Endoreduplication and fruit growth in tomato: evidence in favour of the karyoplasmic ratio theory

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Abstract

The growth of a plant organ depends upon the developmental processes of cell division and cell expansion. The activity of cell divisions sets the number of cells that will make up the organ; the cell expansion activity then determines its final size. Among the various mechanisms that may influence the determination of cell size, endopolyploidy by means of endoreduplication appears to be of great importance in plants. Endoreduplication is widespread in plants and supports the process of differentiation of cells and organs. Its functional role in plant cells is not fully understood, although it is commonly associated with ploidy-dependent cell expansion. During the development of tomato fruit, cells from the (fleshy) pericarp tissue become highly polyploid, reaching a DNA content barely encountered in other plant species (between 2C and 512C). Recent investigations using tomato fruit development as a model provided new data in favour of the long-standing karyoplasmic ratio theory, stating that cells tend to adjust their cytoplasmic volume to the nuclear DNA content. By establishing a highly structured cellular system where multiple physiological functions are integrated, endoreduplication does act as a morphogenetic factor supporting cell growth during tomato fruit development.

Key words: Cell cycle control, cell size, endoreduplication, fruit, growth, karyoplasmic ratio theory, tomato.

Introduction

In the current worldwide socio-economic context and to cope with its anticipated evolution, the increase in productivity of agricultural crops for food supply is a major challenge to meet the demand of a growing population. Improving plant yield is an important process for human nutrition, but it should also be considered in a more global approach to provide the industry with natural plant resources that may be transformed into chemicals, pharmaceuticals, and renewable energy.

Classical plant breeding schemes require the use of molecular markers to facilitate the investigation of the genetic basis of complex quantitative traits, such as those governing plant yield and organ size (for instance grain and fruit sizes). As a result, many quantitative trait loci (QTLs) were identified in the last decade in plant species of great agronomical importance such as rice, maize, or tomato (Tanksley, 2004; Thévenot et al., 2005; Xing and Zhang, 2010). Genetic and molecular approaches provide the means towards the identification of genes underlying these QTLs which may ultimately allow their use in selection and breeding schemes. Model plants such as Arabidopsis thaliana offer the means to characterize the involvement of a great many genes in the regulation of agronomically important traits (Gonzalez et al., 2009, 2010). Therefore, unravelling the regulatory networks affecting plant and organ growth became a major issue for plant biologists in the last decades.
Plant yield and organ size depend upon plant growth and development which involve fundamental cellular processes such as cell division and cell expansion in close interaction with genotype and environmental cues (Beemster et al., 2003). The cell division activity provides the building blocks, setting the number of cells that will make up an organism, whereas the cell expansion activity then determines its final size.

How the size of eukaryotic cells is determined is a long-standing question for biologists. Information derived from the completion of genomes informed us of all the genes that control the cell cycle and cell division, but do not clearly identify the molecular determinants of cell size control. Early in the twentieth century, the karyoplasmic ratio theory stating that cells tend to adjust their cytoplasmic volume to the nuclear DNA content was formulated by Theodor Boveri and colleagues as a constant characteristic (reported in Wilson, 1925). This theory was then enriched by observations in multiple organisms and species of positive correlations among nuclear size and cell size. Consequently, the increase in ploidy level was described as influencing the final size of the cell, which tends to adjust its cytoplasmic volume to the nuclear DNA content (Cavalier-Smith, 2005). Endopolyploidy is a widespread process in eukaryotes that leads to an increase in the cell ploidy level. This phenomenon can result from several mechanisms such as the generation of multinucleate cells originating from acytokinetic mitosis, nuclear fusion, endomitosis, or endoreduplication. During development, the canonical cell cycle leading to mitotic cell division can be replaced by an altered cell cycle where mitosis is bypassed. This modified cell cycle called the endoreduplication cycle consists of one or several rounds of DNA synthesis in the absence of mitosis, and participates in the control of the cell expansion process, consequently contributing to plant organ growth and thus plant yield (Chevalier et al., 2011).

Endoreduplication in plants can occur in many cell types, especially those undergoing differentiation, putatively related to peculiar metabolic properties. Although accumulating data reveal that this process is developmentally regulated, the adaptive value of endoreduplication during plant development is not fully understood so far and its physiological role remains unclear, in particular in the way in which it can contribute to cell and organ growth. Various functional hypotheses have arisen in the literature: endoreduplication is thought to provide a means to sustain growth under adverse environmental conditions, such as genotoxic stress (Hase et al., 2006; Adachi et al., 2011), saline stress (Ceccarelli et al., 2006), water deficit (Cookson et al., 2006), and low temperatures (Barow, 2006).

This review aims to describe the various processes involved in organ growth: namely cell division and cell cycle regulation, and cell expansion. Among the various mechanisms governing the determination of cell size, particular attention is paid to the influence of endoreduplication and nuclear ploidy/DNA content on cell size. Recent advances in the elucidation of the functional role of endoreduplication in organ growth are described, especially those gained from tomato fruit organogenesis supporting the karyoplasmic ratio theory.

### Molecular control of cell division and endoreduplication

Cell division or mitosis is the ultimate step in the cell cycle that leads to the transmission of the genetic information from one mother cell to two daughter cells. The mitotic cycle in eukaryotic cells is comprised of four distinct phases: an undifferentiated DNA pre-synthetic phase with a 2C nuclear DNA content, termed the G1 phase; the S phase during which DNA is synthesized, with a nuclear DNA content intermediate between 2C and 4C; a second undifferentiated phase (DNA post-synthetic phase) with a 4C nuclear DNA content, termed the G2 phase; and the ultimate M phase or mitosis. The classical cell cycle thus involves the accurate duplication of the chromosomal DNA stock during the S phase and its subsequent equal segregation in the nascent daughter cells following cytokinesis at the end of the M phase.

