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RESEARCH PAPER

Ectopic expression of *Arabidopsis CYCD2* and *CYCD3* in tobacco has distinct effects on the structural organization of the shoot apical meristem

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Abstract

Transgenic tobacco lines expressing *Arath-CYCD2* or *Arath-CYCD3* genes under a cauliflower mosaic virus 35S promoter are modified in the timing of their development, but not in the phenotype of their vegetative organs. They display an increased rate of leaf initiation, which is shown to be associated with distinct changes in the structural organization of their shoot apical meristem (SAM). Constitutive expression of *Arath-CYCD2* leads to a progressive modification of the SAM structural organization with predominant periclinal divisions in the L3 layer and to the loss of the classical cytophysiological zonation, the central zone being reduced to the central cells of the L1 and L2 layers. These changes reveal a particular sensitivity of the corpus cells (L3) to *Arath-CYCD2* over-expression and suggest a role for CYCD2 in controlling the planes of cell division in these cells. The SAM structural modifications in the *Arath-CYCD3* over-expressing lines are less drastic; only an increased cell number together with a reduced cell size, particularly in the L1 layer, characterizes the peripheral zones. This could be related to the shortening of the G_1-phase duration that renders cell growth incomplete between successive mitoses. Cell proliferation continues beyond the SAM in the developing internodes and confers a delayed senescence to *Arath-CYCD3* over-expressing juvenile tissues. In addition, the ploidy levels of mature stem tissues in both types of transgenic lines are unaffected, suggesting that the studied G_1 to S cell-cycle genes have no effect on the extent of endoreduplication in tobacco stem tissues.

Key words: D-type cyclins, morphogenesis, plant cell cycle, shoot meristem, tobacco.

Introduction

Plant D-type cyclins have been described as G_1-specific cyclins on the basis of their capacity to rescue yeast mutants lacking endogenous G_1 cyclins (Soni *et al.*, 1995), although their transcript levels are not strongly regulated during the cell cycle (Meijer and Murray, 2000). In addition, mitotic accumulation of transcripts from two tobacco *CYCD* homologues has been reported in synchronized BY-2 tobacco cells (Sorrell *et al.*, 1999), suggesting a possible involvement of these D-type cyclins for entry into or progression through mitosis. Plant CYCDs are a relatively divergent group of genes, represented by 10 genes in the case of *Arabidopsis*, which fall into three major sub-groups (*CYCD1*, *CYCD2*, and *CYCD3*) and three additional single genes (Oakenfull *et al.*, 2002). The similarity between members of different sub-groups is around 45% (30–35%

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Abbreviations: BAP, benzylaminopurine; CDK, cyclin-dependent kinase; CYC, cyclin; GLM, Generalized Linear Model; MS, Murashige and Skoog; SAM, shoot apical meristem; WT, wild type.

identity), whereas similarity between homologous genes in different species is higher, for example, 53% identity (59% similarity) between *Arath-CYCD2;*1 and *Nicta-CYCD2;*1, and 58% identity (66% similarity) between *Arath-CYCD3;*1 and its nearest known tobacco homologue also called *Nicta-CYCD3;*1.

Plant CYCDs interact with the retinoblastoma (RB) protein through the conserved LxCxE motif (Ach *et al.*, 1997; Huntley *et al.*, 1998), which indicates that CYCD may control the G_1 to S transition through RB phosphorylation and E2F transcription factor activation (for a review see Meijer and Murray, 2000). Indeed, cyclin-dependent kinases (CDKs) associated with CYCD can phosphorylate RB protein (Nakagami *et al.*, 1999, 2002), and by analogy with the case in animals this is likely to allow the dissociation between RB and E2F, and the consequential activation of genes under E2F control could then promote S-phase entry (de Jager and Murray, 1999). Most D-type cyclins also possess the PEST sequences, which are characteristic of proteins with a rapid turnover (Rogers *et al.*, 1986).

Based on the timing of their expression when cell cultures re-enter the cell cycle, cyclins of the CYCD3 group are considered to have a role in promoting S-phase entry in response to hormonal and environmental signals (Riou-Khamlichi *et al.*, 1999), although direct mechanistic links have not yet been established. In an *Arabidopsis* cell suspension synchronized in G_1, *Arath-CYCD3;*1 expression is induced by cytokinins with a particular sensitivity to zeatin and benzylaminopurine (BAP) (Murray *et al.*, 1998; Riou-Khamlichi *et al.*, 1999), and by sucrose (Riou-Khamlichi *et al.*, 2000). *Arath-CYCD3;*1 transcripts are also up-regulated by brassinosteroids which can substitute for cytokinins in promoting cell division (Hu *et al.*, 2000), as well as by other mitogenic hormones (Oakenfull *et al.*, 2002). In snapdragon shoot apical meristems, specific expression patterns were observed for different *CYCD3* genes. *Arath-CYCD3b* gene expression is found throughout the meristem and, indeed, in other dividing cells, while *Arath-CYCD3a* is expressed only in the very lateral cells and is locally regulated by the *CYCLOIDEA* gene (Gaudin *et al.*, 2000). In addition, calluses derived from *Arath-35S-CYCD3* transgenic leaves cultured *in vitro* are induced and able to divide in the absence of exogenous cytokinins (Riou-Khamlichi *et al.*, 1999). The *Arabidopsis amp1* mutant, which has a level of cytokinins 5-fold higher than the wild type (WT), is characterized by an up-regulation of *CYCD3;*1 (Riou-Khamlichi *et al.*, 1999; Helliwell *et al.*, 2001). *CYCD2* is activated earlier in G_1 and is induced only by sucrose (Murray *et al.*, 1998; Riou-Khamlichi *et al.*, 2000); this gene is therefore considered as a candidate upstream regulator of cell division (Cockcroft *et al.*, 2000). There are also notable differences in the regulation of protein levels between CYCD2;1 and CYCD3;1, with the former exhibiting greater stability and post-translational regulation (Healy *et al.*, 2001).

Most of the data concerning cell cycle gene expression have been performed on experimental systems such as tobacco BY-2 or *Arabidopsis* cell suspensions, and little information is available about their effects at the whole plant level. It has been demonstrated that over-expressing *CYCD* genes can provide a means to control growth rate (Cockcroft *et al.*, 2000). Constitutive expression of the *Arath-CYCD2;*1 gene in tobacco leads to an increased rate of leaf initiation and accelerated development in all stages from seedlings to maturity due to an increase in the rate of cell division (Cockcroft *et al.*, 2000). Very high level over-expression of the *Arath-CYCD3;*1 gene in transgenic *Arabidopsis* lines induces phenotype modifications such as leaf curling and delayed senescence, associated with the hyperproliferation of cells and inhibited cellular differentiation (Riou-Khamlichi *et al.*, 1999; Dewitte *et al.*, 2003). Such data establish molecular links between plant morphogenesis and cell cycle activity as previously suggested by cytological approaches (Nougarède and Rembur, 1985). In addition, *Arath-CYCD3;*1 over-expression is also increased in hormone autotrophic fast-growing lines of *Arabidopsis*, and this could indicate that *Arath-CYCD3;*1 is the limiting agent for growth responses (Frank *et al.*, 2000). CYCD3;1 and CYCD2;1 proteins share only 31% identity (45% similarity), and this, coupled with the evidence for their differential regulation and stability, instigated the detailed study of the effects of their over-expression *in planta* and to find original effects, particularly on the structural organization of the shoot apical meristem.

