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Tobacco BY-2 cells expressing fission yeast *cdc25* bypass a G2/M block on the cell cycle

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Summary

The mitotic inducer gene from *Schizosaccharomyces pombe*, *Spcdc25*, was used as a tool to investigate regulation of G2/M in higher plants using the BY-2 (*Nicotiana tabacum*) cell line as a model. *Spcdc25*-expressing BY-2 cells exhibited a reduced mitotic cell size through a shortening of the G2 phase. The cells often formed isodiametric double files both in BY-2 cells and in cell suspensions derived from 35S::*Spcdc25* tobacco plants. In *Spcdc25*-expressing cells, the tobacco cyclin-dependent kinase, NtCDKB1, showed high activity in early S phase, S/G2 and early M phase, whereas in empty vector cells CDKB1 activity was transiently high in early S phase but thereafter remained lower. *Spcdc25*-expressing cells also bypassed a block on G2/M imposed by the cytokinin biosynthetic inhibitor lovastatin (LVS). Surprisingly, cytokinins were at remarkably low levels in *Spcdc25*-expressing cells compared with the empty vector, explaining why these cells retained mitotic competence despite the presence of LVS. In conclusion, synchronised *Spcdc25*-expressing BY-2 cells divided prematurely at a small cell size, and they exhibited premature, but sustained, CDKB1 activity even though endogenous cytokinins were virtually undetectable.

Keywords: *Nicotiana tabacum*, cell size, cytokinins, *BY-2 cell line CDKA/B*, plant cell cycle.

Introduction

The plant cell cycle is regulated by cyclin-dependent protein kinases (CDKs), which are themselves phosphoregulated (Joubes *et al.*, 2000). In fission yeast (*Schizosaccharomyces pombe*), *Spwee1* and *Spcdc25* are phosphoregulators of the G2/M transition. *Spwee1* encodes a protein kinase that in G2 phosphorylates the Y15 residue near to the NH_2-terminus of the Cdc2 protein; this suppresses the kinase activity of the CDK (Gould and Nurse, 1989; Russell and Nurse, 1987). *Spcdc25* encodes a tyrosine phosphatase that in late G2 dephosphorylates the Cdc2 kinase on the same tyrosine residue (Y15) (Russell and Nurse, 1986). Following binding of Cdc2 with a B-type cyclin, dephosphorylation of Cdc2 by Cdc25 is the final all-or-nothing signal that triggers Cdc2 kinase activity driving cells into division (see O'Farrell, 2001).

In *S. pombe*, overexpression of *cdc25* induced a short-cell phenotype. The cells divided prematurely through a shortening of the G2 phase, but surprisingly there was little change in the overall length of the cell cycle (Russell and Nurse, 1986). In this case, a lengthening of G1 compensated for the shortened G2. Overexpressing *wee1* had the converse effect, resulting in a long-cell phenotype (Russell and Nurse, 1987). This suggests strongly that by competing for the same substrate Wee1 and Cdc25 regulate cell size at division, although *wee1* is regarded as the main genetic element in this control (Sveiczer *et al.*, 1996); presumably, other size-related signals impinge on this complex that finally enable Cdc25 to out-compete Wee1.

In plants, a homologue to *wee1* has been identified in *Zea mays* (Sun *et al.*, 1999) and in *Arabidopsis thaliana* (Sorrell *et al.*, 2002). Most recently, a gene has been identified that encodes a small CDC25 protein (*Arath; CDC25*) that lacks a regulatory domain but can dephosphorylate plant CDKs (Landrieu *et al.*, 2004). *Spcdc25* also dephosphorylated tobacco CDC2 kinase *in vitro* (Zhang *et al.*, 1996). In wild-type (WT) cultures of *Nicotiana plumbaginafolia* that were depleted of exogenous cytokinin cells were arrested in G2 and CDC2 kinase was inactivated. However, the addition of cytokinin (kinetin) led to dephosphorylation of CDC2 and entry of cells into mitosis (Zhang *et al.*, 1996). Moreover, induction of *Spcdc25* expression in the same culture system could induce cell division in cultures depleted of auxin and cytokinin (Zhang *et al.*, 2005). These data suggest strongly that there is a cytokinin-mediated signal transduction chain that regulates the G2/M transition through activation of a Cdc25-like phosphatase (Zhang *et al.*, 2005). However, the levels of endogenous cytokinins were not reported on. Interestingly, WT BY-2, cells are dependent on zeatin and zeatin riboside synthesis at the G2/M transition because suppression of their synthesis by lovastatin (LVS) blocked the G2/M transition (Laureys *et al.*, 1998; Redig *et al.*, 1996). Lovastatin inhibits the isoprenoid pathway of cytokinin biosynthesis (Crowell and Salaz, 1992).