The progression within the eukaryotic cell cycle is regulated by a class of conserved heterodimeric protein complexes consisting of a catalytic subunit referred to as cyclin-dependent kinase (CDK) and a regulatory cyclin (CYC) subunit whose association determines the activity of the complex, its stability, its localization, and its substrate specificity (Inzé and De Veylder, 2006). The CDK–CYC complexes operate at the boundaries between the G1 and S phases, and between the G2 and M phases, to phosphorylate target proteins whose inhibitory or activatory post-translational modifications are essential for passing these cell cycle checkpoints (Joubès et al., 2000; De Veylder et al., 2003) (Fig. 1). The progression along the various cell cycle phases and at the phase transitions is regulated by specific CDK–CYC complexes. The commitment to S phase is dependent upon the activities of the CDKA–CYCD complexes which phosphorylate the RETINOBLASTOMA-RELATED 1 (RBR1) protein (Gutierrez et al., 2002). The hyperphosphorylation of RBR1 leads to the release of sequestered E2F transcription factors required to drive the expression of S-phase genes. Then CDK–CYCA complexes control the progression through the S phase and the commitment to mitosis whose proper completion depends on CDKA–CYCB complex activities. The kinase activity of the complexes is not only dependent on the presence of a regulatory CYC subunit, but is also finely tuned by the phosphorylation/dephosphorylation status of the catalytic (kinase) subunit itself. Indeed the CDK activity requires its phosphorylation by a CDK-activating kinase (CAK) on a conserved threonine residue (e.g. Thr161 in plant CDKAs) (Harashima et al., 2007). Prior to its timely programmed activity at the onset of mitosis, the activated CDK–CYC complex is kept silent by inhibitory phosphorylation of Thr14 and Tyr15 residues of the CDK mediated by the WEE1 kinase, until the CDC25 phosphatase dephosphorylates these residues to activate the CDK–CYC complex. In plants, this sequential activation of CDKs is probably lacking or has evolved in a different way, since the CDC25 gene seems to be absent from plant genomes (Boudolf et al., 2006). The existing WEE1 function is restricted to the S phase to block the CDK–CYC activity unless DNA damage resulting from DNA replication defects or genotoxic stress has been
Fig. 1. Integrative model for the plant cell cycle control and TOR kinase pathway. During G₁, growth factors, such as sugars and auxin, regulate the expression of D-type cyclins (CYCDs) that associate with their catalytic subunit CDKA. The phosphorylation of the Thr160 residue of CDKA by the CDK-activating kinase (CAK) activates the CDKA–CYCD complex. The active CDKA–CYCD complex initiates the phosphorylation of RETINOBLASTOMA-RELATED 1 (RBR1) protein during late G₁ phase, thereby releasing the E2F–DP complex. The active E2F–DP complex then promotes the transcription of S-phase genes. The presence of a favourable nutrient and energy supply, namely sugar availability, activates the TOR kinase within the TOR complex. As part of the cell size checkpoint, the phosphorylation of S6K by TOR activates its kinase activity on ribosomal protein S6 whose phosphorylation activity tunes the
corrected (De Schutter et al., 2007; Gonzalez et al., 2007; Cools et al., 2011). The loss of CDK–CYC complex activity is then required to exit from mitosis. This occurs upon the proteolytic destruction of the cyclin moiety via the ubiquitin–proteasome system (UPS), involving a specific E3-type ubiquitin ligase named the anaphase-promoting complex/cyclosome (APC/C) which is activated through its association with the CCSS2 protein (Heyman and De Veylder, 2012). Additionally the CDK–CYC complexes are inactivated by the specific binding of CDK inhibitors of the Kip-related protein (KRP) (Torres Acosta et al., 2011) and SIAMESE-related (SMR) type (Churchmann et al., 2006; Van Leene et al., 2010). The CDK inhibitors are also subject to specific degradation mechanisms involving the UPS (Marrocco et al., 2010).

As part of developmental programmes or in response to environmental constraints, cells are able to modify the typical cell cycle into the endoreduplication cycle or endocycle where mitosis is lacking (Joubès and Chevalier, 2000; Edgar and Orr-Weaver, 2001; De Veylder et al., 2011). During endoreduplication, successive rounds of DNA duplication occur in iterative S phases separated by an undifferentiated G phase, leading to the production of polytenic chromosomes with multivalent (2, 4, 8, 16...) chromatids without any change in chromosome number (Edgar and Orr-Weaver, 2001; Bourdon et al., 2012). As a consequence, high nuclear DNA contents/ploidy levels can be reached, thus impacting the morphology of both the nucleus and the cell (Bourdon et al., 2012).

Endoreduplication is a widespread and evolutionarily selected process in angiosperms (Nagl, 1976; D’Amato, 1984; Kondorosi and Kondorosi, 2004; Chevalier et al., 2011; De Veylder et al., 2011; Sabelli et al., 2013). As recently reviewed by De Veylder et al. (2011), the proper unfolding of the cell cycle and the commitment to endoreduplication are determined by CDK–CYC activity levels. The progression through the G2–M transition requires the activity of a mitosis-inducing factor (MIF) above a certain threshold. The absence or reduced activity of this MIF is sufficient to drive cells into the endoreduplication cycle (Inzé and De Veylder, 2006; Vliege et al., 2007). In planta functional analyses identified the M-specific CDKB1;1 as the likely candidate kinase to be part of the MIF when bound to the A-type cyclin CYCA2;3, able to inhibit endoreduplication when fully active (Yu et al., 2003; Boudolf et al., 2004, 2009). The stability of the regulatory CYCA2;3 is the key process in the regulation of CDKB1;1 activity, since the selective degradation of CYCA2;3 achieved by the CCSS2A-mediated activation of APC provokes the commitment to endoreduplication by reducing or suppressing the MIF activity.

