The mitotic inhibitor *ccs52* is required for endoreduplication and ploidy-dependent cell enlargement in plants

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Plant organs develop mostly post-embryonically from persistent or newly formed meristems. After cell division arrest, differentiation frequently involves endoreduplication and cell enlargement. Factors controlling transition from mitotic cycles to differentiation programmes have not been identified yet in plants. Here we describe *ccs52*, a plant homologue of APC activators involved in mitotic cyclin degradation. The *ccs52* cDNA clones were isolated from *Medicago sativa* root nodules, which exhibit the highest degree of endopolyploidy in this plant. *ccs52* represents a small multigenic family and appears to be conserved in plants. Overexpression of *ccs52* in yeast triggered mitotic cyclin degradation, cell division arrest, endoreduplication and cell enlargement. In *Medicago*, enhanced expression of *ccs52* was found in differentiating cells undergoing endoreduplication. In transgenic *M.truncatula* plants, overexpression of the *ccs52* gene in the antisense orientation resulted in partial suppression of *ccs52* expression and decreased the number of endocycles and the volume of the largest cells. Thus, the *ccs52* product may switch proliferating cells to differentiation programmes which, in the case of endocycles, result in cell size increments.

Keywords: cell differentiation/cell size control/fizzy-related/plant cell cycle/WD-repeat proteins

Introduction

Duplication of the genome without mitosis (endoreduplication) is a common process in eukaryotes (Nagl, 1978). Endoreduplication results in an increase in the nuclear DNA content, permitting amplification of the genome of specialized cells. Moreover, the increase in the ploidy level correlates with an increase in nuclear volume and cell size, suggesting that elevated nuclear DNA content is required to maintain larger cells. In animals, endoreduplication is most frequent in arthropods, where the highest values of endopolyploidy occur in the Malpighian tubules and salivary glands of various dipters. In mammals, endopolyploidy is rare and evident only in the trochoblast,

decidua and myocardial cells (Conlon and Raff, 1999). In contrast, endoreduplication is widespread in plants, particularly in angiosperms, and may occur in any cell types except the gametes, the meristematic and guard cells (Nagl, 1978). The constant tissue-specific pattern of endopolyploidy in different organs suggests that endoreduplication cycles in plants constitute an essential part of the developmental programmes that are necessary for differentiation and for specialized function of given cells and tissues. Regulation of endopolyploidy may operate at multiple levels. It requires exit from the mitotic cycle and transformation of the cell cycle to the endocycle by inhibition of the G₂–M transition and cell- or tissue-specific control of the number of endocycles. Thus, formation of differentiating polyploid tissues is likely to be controlled by components of the cell cycle machinery. Indeed, it was shown recently that endoreduplication in maize endosperm cells involves the inhibition of the M phase-promoting factor (MPF) composed of cyclin-dependent kinases (CDKs) and mitotic cyclins, as well as the stimulation of S phase CDKs and phosphorylation of retinoblastoma homologues (Grafi and Larkins, 1995; Grafi *et al.*, 1996). However, the nature of the mitotic inhibitors inducing endoreduplication in plants remains unknown.

The root nodules of leguminous plants induced by the *Rhizobium* soil bacteria represent a striking example of high somatic ploidy (Truchet, 1978). Nod factors produced by rhizobia act as external mitogenic signals that induce cell division in the root cortex, leading to the formation of nodule meristem (Truchet *et al.*, 1991). The meristematic activity is maintained in the indeterminate nodules where cells exit continuously from the persisting apical meristem and enter the nodule differentiation programme by division arrest, followed by several rounds of endocycles and a gradual increase in the cell volume. These endoreduplicated and enlarged cells can be invaded and can host the bacteroids, the nitrogen-fixing form of rhizobia, while diploid cells remain uninfected (Truchet, 1978). By studying the molecular basis of root nodule organogenesis in *Medicago*, the symbiotic partner of *Rhizobium meliloti*, we have identified *ccs52*, a cell cycle switch gene encoding a 52 kDa protein, that appears to be an ubiquitous regulator of cell cycle transition to differentiation.

Results

Identification of *ccs52* encoding a WD-repeat cell cycle regulatory protein

In the course of screening of a young nodule cDNA library for genes involved in nodule organogenesis in *Medicago sativa* (alfalfa), we found a cDNA clone, *ccs52*, exhibiting stimulated expression during nodule development. Sequence analysis revealed an open reading frame encoding a 52 kDa polypeptide of 475 amino acids