In *Nicotiana tabacum*, constitutive *Spcdc25* expression resulted in a small mitotic cell size (Bell *et al.*, 1993) and induction of *Spcdc25* in cultured roots also resulted in a small mitotic cell size (McKibbin *et al.*, 1998). Induction of *Spcdc25* expression in tobacco leaves resulted in variable cell size and cell proliferation in the lamina margins followed by alterations in leaf shape (Wyrzykowska *et al.*, 2002).

The aim of the work reported here was to use *Spcdc25* as a tool to investigate regulation of the G2/M transition in the plant cell cycle using the tobacco BY-2 cell line as a model (Nagata *et al.*, 1992). We tested whether the *Spcdc25*-induced reduction in cell size reported by McKibbin *et al.* (1998), was because of a shortening of the S phase or G2, or both. We also assayed CDKA and CDKB1 to examine whether either enzyme was precociously active in the cell cycle of synchronised *Spcdc25*-expressing cells. Another important aim was to measure endogenous cytokinins in *Spcdc25*-expressing cells compared with an empty vector $\pm$ LVS. This provided an independent test of Zhang *et al.*'s (2005) cytokinin-regulated model of the G2/M transition in the plant cell cycle.

Spcd25-expressing cells not only divide prematurely but show early and persistently high CDKB1 activity and altered planes of cell division. Moreover, synchronised entry of *Spcdc25*-expressing cells from G2 to mitosis was unaffected by LVS, and, most surprisingly, *Spcd25*-expressing cells have an extremely low endogenous cytokinin content.

Results

Spcdc25 *is expressed but the lines are less stable than empty vector controls*

The BY-2 cell line was transformed through a modified CaMV 35S promoter (BIN-HYG-TX) that has an attenuated level of expression compared with the conventional CaMV 35S promoter (Gatz *et al.*, 1992). Periodically, it became necessary to generate new transgenic lines because after about 12–15 months in culture *Spcdc25*-expressing cells unexpectedly stop growing. The mortality of *Spcdc25*-expressing lines, which was not incurred with comparable empty vector (EV) lines, suggests strongly that *Spcdc25* expression eventually becomes lethal in BY-2 cells (see Discussion).

RT-PCR confirmed the expression of *Spcdc25* RNA (Figure 1) and Western blots confirmed the expression of *Spcdc25* at the protein level (Figure 1). The Spcdc25 antiserum also revealed a weaker signal in EV cells (Figure 1). Notably, Zhang *et al.* (2005) observed CDC25-like phosphatase activity in their WT tobacco cultures. The weak signal (Figure 1), which was also detected in WT (data not shown), may be due to the expression of endogenous tobacco *CDC25.* However, if this is the case, the tobacco CDC25 protein is substantially larger than the predicted size of the Arabidopsis CDC25 protein (Landrieu *et al.*, 2004; Sorrell *et al.*, 2005).

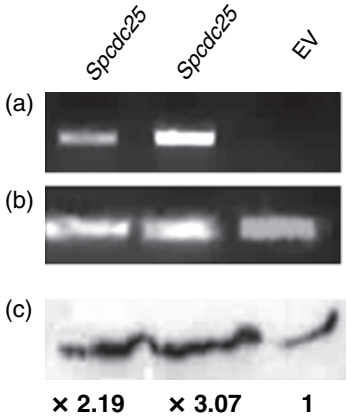

Figure 1. Spcdc25 cells express *Spcdc25* at the mRNA and protein levels.
RT-PCR and Western analysis of *Spcdc25* expression in the BY-2 cell lines used in this study.
Top panel (a): expression of *Spcdc25* (product size 718 bp), in two independent lines carrying the *Spcdc25* construct; EV is the empty vector.
Middle panel (b): 18S RNA control (product size 459 bp).
Bottom panel (c): Western blots of protein from the same *Spcdc25*EC compared with EV, probed with an antibody to *S. pombe* Cdc25. The Western signals were quantified in relation to the EV signal.

Spcdc25 *induces premature cell division through a shortening of the G2 phase but not the S phase*

Spcdc25-expressing cells (referred to hereafter as *Spcdc25*EC) synchronised with aphidicolin exhibited a first peak in the mitotic index (the sum of prophase, metaphase, anaphase and telophase as a percentage of all cells) at 6 h, compared with a first peak at 10 h in corresponding EV cells (Figure 2). The initial rise in the mitotic index is the time taken for cells previously blocked in late S phase to reach mitosis. Hence the G2 phase in *Spcdc25*EC was 2 h and in EV 6 h (Figure 2). Patterns of alteration in amounts of DNA, obtained by microdensitometry, are consistent with a shorter G2 in *Spcdc25*EC compared with EV (upper- and lowermost panels in Figure 2).

Expression of histone H4 is a marker of the S phase, and in both *Spcdc25*EC and EV a strong H4 profile was evident from 0–4 h followed by a drop to less than 20% of the maximum (Figure 2). Hence, the S phase lasts for 4 h in both *Spcdc25*EC and in EV cells. The interval between peaks in the mitotic index is the cell cycle time: 11 and 12 h for *Spcdc25*EC and EV, respectively. Note that in the *Spcdc25*EC the distinctive and highly repeatable change to the cell cycle was a reduction in the duration of G2.