Role of cell number in determining organ size

Plant growth is the result of cell proliferation within meristems and cell enlargement outside these proliferative zones. The competence of cells to divide within meristems obeys internal and environmental signals, and is obviously under a tight genetic control involving a complex gene network. A large array of mutants has allowed the identification of such genes that influence organ cell number and organ growth duration positively or negatively (for a review, see Powell and Lenhard, 2012). The following genes were identified as positive effectors of cell proliferation during organ growth: AINTEGUMENTA (Mizukami and Fischer, 2000), ANGUSTIFOLIA (Autran et al., 2002), ARGOS (Hu et al., 2003), JAGGED (Dinneny et al., 2004), GROWTH-REGULATING FACTORS (Horiguchi et al., 2005), STRUWWELPETTER (Clay and Nelson, 2005), KLUGH (Anastasiou et al., 2007), and ORGAN SIZE RELATED1 (Feng et al., 2011). Indeed mutations of these genes produced smaller organs as the cell proliferation period was reduced; conversely, these genes when ectopically expressed induced an extension of the cell proliferation period, leading to the formation of larger organs with more cells. AUXIN RESPONSE FACTOR2 (Schruff et al., 2006), BLADE ON PETIOLE1 (Ha et al., 2003), PEAPOD1/2 (White, 2006), and BIG BROTHER (Disch et al., 2006) can be cited as negative effectors of cell proliferation, since mutations in these genes produced larger organs because of increased cell number. The tomato mini zinc finger protein INHIBITOR OF MERISTEM ACTIVITY (IMA) is another example of such negative effectors (Sicard et al., 2008). IMA is involved in the control of plant growth at the crossroads of hormonal signaling pathways and cell cycle regulation, by preventing cells from entering into proliferation. However, how the mechanisms by which these different effectors affect the cell cycle regulatory genes remains to be completely unravelled.
The best characterized target of such effectors is CYCD3;1 which is regulated by AINTEGUMENTA (Mizukami and Fischer, 2000). CYCD3;1 in complex with CDKA;1 phosphorylates RBR, thereby releasing the RBR-sequestered E2F transcription factors required for the entry into the S phase and subsequent full cell cycle commitment. The overexpression of CYCD3;1 caused a premature cell cycle progression, leading to hyperproliferation, but also the inhibition of organ growth by repressing cell differentiation and development (Dewitte et al., 2003). The central role of CYCD3 in the balance between cell proliferation and cell differentiation was further highlighted by the observed phenotype of a triple mutant cycd3;1 cycd3;2 cycd3;3 which produced smaller organs with fewer cells (Dewitte et al., 2007).

Interestingly manipulating the downstream effectors of CYCD3 action, i.e. the genes encoding the E2FA (Magyar et al., 2012) and E2FB (Magyar et al., 2005) transcription factors, led to phenotypes of overproliferation, thus confirming that they were required for maintaining cells in a proliferative state.

The TOR pathway, at the crossroads of cell proliferation and cell expansion

Like their animal counterparts, growth and proliferation of plant cells are tightly linked to the perception of available nutrients and energy, developmental signals, and stress factors. All these signals and external factors are integrated through the target of rapamicin (TOR) kinase and sucrose-non-fermenting 1 (Snf1)-related kinase (SnRK1) signalling pathways that act antagonistically (Robaglia et al., 2012). The TOR kinase is activated in the presence of favourable nutrient and energy supply conditions, thereby promoting a signalling cascade that will control growth and development positively, and senescence negatively (especially the process of autophagy): as a result, mRNA translation or cell proliferation is promoted (Robaglia et al., 2012). Conversely, the SnRK1 kinase is activated in low energy level conditions or by carbon starvation, thereby blocking growth as a central node in the sugar-sensing network (Smeekens et al., 2010).

One major target of the TOR kinase complex is the 40S ribosomal protein S6 kinase (S6K) (Mahfouz et al., 2006) (Fig. 1). The phosphorylation of S6K by TOR activates its kinase activity on ribosomal protein S6 whose phosphorylation activity is pivotal for fine-tuning of the translational capacity of cells (Meyuhas, 2008). As a conserved feature in eukaryotic cells, TOR and S6K are the major players in the cell size checkpoint: when activated, they delay the entry into mitosis, allowing cells to increase their size. At the level of cell cycle control, it was recently shown that the Arabidopsis S6K is indeed a negative regulator of the commitment to mitosis during cell size control (Henriques et al., 2010). This functional analysis provided evidence for a new role for plant S6K in elongation-driven cell growth, through the RBR1–E2F pathway. Indeed S6K interacts with the RBR1–E2FB complex, mediates the nuclear re-localization of RBR1 (thus counteracting the CycD3;1 action), and contributes to the repression of key cell cycle regulatory genes such as those encoding CDKA, CDKB, E2FB, and DPA (Henriques et al., 2010).

E2Fa was recently identified as a novel TOR kinase substrate (Xiong et al., 2013). The TOR kinase-mediated phosphorylation of E2Fa was found to activate the transcription of S-phase genes as primary glucose–TOR target genes. The direct TOR–E2F link and consequent unconventional activation of S-phase genes (i.e. apart from the evolutionarily conserved CDK/CYC–RBR–E2F pathway) may represent an alternative entry point into the cell cycle linking cell cycle progression, photosynthesis-driven glucose perception, meristem activity, and plant growth (Xiong et al., 2013).

Another major target of the TOR kinase complex is the ErbB-3 epidermal growth factor receptor-binding protein (EBP1), a nucleolar-localized protein part of ribonucleoprotein complexes that links ribosome biosynthesis, cell proliferation, and cell growth (Squatrino et al., 2004). The potato and Arabidopsis orthologues of EBP1 have been isolated and characterized (Horvath et al., 2006). In both plant species, modulating the level of EBP1 modified organ growth: antisense plants showed dwarfism with smaller leaves and smaller abnormal tubers in the case of potato, while overexpressing plants were larger than the wild type. Correlatively, the mean cell size was respectively smaller and larger in these transgenic plants. EBP1 positively regulates cell cycle effectors required for the commitment to the S and M phase, namely CycD3;1, RNR2, and CDKB1;1, in a dose- and auxin-dependent manner, and negatively regulates RBR1, thus inducing cell proliferation (Fig. 1). However, depending on the developmental stage, EBP1 exerts converse effects on cell size determination: in the early stages of development, EBP1 increases the number of cells by lowering the cell size threshold at which cells divide; in post-mitotic development, EBP1 enhances cell expansion required for organ growth (Horvath et al., 2006).