Materials and methods

Material and growth conditions

Transgenic lines of *Nicotiana tabacum* var. Xanthi carrying either the *Arath-CYCD2;*1 (C8T1-2 and C8T1-5, heterozygous) or *Arath-CYCD3;*1 (1K9, homozygous and 17K9, heterozygous) coding sequences under the control of the CaMV 35S promoter were selected by placing sterilized seeds on solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 50 mg l^{-1} kanamycin for 2 weeks, then the selected seedlings and the WT seeds (homozygous XHFD8 line) were grown in a glasshouse with day and night temperatures of about 25 °C and 20 °C, respectively, and a 16 h photoperiod consisting of natural daylight supplemented with Mazdafluor and Durolux Truelite tubes dispensing 90 μE m^{-2} s^{-1}. Plants were subcultured for 2 weeks after sowing. Growth parameters from sowing to flowering were determined on samples of 20 plants. At each time, mean values of stem height and leaf number were compared in a pairwise manner by using the Tukey's HSD test (Quinn and Keough, 2002). In parallel, the effects of the age of the plants and the type of lines on the distribution of the measurements were tested by reconstructing a Generalized Linear Model (GLM) and by treating the provided model with a two way ANOVA (Quinn and Keough, 2002). This analysis allows the difference in growth rate between lines to be tested precisely. GLM analyses were performed by using the nmle package of the 'R' distribution (R Development Core Team, 2004). *In vitro* culture of leaf explants (1 cm^2) and excised internodes from the various lines was performed in Petri dishes containing gelified MS medium supplemented with 1 mg l^{-1} naphthalene acetic acid (NAA) and

0.2 mg l^{-1} BAP or without BAP. They were grown in a growth chamber under a 16 h photoperiod at 25±2 °C with 70 μE m^{-2} s^{-1} photon flux dispensed by the same type of tubes as indicated above.

Lines over-expressing *Arath-CYCD2* have been renamed A1 for C8t1-2 and A2 for C8T1.5, and lines over-expressing *Arath-CYCD3*,B1 and *Arath-CYCD3*,B2 for 1K9 and 17K9, respectively.

Cytochemistry and immunocytochemistry

Staining of meristematic areas was performed on 8 μm sections of paraffin-embedded samples fixed for 24 h with a 95% ethanol: 35% formalin:acetic acid mixture (85:10:5, by vol.) (Lison, 1960). Ten independent samples from each line at each developmental stage were used. Pyroninophily of cells after staining with methyl green–pyronin reflected the intensity of meristematic activity (Lance, 1954). These sections were also used to measure meristem and cell sizes. Mitotic indices in shoot apices were determined on samples fixed by ethanol:acetic acid (3:1, v/v) then paraffin-embedded and sectioned at 8 μm. The sections were stained according to the Feulgen reaction (1 N HCl hydrolysis for 10 min at 60 °C then Schiff's reagent for 2 h), and mitotic indices were estimated on three axial sections from each apex. The Feulgen reaction also allowed the various zones to be distinguished, as the nuclei of the central zone in tobacco display a weaker staining (Brossard, 1975). Concerning the size of the transgenic SAMs, they were measured on median longitudinal sections passing by the plane of maximal areas (i.e. at the time where leaf initiation was in preparation). Sections from five different plants for each condition were selected and the distance was measured between the bases of left and right peripheral zones, eliminating the cells belonging to the future internodes.

In situ immunolocalization of endogenous CYCD3 was assayed without embedding according to Dewitte *et al.* (1999), using a rabbit antibody raised against the N-terminal MGIQHNEHNQDQT of the NtCYCD3,1 protein (Nakagami *et al.*, 1999; now renamed Nicta; *CycD3;*3; Nakagami *et al.*, 2002) diluted at 1:400 in blocking buffer.

Nuclear DNA imaging analysis

Shoot apices were fixed in acetic acid:alcohol (1:3, v/v), rinsed with 70% (v/v) EtOH, rehydrated, and stained by the Feulgen reaction as described above. Stained apical meristems were excised under a microscope, then flattened and mounted in DePex (Gurr, BDH, Poole, UK). An image-analysis system fitted with Ploidy 4.04 software (SAMBA 2005, Alcatel, TITN, Grenoble, France) performed nuclear DNA quantification. The mean 2C reference value ±2 SD was determined by analysis of half-telophases and submitted to the Kolmogorov–Surinov test of normality. For each condition, around 500 interphase nuclei were analysed and ranged into histograms. The relative frequency of G_0–G_1 nuclei was established on the basis of their position between the limits of 2C distribution.

DNA flow cytometry

Nuclear DNA contents at various stages of stem differentiation were assessed by flow cytometry (Service of Cytometry, CNRS, Gif-sur-Yvette, France). Isolation of nuclei was carried out by mechanical chopping with a razor blade in Galbraith's buffer (Galbraith *et al.*, 1983) with the addition of 1% Triton X-100, 10 μl ml^{-1} Na metabisulphite, and 7 μg ml^{-1} DAPI. The extracted nuclei were filtered through a 75 μm nylon mesh and analysed with an EPICS V cytofluorimeter (Coulter, Hilaeah, FL) fitted with an argon ion laser (Spectra-Physics, Les Ulis, France) using 60 mW at 351–364 nm. The blue emission of the DNA–fluorochrome complex was collected through 418 nm long-wave pass and 530 nm short-wave filters. *Petunia hybrida* leaf nuclei (2C=2.85 ρg) were used as the internal reference (Marie and Brown, 1993). Four thousand nuclei were analysed per sample.

Electron microscopy

Excised shoot apices were fixed in a mixture of 2% glutaraldehyde–1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.5 for 6 h at room temperature. After 6 h of washing with four changes of buffer, the material was post-fixed in 1% buffered osmium tetroxide for 6 h at room temperature, rinsed, dehydrated in an ethanol–propylene oxide series, infiltrated with increasing concentrations of Araldite–epon resin over a period of 40 h and embedded in fresh resin. Ultra-thin sections (80–90 nm) were cut with an OmU3 ultramicrotome (Reichert, Vienna, Austria) and collected on uncoated 200 hexagonal mesh copper grids. Sections were stained with a saturated solution of uranyl acetate in 50% EtOH and subsequently with lead citrate (Reynolds, 1963) for 15 min each. Grids were then observed at 80 kV on a Philips EM 201 transmission electron microscope.

