing the distance between the nuclear compartment and its envelope communicating with the cytoplasm.

As a membrane-specific dye, $DiOC_6(3)$ revealed the presence of numerous mitochondria in the close vicinity of the nuclear envelope. Interestingly the number of mitochondria was proportionally higher within the numerous grooves characterizing the highly polyploid nuclei. In order to quantify the number of mitochondria, we then used Rhodamine-123 as a stain labeling mitochondria in living cells (Emaus et al. 1986). The mean number of mitochondria surrounding a nucleus was found to be proportional to its size and ploidy level. This was confirmed by counting mitochondria close to the nuclear envelope on TEM images. Interestingly many mitochondria co-localized with the nuclear envelope invaginations of highly polyploid nuclei. Altogether these observations suggest an increase of the exchange ability between the inside and the outside of the nucleus for efficient nucleus-to-cytoplasm communications, and more particularly a bigger exchange between mitochondria and nucleus, probably to provide a greater energy supply for nuclear biochemical processes such as DNA replication or transcriptional activity during endoreduplication.

Endoreduplication triggers an enhanced nuclear transcriptional activity

The numerous mitochondria observed in the vicinity of polyploid nuclei and within their envelope invaginations suggest an enhanced requirement of energy for the nuclear activity. Since the main nuclear activity is transcription, we assessed the transcriptional activity according to ploidy levels using three complementary different approaches.

It would be expected that an increased transcriptional activity could lead to an increase in protein translation and thus in a sustained ribosome biogenesis required for translation (Rudra and Warner 2004). To reveal the ribosome biogenesis, we assessed the transcription of 5.8S rRNA. We used a 5.8S rRNA probe in a RNA FISH experiment. We could observe an increase in the hybridisation signal for the 5.8S rRNA which was positively correlated with the nucleolar area, and thus with the ploidy level (Bourdon et al. 2011).

In order to visualize the transcriptional activity inside endoreduplicated nuclei, we used a specific antibody recognizing the active phosphorylated form of RNA Pol II to perform an immunocytolocalization on fruit pericarp transverse sections. The spatial distribution of the active RNA Pol II was found homogeneous and very similar whatever the position of the detected nucleus in the pericarp, i.e. whatever the ploidy level. Interestingly the active RNA Pol II was excluded from the nuclear periphery and more generally from intense DAPI-stained regions corresponding to dense heterochromatic regions. The quantification of active versus inactive forms of RNA Pol II was determined using a dually

immunolabelled nuclear suspension and specific antibodies raised against the active phosphorylated form and the unphosphorylated inactive form of RNA Pol II. The fluorescence intensities resulting from each immunolabelling were simultaneously quantified for each ploidy class by flow cytometry. The two forms of RNA pol II were both positively correlated to ploidy levels, showing that the protein level for RNA pol II increased proportionally with ploidy.

Finally, we investigated the gene expression during endoreduplication. We selected five single-copy genes coding for the following proteins from tomato: RPB1, the large subunit of RNA pol II; CCS52A and WEE1, two key proteins involved in the regulation of the endocycle (Gonzalez et al. 2007; Mathieu-Rivet et al. 2010); two mitosis-associated proteins: CDKB2, a cyclin-dependent kinase specific to the G2-M transition (Chevalier 2007) and the kinetochore MIS12 protein (Sato et al. 2005). The genes encoding these two mitosis-specific proteins were thus used as negative controls of gene expression in endoreduplicated nuclei. As a first step, we checked by quantitative PCR on genomic DNA that the numbers of gene copy does increase proportionally to ploidy levels. Then we assessed by qRT-PCR the transcript accumulation for this set of candidate genes using total nuclear RNA extracted from sorted nuclei at 4C, 16C and 32C ploidy levels. The amount of *RPB1*, *CCS52A* and *WEE1* transcripts increased accordingly to ploidy levels, whereas CDKB2 and MIS12 transcripts were undetectable as expected for mitosis-associated genes.

Taken together these three different approaches demonstrated that the nuclear transcriptional activity increases according to the ploidy level, while keeping the fine tuning of gene regulation.

Conclusions

Endopolyploidy is of widespread occurrence in plants and endoreduplication represents the most common mode of cell endopolyploidization in plants with more than 90% of Angiosperms species concerned. To circumvent the scarcity and practicality of available methods for investigating the spatial distribution of endoreduplication in complex tissues, we developed an innovative BAC-FISH method. The use of this method