CDKB1 activity is consistently high in S-phase S/G2 and G2/M in Spcdc25-*expressing cells*

If *Spcdc25* cells have a short G2 phase as a direct result of increased Cdc25 phosphatase activity, then CDKs that propel cells into division ought to be highly active at earlier sample times compared with EV cells. This hypothesis was tested by assaying CDKA and CDKB1. Note that samples were taken from 1–9 h (*Spcdc25*EC) or 1–14 h (EV), i.e. before, during and after the first mitotic peak (the mitotic index amplitude) (Figure 3).

In the *Spcdc25*EC lines, CDKB1 activity was consistently high at 1 h (early S phase) and at 3–4 h (90% of maximum

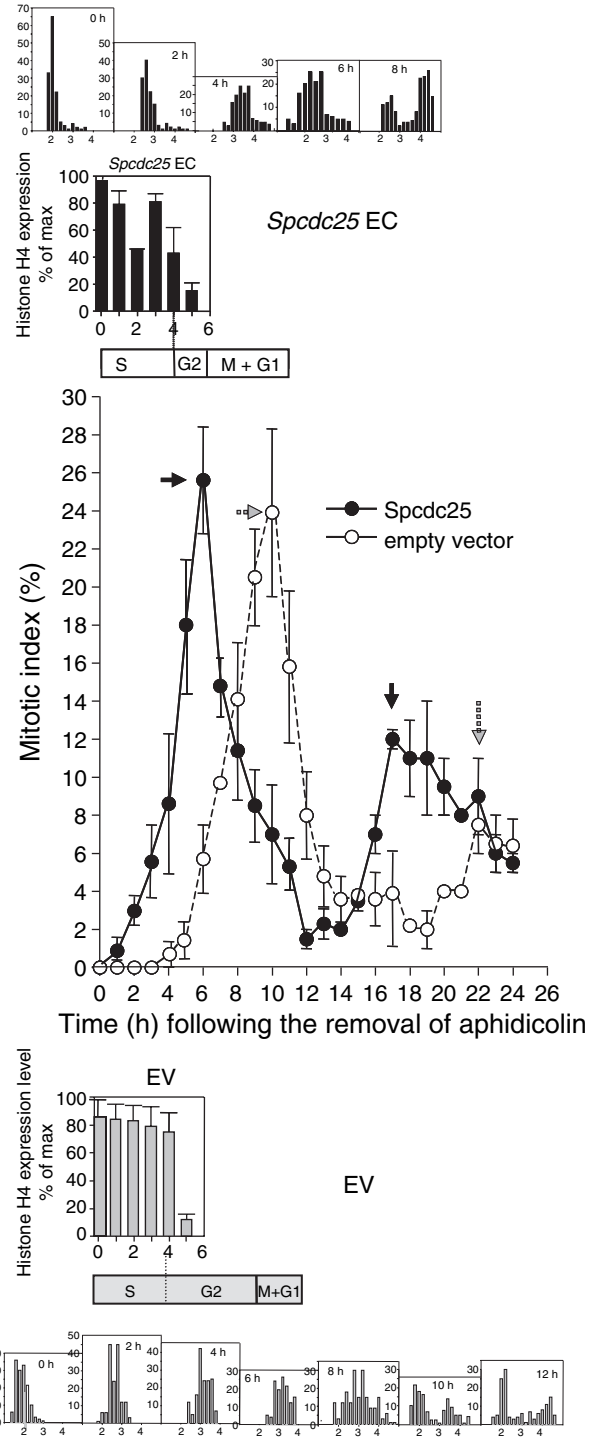

Figure 2. Synchronised *Spcdc25*-expressing BY-2 lines exhibit a premature rise in the mitotic index and a short G2 phase.
The mitotic index (means ± SE) from independent *SpCDC25* lines and corresponding means from EV, following release from aphidicolin ($n = 3$). The earlier rise in the mitotic index in *Spcdc25*EC is consistent with a shorter G2 (2 h) compared with EV (G2 = 6 h = the first significant rise in the mitotic index). Arrows indicate the peaks of mitotic index which is a measure of the duration of the cell cycle: *Spcdc25*EC = 11 h (6–17 h on the *x*-axis) and in EV = 12 h (10–22 h). Immediately above and below the mitotic index data are mean (±SE) histone H4 expression levels from replicates ($n = 2$). The S phase is measured from zero to the sample time immediately before a signal that was <20% of the maximum (S phase = 4 h in both). The durations of G2 and G1 + M are added to the horizontal bar charts. In summary G1 + M was obtained by difference (i.e. *C* – (G2 + S):

	*SpCDC25*EC	EV
S phase	4	4
G2	2	6
G1 + M	5	2
C	11	12

The uppermost and lowermost bar charts, indicate temporal changes in nuclear DNA amounts (*C* values) in interphase cells following the release from aphidicolin: ordinate = frequency, abscissa = amounts of nuclear DNA (*C* values). Each density value was normalised against prophase (4*C*) and half-telophase (2*C*) mitotic figures. Note that in histograms lacking 4*C* values, those samples and 'mitotic' samples were stained simultaneously ($n = 150$).