Northern and western blot analysis and histone H1 kinase assays

RNA extraction, northern blot and histone H1 kinase assays were performed according to Riou-Khamlichi *et al.* (2000). Procedures for western blot were described previously by Cockcroft *et al.* (2000) and Healy *et al.* (2001).

Results

*Characterization of transgenic tobacco lines expressing A*rath-CYCD2;*1 and* CYCD3;*1*

Transgenic tobacco lines over-expressing *Arath-CYCD2;*1 were previously described and analysed for growth and cell cycle effects (Cockcroft *et al.*, 2000). The progeny of these lines (C8-2 and C8-5) was selected for further analysis and named C8T1-2 (A1) and C8T1-5 (A2), respectively. These two lines showed similar levels of CYCD2;1 protein and associated kinase activity (Cockcroft *et al.*, 2000). A number of lines expressing *Arath-CYCD3;*1 were also generated under the CaMV 35S promoter, which exhibited various levels of expression of *CYCD3;*1 (Fig. 1). Among these lines, lines B1 and B2 were selected for further analysis, line B1 showing a higher expression level than line B2.

Growth parameters in relation to the levels of CYCD2 *and* CYCD3 *over-expression*

Stem height and leaf number were measured over a 5 month period for the four transgenic lines and the WT. All the

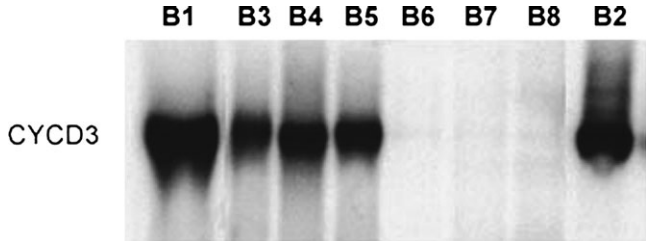

Fig. 1. Northern blot of mRNA extracted from T0 founder plants of transgenic *CYCD3;*1 lines. Note that the *Arabidopsis CYCD3;*1 probe does not cross-react with the tobacco genes, as seen in the non-expressing lines such as B6. It is thus not possible to relate the degree of over-expression to the endogenous tobacco CYCD3 levels. The RNA was controlled by optical density measurement and the same amount was loaded in each lane.

transgenic lines displayed a transient increase of growth. At 2.5 months, lines A1 and B1 were significantly higher than the WT and other transgenic lines (Table 1; Fig. 2a). After 3 months, all the transgenic lines were still significantly higher than the WT (Table 1). However, after 5 months, only the line A1 remained higher than the WT. A correlation analysis performed on the whole data set between stem height and leaf number showed that both data were significantly correlated (r^2=0.8848, P<0.001). That allowed a GLM analysis to be performed on the leaf number only, this statistical approach being convenient for counted values. No interaction was found between age and lines (P=0.79) indicating that, on average, during the whole growth period (>5 months), growth rates were not significantly different between lines. This was confirmed by the relatively similar slope for the increase in log of leaf number for all the lines (Fig. 2b). The growth patterns of *35S:CYCD2* lines compared with the WT were not fully similar with the observations of Cockcroft *et al.* (2000). This was probably due to both the use of different transgenic lines and different growth conditions, and reflects the high degree of variation among the lines. Be that as it may, both the lines used in the present study and those used by Cockcroft *et al.* (2000) had elevated overall growth rates and increased rate of leaf initiation. After 5 months of vegetative growth, similar leaf morphologies were observed in all the lines but the number of leaves initiated was different, with mean values significantly higher for both *Arath-CYCD2* over-expressing lines compared with the WT; one *CYCD3* over-expressing line (B1) also produced more leaves than the WT (Fig. 2b). In both *35S-CYCD2* lines, the first flower buds were formed after 20 weeks, i.e. 2–3 weeks earlier than in one of the *35S-CYCD3* lines (B1) which was itself more precocious than the other *35S-CYCD3* line (B2) and the WT. This suggested that the flowering transition occurs in cv. Xanthi as in other tobacco cultivars, i.e. when plants have initiated a determined number of leaves. Under the growth conditions used, this number (45±3) was reached earlier in both *35S-CYCD2;*1 lines and in one (B1) of the *35S-CYCD3*;1 lines, correlating with the early flowering in these lines. The earlier flowering and faster growth of B1 compared with its sister *Arath-CYCD3;*1 over-expressing line reflects the higher expression level of *Arath-CYCD3;*1 in B1.

Immunodetection of the endogenous tobacco Nicta-CYCD3;3 protein (Nakagami *et al.*, 2002) in 3-month-old plants showed a moderate and patchy signal in the SAM, the leaf primordia, and the differentiating internodes of WT (Fig. 3a) and transgenic *35S-CYCD2* lines (Fig. 3b). The B2 (*35S-CYCD3*) line reacted similarly (not shown). By contrast, the homozygous B1 (*35S-CYCD3*) line showed a stronger signal (Fig. 3c). Although immunochemistry does not provide quantitative information, the signal in B1 is unambigously increased and confirmed the result of the northern blot (Fig. 1).

Since the antibody used is against an N-terminal peptide of tobacco Nicta-CYCD3;3 that has no homology to the protein encoded by the over-expressed *Arath-CYCD3;*1 gene, this indicates a possible positive feedback resulting in increased tobacco Nicta-CYCD3;3 in response to increased levels of Arath-CYCD3;1 from the transgene. The difference in immunodetected Nicta-CYCD3;3 in this line may correlate with the differences observed between both *35S-CYCD3* lines and the more drastic effects of the over-expression in line B1, which also expresses a higher level of the *Arath-CYCD3;*1 transgene. No signal was observed in the control sections incubated without the primary antibody (Fig. 3d). Differences in the immunosignal of differentiated internodes were not observed (Fig. 3e–h).

Further characterization of the regenerative capacities of the various types of lines was performed using *in vitro* culture of stem and leaf explants on a medium containing an auxin:cytokinin ratio previously determined as optimal for bud regeneration for WT explants and on a BAP-free medium. No bud regeneration was obtained on the BAP-free medium. By contrast, the *35S-CYCD3* and, to a lesser extent, the *35S-CYCD2* leaf and internode explants gave rise to an increased number of regenerants compared with the WT (average numbers of 15 buds per *35S-CYCD3* explant instead of 12 for *35S-CYCD2* and seven for the WT after 3 weeks) on hormone-containing medium. This suggested that the transgenic explants have a higher sensitivity to the culture conditions, but do not possess sufficient endogenous cytokinins to regenerate without exogenous BAP.