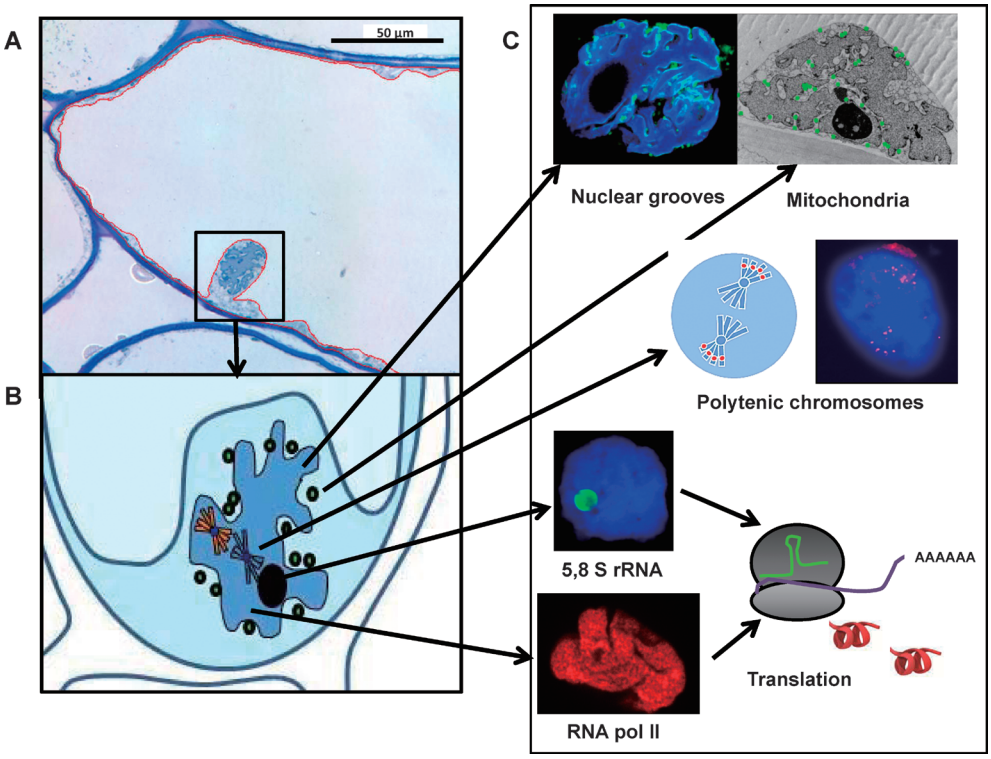

Figure 2. Endoreduplication triggers the establishment of a highly structured cellular system with multiple integrated physiological functions. A. Representative endoreduplicated nucleus showing a complex irregular shape (enclosed in black square) taken from an image of a resin-embedded pericap section stained with toluidine blue. The cytoplasm excluding the nuclear and vacuolar compartments was delineated in red. Using the dedicated imaging software Image-ProPlus, we used this delineation to determine the whole cytoplasmic area of cells from pericarp sections and demonstrate that the experimentally measured cytoplasmic area increases according to cell size according to the karyoplasmic theory. B. Schematic representation of an endoreduplicated nucleus with its associated characteristics. C. Summarized description of multiple integrated physiological functions induced by endoreduplication. Endoreduplication inside enlarged nuclei induces optimized nucleus-to-cytoplasm communications with the development of nuclear grooves, availability of energy-producing mitochondria at the vicinity of nuclear envelope, the production of polytenic chromosomes with enhanced gene copy number, the increased transcription of ribosomal RNA at the nucleolus and the increased transcription of messenger RNA as revealed by RNA Polymerase II activity (these latter two resulting in probable increased ribosome synthesis and translation).

allowed us to draw a map of ploidy levels in the pericarp tissue of tomato mature green fruits. We have been able to demonstrate at the cellular level that even if it is associated to cell size determination, endoreduplication does not regulate alone cell growth, as a clear tissue context/cell layer effect is also involved.

The use of the FISH technique and the resulting ploidy mapping associated to cytological and molecular analyses allowed us to present a detailed structural and molecular analysis of endoreduplicated nuclei in tomato fruit. We were able to provide a first comprehensive survey on the structural aspects of endoreduplicated nuclei and the most persuading quantitative evidence up to date for the karyoplasmic ratio theory, stating that the cytoplasmic size of a cell is determined by its DNA content. Various levels of specific structural and functional organization occur in endoreduplicated cells (Figure 2). Endoreduplication contributes to the establishment of a highly structured cellular system where multiple physiological functions are integrated: optimized nucleus-to-cytoplasm communications with the development of nuclear grooves, availability of energy-producing mitochondria at the vicinity of nuclear envelope, polytenic chromosomes with enhanced gene copy number, increased overall transcription activity with both messenger RNA and ribosomal RNA transcription (resulting in highly probable increased ribosome synthesis and translation).

Among the various postulated hypotheses related to the functional role of endoreduplication in plant development (Chevalier et al. 2011), our recent studies provided new data in favor of the karyoplasmic theory. Endoreduplication does act as a morphogenetic factor supporting cell growth during tomato fruit development. In addition and related to the karyoplasmic homeostasis control, we provide the first demonstration for the involvement of endoreduplication in a quantitative and qualitative regulation of transcription.

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