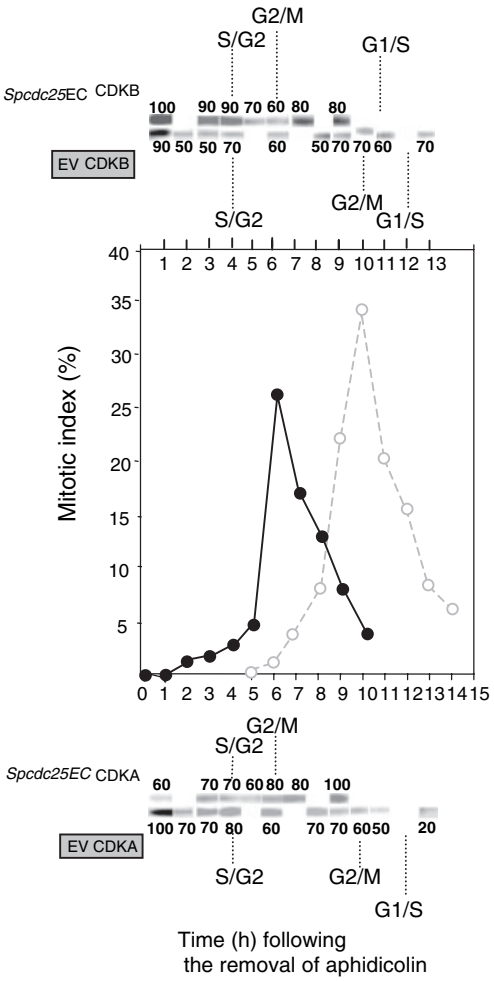

Figure 3. Premature CDKB1 activity in S phase, G2/M and M in *Spcdc25*EC.
*Spcdc25*EC and EV cells were synchronised as explained earlier, and mitotic indices recorded for between 12 and 14 h following the release from aphidicolin. (Note that the timings of the increases and peaks in these new mitotic index data are identical to those shown in Figure 2.)
The mean histone H1 kinase activities of the CDKA and CDKB1 immunoprecipitates are quantified as a percentage of maximum activity for each assay ($n = 2$).
The quantitative data, either rounded up or down to the nearest 10, are presented for each gel image. Cell cycle phase transitions are added to each row of gel images. Note that the gel images are *representative* of images the two replicate experiments and that key phase transitions are inserted as shown by vertical dotted lines.

(S/G2)). It dipped to 60–70% of maximum at 5 and 6 h before rising again at 7 h (80% of maximum at early M phase) and 9 h (80% of maximum). In EV, CDKB activity was transiently high at 1 h (early S phase), but from there on it was between 50 and 70% of maximum. Note that M phase in WT BY-2 cells is between 1 and 2 h (Francis *et al.*, 1995; Herbert *et al.*, 2001; Porceddu *et al.*, 2001; Sorrell *et al.*, 2001), which would place the 9 h signal for *Spcdc*25EC (80% maximum) at M/G1 and the 13 h signal for EV in the S phase of the *next* cell cycle.

In the *Spcdc25*EC, CDKA activity was relatively constant between 1 and 5 h (60–70% maximum, S phase and early G2), but increased to 80% at 6 h (G2/M) and was 100% of the maximum at 9 h ('M/G1'). In the EV, CDKA activity was maximal at 1 h (early S phase) and was also high at 4 h (80% maximum, S/G2). Between 6 and 11 h it was between 60 and 70% of maximum, before dropping to 20% of maximum at 13 h (S phase of the next cell cycle).

To summarise, a clear and repeatable feature of these assays was premature and persistent CDKB1 activity in *Spcdc25*EC compared with EV.

Mitotic cell size is smaller in Spcdc25*-expressing lines*

Overexpression of *Spcdc25* in fission yeast and in tobacco plants resulted in a decrease in mitotic cell size (McKibbin *et al.*, 1998; Russell and Nurse, 1987). Indeed, in the latter it was the most characteristic feature of the transgenic plants (see Introduction). The shortened G2 phase in the BY-2 cells expressing *Spcdc25* would suggest premature cell division at a reduced cell size. In *Spcdc25*EC, the mitotic cell area was indeed significantly smaller compared with EV and compared with hitherto unpublished data for WT (Figure 4, $P < 0.001$). However, the mean mitotic cell area for EV cells was not significantly different from WT ($P > 0.05$). In other words, neither the transformation process *per se* nor expression of the antibiotic resistance gene affected mitotic cell size.