Table 1. *Multiple pairwise comparison analyses (Tukey's test) between lines for stem height and leaf number at several growth times*

At each time, values followed by the same letters means that they are not significantly different (P <0.05).

Time (months)	Lines		Stem height (cm)		Leaf number	
			Mean	SD	Mean	SD
1.5	WT		2.7 1 a	1.56	6.55 a	1.44
	35S-CYCD2	A1	4.90 ac	0.36	7.33 a	0.58
		A2	3.50 ac	1.32	7.67 a	1.15
	35S-CYCD3	B1	5.31 bc	1.44	8.33 a	1.37
		B2	2.92 a	0.55	6.60 a	0.89
2.5	WT		7.40 a	3.34	9.08 a	1.83
	35S-CYCD2	A1	13.67 bcd	1.15	13.00 b	1.00
		A2	12.67 acd	3.21	10.67 ab	3.21
	35S-CYCD3	B1	15.42 bc	2.03	13.33 b	2.25
		B2	8.70 ad	2.31	10.02 ab	0.45
3	WT		23.67 a	7.03	14.07 a	2.89
	35S-CYCD2	A1	42.43 b	8.80	22.17 b	3.08
		A2	39.09 bc	10.91	20.23 bc	3.85
	35S-CYCD3	B1	34.17 c	8.07	18.33 c	2.57
		B2	31.60 c	5.43	18.40 c	2.67
5	WT		62.00 a	10.41	31.60 a	2.95
	35S-CYCD2	A1	94.85 bc	27.17	48.00 b	3.38
		A2	81.71 ac	18.14	44.14 bc	6.04
	35S-CYCD3	B1	94.20 ac	32.98	41.60 bc	3.13
		B2	66.33 ac	16.04	35.67 ac	9.71

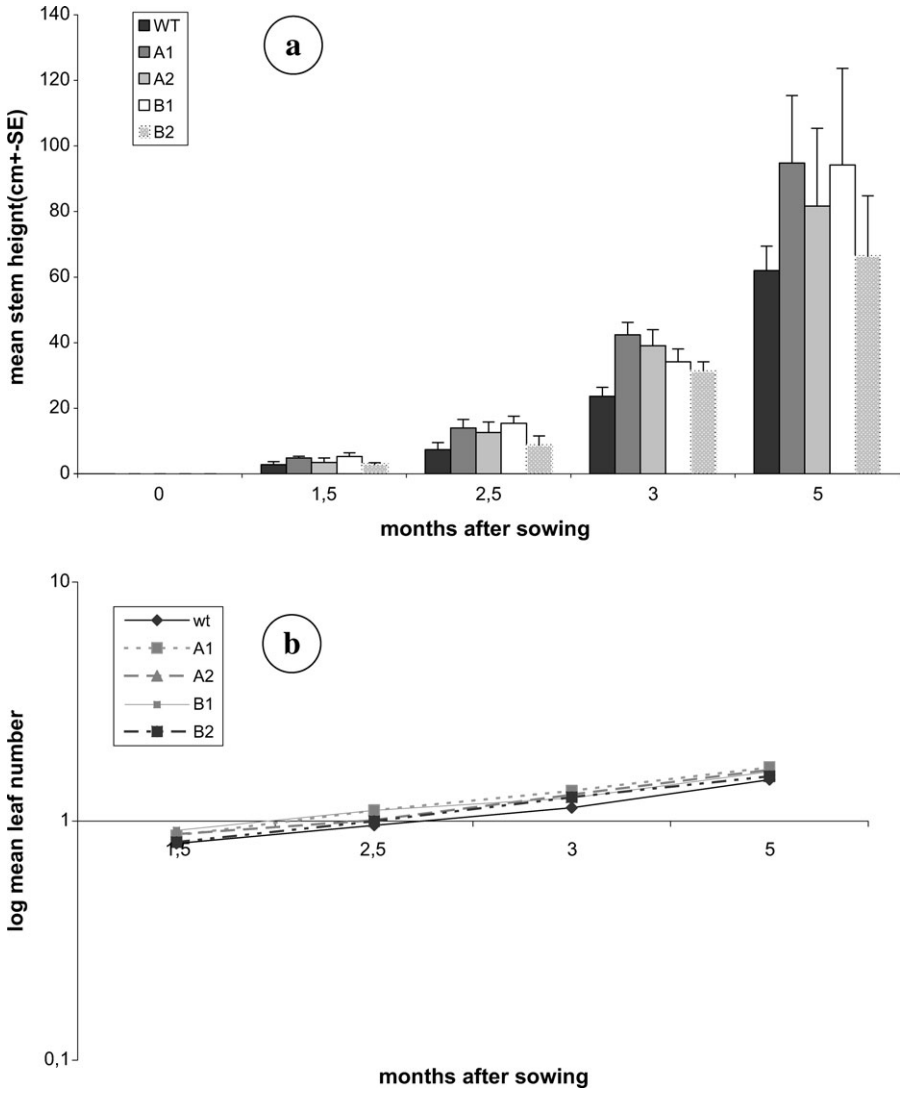

Fig. 2. Comparaison of mean stem height (a) and leaf number (b) of wild-type (WT) and transgenic tobacco lines over-expressing *CYCD2* (lines A1 and A2) or *CYCD3* (lines B1 and B2) from sowing to flowering. Growth parameters were determined on a sample of 20 plants.

Structural organization of the WT and transgenic vegetative shoot apical meristems

In order to determine whether the higher rate of leaf initiation in the transgenic lines was associated with changes in the functional organization of the SAM, the structure of A1 (*35S-CYCD2;*1) and B1 (*35S-CYCD3;*1) shoot apices was examined under light and electron microscopes at different times of the vegetative phase, both lines being selected on the basis of their more pronounced differences to the WT.

Similar SAM structures and cytophysiological zonation were observed for the three types of lines during the first month (not shown). After 6 weeks, some differences began to be observed between the three types of SAMs. While the WT SAMs displayed a normal organization marked by a higher pyroninophily in the peripheral zones (Fig. 4a) and a regular arrangement into layers (L1, L2, and L3) exhibiting the usual planes of cell division, the *35S-CYCD2* SAMs became enlarged with an attenuated zonation and frequent mitoses in the corpus (Fig. 4b, arrowheads). In addition, an increased pyroninophily and unusual periclinal divisions were sometimes observed in the peripheral zones. In the *35S-CYCD3* SAMs, zonation and regular arrangement into layers were present, but the SAM surface was flatter and enlarged due to the enlargement of the peripheral zones (Fig. 4c).