Role of cell size in determining organ size

Genetic factors regulating cell size

Examples of genetic factors regulating organ growth by cell expansion are not that numerous, in contrast to those exerting their effects on cell number. The ROTUNDIFOLIA3 and LONGIFOLIA1/2 genes both regulate leaf morphology by positively promoting longitudinal polar cell elongation in Arabidopsis (Kim et al., 1998; Lee et al., 2006). Members of the GROWTH-REGULATING FACTOR gene family are putative transcription factors that regulate cell expansion in cotyledon, leaf, and petiole tissues in Arabidopsis (Kim et al., 2003): AtGRF overexpressors harbour larger organs, while triple mutants of AtGRF1–AtGRF3 have smaller ones, as a result of increased or decreased cell size, respectively. The basic helix-loop-helix (bHLH)-type transcription factor BIGPETALp was identified as a negative regulator of cell expansion in Arabidopsis petals: when BIGPETALp is lacking, petals are enlarged as a result of increased cell size (Szécsi et al., 2006).
Vacuole enlargement and cell homeostasis

Plant cells are characterized by the presence of a large vacuole whose volume occupancy is classically reported to correspond to >90% of that of a differentiated cell (Taiz, 1992). As a multifunctional organelle, the vacuole is involved in many processes related to pH homeostasis and osmoregulation, detoxification, and lytic processes, solute accumulation, and regulation of cytoplasmic ion concentrations (Sze et al., 1992; Barkla and Pantoja, 1996; Marty, 1999). Since most of the plant cell volume corresponds to the vacuolar compartment, the vacuole plays an important part in cell expansion which is driven by the internal turgor pressure created by osmotic water and solute uptake. Conversely, the process of turgor-driven cell expansion is restricted by the extensibility of the cell wall which requires an integrated regulation of the synthesis, incorporation, and cross-linking of new cell wall components (Cosgrove et al., 2005). As a result, the process of turgor-driven cell expansion occurs according to a subtle mechanism linking cell wall relaxation/stiffening and mechano-sensing feedback loops (Wolf et al., 2012).

A major role for the central vacuole is to maintain turgor pressure against the cell wall. Both tonoplast and plasma membrane proton pump activities contribute to establish the pH gradient that is required for pH homeostasis. Upon auxin induction, rapid cell growth is mediated at least in part by the activation of plasma membrane ATPase proton pumps: protons are excreted, resulting in a decrease in the apoplastic pH which subsequently activates cell wall-loosening processes (Cleland, 2004), involving a battery of cell wall-modifying enzyme activities and a complex signal transduction pathway leading to cell wall extension required for cell growth (for a review, see Wolf et al., 2012).

The tonoplast of the plant cell vacuole harbours two distinct proton pumps: the vacuolar H⁺-ATPase (V-ATPase) and H⁺-pyrophosphatase (V-PPase), using ATP and PPI, respectively, as an energy source to create a pH gradient as well as a membrane electrochemical gradient between the cytosol and the vacuole lumen. These gradients are used to energize secondary solute transport processes (Rea and Poole, 1993), both being necessary for subsequent water uptake. The involvement of these enzymes in relation to cell size and/or organ size has been reported for many years. For instance, the inhibition of V-ATPase expression in carrot limits leaf cell expansion and thus leaflet growth at early stages of development (Gogarten et al., 1992). In Arabidopsis thaliana, the mutation of a V-ATPase subunit C in the det3 mutant confers a reduced hypocotyl growth due to the altered cell elongation (Schumacher et al., 1999), as well as the inhibition of V-ATPase subunit C1 and C3 expression during root growth (Padmanaban et al., 2004). Interestingly, the fruit-specific suppression of V-ATPase in tomato transgenic plants resulted in reduced fruit size displaying a thinner pericarp (Amemiya et al., 2006), most probably due to altered cell expansion. In planta functional analyses of V-PPase are much less documented. In tomato as well, RNA interference (RNAi) lines in which the expression of genes encoding V-PPase is fruit specifically repressed exhibited fruit growth retardation at an early stage of development (Mohammed et al., 2012). Li et al. (2005) reported a novel role for the Arabidopsis type-I V-PPase AVP1, namely the facilitation of auxin transport and consequent regulation of cell division at the onset of organ formation. The avp1-1 null mutant exhibited severely disrupted root and shoot development, together with reduced auxin transport (Li et al., 2005). Conversely, transgenic plants overexpressing the Arabidopsis AVP1 displayed increased cell division, hyperplasia, and increased auxin transport (Li et al., 2005). Gaxiola et al. (2012) recently reviewed the promising applications in genetically manipulating V-PPase so as to improve plant yield, but also so as to enhance drought and salt tolerance under limiting Pi conditions.