that contained a putative PEST protein degradation motif (PEST find score: +18.4) at the N-terminal region, several putative CDK phosphorylation sites and, in the C-terminal part, seven WD40 repeats. Using an *M.sativa* *ccs52* cDNA probe, we isolated the *ccs52* gene also from the diploid *M.truncatula*. The deduced sequences of *M.sativa* and *M.truncatula* CCS52 proteins were 99.6% identical and differed only in two residues at positions 16 (R/G) and 141 (V/I). The WD-repeat proteins found in eukaryotes constitute a superfamily of regulatory proteins controlling diverse cellular functions. Functional homology between different polypeptides is reflected by the same number of WD repeats, by higher similarity of WD repeats at equivalent positions than to any other repeats in the same protein, and by sequence similarity at the N- and C-terminal extensions (Neer *et al.*, 1994). The deduced CCS52 protein sequence showed the highest homology with the WD-repeat cell cycle regulatory proteins (Figure 1A). Within this family, CCS52 was more homologous to FZR (fizzy-related) from *Drosophila melanogaster* (57% amino acid identity) (Sigrist and Lehner, 1997), to SRW1/STE9 from *Schizosaccharomyces pombe* (52%) (Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998), to HCT1/CDH1 from *Saccharomyces cerevisiae* (46%) (Schwab *et al.*, 1997; Visintin *et al.*, 1997) and to other putative FZR/CDH1 products from animals and human than to the CDC20/FZY (fizzy) proteins. On the basis of the *Medicago ccs52* cDNA sequence, we assembled two AtCCS52 polypeptides (A and B) from the available *Arabidopsis thaliana* genomic sequence database. These polypeptides exhibited higher similarity (80 and 64% identity, respectively) to the *Medicago* CCS52 than to the yeast and animal homologues, indicating a strong conservation of the CCS52 proteins in the plant kingdom. Comparing the N-terminal sequences of the WD-repeat cell cycle regulatory proteins has led us to identify the conservation of an oligopeptide in box II that appears to be specific for the CCS52/FZR/SRW1(STE9)/HCT1(CDH1) proteins and not conserved in the CDC20/FZY proteins, as shown for the *Drosophila* and the *Arabidopsis* FZY (Figure 1B). In contrast, box I as well as similarity of the equivalent WD40 repeats were detected in all these proteins.

Conservation of the *ccs52* gene was tested by genomic Southern blot analysis in tetraploid and diploid *Medicago* species and in various other plant species including *Arabidopsis*, tobacco, tomato, potato, soybean, wheat and rice (data not shown). In the genome of the tetraploid *M.sativa* lines, two fragments hybridized strongly and three to five fragments more weakly to the *ccs52* probe, suggesting that *ccs52* represents a gene family. Similarly, a few hybridizing bands were detected in *M.truncatula* as well as in most of the plant species tested.

CCS52 is a functional homologue of SRW1 in fission yeast

The FZR, SRW1/STE9 and HCT1/CDH1 proteins have been shown to control degradation of specific mitotic cyclins and exit from cell division (Schwab *et al.*, 1997; Sigrist and Lehner, 1997; Yamaguchi *et al.*, 1997). To demonstrate functional homology of CCS52 to these proteins, in addition to its structural conservation, the effect of overexpression of *ccs52* in *S.pombe* was compared with that of *srw1*⁺. In fission yeast, overexpression of *srw1*⁺

from the thiamine-repressible expression vector pREP1 has led to endoreduplication and cell enlargement as a consequence of successive round(s) of cell cycles without mitosis (Yamaguchi *et al.*, 1997). The *ccs52* coding sequence was inserted similarly in pREP1, yielding pREP1-*ccs52*. This plasmid as well as pREP1 and pREP1-*srw1*⁺ (Yamaguchi *et al.*, 1997) as negative and positive controls, respectively, were transformed into *S.pombe*. Upon withdrawal of thiamine, the growth of the cells carrying pREP1 was not affected; however, expression of either the *srw1*⁺ or the *ccs52* gene resulted in growth arrest (Figure 2B compared with A). Similarly, overexpression of the *D.melanogaster* or *S.cerevisiae* homologues in their respective organisms provoked cell division arrest by inhibiting mitosis. Moreover, *S.pombe* cells carrying pREP1-*ccs52* became abnormally elongated and contained enlarged nuclei (Figure 2D). In contrast, cells carrying only pREP1 (Figure 2C) exhibited no morphological changes and were like the wild-type cells. To investigate whether these responses were specific for *ccs52*, we cloned the *Arabidopsis* *fzy* gene in pREP1 followed by transformation in *S.pombe*. The *S.pombe*(pREP1-Atfzy) transformants behaved similarly to the control *S.pombe*(pREP1) cells, demonstrating that growth inhibition and cell enlargement were due to *ccs52* expression. Analysis of the nuclear DNA content by flow cytometry revealed the presence of 4C and 8C nuclei in *S.pombe*(pREP1-*ccs52*) cells (Figure 2F). Overexpression of *srw1*⁺ also produced 4C nuclei (not shown; Yamaguchi *et al.*, 1997), in contrast to the control *S.pombe*(pREP1) cells where no nuclei >2C were detected (Figure 2E). These data demonstrated that overexpression of either *ccs52* or *srw1*⁺ resulted in the same cellular response: inhibition of mitosis and endoreduplication of nuclear DNA.