From 8 weeks, differences became more pronounced. While the WT SAMs were developing normally (Fig. 4d), the *35S-CYCD2* SAMs (Fig. 4e) were enlarged (177% of the WT), due to the enlargement of both the peripheral and the central zones, and displayed an unusual appearance. The L1 and L2 layers appeared quite normal and displayed the normal pattern of zonation into peripheral and central zones. By contrast, the corpus (L3) was strongly modified and replaced by four or five layers of flat cells resulting

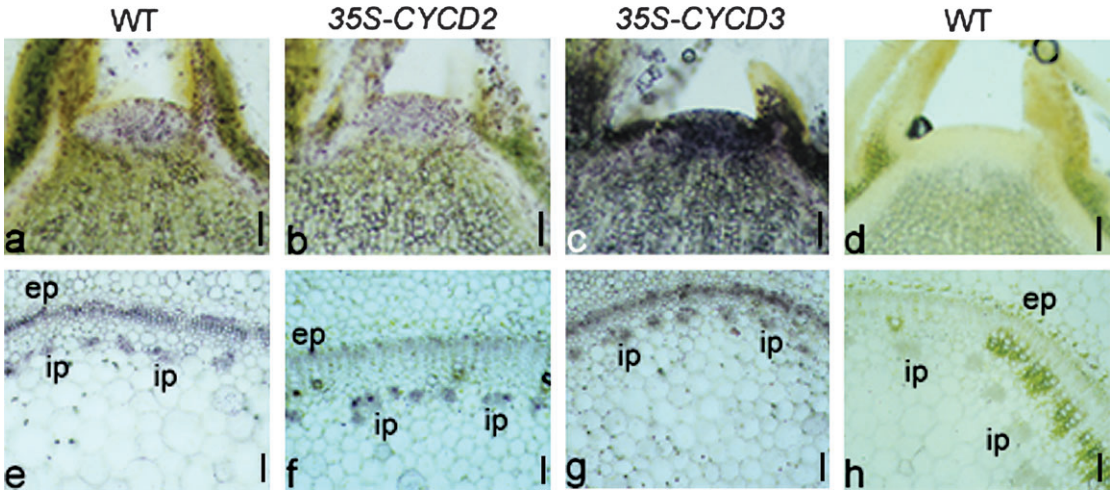

Fig. 3. Immunolocalization of Nicta-CYCD3 in WT and transgenic shoot apices (a–d) and internodes (e–h). (d, h) Control sections incubated without the primary antibody. ep, External phloem; ip, internal phloem. Bars=100 μm (a–d) and 10 μm (e–h).

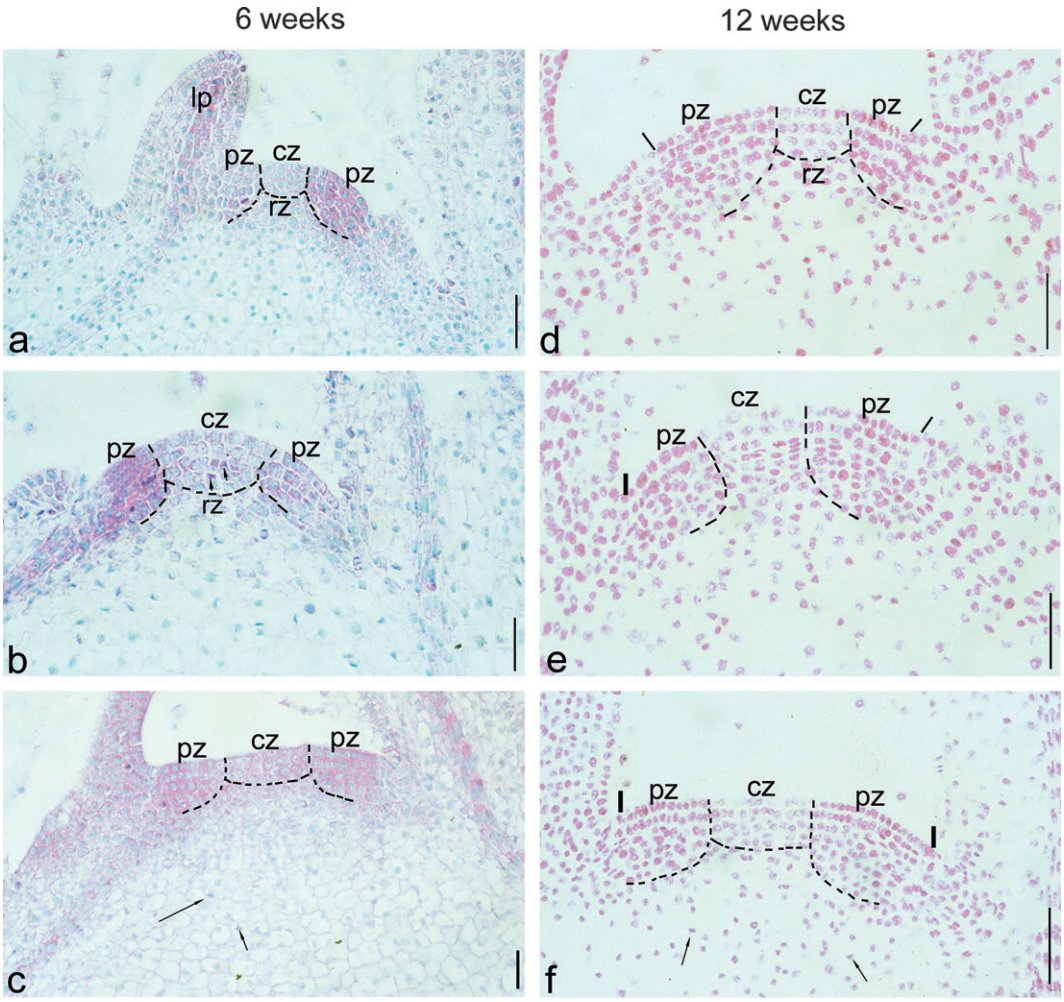

Fig. 4. Longitudinal sections of 6- and 12-week-old SAMs of WT (a, d), *35S-CYCD2* line A1 (b, e) and *35S-CYCD3* line B1 (c, f) stained by methyl green-pyronine (a–c) or by Feulgen's reaction (d–f). cz, Central zone; lp, leaf primordium; pz, peripheral zone; rz, rib meristem. Arrows in the pith on (c) indicate mitotic figures; arrowheads on (b) indicate unusual mitosis in the corpus. Note also the particular periclinal stratification of the corpus in (e). Bars=100 μm.

apparently from co-ordinated periclinal divisions. The onset of these periclinal divisions in layer L3 started from the periclinal zones after 6 weeks of growth. Later, all the cells of the central zone also divided periclinally so that the 8-week-old corpus had an entirely stratified appearance. Consequently, the central zone became reduced to the L1 and L2 layers and a classical corpus was no longer present.