Cells expressing Spcdc25 *form doublets of small cells*

Not only did we observe a reduced cell size at division, but the cells also exhibited a tendency to form in doublets as

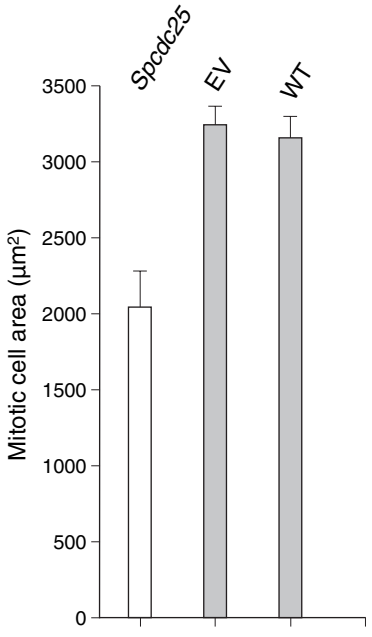

Figure 4. The mean (±SE) mitotic cell area (μm^2) is smaller in *Spcdc25*EC than in EV and WT ($n \geq 50$).

opposed to the single-cell filaments characteristic of WT and EV lines (Figure 5). We quantified this observation by scoring the frequency of doublets in *Spcdc25*EC during normal log-stationary phase growth. On each day of the 7-day culture period, filaments of six cells or more in length were scored as either single or double. Notably, between 19 and 34% of filaments were present as doublets in *Spcdc25*EC on day 4 compared with singlets in EV (Table 1). Although the frequency of doublets in *Spcdc25*EC varied, such doublets were either observed rarely or not at all in EV (Figure 5, Table 1).

A cell suspension established from internodal tissue from 35S::*Spcdc25* tobacco also exhibited small isodiametric doublets of cells (Figure 5d), confirming our observations in BY-2 cells.

Spcdc25*EC* overcome a block imposed by lovastatin

Zhang *et al.* (1996) reported that both *Spcdc25* and cytokinin treatment could dephosphorylate plant Cdc2 in late G2 of the cell cycle (see Introduction). We therefore tested whether *Spcdc25* expression is dependent on a cytokinin signal by treating BY-2 cells with LVS, a known inhibitor of cytokinin biosynthesis in late G2. Cells were synchronised, LVS was added 1 h following the removal of aphidicolin and samples were taken during mitotic index

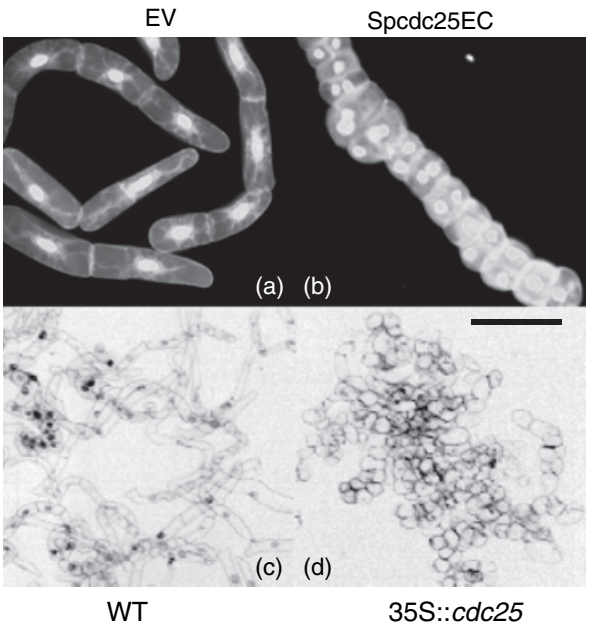

Figure 5. *Spcdc25*-expressing BY-2 cells form in isodiametric double filaments.
Interphase BY-2 cells stained with Hoechst viewed by fluorescence (a,b) or bright field optics (c,d): (a) EV, (b) *Spcdc25*EC, (c) cell suspension established from tobacco internodal tissue and (d) a cell suspension established from internodal tissue of 35S::Sp*cdc25* tobacco. The bar scale = 100 μm in all cases.

Table 1 The range of percentage frequency of doublets of BY-2 cells (double filaments six cells or more in length) in the *Spcdc25*EC compared with EV, during the normal 7 days of BY-2 batch culture at 27°C ($n = 300$)

	Range % frequency of doublets in:	
Day of culture	*Spcdc25*EC	EV
1	2–6	0
2	7–10	1
3	18–24	0
4	19–34	0
5	9–26	0
6	3–17	0
7	2–4	0

amplitudes so as to compare the G2/M transition in both *Spcdc25*EC and EV. In *Spcdc25*EC, the addition of LVS did not affect the mitotic index compared with the control (Figure 6). However, in the EV, LVS inhibited the mitotic index so that an expected curve was suppressed. Hence, the data are consistent in showing that in progressing from G2 to mitosis cells in the *Spcdc25*EC can escape a cell cycle block imposed by LVS.

