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Minireview

Endoreduplication and activation of the anaphase-promoting complex during symbiotic cell development

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Abstract Postembryonic development of plant organs requires a constant interplay between the cell cycle and the developmental programs. Upon endo- and exogenous signals, plant cells can enter, exit or modify the cell cycle. Alteration of mitotic cycles to endoreduplication cycles, where the genome is duplicated without mitosis, is common in plants and may play a role in cell differentiation. The switch from the mitotic to endocycles is regulated by Ccs52A, a plant orthologue of the yeast and animal Cdhl proteins, acting as substrate-specific activator of the anaphase-promoting complex E3 ubiquitin ligase. Here, several aspects of endoreduplication are discussed with special attention on nitrogen-fixing nodule development where endoreduplication is an integral part of symbiotic cell differentiation.
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1. Introduction

Plants have unique growth characteristics, developmental patterns and body architecture. In contrast to animals, organogenesis starts at the end of embryogenesis and results mainly in postembryonic development of higher plants. The formation of new organs, such as lateral roots, shoots, leaves or flowers, and determination of tissue specificity are prolonged during the entire life time of the plants. This continuous organ development necessitates constant coordination of cell proliferation with the various differentiation programs. Cell division in plants is restricted to meristems, however, most cells maintain their ability to re-enter or modify the cell cycle under the control of developmental programs or in response to abiotic and biotic signals. This plasticity of the plant cell cycle is essential for the sessile life style, for better adaptation to the environment and largely contributes to the regular postembryonic body remodelling. Cells in the meristems are indeterminate, whereas differentiation transforms the actively dividing cells into non-dividing cells with specialised functions. These cells may lose the cell cycle activity and become quiescent or enter endoreduplication cycles, representing an altered form of cell cycle where the genome is duplicated while mitosis is inhibited. Single or repeated rounds of endoreduplication cycles, known also as endocycles, lead to polyploidisation of cells that is widespread in plants and can occur in any somatic cell type. The inherited pattern of endoploidy, characteristic for the different organs, tissues or cell types in a given species, suggests that multiplication of the genome might contribute to cell differentiation as part of the developmental programs.

Endocycles are composed of an S-phase and a gap period, however, the mechanisms and signals required for the initiation and maintenance of endocycles are largely unknown. Recently, our studies on the organogenesis of *Medicago* root nodules, a symbiotic organ where endocycles persist in a limited region, have led to the identification of the cell cycle switch gene *ccs52* that by inhibiting mitosis might be a major regulator of the endoreduplication cycles [1].

2. Functional benefits of endoreduplication

The physiological significance of endoreduplication is still not well understood. It is not clear whether endoreduplication is genetically programmed or a consequence of the differentiation. There is an ancient observation on the correlation between cell size and nuclear volume in eukaryotes, which led to the "nuclear–cytoplasmic ratio" theory [2], establishing a direct relationship between nuclear DNA content and cell size in endoreplicative tissues. In animals, the ploidy levels do not affect the constant size of organs or the organism that are controlled with astonishing precision. In tetraploid mice, the increase in cell size is compensated with the decrease of cell number to conserve the constant mass of the organism [3–5]. Though similar control mechanism exists in plants, it is not so strict as in animals and significant variations in organ and organism sizes may exist without affecting viability. Increased ploidy levels in plants frequently result in an increase in the size of the organs or the whole plant. While animal cells are rather uniform in their size, plant cells exhibit extreme variations in their size. This uneven enlargement of plant cells is one of the most striking features of plant development that is often coupled to somatic endoploidy, which indicates that the increased genome size may be required for the formation of large plant cells [6].

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Abbreviations: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; RB, retinoblastoma; SCF, Skpl-Cullin-F-box protein

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During endoreduplication, the chromosomes do not condensate. This together with the increased gene dosages may enhance the transcriptional as well as metabolic activities in polyploid cells. In the endocycles, replication of the gene-rich euchromatin precedes replication of the heterochromatin, which in the case of multiple endocycles may result in loss of heterochromatin regions and preferential amplification of the entire or part of the euchromatin. Multiple gene copies may also be advantageous to overcome DNA damages caused by environmental factors and genetic errors linked to chromosome segregation are also limited during endoreduplication [7,8]. Moreover, the endocycles do not require the reorganisation of the cytoskeleton and may allow faster growth, for example, in the case of fruits or grains, than growth by cell proliferation. Cell enlargement linked to endoreduplication may also be required for specific morphology of cells like in the case of trichomes.