The *35S-CYCD3* SAMs (Fig. 4f) were differently modified. They became enlarged (171% of the WT), again due to the enlargement of both the peripheral and central zones. However, the zonation was preserved, although few dividing corpus cells were present, and the enlarged peripheral zones contained an increased number of cells in the L1 and L2 layers that was smaller than in the WT. This led to an increased density of nuclei per surface unit (Fig. 4f). In addition, mitotic figures were frequently observed in the young pith cells located behind the SAM (Fig. 4c, f, arrows) while they were rare in the WT, and the differentiating cells of the internodes were smaller than in the WT (80% of the WT for cells located 300 μm below the apex).

The observations were further analysed by electron microscopy (Fig. 5a–f). Meristematic cells of the WT SAMs displayed spherical nuclei with reticulate heterochromatin and sparsely vacuolated cytoplasm containing numerous ribosomes (Fig. 5a, d). The vacuoles contained electron-dense material identified previously as phenolic compounds by cytochemistry (Brossard, 1975) and the proplastids of L1 cells contained small, electron dense protein bodies (Fig. 5a, asterisk) as already described in another tobacco cultivar (Brossard, 1975).

Changes in nuclear shapes and ultrastructure were observed in *35S-CYCD2* (Fig. 5b, e) and in *35S-CYCD3* (Fig. 5c, f) apices. In both transgenic lines, nuclei had a greater proportion of dense chromatin, which could be indicative of a different position in the cell cycle, G_2 nuclei being usually characterized by an increased chromatin density (Barlow, 1977). In addition, cells and nuclei in the L1 layer of *35S-CYCD3* peripheral zones (Fig. 5c) were narrower than their WT counterpart, while cells and nuclei of the re-divided L3 layer of *35S-CYCD2* SAM (Fig. 5e) were flat and enlarged. In both transgenic lines, accumulation of electron-dense phenolics in the vacuole was reduced and protein bodies in the proplastids of the L1 cells were more developed (Fig. 5b, c, asterisks). Both these features are suggestive of more juvenile and hence less differentiated cell characteristics.

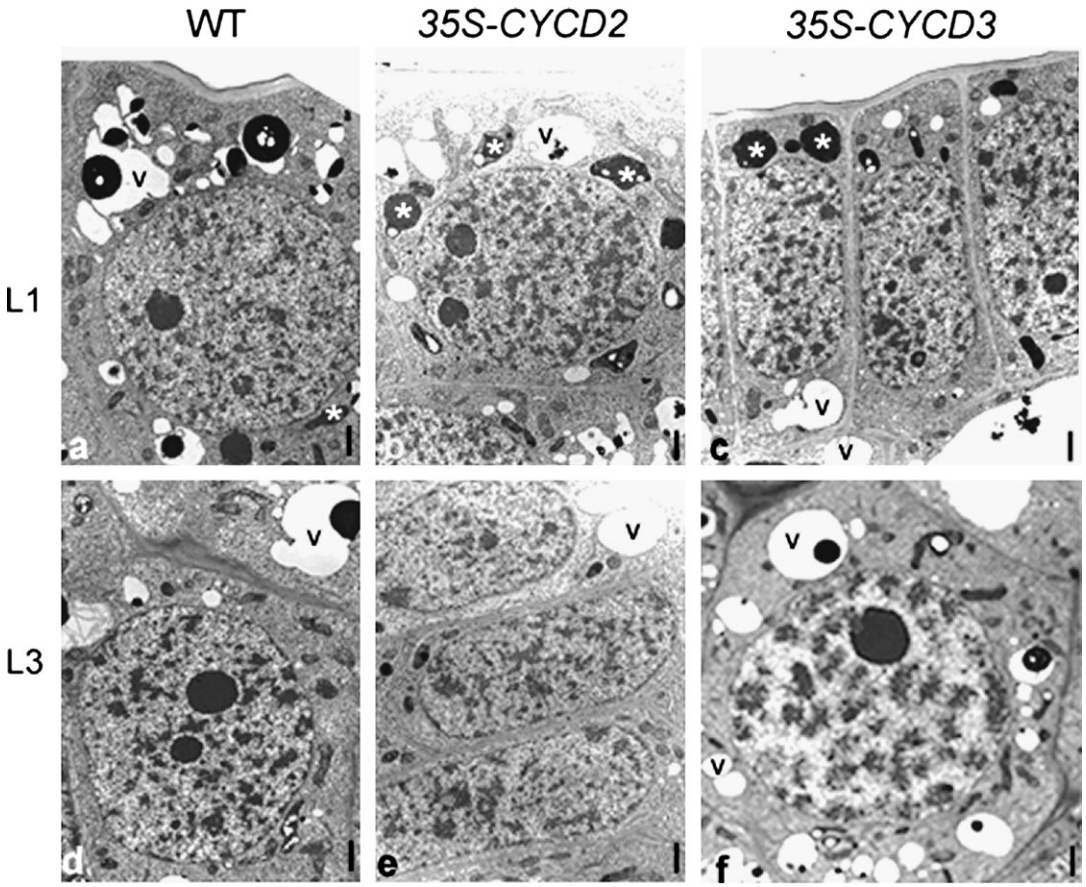

Fig. 5. Electron micrographs of L1 and L3 peripheral cells of the WT (a, d) and transgenic shoot apical meristems over-expressing *CYCD2* (b, e) or *CYCD3* (c, f). v, Vacuole. White asterisks in (a), (b) and (c) indicate proteic inclusions in proplastids. Bars=1 μm.

Mitotic activity and cell cycle parameters at the SAM level

Mitotic index was estimated on longitudinal axial sections of 3-month-old SAMs from WT and transgenic lines (Table 2). Compared with the WT, an increased mitotic index was observed in the peripheral zones of all transgenic lines, but this was significant only in the *Arath-CYCD2;*1 over-expressing A2 and *Arath-CYCD3;*1 over-expressing B2 lines. Although apparently higher, the mitotic indices in the central zones of the various transgenic lines were not found significantly different according to the binomial probability distribution, probably due to the lower number of cells in these zone. No significant change was observed in the level of the PSTAIRE CDK (CDKA, Fig. 6a), but increased mitotic activity in the *CYCD2;*1 over-expressing lines was found to be correlated with a slight increase in the level of CDKB protein (Fig. 6b) and by a stronger increase in total CDK activity (Fig. 6c). Since CDKB is only present in cells in G_2 and mitosis (Sorrell *et al.*, 2001), its increase would also be consistent with higher mitotic activity.