Cell ploidy/DNA content and cell expansion

It has long been observed that the size of the nucleus is related to that of the cell, according to a merely constant nuclear-to-cell volume ratio. These frequent observations led to a theory commonly referred to as the ‘karyoplasmic ratio theory’, first formulated by Theodor Boveri and reported by Wilson (1925), stating that there is a causal relationship between nuclear and cytoplasmic growth (and thus cell volume) (Sugimoto-Shirasu and Roberts, 2003). This theory has been supported by a large number of studies in animals (Horner and Macgregor, 1983; Cavalier-Smith and Beaton, 1999; Henery and Kaufman, 1992; Cavalier-Smith, 2005) and in plants (Price et al., 1973; Jovtchev et al., 2006; Beaulieu et al., 2008; Knight and Beaulieu, 2008; Hodgson et al., 2010), as well as in diatoms (Connolly et al., 2008). The origins of this positive correlation between DNA content and cell size have been explained in different ways (reviewed by Gregory, 2001) and are still a matter of debate. As a result of endopolyploidyization (including endoreduplication), the correlation between the ploidy level (i.e. DNA content) and cell size has also been reported for some time in yeast (Galitski et al., 1999), plants (Melaragno et al., 1993; Chenielet et al., 2005; Jovtchev et al., 2006; Chevalier et al., 2011), and animals (Hancock et al., 1993; Anisimov, 2005; Lilly and Duronio, 2005; Lozano et al., 2006; Hemberger, 2008), suggesting a strong positive impact of DNA content on the determination of cell size. As far as plants are concerned, this assumption was investigated by manipulating the extent of endoreduplication through the alteration of cell cycle gene expression. The induction of endoreduplication was obtained by hampering the commitment to mitosis via the down-regulation of mitosis-specific CDKs (Boudolf et al., 2009), A-type cyclins (Cebolla et al., 1999), or a mild up-regulation of KRP s (Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002; Zhou et al., 2003). As a consequence, the premature blockage of mitosis induces endoreduplication, generating plants with fewer cells of larger size (Cebolla et al., 1999; Wang et al., 2000; Jasinski et al., 2002) or of approximately equal size to those in wild-type plants (De Veylder et al., 2001; Zhou et al., 2003). As an example of a cell cycle regulatory protein exerting a dual function, E2FA not only maintains cell proliferation, as mentioned above, but also enhances cell expansion required for organ growth in
differentiating cells by promoting endoreduplication (Magyar et al., 2012). Interestingly, Roeder et al. (2010) were able to demonstrate, using computational modelling supported by time-lapse imaging, that the timing of entry into endoreduplication determines the variation in cell size in Arabidopsis epidermal sepal cells.

However, a modified extent of endoreduplication does not always confer an alteration in cell size. We can report two striking examples describing the uncoupling of cell size and endoreduplication. First, the overexpression of a dominant-negative form of CDKA in maize kernel endosperm resulted in a decreased endoreduplication independently from cell size (Leiva-Neto et al., 2004). Secondly, Nafati et al. (2011) reported similar results by overexpressing a tomato KRP in a fruit-specific and cell expansion-specific manner. Interestingly, these two examples made use of promoters specifically expressed in the endoreduplication phase of maize endosperm and tomato pericarp development, respectively, the 27 kDa γ-zein and the PhosphoenolPyruvate Carboxylase 2 (PEPC2) promoters. In both cases, endoreduplication was not totally impaired, as the overexpression of these negative effectors did not produce a radical effect or because the process already starts early in development and to some extent concomitantly with cell divisions: thus it is likely that enough ploidy still occurred to support cell growth. This would infer that endoreduplication does not exert a strict direct control on cell growth but would rather be a limiting factor for cell growth, in accordance with previous studies (Schmittger et al., 2003; Mathieu-Rivet et al., 2010).

The influence of the DNA content on nuclear size and cell size, as formulated by the karyoplasmic ratio theory, thus constitutes a complex issue. If strong correlations exist between the DNA content and cell size, the causal link between these two parameters is hard to demonstrate and seems to be submitted to multifactorial origins. Nevertheless, a common aspect to all eukaryotic models influencing cell size positively is the ability ‘to produce’ cytoplasm for a given DNA content. This ability can be reflected by ribosome biogenesis, an important factor for cytoplasm production in the cell growth process (Sugimoto-Shirasu and Roberts, 2003). In most eukaryotic organisms, the frequency of rDNA repeats is positively correlated to genome size (Prokopowich et al., 2003). This amplification of rDNA gene copies is likely to provide to large genome-sized cells a great potential for ribosome biogenesis, even though all rDNA copies may not be expressed. In tomato, it was demonstrated that each round of conservative DNA replication increasing the ploidy level generates a doubled bulk of rDNA repeats (Bourdon et al., 2011, 2012) to be used for ribosome biogenesis. Interestingly, increasing the ploidy levels in tomato fruits resulted in significantly sustained protein content and a global modification of nitrogen metabolism rather than a particular amino acid biosynthetic pathway, when compared with wild-type fruits (Mathieu-Rivet et al., 2010). These modifications were found to correlate with the observed discrepancy in fruit growth between the endoreduplication-induced lines and the wild type.

**Tomato fruit development as a model to investigate organ size control in plants**

As a highly valuable crop, tomato has been used as the model species for all fleshy fruits because of its favourable biologcal properties (short life cycle, high multiplication rate, easy self-crossing) and the development of genetic resources and genomic tools, such as marker-assisted mapping of QTLs, collections of mutants, in planta functional analyses of candidate genes, and the recent release of the complete tomato genome sequence (Tomato Genome Consortium, 2012). Over the last two decades, a major goal for plant breeders has been to unravel the links between the quantitatively determined fruit size/fruit growth trait (by increasing cell number and/or cell size) and final fruit composition to comply with consumers’ demands. For instance, these efforts led to the detection of 30 QTLs governing fruit size/weight in the tomato genome (Grandillo et al., 1999), and in particular that called fw2.2 (Fruit Weight QTL of chromosome 2, number 2) accounting for as much as 30% difference in fruit fresh weight between the domesticated tomato and its wild relatives (Alpert et al., 1995). FW2.2 was cloned, characterized at the molecular level, and shown to code for a small protein (22 kDa) acting as a negative regulator of cell divisions and thus affecting fruit development and final size (Frary et al., 2000). However, the identity and real mode of action of FW2.2 on cell division/cell cycle regulation so far remains totally elusive.