The first molecular evidence demonstrating the biological role of endoreduplication comes from the comparison of the expression profiles of the haploid, diploid, triploid and tetraploid yeast genomes. In these isogenic strains, Galitski et al. [9] showed ploidy-dependent expression of a subset of the genome that might control and specify cell functions.

3. Endoreduplication and differentiation in plants

In plants, endoploidy is most common in angiosperms but found also in algae and mosses [10,11]. Polyploidy can occur in any tissue or organ and might be part of the differentiation of a single cell such as the *Arabidopsis* trichome. These single cells undergo four cycles of endoreduplication, resulting in 32C DNA content and develop three branches [12]. Several mutants affected in trichome development were also altered in ploidy levels and suppression of endoreduplication cycles resulted either in multicellular trichomes or reduced cell size and induced cell death, indicating that endoreduplication is tightly linked to differentiation of trichome cells [13,14]. As the ploidy level may determine the volume and storing capacity of cells, special attention is paid to endoreduplication cycles in maize kernels. During seed development, after an initial mitotic proliferation period, endocycles occur from 10 to 20 days after pollination in the endosperm, leading to 24C, 48C and 92C DNA content which in a few cells can even reach 384C values [15,16]. Similarly, development of large cells in fruits, for example, in tomato, involves also endocycles [11,17].

While the above examples of endocycles are controlled by developmental programs and reviewed recently [8,11,18], endoreduplication cycles can also be induced during pathogenic and symbiotic plant interactions. Here, we focus on endosymbiotic interactions of the model legume *Medicago truncatula* and the cultivated alfalfa *Medicago sativa* with the soil bacterium *Sinorhizobium meliloti* and the endoparasitic root knot nematode *Meloidogyne incognita.*

The symbiosis between *S. meliloti* and its host plants *M. truncatula* or *M. sativa* results in the formation of a particular plant organ, the nitrogen-fixing root nodule [19] which represents the highest level of somatic endoploidy in these plants. Nodule organogenesis is triggered by *S. meliloti* Nod factors in the emerging root hair zone at limitation of combined nitrogen. The Nod factors are lipochitooligosaccharide signal molecules that reactivate the cell cycle in the differentiated G0-arrested cortical cells, which leads to cell division in the inner cortex and de novo formation of the nodule meristem [20]. The nodule primordium, after its outgrowth of the root, differentiates into various nodule cell types resulting in a complex nodule structure [21] (Fig. 1). The meristem (zone I) persists in the apical region, whereas the downstream central region of a nitrogen-fixing nodule is composed of the infection zone II and the nitrogen fixation zone III. Infection of plant cells and differentiation of symbiotic cells take place in zone II. In this zone, the bacteria still produce Nod factors and although the cells do not divide, cell cycle activities necessary for DNA synthesis are maintained [1,20] and the cells undergo successive rounds of endoreduplication cycles. As a consequence, the nuclear DNA content increases from 2C up to 64C and, proportional to the genome size, the cells enlarge drastically as they become older and more distant from the meristem during the longitudinal nodule growth [1,22].

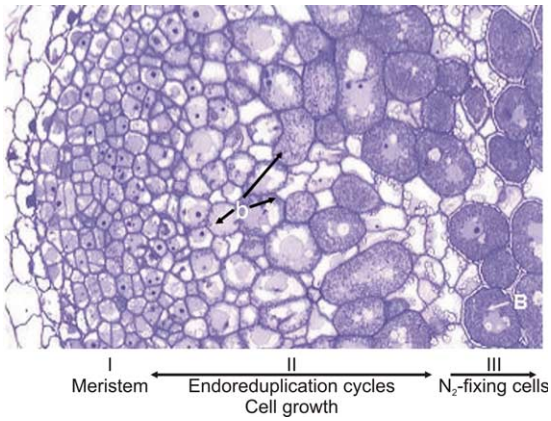

Fig. 1. Growth and differentiation of symbiotic cells in *M. truncatula* root nodule. Longitudinal section shows the apical nodule region, the persistent apical meristem (I), the infection zone (II) and the upper part of the nitrogen-fixing zone (III). In zone II, symbiotic cells enter successive endoreduplication cycles which correlate with the gradual growth of the cells. In zone III, the symbiotic cells are terminally differentiated, packed with bacteria and highly specialised for symbiotic functions. Arrows indicate bacteroids at the early (b) and late (B) stages of their development.