In *S.pombe*, SRW1 is essential for the degradation of the mitotic cyclin CDC13 (Yamaguchi *et al.*, 1997). To find out whether CCS52 also acts via mitotic cyclin degradation, we investigated the protein level of CDC13 in the Δ *srw1* derivative of *S.pombe* (SY1) which is unable to degrade CDC13. SY1 was transformed with pREP1 as well as with (pREP1-*ccs52*), and protein extracts of two independent transformants from each cell type were tested by Western blot analysis (Figure 2F). As compared with the control SY1(pREP1) cells, expression of *ccs52* in SY1(pREP1-*ccs52*) resulted in a drastically reduced level of the CDC13 protein. In contrast, the level of the cyclin-dependent kinase CDC2 protein detected by an anti-PSTAIR antibody or that of α -tubulin were not affected by *ccs52*. Thus, the observed effects of *ccs52* expression, namely growth arrest, endoreduplication, cell elongation and mitotic cyclin degradation, indicate that CCS52 is functionally equivalent to SRW1.

Expression of *ccs52* is linked to differentiation and endoreduplication of plant cells

In *Drosophila*, *fzr* expression is under developmental control, exhibiting temporal and spatial regulation during embryogenesis, and is required for cyclin degradation during G₁ when the embryonic epidermal cell proliferation stops, and during G₂ preceding salivary gland endoreduplication (Sigrist and Lehner, 1997). If *ccs52*, similarly to *fzr* in *Drosophila*, is involved in cell differentiation, its expression may be regulated differentially during develop-

ment of the distinct plant organs or tissues. Northern analysis with RNA from various organs of *M.truncatula* demonstrated that *ccs52* expression is not restricted to nodulation (Figure 3A). Although the highest expression was found in nodules (lane 7), the *ccs52* transcript was also detected in other organs. In roots, the expression was localized predominantly in the tip region (lanes 6 and 8). Since *ccs52* expression in fission yeast evoked endoreduplication, we tested whether a correlation exists between endopolyploidy and *ccs52* expression. The nuclear DNA content was measured in different organs of the diploid *M.truncatula* by flow cytometry (Figure 3B). All of the vegetative organs tested, except the leaves, contained polyploid nuclei, albeit with different levels of endopolyploidy. The endopolyploidy was high in petioles (2C 45%, 4C 35%, 8C 16%, 16C 4%, 32C 0.3%) and nodules (2C 8%, 4C 68%, 8C 10%, 16C 3%, 32C 11%), and lower in roots (2C 49%, 4C 49%, 8C 2%). Thus, the highest degree of endoreduplication detected in nodules coincided with the highest level of *ccs52* expression, suggesting that in plants the degree of endopolyploidy and the level of *ccs52* expression are directly correlated. In the leaves, most of the cells maintained the 2C DNA content (95%) and only a few 4C nuclei (5%) were detected, suggesting that expression of *ccs52* in leaves might reflect a function similar to that of *fzr* in the epidermal cells required to exit from the cell cycle in G₁ phase.

To obtain further insight into the involvement of *ccs52* in cell differentiation and endoreduplication, the *ccs52* transcript was localized in nodules and root tips by *in situ* hybridization (Figure 3C, D and H). *Medicago* nodules contain three major zones differing in the level of cell differentiation: the meristematic zone (zone I); the pre-fixation zone (zone II), where cell division is arrested and the plant cells undergo differentiation and become infected by the rhizobia; and the nitrogen fixation zone (zone III), where both the plant cells and the bacteria are terminally differentiated for symbiotic functions (Figure 3C). In *M.sativa* nodules, the meristematic cells were shown to contain 2C–4C DNA, corresponding to G₁–G₂ nuclei, while cells in zones II and III were endoreduplicated (Truchet, 1978). Expression of *ccs52* shown for the digoxigenin-labelled antisense RNA probe (red) was detected in zones I and II (Figure 3C). A dramatic and sharp change in *ccs52* expression was observed, however, at the boundary of zones II and III (Figure 3D). The expression of *ccs52* was high in the cells of zone II, just preceding the final cell enlargement and differentiation events required for symbiotic nitrogen fixation, while in the adjacent cells belonging to zone III, *ccs52* expression decreased to background levels. Hybridization with ³⁵S-labelled probe resulted in the same pattern of *ccs52* expression, albeit that the signal was weaker, while no signal was detected with either the digoxigenin- or the ³⁵S-labelled sense probes (data not shown). Thus, the *ccs52* transcript in the nodule was found in those regions

where cells were entering (zone I) or undergoing (zone II) differentiation.

In order to localize the sites where endoreduplication occurs, the nodule sections were hybridized with ³⁵S-labelled antisense histone H3 RNA probe (Wu *et al.*, 1989) marking the S-phase cells (Figure 3F). In contrast to the homogenous expression pattern of *ccs52*, expression of histone H3 exhibited a patchy pattern. It was found in the meristem and in certain cells in zone II (yellow spots) but not in zone III. Similarly, the transcript of the B2-type mitotic cyclin, *cycIIIMs* (Savouré *et al.*, 1995), referred to as *Mscyc2;3* according to the new nomenclature of plant cyclins (Renaudin *et al.*, 1996), was also localized in the meristem and in a few cells of zone II (red spots), corresponding to cell cycle-regulated expression of mitotic cyclins (Figure 3G). Lack of expression of these cell cycle marker genes in zone III indicated that cell cycle activity ceased when cells were committed to symbiotic nitrogen fixation. Furthermore, it also demonstrated that endocycles could occur in zone II where cell cycle genes are expressed but cell division is arrested. The S-phase cells expressing histone H3 in zone II may represent those which undergo endoreduplication. As cells are moving away from the meristem and getting closer to zone III, they may enter multiple rounds of endocycles consistent with the observed gradual increase in nuclei in this region (Figure 3E).