Nuclear DNA imaging analysis was performed on squashed and Feulgen-stained SAMs excised from 3-month-old plants. This allowed an estimation to be made of the percentage of G_0–G_1 and S+G_2 nuclei in wild and transgenic apices (Table 3). All the *35S-CYCD2* and *35S-CYCD3* transgenic SAMs displayed a significant decrease in the G_0–G_1 population concomitant with an increased S+G_2 population. The lower frequency of 2C nuclei observed in the B1 SAMs correlates with the higher *CYCD3;*1 transgene expression (and expression of endogenous tobacco *CYCD3;*3, see above) as well as the increased growth rate of this line. To a lesser extent, the actively growing line A1 also differs from A2 by a weak but significant decrease of the 2C nuclear population in the SAM.

Effects of CYCD2 and CYCD3 over-expression on ploidy levels in the stem

Since both *CYCD2;*1 and *CYCD3;*1 genes are considered as potentially having a role in regulating the G_1-to-S transition, the effect of their constitutive expression on DNA endoreduplication during stem differentiation was examined using flow cytometric analysis of nuclear DNA contents (data not shown). No increase in ploidy level in transgenics compared with WT was found whatever the level of internodes examined. Only weak differences could

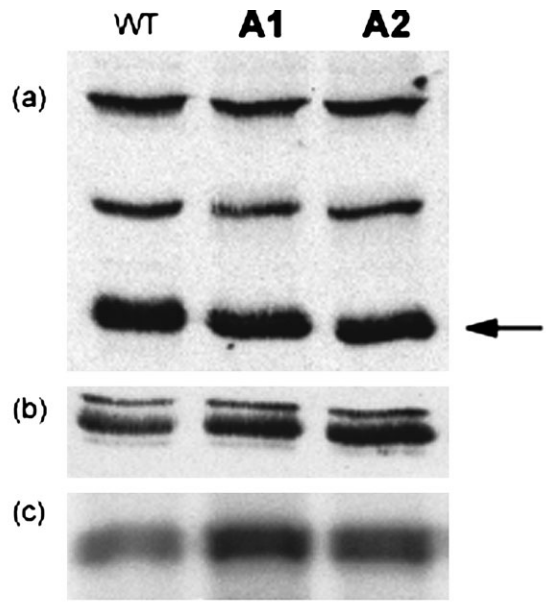

Fig. 6. CDC2a (CDKA) (a) and CDC2b (CDKB1) (b) western blots in WT and *CYCD2;*1 over-expressing lines. (c) Histone H1 kinase assay of total CDK bound to suc1 beads of WT and two *CYCD2;1* over-expressing lines. Extracts were prepared from T2 seedlings derived from homozygous T1 plants of lines 5 (centre) and 2 (right). Arrow in (a) shows CDKA band; other cross-reacting bands are shown as controls for loading. All experiments were carried out on equal amounts of the same extracts.

Table 2. *Mitotic indices in the peripheral and central zones of 3-month-old WT and transgenic SAMs*

Ten independent SAMs were examined for each line.

		Peripheral zone	Central zone[a]
WT		2.65%	0.95%
35S-CYCD2	A1	3.32%	2.85%
	A2	3.88%[b]	0.85%
35S-CYCD3	B1	3.21%	1.53%
	B2	5.99%[b]	2.06%

[a] Only the L1 and L2 cells of the central zone were considered as the L3 was not participating in this zone in the *35S-CYCD2* lines.
[b] Significantly different according to the binomial probability distribution.

Table 3. *Percentages of G_0–G_1 and S+G_2 nuclei established from nuclear DNA imaging analysis on squashed and Feulgen-stained WT and transgenic SAMs*

Five hundred interphasic nuclei from 10 independent 10-week-old plants were analysed for each line.

		G_0–G_1 nuclei (%)	S+G_2 nuclei (%)	Mean nuclei surface (μm^2)
WT		89.1	10.9	33.7
35S-CYCD2	A1	82.1[a]	17.9[a]	40.6[a]
	A2	83.2[a]	16.7[a]	43[a]
35S-CYCD3	B1	81.7[a]	18.2[a]	48[a]
	B2	84.7[a]	15.3[a]	50[a]

[a] Significantly different from the WT according to the binomial probability distribution.

be observed in comparing the DNA levels in apical internodes of WT and both *CYCD3* over-expressing lines. The latter displayed a tendency to have reduced 2C and increased 4C values, which is, however, unlikely to be due to an earlier stimulation of tetraploidy, but rather to an increase in G_2 nuclei of the diploid population as mitotic activity was still present in the developing internodes.

Discussion

The aerial part of plants is built from shoot apical meristem cells set aside during embryogenesis at the tip of the embryo axis. Two main architectural features are recognized in the shoot apical meristem: stratification into three clonally distinct layers (L1, L2, and L3) (for review, see Steeves and Sussex, 1989) and zonation into cytophysiologically distinct regions (Nougarède, 1967; Bowman and Eshed, 2000). Layer L1 is a single layer that usually divides anticlinally and gives rise to the epidermis; L2 is also a single layer that divides anticlinally except at the sites of leaf formation (Cunninghame and Lyndon, 1986) and gives rise to ground tissue. Cells interior to the L2 constitute the corpus (L3) and divide in various planes to initiate the innermost tissues of the stem. Superimposed on the stratified organization, a zonal organization also characterizes the vegetative SAMs. The peripheral zone is composed of cells that divide actively to produce leaf primordia. The central zone has lower cell division activity and contains a core of stem cells both for the two superficial layers (L1 and L2) and for the underlying corpus (L3). A rib zone corresponding to the lower L3 cells gives rise to the pith tissue.

This correct organization seems to be required for normal development as mutations in genes involved in identity and/or functioning of the various zones or layers usually leads to altered phenotypes through alterations of co-ordinated patterns of SAM development (Fletcher and Meyerowitz, 2000; Schoof *et al.*, 2000; Frank *et al.*, 2002). Cell division appears to be essential to ensure the co-ordinated functioning of the various domains, although little is known about how local patterns of cell division are generated and maintained (Meyerowitz, 1997). In addition, communication between the various domains of the SAM appears important for co-ordination of cell division and growth activities (Lucas *et al.*, 1995; Fletcher *et al.*, 1999), but the effects of local modifications in cell cycling activity within the meristem have not been studied very much.