Meloidogyne incognita, an endoparasitic root knot nematode induces re-differentiation of root cells to nematode feeding sites. Infection occurs usually in the vicinity of the root tip where second-stage infective juveniles penetrate the roots and migrate toward the vascular cylinder. Close to the xylem, the nematodes trigger the development of a few giant cells characterised by nuclear and cellular hypertrophy generated via endoreduplication cycles [23]. Formation of giant cells and division of the neighbouring root cells result in the formation of root-knots or galls.

4. Regulation of endocycling

The endoreduplication cycle represents a simplified version of the mitotic cell cycle. It is composed of two phases; an S-phase and a gap period in contrast to the G1, S, G2 and M phases of the mitotic cycles (Fig. 2). In all eukaryotes, the cell cycle is controlled by sequential activities of cyclin-dependent kinases (CDK), which form complexes with different cyclins that regulate the timing, substrate specificity and the

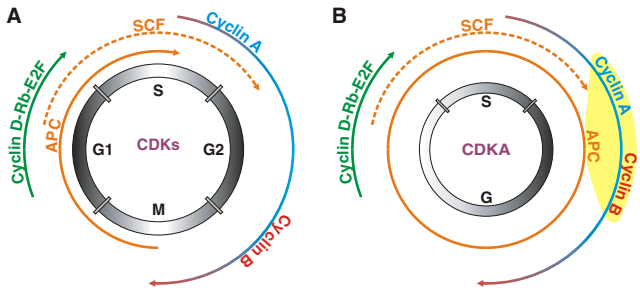

Fig. 2. Mitotic cycle (A) versus endocycle (B).

localization of CDK activities. Periodic activities of the different CDK–cyclin complexes are regulated at multiple levels, including cell cycle-regulated expression, interactions of CDKs with inhibitor proteins and posttranslational modification of cell cycle proteins by phosphorylation or their irreversible degradation by the ubiquitin-proteasome pathway via the anaphase-promoting complex (APC) and the Skpl-Cullin-F-box protein (SCF) E3 ubiquitin ligases [24]. It is suggested that endoreduplication requires nothing more than loss of M-phase and oscillations in the activity of S-phase CDK [8]. In the following, our present view on the control of DNA replication and mitosis inhibition will be described.

4.1. G1–S control and DNA replication

Oscillation of S-phase-dependent kinase activity is suggested to re-replicate chromosomes. The endocycles appear to use much of the same machinery as mitotic cycles to re-enter the S-phase. G1–S transition is controlled by the retinoblastoma (RB)-E2F pathway [25,26]. The E2F proteins interacting with DP form heterodimeric transcription factors which regulate the expression of a wide variety of genes including those required for DNA replication or encoding structural proteins of chromatin. E2F activity is negatively regulated by RB which in its hypophosphorylated form binds to E2F and blocks its activation domain. CDKA–cyclin D complexes phosphorylate RB (known as retinoblastoma-related, RBR in plants), which results in the release and activation of E2F. Overexpression of E2Fa and DPa in transgenic *Arabidopsis* plants promoted endoreduplication and upregulation of key S-phase initiation genes such as ORC, CDC6, CDT1 and MCM [26,27].

In maize endosperm, the Rb-related protein undergoes changes in the level and the phosphorylation state concomitant with endoreduplication and the activity of S-phase CDKs increases substantially with the initiation of endoreduplication [28]. Involvement of CDKA in endoreduplication was shown by overexpression of CDK inhibitor proteins (ICK), known also as Kip-related proteins (KRP). Overproduction of KRP2 in *Arabidopsis* resulted in a decrease in CDK activity and reduction in the endoreduplication levels in older leaves [29]. Similarly, overexpression of NtKISla, a tobacco CKI, interfered with endoreduplication in *Arabidopsis*. In 35S::NtKJSla rosette leaves, most cells displayed 2C and a small fraction of 4C DNA contents, whereas endoreduplicated cells with 8C, 16C and 32C nuclei were absent [30]. Misexpression of KRP1 in single-celled *Arabidopsis* trichomes reduced endoreduplication and cell size [14]. Ectopic expression of AtCDC6, one of the E2F regulated genes that is essential for activation of DNA replication origins, increased the proportion of 16C cells in transgenic *Arabidopsis* leaves indicating that Cdc6 may be one of the factors required for the maintenance of endoreduplication cycles [31].