In the root tip, *ccs52* expression was found predominantly in cell files leading to the differentiation of the epidermis, the root cortex, the endodermis and the pericycle (Figure 3H). Expression of histone H3 and *MscycB2;3* was detected in the same root region as that of *ccs52*; however, expression of these genes was localized in individual, differentiating cells rather than in continuous cell files (Figure 3I and J). Thus, the expression pattern of *ccs52* in *Medicago* nodules and roots correlated with its involvement in cell cycle control during the differentiation process. Expression of *ccs52* overlapped that of the mitotic cyclin *MscycB2;3*, which was consistent with the function of the CCS52 homologue, FZR, which did not affect the transcription of mitotic cyclins but played a role in their proteolysis (Sigrist and Lehner, 1997).

Alteration of *ccs52* expression affects endopolyploidy and cell size in *M.truncatula*

Expression of *ccs52* in different organs as well as the presence of multiple *ccs52* gene copies suggested that in addition to its function in nodules, *ccs52* could play a role in the development of other organs as well. The involvement of *ccs52* in plant organ development was tested in transgenic *M.truncatula* plants overexpressing a *ccs52* cDNA fragment in the antisense orientation from the constitutive 35S promoter of the cauliflower mosaic virus (CaMV). Petioles exhibiting a high degree and reproducible pattern of endopolyploidy (Figure 3B) were chosen to study the correlation between reduction of endogenous *ccs52* transcript levels and changes in somatic ploidy. Out of 38 regenerated plants, three T0 lines, A4,

Fig. 1. Relationship of CCS52 to WD40-repeat cell cycle regulatory proteins. (A) Dendrogram of CCS52 homologues generated with the PILE UP program of the GCG software package. Abbreviations: At, *A.thaliana*; Ce, *Caenorhabditis elegans*; Dc, *Daucus carota*; Dm, *D.melanogaster*; Hs, *Homo sapiens*; Ms, *M.sativa*; Mt, *M.truncatula*; Sc, *S.cerevisiae*; Sp, *S.pombe*; Rn, *Rattus norvegicus*; Xl, *Xenopus laevis*. Accession numbers are shown in parentheses. The FZR protein family is framed by a dashed line. (B) Alignment of the plant CCS52 proteins with FZR, SRW1, HCT1 as well as with FZY from *Arabidopsis* and *Drosophila*. Conserved sequences at the N-terminus in boxes I and II are overlined and the positions of WD repeats are indicated.

Fig. 2. Overexpression of *ccs52* in *S. pombe* results in growth inhibition, endoreduplication, cell enlargement and a decreased amount of CDC13. (A) Growth of *S. pombe* transformed with: (1) pREP1, a thiamine-repressible promoter p_{nmt1} vector; (2) pREP1-*ccs52*; and (3) pREP1-*srw1*⁺ in the presence of thiamine. (B) Growth of *S. pombe* transformants 1–3 in the absence of thiamine. (C and D) DAPI-stained cells of *S. pombe*(pREP1) and *S. pombe*(pREP1-*ccs52*), respectively, grown in the absence of thiamine. (E and F) Flow cytometric analysis of the nuclear DNA content of *S. pombe*(pREP1) and *S. pombe*(pREP1-*ccs52*) shown in (C) and (D), respectively. (G) Protein extracts from SY1(pREP1) (lanes 1 and 2) and from SY1(pREP1-*ccs52*) (lanes 3 and 4) separated by SDS-PAGE and immunoblotted with anti-CDC13, anti- α -tubulin and anti-PSTAIR antibodies.

A7 and A32, exhibited reduction in the endogenous *ccs52* transcript levels compared with wild-type and control transgenic plants (C_{2n}) carrying a similar construct with the *uidA* (β -glucuronidase) gene instead of the antisense *ccs52* DNA (Figure 4A). In the A4, A7 and A32 transgenic plants, the petiole ploidy level was also significantly reduced and 16C nuclei were either absent or their proportion was strongly reduced (Figure 4A). A further dramatic decrease in the number of 8C nuclei was found in the line A4 and, to a lesser extent, in the line A32. The 22 control transgenic plants (C_{2n}) exhibited no significant deviation in ploidy from the wild-type petioles (Figure 4A and B). The A4 plant had a slender appearance compared with the control plants due to the formation of fewer side branches, but otherwise it was normal. In the T1 generation of the A4 line, 17 out of 59 germinated plants did not contain 16C nuclei, as measured in the petioles in 1-month-old plants. Partial suppression of *ccs52*