The early fruit development in tomato can be divided into three distinct phases (Gillaspy et al., 1993). During the first phase, the ovary develops and ‘takes’ the decision to set fruit upon pollination and fertilization. Then the ovary walls enlarge through an intense activity of cell divisions in the second phase. Thereafter, fruit growth corresponding to the third phase is mainly sustained by cell expansion leading to a fruit which exhibits its almost final size and is able to ripen. At the end of the cell expansion phase, individual cells in the fleshy part (mesocarp tissue) of the fruit can reach spectacular levels in volume: >30 000-fold increase from the initial cell volume, sometimes corresponding to >0.5 mm in diameter (Cheniclet et al., 2005). Of importance, this spectacular cell hypertrophy is closely correlated with an increase in nuclear DNA ploidy levels due to endoreduplication. Indeed, high levels of endoploidy occur in the course of fruit development within the mesocarp and the jelly-like tissue in which the seeds are embedded (Bergervoet et al., 1996; Joubès et al., 1999; Cheniclet et al., 2005; Bertin et al., 2007). The typical ploidy levels encountered in tomato fruit can reach up to 512C (where C is the haploid DNA content); these values are unmatched by other species such as Arabidopsis, maize, or Medicago, classical model plants in which endoreduplication has been studied (Melaragno et al., 1993; Vilhar et al., 2002; Kondorosi et al., 2005). Interestingly, a remarkable conservation of pericarp pattern, including cell layer number and cell size, is observed at anthesis in tomato genotypes varying over a large range of fruit weight at maturation (Cheniclet et al., 2005). The large variation in fruit weight correlates with the mean ploidy level achieved in pericarp cells which itself correlates with the mean cell size, thus highlighting the
contribution of cell size to final fruit weight and the putative role of endoreduplication in driving fruit growth (Bourdon et al., 2010; Chevalier et al., 2011).

Endoreduplication is such an important process during tomato fruit development that modifying the expression of genes involved in the regulation of the cell cycle and commitment to endoreduplication was expected to affect fruit growth and thus alter fruit size. The various phenotypes of such tomato transgenic fruits reported in the literature are illustrated in Fig. 2.

The fruit-specific down-regulation of the canonical CDKA using an artificial microRNA (amiCDKA) in tomato resulted in the production of smaller fruits, characterized by a reduced pericarp thickness due to an overall decreased number of cell layers (Czerednik et al., 2012). However, this phenotype only impacted the outer pericarp of amiCDKA fruits (displaying the smallest cells), while the mesocarp displayed normally enlarged cells without any significant difference in ploidy levels. The fruit-specific down-regulation of CDKA1 phenocopied the fruit-specific overexpression of both mitosis-associated CDKB1 and CDKB2 (Czerednik et al., 2012): fruits were indeed smaller, with a thinner pericarp of an almost identically modified structure. The molecular characterization showed that the CDKA expression is greatly repressed in the overexpressing CDKB1 and CDKB2 lines, compared with that in control fruits. However, in these CDKB1 and CDKB2 overexpressor lines, a detailed cytological analysis revealed that cell sizes are reduced together with a reduction in ploidy levels. The phenotypes observed in tomato fruits modified for CDKA or CDKB1/2 expression are complex to interpret, especially in light of the differential effects on cell division, cell expansion, and endoreduplication. This is probably due to the use of the fruit-specific TPRP promoter (Fernandez et al., 2009) that drives the maximum of the transgene expression during the cell expansion phase of fruit development (Czerednik et al., 2012); that is, outside the natural timing of expression for CDKB1 and CDKB2 (Joubès et al., 1999, 2001). Then the availability of regulatory cyclins may not be fully respected for the proper CDK–CYC complex composition, and/or the misexpressed CDKs may compete for regulatory cyclins during tomato fruit development.

The commitment towards endoreduplication requires the loss of mitotic CDK–CYC complex activity via the UPS-mediated selective proteolytic destruction of the cyclin subunits involving the APCCCS52A E3 ubiquitin ligase. The ectopic down-regulation of CCS52A in transgenic tomato plants led to the production of smaller fruits than wild-type plants (Mathieu-Rivet et al., 2010). The ploidy levels in these fruits were shifted towards lower levels, correlated with a decrease in mean cell size and an increase in cell number. In tomato, SICCS52B encodes a second APC/C activator which is preferentially expressed during cell division, while SICCS52A is involved in the endoreduplication-driven cell expansion (Mathieu-Rivet et al., 2010). Surprisingly, the ectopic down-regulation of SICCS52B gave an opposite phenotype to that of antisense SICCS52A plants, namely larger fruits (personal unpublished data). The cytological and molecular characterization of antisense SICCS52B fruits revealed that this phenotype originates from higher ploidy levels and larger cell sizes induced by the compensatory overexpression of the endogenous SICCS52A gene. The involvement of SICCS52A in the regulation of endoreduplication was further confirmed by gain-of-function transgenic tomato plants. When SICCS52A was ectopically overexpressed, fruits grew much more slowly than wild-type fruits, but resumed growth and were able to reach almost the size of wild-type fruit at the end of the growing period (~35 days post-anthesis, dpa). A careful kinetic analysis of the appearance of polyploid nuclei during fruit growth revealed that endoreduplication was increased at 20 dpa and afterwards due to a burst in the appearance of 32C and 64C nuclei. This production of highly polyploid nuclei generated cells of an increased size inside the pericarp of SICCS52A-overexpressing fruits, which drove growth to resume and reach almost the final size of wild-type fruits (Mathieu-Rivet et al., 2010). Remarkably, the fruit- and cell expansion-specific mediated overexpression of SICCS52A using the tomato PEPC2 promoter (Fernandez et al., 2009) greatly affected fruit enlargement: fruits started to enlarge from 10 dpa and continued afterwards (until the end of the cell expansion phase, and according to the expression window of the PEPC2 promoter), leading to significantly larger fruits with thicker pericarp and a much higher endoreduplication index (EI; personal unpublished data).