While all the above mentioned genes are active both in mitotic and endocycles, there are some examples when the machinery of mitotic and endocycles is different. In *Drosophila*, MCM4/dpa, a member of the evolutionarily conserved MCM family required for DNA replication, is involved in mitotic cycles but not in endoreduplication cycles [32].

Cyclin A2 from *M. sativa* and *M. truncatula* is another example [33]. This cyclin, structurally characterised as a mitotic A2-type cyclin, is present from late G1 until prophase in the mitotic cell cycle and interacts with CDKA and RBR. The CycA2-associated kinase activities peak in mid S-phase and at the G2/M transition. *CycA2* is amongst the earliest genes induced during lateral root or nodule development. CycA2 is present in the meristems but absent in postmitotic cells undergoing endoreduplication [34]. *CycA2* is not expressed during root-knot nematode-induced gall development, which involves endoreduplication but not secondary meristem formation suggesting that CycA2 might be required for meristemaric activities but dispensable for cell differentiation and might be incompatible with endocycles.

Recent data from three independent laboratories point to the importance of DNA topoisomerase VI in endocycles beyond 8C level in *Arabidopsis*. In all organisms, type II DNA topoisomerases are essential for untangling chromosomal DNA [35]. DNA topoisomerase VI is an archaeal type II topoisomerase composed of two subunits, TOP6A and TOP6B forming a heterotetramer, A_2B_2. Mutations in TOP6A and TOP6B subunits were identified in two sets of *Arabidopsis* dwarf mutants *root hairless2(rhl2)/brassinosteroid insensitive5 (bin5)/At sporulation11-3 (atspo11-3)* and *hypocotyl6 (hyp6)/bin3/attop6b* carrying mutations in the TOP6A and TOP6B subunits, respectively [36–38]. In these mutants, the mitotic cycles and endoreduplication up to 4C and 8C levels were not affected, but the higher ploidy levels were reduced. This indicates that DNA topoisomerase VI might be required to decatenate DNA during the successive rounds of endoreduplication [36,37]. Moreover, the failure to increase ploidy resulted in smaller cell size, supporting the nuclear–cytoplasmic ratio theory.

4.2. Inhibition of mitosis: the switch to endocycles involves $APC^{cdh1/Ccs52A}$

M-phase progression is controlled by successive functions of cyclin A- and cyclin B-associated CDK complexes. These activities should be inhibited or limited during endocycles and might be controlled at multiple levels. There are examples when expression of mitotic cyclins is switched off during endocycles, however, in many cases mitotic cyclins are expressed during endocycles, as it has been demonstrated for cyclin B in *Medicago* nodules [34] indicating that inactivation of the mitosis-promoting factor might be controlled either by CKIs or by altered, premature degradation of mitotic cyclins. Mitotic cyclins are known as unstable proteins, which contain in their N-terminal region a Destruction (D-box; RxxLxxxxN) [39] sequence that targets their degradation via the ubiquitin-proteasome pathway. Recent work on *Medicago* root nodules provided molecular evidence for the involvement of this proteolytic pathway in the endoreduplication cycles.

The ubiquitin-dependent proteolysis ensures that specific protein functions are turned off at the right time, in the right place, and in a unidirectional fashion. Polyubiquitylation of

proteins involves at least three enzyme activities. The ubiquitin-activating enzyme (E1) forms a high-energy bond with ubiquitin, which then is transesterified to an ubiquitin conjugating enzyme (E2). The transfer of ubiquitin to the target protein substrate requires an ubiquitin protein ligase (E3). Polyubiquitylation of a protein is sufficient to target its degradation by a large ATP-dependent multicatalytic protease, the 26S proteasome. The selection and specific timing of polyubiquitination of the target proteins are conferred by different E3 ubiquitin ligases. In the cell cycle, two structurally related cullin-dependent multi-component ubiquitin ligases, the APC and the SCF complexes, have essential and complementary functions [24].