Fig. 3. Expression of *ccs52* is linked to cell differentiation and endopolyploidy. (A) Northern blot analysis of *ccs52* expression in different plant organs. A 20 μ g aliquot of total RNA was used from (1), stems, (2) hypocotyls, (3) leaves, (4) flower buds, (5) flowers, (6) roots without the root tip, (7) nitrogen-fixing nodules and (8) root tips. As a control for RNA loading, the filter was rehybridized with the constitutive *Msc27* probe. (B) Somatic ploidy in different organs of *M. truncatula*. (C, D and F–J) RNA *in situ* hybridization of nodule or root sections with antisense digoxigenin- (C, D and H) and ³⁵S-labelled (F, G, I and J) RNA probes of *ccs52* (C, D and H), the S-phase-specific histone H3 (F and I) and the B-type cyclin *MscycB2;3* (G and J). (E) Nodule structure by autofluorescence. ZI, meristematic zone; ZII, differentiation zone; ZIII, nitrogen fixation zone. The thin arrow in (D) points to *ccs52* transcript accumulation in a zone II cell compared with the adjacent nitrogen-fixing cell (thick arrow) in zone III. The arrows in (H) show the differentiating cell files expressing *ccs52*. Bars correspond to 100 μ m, except in (D) where it is 50 μ m.

Fig. 4. Ploidy levels and cell size patterns in petioles of transgenic *M. truncatula* plants correlate with the expression level of *ccs52*. (A) Measurements of endogenous *ccs52* transcript levels in petioles of independent, antisense *ccs52* transgenic plants (A1, A3, A4, A7 and A32) and in the control transgenic plant (C_{2n}) by RT-PCR as well as by Northern analysis in A4 and C_{2n} plants. The means of three independent experiments, normalized by the *Msc27* signal and expressed as a percentage of the control C_{2n} value, are displayed with the corresponding standard error of the mean (SEM). Open bars show the results of RT-PCR. One representative experiment is shown under the bars. Filled bars correspond to the Northern analysis. The percentage of polyploid nuclei is shown for each plant line. (B) Flow cytometric analysis of petioles from the control diploid (C_{2n}) and tetraploid (C_{4n}) plants and from the line A4 (A_{42n}). (C) Comparison of cell sizes in the petiole parenchyma in the C_{2n}, C_{4n} and A_{42n} plants. x, the mean of cell areas (= 9% SEM) corresponding to the 15 largest cells/petiole. Petiole parenchyma cells from the C_{4n}, C_{2n} and A_{42n} plants are shown at the same magnification, and bars correspond to 100 μ m.

expression in the petioles of transgenic T1 siblings was confirmed by Northern blot analysis and was found to be in a range similar to that in the A4 petioles (Figure 4A). Thus, analysis of T0 and T1 transgenic plants showed a direct correlation between reduced endogenous *ccs52* transcript level and the decrease in the degree of endoploidy in the petioles (Figure 4A). The lack of transgenic plants with a more pronounced reduction of *ccs52* transcript level than detected in the line A4 suggests that stronger suppression of this gene might inhibit embryogenesis, and it could be lethal.

In addition to the petioles, down-regulated expression of *ccs52* also resulted in a lower degree of endopolyploidy

in the hypocotyls and roots of 2- and 3-day-old seedlings measured in two lines of the A4 T2 generation. The C_{2n} hypocotyls contained on average 20% of endoreduplicated (>4C) nuclei, while their ratio in these A_{42n} T2 lines was only 3.8%; moreover, 16C nuclei were absent. A similar but less dramatic decrease was observed in the content of >4C nuclei of seedling roots. While the content of nuclei of >4C in the C_{2n} seedling roots was 4%, suppressed expression of *ccs52* in the A_{42n} plants reduced it to 1.5% and in some plants resulted in the lack of >4C nuclei. In contrast to the endoreduplicated organs, the leaves containing virtually only diploid cells were not affected in their nuclear DNA content by the reduced expression level of *ccs52*.

In *Arabidopsis*, a definite relationship was found between cell size and ploidy level (Melaragno *et al.*, 1993), which raised the possibility that the *ccs52*-mediated changes in endoploidy may also affect the cell size. To analyse the morphological consequences of altered ploidy levels, the cell surface areas were measured in cross-sections of petioles collected from transgenic plants exhibiting either normal (three independent C_{2n} lines) or reduced ploidy (A_{42n} and seven lines from the T1 generation) as well as those from two control tetraploid (C_{4n}) lines. Since down-regulation of *ccs52* abolished or reduced the formation of the 32C, 16C and 8C nuclei, changes in cell size were expected in the largest cells. Indeed, the size of the smallest cells was similar in the C_{2n} and A_{42n} petioles, while the largest parenchyma cells were absent in the A_{42n} petioles. The 15 largest cells were selected and ranked in each petiole section, then the mean areas of the corresponding cells were determined in the A_{42n}, C_{2n} and C_{4n} plants and plotted (Figure 4C). Comparison of the A_{42n} and C_{2n} lines revealed that the parenchyma cells corresponding to the mean values of the four largest cells in C_{2n} exceeded the average size of any parenchyma cells in A_{42n}, correlating with the absence of 16C and 32C cell populations in these A4 plants. The tetraploid lines of *M. truncatula*, obtained in the course of regeneration of leaf explants from the original wild-type line, exhibited a shifted pattern of petiole endoploidy (2C 0%, 4C 62%, 8C 28%, 16C 9%, 32C 1%) (Figure 4B) and served as a control for correlating cell size and polyploidy. In these C_{4n} lines, the mean surface areas of 11 petiole parenchyma cells out of 15 were larger than those of cells in the control C_{2n} plants (Figure 4C). In the lines tested, the cell surface areas on longitudinal sections of petioles showed a similar correlation with the degree of endopolyploidy (data not shown), indicating that the whole-cell volume was dependent on the DNA content of nuclei. These results support our conclusion that CCS52 plays a general role in organ developmental processes by linking cell cycle control to cell differentiation.