Thus this functional analysis highlighted the important regulatory role of SICCS52A in endoreduplication triggering fast fruit growth in tomato.

The ectopic down-regulation of WEE1 in tomato transgenic plants (plants referred to as Shwee145) provoked an enhanced CDK–CYC histone H1 kinase activity, resulting from a strong decrease in the phosphorylation of the Tyr15 in CDKA (Gonzalez et al., 2007). The enhanced CDK–CYC activity led to the production of smaller fruits displaying a reduction in pericarp thickness because of a significant reduction in cell size, together with a strong reduction in ploidy levels. Thus the CDK phosphorylation status controlled by the activity of WEE1 appears to be an important mode of regulation for the promotion of endoreduplication during tomato fruit development. As a consequence, the WEE1 activity contributes to cell size determination at the interplay between normal cell cycle events and endoreduplication, and thus influences final fruit size (Chevalier et al., 2011).

In transgenic fruits where the expression of the SKRPI gene under the control of the PEPC2 promoter was up-regulated similarly in a fruit- and cell expansion-specific manner, DNA ploidy levels were significantly lowered in the mesocarp, as a result of the effect of a high expression of SKRPI (Nafati et al., 2011). However, the PEPC2-driven overexpression of SKRPI during the phase of cell expansion uncoupled DNA ploidy from cell size: indeed final fruit size and mean pericarp cell size were not affected in SKRPIOE plants despite the significant negative impact on endoreduplication. Though being reduced, endoreduplication was not totally impaired in these transgenic fruits, suggesting that enough DNA ploidy still occurred to support cell growth. Hence endoreduplication per se would not determine the exact size of a cell, but would rather act as a limiting factor for cell...
Fig. 2. Phenotypes of tomato transgenic fruits modified for the expression of cell cycle or endocycle regulatory genes. The down-regulation of *SlWEE1* (Gonzalez et al., 2007), *SlCSS52A* (Matthieu-Rivet et al., 2010), and *SlCDKA1* (Czerednik et al., 2012) using antisense constructs (*Slwee1AS*, *Slccs52AAS*) or artificial miRNA (*Slcdka1ami*), respectively, and the overexpression of *SlCDKB1* and *SlCDKB2* (*SlCDKB1/2OE*) (Czerednik et al., 2012) produced smaller fruits (illustrated in the red-framed pictures). The mean nuclear ploidy level (represented by the abbreviation EI for endoreduplication index) and cell size in these fruits were accordingly reduced (red downward-pointing arrow), except for *Slcdka1ami* fruits where the reduction in fruit size originates from a decreased number of cell layers in the outer pericarp. The fruit- and cell expansion-specific overexpression of *SlCCS52A* (*SlCCS52AOE*) resulted in enlarged fruits (illustrated in green-framed pictures) with an increased EI (green upward-pointing arrow). Similarly the repression of *SlCCS52B* (*Slccs52BAS*) produced enlarged fruits due to the compensatory overexpression of endogenous *SlCCS52A*. The fruit- and cell expansion-specific overexpression of *SlKRP1* (*SlKRP1OE*) did not modify the final fruit size, though the EI was decreased. For more details, see the text.
growth, setting up a potential for growth (Schnittger et al., 2003; Nafati et al., 2011).

Quantitative evidence supporting the role of endoreduplication in the karyoplasmic ratio theory during fruit development

As already mentioned, endoreduplication is part of the natural developmental programme in tomato fruit. It occurs in all Solanaceae species so far tested (Bourdon et al., 2010), displays a strong positive correlation with cell size as described for a set of wild-type tomato fruit accessions (Cheniclet et al., 2005), and clearly contributes to fruit development and the determination of final fruit size (Gonzalez et al., 2007; Mathieu-Rivet et al., 2010). However, and despite these advances, the physiological role of endoreduplication remains elusive.

An in situ and direct determination of the DNA ploidy level of individual nuclei (i.e. at the cellular scale) is a pre-requisite to decipher the functional role of endoreduplication. Bourdon et al. (2011) developed such a fluorescent in situ hybridization (FISH)-based methodology, which allowed the establishment of a ploidy map of mature green tomato pericarp. A positive correlation between cell size and ploidy level was thus demonstrated, and three contrasting areas in terms of ploidy levels could be observed: the outer epidermis (OE, consisting of the smallest cells) displayed very low ploidy levels (2C and barely 4C); the inner epidermis (IE, composed of medium-sized cells) displayed mid-ploidy levels (8C–32C); and the mesocarp (M) displayed the largest range of ploidy levels (from 4C to 256C), with the highest ones present within the largest cells. This ploidy mapping analysis revealed that cell size is dependent not only on ploidy levels but also upon the position of the cell within the pericarp; clearly a precise cell size may be associated with various ploidy levels. In agreement with Nafati et al. (2011), this suggests that (i) endoreduplication would favour a larger range of cell sizes rather than any particular class; and (ii) endoreduplication would precede cell expansion, providing the potential to support a range of cell sizes, thus triggering organ growth.

The availability of a ploidy map across tomato fruit pericarp was used to advantage to investigate the consequences of endopolyploidization on the morphology of endoreduplicated nuclei (Bourdon et al., 2012). A clear relationship between the complexity of the nuclear shape and its DNA content was established (Bourdon et al., 2012). Within the three most contrasted regions in terms of ploidy levels across the pericarp (as defined above), the less polyplloid nuclei from OE are small with an almost perfectly round shape; the mid-ploidy nuclei from IE are elongated and slightly grooved; and the highly polyplloid nuclei from M are large and display a complex shape with numerous invaginations. Therefore, the nuclear morphology of endoreduplicated cells within tomato fruit pericarp is strongly affected by endopolyploidization. Such nuclear invaginations were previously reported in plants (Collings et al., 2000), but the relationship with ploidy levels was never established before.