The APC is nuclear and has fundamental roles in the metaphase–anaphase transition, exit from mitosis, and control of DNA replication by ordered destruction of various cell cycle proteins including mitotic cyclins [40,41]. The core APC components are also present in postmitotic cells (e.g., terminally differentiated neurons) [42], however, the role of APC outside the cell cycle, including the endoreduplication cycle, is largely uncovered. Temporal and spatial control on the activity and substrate specificity of the APC are defined by two WD40-repeat activator proteins, Cdc20 (also known as Slpl, Fzy, $p55^{CDC}$) and Cdhl (also known as Hctl, Ste9/Srwl in yeast, Fzr in *Drosophila*). Expression of *cdc20* is restricted to the mitotic cycle from late S-phase to M-phase, while *cdh1* is constitutive and active both in mitotic and postmitotic cells [42,43]. These proteins have an extreme capacity for protein interactions via the seven WD40 repeats and the N-terminal region. Their binding to APC requires an N-terminal C-box sequence and the C-terminal IR residues [44,45]. The Cdhl proteins have in addition a Cdhl-specific motif that is also required for APC interaction [46]. Phosphorylation of Cdhl by CDKA–cyclin A decreases APC^{Cdh1} activity during S and G2 by preventing the association of Cdh1 with the APC [46,47] and leading to its nuclear export [48,49]. Both Cdc20 and Cdhl recognize D-box proteins as APC substrates and have a conserved cyclin binding RVL motif in their C-terminus. Thus, APC^{Cdc20} as well as APC^{Cdh1} mediate degradation of mitotic cyclins, however, differing in the timing and spatial control on cyclin destruction. Cdhl interacts with a wider range of APC substrates that contain KEN- [50], A- [51], or GxEN-boxes [52].

APC functions are unexplored in plants. Most of the APC subunits are evolutionarily conserved and the APC subunits could be predicted on the basis of homology in *Arabidopsis* [53], however, APC has not been purified yet from plants and the exact composition remains to be identified. Not only the core components, but the APC activators are also conserved, however, in plants Cdc20 and Cdhl proteins are encoded by several genes in contrast to single gene copies in most animals [46].

In plants, two classes of the Cdhl-type proteins were identified: Ccs52A that appears to be an orthologue of the yeast and animal Cdhl-type proteins and Ccs52B that is plant-specific [46]. *ccs52A*, the first plant orthologue of *Cdh1*, was identified from *M. sativa* nodules as a cell cycle switch gene, involved in conversion of mitotic cycles to endocycles [1]. In fission yeast, Ccs52A but not Ccs52B was able to interact with the yeast APC and to elicit degradation of the mitotic cyclin Cdc13 resulting in M phase and growth inhibition, repeated