Discussion

In plants, little is known about the mechanisms responsible for cell division arrest and the integration of cell cycle machinery into the appropriate stages of developmental programmes. Here we describe *ccs52* as the first plant cell cycle regulator that links cell proliferation to cell differentiation and promotes endoreduplication and cell enlargement during organ development (Figure 5).

Fig. 5. Model for the role of CCS52 in the control of endocycles and cell enlargement. In proliferating cells, where cell growth is controlled by the G₁ and G₂ checkpoints, the volume of the 2C and 4C cells increases during interphase. Exit from the mitotic cycle in either the G₁ or G₂ phase may require CCS52. CCS52 may block mitosis by degradation of mitotic cyclins via activation of the APC proteolytic machinery, leading to the conversion of the mitotic cycle to endocycles comprised of G₁–S–G₂ phases. Single or repeated rounds of endocycles result in the amplification of the genome, allowing a rapid increase in the cell volume. n, number of endocycles.

In eukaryotes, exit from the cell cycle may be achieved by different mechanisms such as by accumulation of negative regulators and/or by inactivation or degradation of the positive regulators of cell cycle progression. The MPF complexes composed of CDKs and the mitotic cyclins are the key regulators of the G₂–M transition, and their inactivation is probably required for cell division arrest prior to differentiation. Ubiquitin-dependent proteolysis of mitotic cyclins, mediated by a ubiquitin protein ligase referred to as anaphase-promoting complex (APC; King *et al.*, 1995) or cyclosome (Sudakin *et al.*, 1995), is one of the mechanisms that inhibits mitotic kinase activity. In yeasts and animals, the APC-dependent proteolysis of target molecules is activated and specified by direct binding of the CDC20/FZY or HCT1/CDH1 proteins to the APC (Fang *et al.*, 1998; Lorca *et al.*, 1998; Zachariae *et al.*, 1998). These WD-repeat proteins provide, however, distinct substrate specificity and temporal control of the APC-dependent proteolysis (Visintin *et al.*, 1997; Fang *et al.*, 1998; Prinz *et al.*, 1998). The CDC20 proteins have a strict requirement for the destruction D-box and they peak at G₂/M phase, in contrast to the FZR-type CDH1 proteins which have a relaxed specificity for the D-box and are present during the cell cycle, acting as the sole known regulators of the APC in G₁ phase (Fang *et al.*, 1998).

Sequence similarity and functional studies in fission yeast suggest that CCS52, like its animal (FZR/hCDH1) and yeast (SRW1/STE9/HCT1/CDH1) homologues, may act as a substrate-specific activator of the APC pathway. As reported here, overexpression of *ccs52* in *S.pombe* resulted in growth arrest and evoked endoreduplication and cell elongation, similarly to that of *srw1*⁺. These responses were specific for *ccs52* and were not elicited by the related plant gene *Atfzy/cdc20*. Since *ccs52* promoted degradation of the mitotic cyclin CDC13 in *S.pombe*, it may also induce mitotic cyclin proteolysis in plants.

Degradation of mitotic cyclins via the CCS52 control at a defined stage of the cell cycle might be responsible for the inhibition of the MPF activity leading to proliferation arrest and induction of endoreduplication cycles during plant development (Grafi and Larkins, 1995). In *Medicago* cell cultures, the *ccs52* transcripts were detected in all phases of the cell cycle (data not shown); therefore, CCS52, similarly to FZR/CDH1, might be responsible for APC activation also in the G₁ phase. CCS52 activity may be regulated post-transcriptionally by either its degradation or phosphorylation. The existence of several putative CDK phosphorylation sites, found also in the yeast and animal homologues, and a putative PEST protein degradation motif found in the N-terminus of the *Medicago* CCS52 proteins support this idea. In budding yeast, it was demonstrated recently that phosphorylation of HCT1/CDH1 by CDK inhibited the binding of HCT1/CDH1 to the APC, resulting in down-regulation of the proteolytic activity (Zachariae *et al.*, 1998).