Endogenous cytokinins are extremely low in Spcdc25 *cells.*

In *Spcdc25* cells, the flow of cells into mitosis was unaffected by LVS, suggesting that *Spcdc25* lines might exhibit an increased level of cytokinin to compensate for LVS inhibition. To test this hypothesis, samples were taken from the *Spcdc25*EC and EV at the same time as those taken to generate the mitotic indices shown in Figure 6. Cytokinins were measured in samples that included the G2/M transition: 2–5 h for *Spcdc25*EC and 0–10 h for EV. Levels of representative cytokinins are presented in Figure 7. Surprisingly, in *Spcdc25*EC the endogenous concentrations of cytokinins were extremely low or below detectable limits regardless of LVS treatment, thereby negating our hypothesis. However, LVS added to EV resulted in a predictable reduction in levels of endogenous cytokinins (Figure 7).

Discussion

Spcdc25 *shortens G2*

In the BY-2 cell line, *Spcdc25* induces a small cell size at mitosis because of a 50% reduction in the G2 phase (Figure 8) This mechanism was only hypothesised in previous work (McKibbin *et al.*, 1998).

In fission yeast, overexpression of *Spcdc25* induces a reduction in mitotic cell length. However, the cell cycle duration was very similar to that in WT with a lengthening of G1 compensating for the shortening of G2 (Russell and Nurse, 1986). This is slightly different from the *Spcdc25*EC

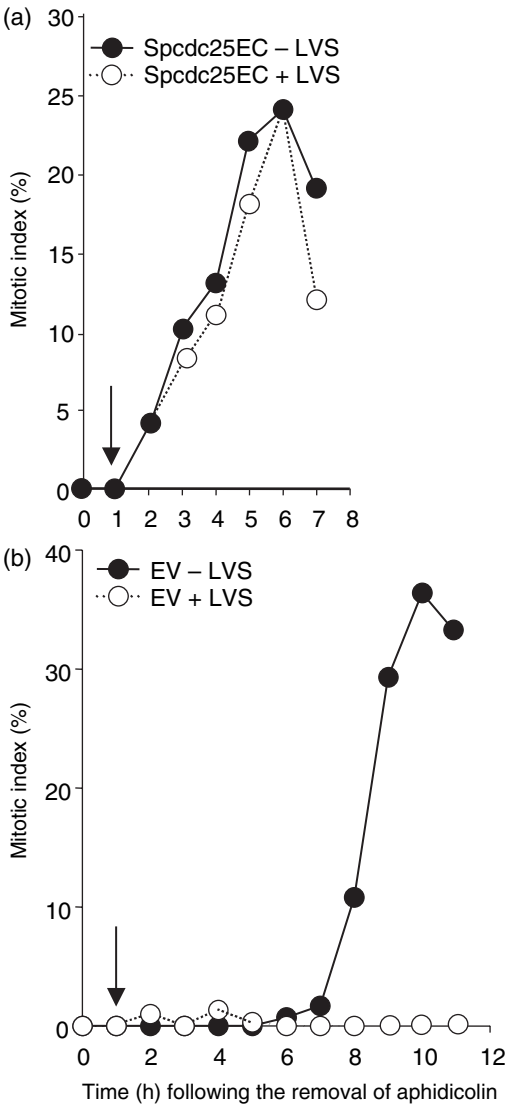

Figure 6. *Spcdc25*EC escape a LVS-induced block.
Changes to the mitotic index ±10 μM LVS given at 1 h following aphidicolin release (↓) in (a) *Spcdc25*EC and (b) EV. (Note that the timings of the increases and peaks of these new data are identical to those shown in Figures 2 and 3.)