The presence of multiple grooves generated by endoreduplication in the nuclear envelope may obviously impact the exchange ability between the nucleus and cytoplasm. The exchange ability between the nucleus and the cytoplasm was found to be maintained during endopolyploidization despite the increase in nuclear volume (Bourdon et al., 2012). This finding suggested that the induced complex morphology of endoreduplicated nuclei with so many invaginations is required to enable the maintenance of an efficient nucleus to cytoplasm communication by minimizing the distance between the nuclear compartment and its envelope at the point of contact of the cytoplasm. Interestingly numerous mitochondria gather in the close vicinity of polyploid nuclei, especially inside grooves: quantifying the number of mitochondria in the proximity of the nuclear envelope revealed that it increases according to the nuclear size and ploidy level (Bourdon et al., 2012). Two additional observations provided new information regarding the relationship between endoreduplicated nuclei and the cytoplasm: first, the number of mitochondria relative to nuclear volume remains constant whatever the ploidy level; secondly, these numerous mitochondria are largely active whatever the pericarp area observed, as revealed by the use of JC-1 dye (Bourdon et al., 2012), which supports the idea that endopolyplloidy may confer high metabolic activities (Larkins et al., 2001; Sugimoto-Shirasu and Roberts, 2003). As part of the endoreduplication process, the increase in nuclear DNA content and consequent increase in nuclear volume were found to match with that of the cytoplasmic area and volume of cells (Bourdon et al., 2012), thus clearly demonstrating the maintenance of the nuclear to cytoplasmic ratio to drive cell growth, consistent with the karyoplasmic ratio theory.

Since ribosome biogenesis and protein synthesis are required for cell growth, it would be expected that an increase in nuclear DNA content during endopolyploidization could lead to an increased transcription of rRNA and mRNA. As revealed by RNA FISH experiments using a 5.8S rRNA probe on isolated mature green fruit pericarp nuclei, the hybridization signal area labelling the nucleolus correlated positively with the nucleus area and thus with the ploidy level, demonstrating that the transcription of the 5.8S rDNA within the nucleolus is increased during endoreduplication.

The active phosphorylated form of the RNA polymerase II (RNA pol II), responsible for mRNA transcriptional activity, associates preferentially with euchromatin and is homogeneously distributed inside endoreduplicated nuclei, whatever the position of a nucleus within the pericarp (i.e. whatever its ploidy level) (Bourdon et al., 2012). In addition, the quantification of RNA pol II by flow cytometry following specific immunolabelling of pericarp endoreduplicated nuclei showed a positive correlation with ploidy levels, demonstrating that the protein level for RNA pol II increased proportionally with ploidy. At the level of specific gene expression, this relationship between endoreduplication and transcription was confirmed since the amount of RPBI transcripts encoding the large subunit of RNA pol II, as well as the endoreduplication-induced CCS32A and WEE1 transcripts accumulated according to ploidy levels, whereas mitosis-associated
transcripts such as CDKB2 and MIS12 were undetectable (Bourdon et al., 2012).

Altogether, the recent results from Bourdon et al. (2012) demonstrated that endoreduplication is associated with a complex cellular organization and contributes to karyoplasmic homeostasis during fruit growth. It provided the first direct evidence that endopolyploidy plays a role in increased transcription of rRNA and mRNA on a per nucleus basis. Endoreduplication leads to larger nuclei with conserved karyoplasmic exchange ability and is associated with efficient nucleus to cytoplasm communication, especially between the mitochondria and nucleus, probably to provide a sufficient energy supply for nuclear biochemical processes such as DNA replication, ribosome biogenesis, transcriptional activity, protein synthesis, and exchange across the nuclear envelope (Fig. 3). During endoreduplication, all these cellular processes must be proportionally enhanced to increase and therefore maintain the homeostasis of cytoplasmic components essential for sustained cell growth.

**Conclusion**

Endoreduplication is of widespread occurrence in plants and concerns >90% of angiosperm species. The mean level of endoreduplication in various plant organs has repeatedly been found to be correlated with organ size. However, the role of endoreduplication in organ growth has long been disputed in the literature, with two opposing mechanisms accounting for organ growth.

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**Fig. 3.** Endoreduplication is associated with a complex cellular organization leading to karyoplasmic homeostasis. (A) Ploidy map of the pericarp tissue from mature green tomato fruits. Cell layers and ploidy levels found in each cell layer are indicated above the section. An example of a polyploid cell (256C) is outlined (in red); V, vascular bundle. (B) Schematic representation of a polyploid cell with its associated characteristics. (C) Nuclear characteristics associated with endoreduplication. From bottom to top: morphology of a nucleus with its complex irregular shape and development of grooves to optimize nucleus to cytoplasm communication; presence of numerous mitochondria in the vicinity of the nuclear envelope especially inside grooves to provide energy for nuclear biochemical processes such as DNA replication and/or RNA transcription; an increase in RNA pol II activity for mRNA transcription; and an increase in rRNA transcription at the nucleolus (~5.8S rRNA).
First, growth itself is the dominant regulator of cell proliferation, cell expansion, and endoreduplication, with compensation if one of the underlying cellular processes is limiting, according to an organismal level of regulation (Mizukami, 2001; John and Qi, 2008; Massonnet et al., 2011). Secondly, the final size of an organ results from a given balance of cell-based (autonomous) growth relying on cell division, expansion, and endoreduplication (Sugimoto-Shirasu and Roberts, 2003). Most of the functional analyses aimed at manipulating cell cycle- and endocyte-regulating factors and resulting in modification of the ploidy patterns were in favour of the latter hypothesis.

As a remarkable feature of tomato fruit, endoreduplication was shown to act as a morphogenetic factor supporting cell growth during fruit development, thus providing quantitative evidence for the karyoplasmic ratio theory: the cytoplasmic size of a cell in tomato fruit is indeed determined by its DNA content.

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