Plant cells, in line with their flexibility to exit or re-enter the cell cycle, seem to have evolved a multitude of cell cycle regulators. *ccs52* probably represents a gene family in plants, as we found in *Arabidopsis* and in diploid and tetraploid *Medicago* species, in contrast to the single *fzr* homologues identified so far in animals. Members of this family might differ in their temporal and spatial expression patterns or provide distinct substrate specificity, which is also suggested by the sequence divergence of the AtCCS52A and B polypeptides, and thereby they might be involved in different developmental programmes. In *Drosophila*, *fzr* is expressed and is of crucial importance at specific stages of embryogenesis when cells terminate cell proliferation (Sigrist and Lehner, 1997). Expression of *ccs52* in *Medicago* nodules is turned on when nodule primordium differentiates (data not shown), and the formation of large differentiated cells with increased ploidy level is accompanied by high expression level of *ccs52*. Thus, similarly to *fzr*, *ccs52* expression marks post-mitotic cells which exit continuously from the meristem and switch from mitotic cell division to endoreduplication cycle(s). To test the involvement of CCS52 proteins in developmental cell cycle programmes, additional experiments for identification of *ccs52* family members, genetic manipulation of *ccs52* expression and a search for T-DNA/transposon-tagged mutants are underway. Besides CCS52, there may exist other mechanisms of MPF inhibition that may contribute to mitotic exit necessary for endoreduplication, such as inhibitory phosphorylation of CDKs at Tyr15 by Wee1-related kinases (Sun *et al.*, 1999).

In contrast to animals, where cell differentiation rarely involves multiple rounds of endocycles, endoreduplication in plants might be a major driving force for cell differentiation and cell expansion. It can produce giant mononuclear cells by multiplying the normal amount of DNA through successive rounds of DNA replication without nuclear and cell division. The most extreme examples in plants are reported for cells in the ovule, in the endosperm haustorium and in the suspensor (Nagl, 1978). Endoreduplication can be an integral part of the differentiation programme of single cells such as in the trichome where branching is regulated by the number of endoreduplication cycles (Hülkamp *et al.*, 1998). Mutants affected in endosperm development in maize (Kowles *et al.*, 1992), in hypocotyl

elongation (Gendreau *et al.*, 1997, 1998) or in trichome development (Hülkamp *et al.*, 1998) in *A.thaliana* are also altered in the level of endopolyploidy. The latter studies showed that environmental factors can affect specific rounds of DNA synthesis and cell volume simultaneously. Since DNA content may determine the limits of variation in the cell volume, endoreduplication seems to be a prerequisite for cell expansion in certain tissues (Lehner and Lane, 1997; Neufeld *et al.*, 1998). Our results support this hypothesis since changes in the expression level of *ccs52* in transgenic *Medicago* plants directly affected both the degree of ploidy and the cell volume. However, endoreduplication is not always coupled with an increase in cell volume and can be, at least partially, uncoupled from cell elongation (Gendreau *et al.*, 1998).

In multicellular organisms, cells of increased size can be advantageous for specialized functions. For example, the differentiated nodule cells have to store extremely large numbers of nitrogen-fixing bacteria. Cells which do not enter the endocycles in the nodule remain small and uninfected. In contrast, cells undergoing multiple rounds of endoreduplication cycles, resulting in, on average, an 8-fold increase in the nuclear volume (Truchet, 1978), have the capacity to increase the whole-cell volume 80-fold according to our estimations. In addition to the space requirement, high and symbiosis-specific metabolic activity is required for nodule cell differentiation which could be again sustained by repeated endoreduplication cycles. Moreover, by causing a short-circuit in the cell cycle (reducing it to G₁–S–G₂ phases), the *ccs52*-mediated endocycles may provide a faster way than cell proliferation to increase the tissue or organ size in the developmental programmes.

Based on its wide conservation in the plant kingdom, *ccs52* may be a ubiquitous regulator of the cell cycle transition in the developmental cell cycle programmes, with significant impact on plant growth. Thus, control of endoreduplication by *ccs52* may provide a means to manipulate the size of specialized cells or the growth of organs of agronomic interest.

Materials and methods

Isolation of *ccs52*

The *ccs52* cDNA clones were obtained by differential screening of a cDNA library of young *M.sativa* ssp. *varia* A2 nodules (Crespi *et al.*, 1994) using nodule versus root cDNAs as probes. Eight clones have been sequenced that were either full or partial derivatives of the same or highly related sequences that could be allelic variants. The *M.truncatula ccs52* clone was isolated from a genomic library constructed by *Mbo*I partial digestion of *M.truncatula* ecotype Ghor genomic DNA and by cloning of the 15–20 kb fragments in the *Bam*HI site of pEMBL4.