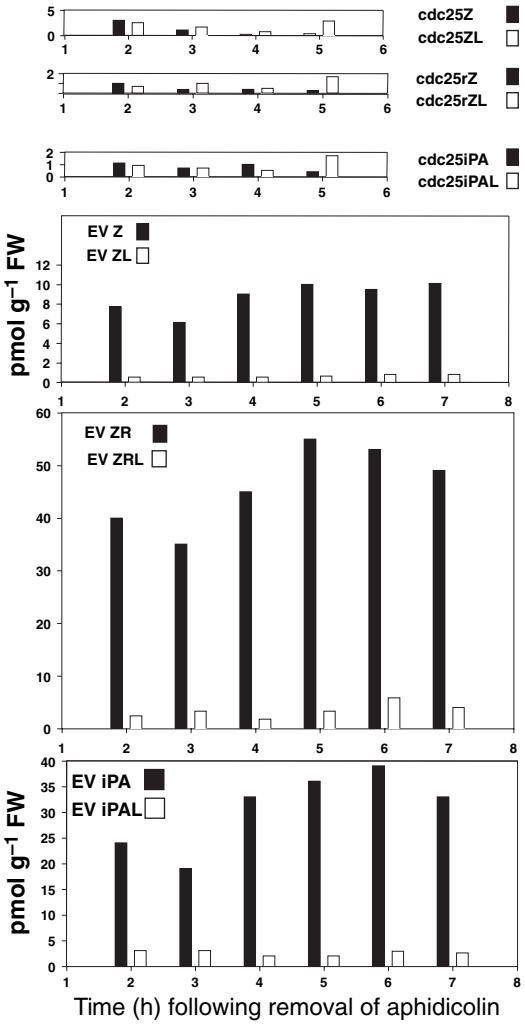

Figure 7. Cytokinins are virtually undetectable in *Spcdc25*EC.
Levels of zeatin (Z), zeatin riboside (ZR) and N^6-(Δ^2isopentenyl)-adenine (iPA) in *Spcdc25*EC and EV ± LVS. Samples were taken hourly from the same experiment used to generate mitotic indices in Figure 6.
Top three panels: *Spcdc25*EC ± LVS. Black bars, cytokinin level; white bars, cytokinin level + LVS.
Bottom three panels: EV ± LVS. Black bars, cytokinin level; white bars, cytokinin level + LVS.

where the duration of the cell cycle was reduced by 1 h compared with EV, although there was a compensatory lengthening in M + G1 (Figure 8).

Premature cell division is preceded by earlier CDK activities in Spcdc25 *cells compared with EV*

In *Spcdc25*EC, we detected persistently high CDKB1 kinase activity in early S phase, S/G2 and early M (Figure 3). However, CDKA activity was relatively constant before peaking in M + G1 (Figure 3a). In EV, CDKA activity was very similar to WT (Porceddu *et al.*, 2001; Sorrell *et al.*, 2001).

In the EV cells, CDKB1 activity data gave an isolated peak in early S phase, whereas in WT CDKB activity peaks in G2/M (Porceddu *et al.*, 2001; Sorrell at al., 2001). Zhang *et al.* (2005) showed that activation of CDK activity is dependent

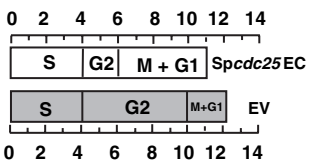

Figure 8. Cell cycle and component phases (h) for *Spcdc25*EC and EV.

on native CDC25-like activity. Also, in early S phase of WT a strong signal can be seen for CDKB1 at the RNA level (Figure 2b of Sorrell *et al.*, 2001). Therefore isolated CDKB1 activity in early S phase in the EV cells may not be completely surprising given that CDC25 activity can be high at G1/S (e.g. mammalian *CDC25B* in Jinno *et al.*, 1994). Also, note that the EV cell cycle has a very short M + G1 of 2 h compared with 5 h in *Spcdc25*EC and WT (Herbert *et al.*, 2001) and that the transient CDKB1 activity coincides with this perturbation.

We have shown that premature and sustained CDKB1 activity can result in premature cell division at a small mitotic size. Intriguingly, *Spcdc25* expression has its greatest influence on tobacco CDKB1. Note that unlike CDKA, CDKB1 activity is highest solely at the G2/M transition in WT BY-2 cells (Porceddu *et al.*, 2001; Sorrell *et al.*, 2001). Also, in Arabidopsis, the B type that has the PPTLRE signature only functions during G2 and M phase and is unique to plants. However, in both tobacco and Arabidopsis, CDKA activity is high during S, G2 and M, and in Arabidopsis CDKA has the strongly conserved PSTAIRE motif also found in yeast and animal CDKAs (Joubes *et al.*, 2000).

Spcdc25 *induces sister filaments of cells*

A strikingly visual result in these transformed lines was the appearance of double filaments of near-isodiametric cells in the *Spcdc25*-expressing lines and in a cell suspension derived from transgenic tobacco plants expressing *Spcdc25* constitutively. This is an unusual occurrence in WT BY-2 cells that typically grow in long single filaments, and cell length ratios are typically between 2:1 and 6:1 (unpublished data). Such filaments of long cells arise by transverse divisions. In *Spcdc25*EC the cells are considerably smaller in area, mainly due to a reduction in cell length resulting in isodiametric cells (Figure 5). Indeed, in some cases the width of the cell exceeds what should be its length as viewed along a filament of cells. Thus, we conclude that *Spcdc25*-induced reduction in cell length permits cells to divide either transversely *or* longitudinally.

When *Spcdc25* transcripts were induced in cultured tobacco roots, there was an increase in the frequency of lateral roots per primary root length (McKibbin *et al.*, 1998). Lateral root primordia are initiated from the pericycle close to the primary apical meristem and immediately adjacent to protoxylem poles (Dubrovsky *et al.*, 2001). Formative divisions are transverse as large 'mother' cells partition into smaller descendants. This is followed by a change in the plane of cell division from transverse to longitudinal so that these youngest primordia appear as a double filament of near-isodiametric cells (Dubrovsky *et al.*, 2001); they are not dissimilar in appearance to the double filaments observed in the *Spcdc25*-expressing BY-2 lines reported here.