Control of *CYCD2* and *CYCD3* expressions by hormones and sucrose have suggested that these genes might play a major role in plant response to external signals in controlling cell division and differentiation. Previous work has shown that over-expression of the *Arath-CYCD2;*1 gene resulted in transgenic lines with accelerated growth and development compared with WT controls, but overtly normal phenotypes (Cockcroft *et al.*, 2000). This faster growth was shown to be the consequence of both a shortening of the G_1 phase of meristematic cells, a substantially greater probability of cells immediately re-initiating a new cell cycle after completion of the preceding division. These shorter cell cycles and a higher growth fraction resulted in faster growth and faster rate of leaf initiation. Meristem size was unaffected in the juvenile stages examined (Cockcroft *et al.*, 2000).

A detailed cytohistological analysis of SAM development in these *Arath-CYCD* over-expressing lines was carried out, and the analysis extended to examine the effects of constitutive *CYCD2;*1 and *CYCD3;*1 expressions. The faster rate of leaf production and growth of *CYCD2;*1 over-expressing lines (Cockcroft *et al.*, 2000) was confirmed, although these differences were less apparent up to 2–3 months after germination. It was also confirmed that the meristems of *CYCD2;*1 over-expressing plants do not noticeably differ from WT meristems in juvenile stages (up to 1 month). However, after 6–8 weeks, it was found that over-expression of *Arath-CYCD2;*1 and *CYCD3;*1 genes in tobacco alters the basic organization of the SAM in distinct ways without noticeably disturbing overall plant phenotypes. Transgenic tobacco lines over-expressing *CYCD2* or *CYCD3* are able to accelerate the rate of leaf initiation through increased cell production as has already been reported for *CYCD2* (Cockcroft *et al.*, 2000). The present data demonstrates that triggering the cell cycle by *CYCD2* and *CYCD3* over-expression in tobacco can lead to an increase in the SAM size due to the enlargement of the peripheral zones and, to a lesser extent, of the central zone, accompanied by an increase in the leaf initiation rate. A relationship between the SAM size and the rate of leaf initiation was also found in the *Arabidopsis* mutants *clavata* (Clark *et al.*, 1993) and *amp1/pt* (Chaudhury *et al.*, 1993; Mordhorst *et al.*, 1998). This suggested that the upstream machinery that regulates the co-ordinated function of the various zones of the SAM may act, in part, through the action of the *CYCD2* and *CYCD3* genes or, alternatively, that an increase in cell proliferation within the meristem usually leads to an increased leaf initiation rate (Cockcroft *et al.*, 2000). Conversely, over-expression of the *CKS1At* gene, an inhibitor of cell cycle progression, leads to reduced meristem size and growth inhibition (De Veylder *et al.*, 2001). All these data argue that meristem size and the rate of cell proliferation are closely interrelated and that both phenomena influence plant growth rate.

Surprisingly, it was found that *CYCD2* over-expression stimulates periclinal cell divisions in the central and peripheral L3 cells. By contrast, the L1 and L2 central cells are not modified, even though both central and peripheral zones are significantly expanded relative to WT SAMs. Perhaps as a consequence, the *35S-CYCD2* SAMs progressively lose cytophysiological zonation, their central zone becomes restricted to L1 and L2 central cells and all the L3 cells reach a particular stratified organization into four or five regular layers of flat cells. Interestingly, unusual SAM

organization is not incompatible with leaf initiation. This indicates a particular plasticity of the SAM, which is seen to tolerate various internal modifications without altering organogenesis. Such behaviour is also reminiscent of what occurs in photoperiod-dependent plants when they are prevented from flowering under non-inductive conditions. In such conditions, the SAMs enter a particular state called the 'intermediate phase' during which they accelerate the rate of leaf initiation and acquire a stratified appearance through activation of the corpus cells, with the central zone becoming reduced to L1 and L2 central cells (Nougarède, 1965; Nougarède *et al.*, 1965). Whether such intermediate meristems are characterized by an increase of *CYCD2* expression has not been studied, but the similar organization of *CYCD2*-over-expressing and intermediate SAMs suggests that the corpus cells are particularly responsive to both external and internal signals and that CYCD2 could mediate such signals. It also suggests that stimulation of the cell cycle in this domain could interfere with genes such as *WUS* (Laux *et al.*, 1996), *CLAVATA* (Clark *et al.*, 1993) and/or *STM* (Barton and Poethig, 1993) that maintain the meristem identity and the low rate of cell division in the central stem cells. As plants appear to have mechanisms that can at least partially compensate defects in proliferation by increasing cell expansion (Hemerly *et al.*, 1995; Smith *et al.*, 1996; Broadhvest *et al.*, 2000), the possibility cannot be excluded that periclinal divisions in the corpus represent compensatory divisions that could maintain SAM integrity in situations of cell cycle activation in the upper L1 and L2 layers. However, this hypothesis seems unlikely because ectopic expression of *CYCD3* also stimulates cell division in the L1 and L2 peripheral cells without inducing periclinal divisions in the L3 cells.

A further hypothesis could be the involvement of CYCD2 in determining planes of cell division in L3 cells. Up to now, only CDC2a (CDKA;1) has been reported as a cell cycle regulator possibly involved in the orientation of cell division due to its association with the preprophase band (Mineyuki *et al.*, 1991; Colasanti *et al.*, 1993). Further studies are still necessary for understanding whether CYCD2 has a possible role in this context.

The *35S-CYCD3* plants show another type of SAM reorganization. The SAM is enlarged as in the *35S-CYCD2* plants and the cytophysiological zonation is maintained although attenuated. The more striking change occurs in the actively dividing peripheral zone, which consists of cells of reduced size, particularly in the L1 and L2 layers. Coordination of cell growth with cell division is important (Francis, 1998; Polymenis and Schmidt, 1999). The increased rate of cell proliferation and the reduction of the G_1 phase duration in the *35S-CYCD3* SAM probably do not allow the daughter cells to grow sufficiently between two successive divisions. The enhanced cell production is also accompanied by smaller cell sizes in the differentiating tissues, which may be a result of delayed differentiation or continued cell cycling. A reduced cell size and increased zone of proliferation were also observed in *Arabidopsis* expressing very high levels of *CYCD3;*1 (Dewitte *et al.*, 2003).

Finally, over-expression of *CYCD2* or *CYCD3* does not stimulate the process of endoreduplication during stem differentiation despite their putative role in the G_1 to S transition. On the contrary, cells of the *35S-CYCD3* developing internodes continue to have a mitotic activity while the corresponding WT cells stop dividing earlier. *CYCD3* over-expression extends the meristematic activity beyond the SAM and to delays the onset of cell cycle arrest and differentiation. Similar results were reported recently in *Arabidopsis* (Dewitte *et al.*, 2003). This effect in delaying cell differentiation senescence is also consistent with a possible link between cell division and cytokinin production (Boucheron *et al.*, 2002). It remains to be established whether these data should be interpreted as the action of *CYCD3* on both DNA replication and cell division as reported by Schnittger *et al.* (2002).

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