Schizosaccharomyces pombe strains, plasmids and transformants

The *S.pombe* SP-Q01 (Stratagene) and SY1 (*h⁻srw::ura4⁺ ura4-D18 leu1-32*) (Yamaguchi *et al.*, 1997) strains were used. pREP1-*ccs52* was constructed by cloning a *Bam*HI–*Eco*RV fragment, containing the *ccs52* cDNA without the first four codons, into the *Bam*HI–*Sma*I sites of pREP1 (Maundrell, 1993). An ATG from the vector was fused in-frame with *ccs52* by opening the plasmid by *Bam*HI digestion, filling in the termini with Klenow fragment and dNTPs, and religation. The intronless *Atfzy* was amplified by PCR from *A.thaliana* ecotype Columbia genomic DNA using *Pfu* and the ATGGATCCGGGTATGAGAGCTACGTGT and CGCCCGGGTTAGTCTTCTCGTTTCAC primers. The PCR fragment was digested with *Bam*HI and *Sma*I and ligated into the *Bam*HI–*Sma*I sites of pREP1. Transformation of *S.pombe* SP-Q01 competent cells,

growth of transformants and induction of *ccs52* expression were carried out according to the Stratagene protocol. The SY1 cells grown in 10 ml of complete (YPAD) medium to OD₆₀₀ = 0.4–0.5 at 30°C were washed with ice-cold water, then with 1 M sorbitol and resuspended in 200 µl of sorbitol. A 100 ng aliquot of DNA was added to 40 µl of cells for 5 min at 0°C and then electroporated (1.5 kV, 132 Ω, 40 µF).

Schizosaccharomyces pombe cells taken at different intervals from liquid cultures after thiamine withdrawal were fixed in 70% ethanol and stained with propidium iodide for flow cytometry using an EPICS V, Coulter flow cytometer and with 4',6-diamidino-2-phenylindole (DAPI) for fluorescent microscopy (Beach *et al.*, 1985) or were used for protein extraction and Western blot analysis according to Yamaguchi *et al.* (1997). The rabbit anti-CDC13 polyclonal antiserum provided by H.Yamano was used at 1:2000 dilution. Mouse monoclonal antibodies against the PSTAIR motif and α-tubulin were purchased from Sigma.

Construction and analysis of transgenic *M.truncatula* plants

Transformation of *M.truncatula* R108 was done according to Trinh *et al.* (1998). A 1.2 kb *Sst*I–*Pvu*II *ccs52* cDNA fragment lacking the N-terminal part of the protein was cloned in the antisense orientation behind the CaMV 35S promoter in the binary vector pB35S that contains the *bar* gene as a selection marker for resistance to the herbicide BASTA (White *et al.*, 1990; Becker *et al.*, 1992). RT-PCR analysis was performed to detect changes in the endogenous *ccs52* transcript level using 1 µg of total RNA isolated with the RNeasy plant mini kit (Qiagen) from petioles that were retrotranscribed in the presence of oligo(dT) primer, M-MLV reverse transcriptase, RNase inhibitor and dNTPs. The primer pairs (P55CL–P55CR, P55BL–P55CR) discriminating the wild-type *ccs52* mRNA from the antisense transcript were: P55BL (TTTGGG-GGTTGATGATTGTG), P55CL (CTCTCTACCGTTCTATCTCTTG-GGA) and P55CR (GGTAAAGATGCTACTTTGGTGGTGT). The *ccs52* and *Msc27* (Györgyey *et al.*, 1991) cDNAs were amplified by 15 cycles at 94°C 20 s, 55°C 30 s and 72°C 1 min and blotted. Northern analysis was performed with 20 µg of total RNA samples, except in the case of petioles of the A4 lines and control plants when 7 µg of total RNA was used. The hybridization signals obtained with the corresponding cDNA probe were quantified by a PhosphorImager (Molecular Dynamics). Siblings of transgenic plants were tested by PCR for the presence of the transgene. Plant materials (e.g. petioles, leaves, roots and nodules) were collected at equivalent positions from wild-type and transgenic *M.truncatula* plants grown under the same condition in the greenhouse or in growth chambers for nodulation assay performed in aeroponic tanks as described previously (Hoffmann *et al.*, 1997). Sterile seedlings for the analysis of hypocotyls and roots were grown for 2 and 3 days on agar plates.

The DNA content of nuclei from different organs was determined by flow cytometry. Freshly collected plant material (100 mg) was immersed in 0.5 ml of Galbraith buffer containing 1% Triton X-100 and sliced with a scalpel to release intact nuclei (Brown *et al.*, 1991). Filtered nuclei were stained with DAPI (5 µg/ml) and 5000 nuclei were analysed per measurement in a minimum of three to five independent experiments.

For cell size measurements, control (C_{2n} and C_{4n}) and transgenic (A4) plants were germinated simultaneously and grown under the same conditions. Petioles were collected from young (5-week-old) plants. Three petioles at equivalent positions from each plant were embedded in 6% agarose. From each petiole, three to five sections of 80–100 µm were photographed under the microscope. The areas of the 20–40 largest cells per section were measured by computer-assisted image processing, and cells in each section were ranked according to their size. The 15 largest cells were numbered in decreasing order, the mean value of each of the corresponding cells in different sections and petioles of the same plant type was calculated and the mean area values for the C_{2n}, C_{4n} and A4 plants were then plotted.

In situ hybridization was performed according to Crespi *et al.* (1994). Localization of the *ccs52* transcripts was performed with both [³⁵S]UTP- and digoxigenin-labelled sense and antisense RNA probes. For detection of the histone H3 and *MscycB2;3* transcripts, [³⁵S]UTP-labelled antisense and sense riboprobes were used. Sigma Fast Red was the substrate for the alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim).

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