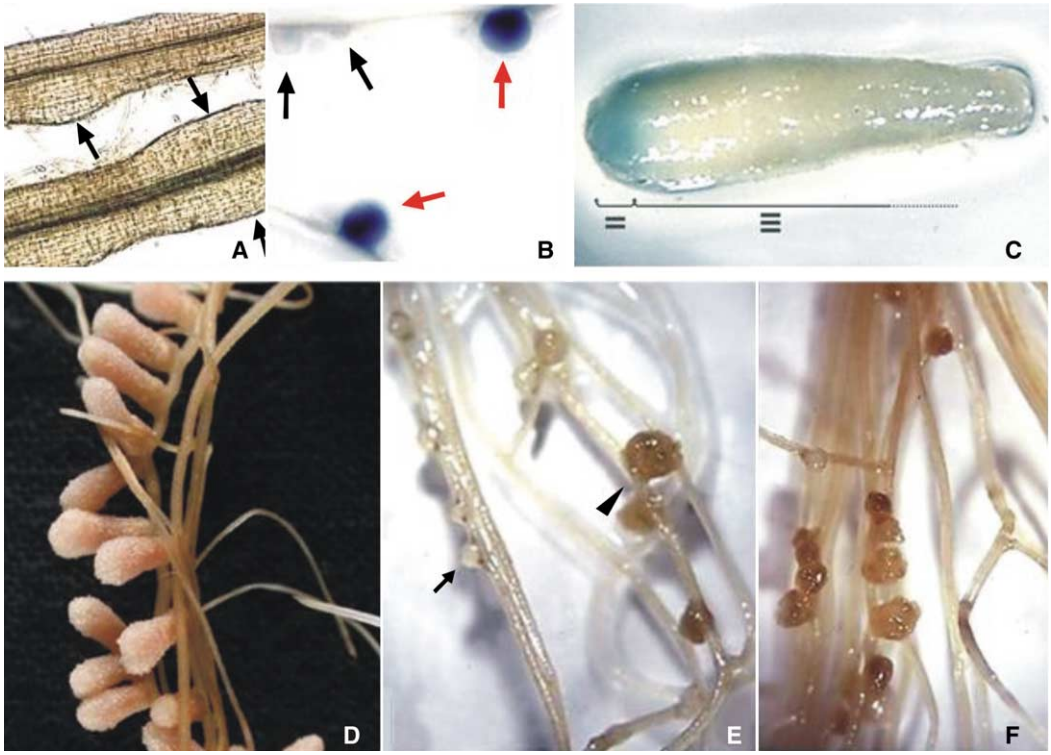

Fig. 3. Ccs52A is required for differentiation of symbiotic nitrogen-fixing cells. A–C: Gus staining during nodule development in transgenic *M. truncatula* carrying the *Mtccs52A* promoter-*uidA* fusion. A: Cell proliferation in the root cortex upon *S. meliloti* infection. Arrows indicate the initiation of nodule primordia. B: Emerging (black arrows) and fully grown (red arrows) nodule primordia. C: Differentiated nitrogen-fixing nodule. D–F: Comparison of wild-type (D) and *ccs52A* antisense (E, F) nodules. In (E) the arrow points to a nodule primordium and arrowhead to an aborted, senescent nodule.

rounds of endoreduplication cycles and elongation of the yeast cells [1,46].

In planta, expression of *ccs52A* was linked to cell differentiation and endoreduplication [46,54]. In *M. sativa* and *M. truncatula*, polyploid cells are present in all organs except in the leaves and expression of *Mtccs52A* was observed transiently during the differentiation of the various organs. In nitrogen-fixing root nodules, *ccs52A* transcript levels were the highest, which correlated with the highest ploidy levels in *Medicago* [1]. *ccs52A* was not expressed during nodule primordium formation indicating that *ccs52A* was not required for the mitotic cycles (Fig. 3A). Similarly, the *Drosophila* Fzr and the chicken Cdhl proteins were also dispensable for cell cycle progression but crucial for cell cycle arrest [55,56]. *ccs52A* was activated prior to nodule differentiation when cells exit from the mitotic cycle and enter endoreduplication (Fig. 3B). In the nodules, *ccs52A* was expressed in the meristem and zone II (Fig. 3C) and the Ccs52A protein was nuclear and present in all endoreduplication competent cells in zone II [54]. The constant presence and nuclear localization of Ccs52A as well as the absence of CycA2, that is potentially involved in negative regulation of Ccs52A, may reflect a constitutive APC activity that might be necessary for the endoreduplication cycles. *ccs52A* has also been induced in endoparasitic-nematode interactions, during the formation of giant polyploid feeding cells in *M. truncatula* [57,58] where similarly to endoreduplicating nodule cells, CycA2 was also absent.

The APC activity has not been reported for endoreduplication cycles, although non-periodic APC^{Cdh1} activity in human cells prevented G2 and M events and caused endoreduplication that was linked to the destruction of several mitotic regulators including cyclin A and cyclin B1 [59]. These data support our hypothesis that non-periodic APC activity might be necessary for endoreduplication cycles.

5. Functional proof for the requirement of Ccs52 in symbiotic nodule development

The biological significance of endoreduplication has been long debated, whether this is a cause or a consequence of the differentiation. This was studied in antisense transgenic *M. truncatula* on nodule development where expression of *ccs52A* was down-regulated [54]. These plants responded similarly to the *S. meliloti* Nod factors as the wild-type plants; cell proliferation and the initial stage of nodule development were unaffected and the nodule primordia appeared with the same kinetics and numbers as in the control plants. In contrast, a drastic difference was observed when differentiation of the nodule primordium started (Fig. 3D–F). In the control plants, the differentiation coincided with endoreduplication cycles and formation of polyploid cells leading to the development of nitrogen-fixing nodules (Fig. 3D). In the *ccs52A* antisense plants, many nodules were halted at the primordium stage and senescence started already in these globular, primordium-like nodules (Fig. 3E). The antisense expression of *ccs52A* did not silence, only reduced the expression of the endogenous gene, therefore a few nodules developed further, but never to nitrogen-fixing nodules (Fig. 3F). These nodules were elongated, bacterial infection and multiplication of the bacteria started in the submeristematic cells, which corresponded to the first cell layers of zone II in wild-type nodules. The differentiation of the infected symbiotic cells was, however, not completed and these cells showed premature senescence, disintegration leading to death of the cells and finally the whole organ.

Measurement of the nuclear DNA content of cells by flow cytometry showed that down-regulation of *ccs52A* correlated with a decrease in nodule ploidy. Compared to the control, there was a 50% reduction in the population of endoreduplicated cells (>4C) in the aborted nodules. This affected particularly the third and fourth endoreduplication cycles, resulting in a sixfold reduction in the proportion of 32C nuclei and the absence of 64C nuclei. The average area of the largest cells was 35% smaller in the aborted nodules than in wild-type nodules. This result was consistent with the decreased production of the highly endoreduplicated cell populations and was in line with the "nuclear–cytoplasmic ratio" theory; adjustment of cell volume with respect to the DNA content of the nucleus.

All these data show a tight linkage between reduced expression of *ccs52A* and decrease in endoploidy and cell size. Moreover, the correlation of reduced ploidy with inefficient nitrogen fixation indicates that endoreduplication cycles do not simply accompany but do play a central role in nodule development. Repeated endoreduplication cycles during symbiotic cell development might have dual roles; in one hand they could ensure extreme enlargement of cells to host the bacteroids and, on the other hand, provide energy and nutrient supply for the bacteroids by increased transcriptional and metabolic activities of the host cell.

If cell cycle activities are provided for DNA replication, upregulation of *ccs52A* is expected to increase ploidy levels, cell and organ sizes. This could not be proven in *M. truncatula*, since transformation via callus formation and somatic embryogenesis did not allow overexpression of *ccs52A*, likely because Ccs52A inhibits cell proliferation. In *Arabidopsis*, slight overproduction of the Ccs52A proteins Was however possible, which confirmed a direct correlation between *ccs52A* expression levels and degrees of ploidy in different cell types and organs (unpublished) supporting an important role for Ccs52A in the regulation of endocycles.

6. Perspectives

During the last few years significant progress has been achieved in the plant cell cycle research. This has led to the identification of cell cycle components at genome level and revealed significantly higher complexity of cell cycle in plants than in other eukaryotes. Functional characterisation of these cell cycle regulatory proteins, attributing specific functions to them in the mitotic and endoreduplication cycles, will be a major task in the coming years. As regulation of G1–S involves the same pathway and mostly identical components during the mitotic and endocycles, the critical step in the conversion of mitotic cycles to endocycles is probably linked to inhibition of M-phase.

It became evident lately that APC controls most cell cycle events. Its activity and the degradation of selected proteins by the ubiquitin-proteasome pathway depend on the APC activator Cdc20 and Ccs52A/Ccs52B subunits. Our studies provided evidence for the involvement of Ccs52A in the induction and maintenance of successive endocycles. This is probably due to the inactivation of mitotic cyclins but APC^{CCS52A} likely

functions much beyond that. In the endocycles, G1–S events might be under the control of APC^{CCS52A} either directly or via its superior control on SCF [60,61]. Moreover, the presence of Ccs52A and Ccs52B proteins in differentiating cells indicates that in addition to cell cycle-related control of development, they may also contribute to the specialisation of different cell types. As more than 5000 *Arabidopsis* proteins might be potential targets of the APC, the exploration of the APC-regulated molecular pathway and cellular processes in differentiating and endoreduplicating cells will be a great challenge in the near future.

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