Spcdc25-*expressing cells overcome a cytokinin requirement at G2/M*

As mentioned in the Introduction, there is accumulating evidence for a cytokinin-regulated G2/M transition in the plant cell cycle: LVS blocks cytokinin biosynthesis and prevents cells from traversing G2/M (Laureys *et al.*, 1998; Redig *et al.*, 1996). Cytokinin treatment activates the G2/M transition (Zhang *et al.*, 1996) and either *Spcdc25* or a cytokinin treatment can dephosphorylate plant CDC2 (Zhang *et al.*, 1996, 2005). Our data show that *Spcdc25*EC can traverse from G2 to M regardless of a LVS block on the cell cycle, whereas just like in WT (Redig *et al.*, 1996) this transition was blocked in EV cells.

We have shown BY-2 proliferative cells that do not rely on endogenous cytokinins. *SpCdc25*EC have remarkably low amounts of cytokinins compared with EV and with WT (Laureys *et al.*, 1998). Indeed, the levels of various cytokinins were virtually below detectable limits. Thus, the reason why *Spcdc25*EC can overcome a block imposed by LVS is because there is nothing for LVS to block. Clearly, EV cells require cytokinins for G2/M progression but *Spcdc25*EC do not. In fact the cytokinin data are consistent with *Spcdc25* expression leading to an inhibition of cytokinin biosynthesis.

In the *Spcdc25*EC cell size is stabilised to be about two-thirds that of the EV, a similar result to that obtained when *Spcdc25* was overexpressed in fission yeast (Russell and Nurse, 1986). However, for about 12–15 months constitutively expressing cells do not divide at a reduced cell size at each division (which would eventually result in mitotic catastrophe). In fission yeast, when the normal mitotic size control is abolished, such as by the overexpression of *Spcdc25*, a G1/S minimum size controller stabilises cell size thereby preventing the continual drift towards smaller cell sizes (Sveiczer *et al.*, 1996). The eventual demise of *Spcdc25*-expressing cultures after about 12 months might be because a cell size checkpoint is eventually over-ridden by *Spcdc25* expression.

In conclusion, in *Spcdc25*EC a shortened G2 phase and a small mitotic cell size are entirely consistent with premature CDKB1 kinase activity. In BY-2 cells small cell size is coincident with either transverse or longitudinal division. Finally, the data from the LVS treatments and cytokinin measurements show that *Spcdc25*EC are not dependent on endogenous cytokinins to progress from G2 to mitosis. The very recent cloning of *Arath;CDC25* (Landrieu *et al.*, 2004), which when overexpressed in fission yeast induces a short cell length (Sorrell *et al.*, 2005), means that a model: cytokinin → native CDC25 → CDKB1 → G2/M, can now be tested *in planta*.

Experimental procedures

The tobacco BY-2 cell line

The tobacco BY-2 cell line was cultured at 27°C in darkness in modified Linsmaier and Skoog (1965) medium and was subcultured in exactly the same way as detailed previously (Herbert *et al.*, 2001).

Transformation of the BY-2 cell line with Spcdc25

Stable transformation of BY-2 cells was achieved using a modified version of the method described by An (1985). Isolated colonies of *Agrobacterium tumefaciens* strain LBA4404 harbouring either the binary vector pBTX (empty vector) or pBTX-CDC25 (McKibbin *et al.*, 1998) were picked from fresh 2YT-kanamycin (50 μg ml^{-1}) plates and cultured overnight in 7 ml 2YT (without antibiotic) in 50 ml conical flasks at 30°C with shaking. Four millilitre aliquots of 6-day-old stationary phase BY-2 cells containing 20 μM of freshly added Acetosyringon (Sigma-Aldrich, Gillingham, UK) were co-cultivated with 100 μl of *Agrobacterium* culture in 90 mm Petri dishes sealed with Nescofilm for 2 days at 27°C in the dark without shaking. Cells were washed with 1 l of BY-2 medium using a cell dissociation sieve fitted with a 100 μm mesh (Sigma-Aldrich) and resuspended in 5 ml BY-2 medium containing 250 μg ml^{-1} Timentin (Melford Laboratories, UK), and 2.5 ml aliquots plated onto solidified BY-2 medium (0.8% agar) supplemented with 250 μg ml^{-1} Timentin and 80 μg ml^{-1} hygromycin. Plates were sealed with Micropore tape and incubated at 27°C in the dark. Isolated hygromycin-resistant calli (each individual callus was considered as an independent clone) appeared after 2–4 weeks, and were harvested and grown for a further 2 weeks on fresh plates. Calli were then transferred to 50 ml BY-2 medium supplemented with 250 μg ml^{-1} Timentin and 80 μg ml^{-1} hygromycin and incubated at 27°C and 75 ***g*** in the dark until the cultures reached stationary phase (1–3 weeks). Cultures were subsequently maintained as described for WT BY-2 cells using medium containing both 250 μg ml^{-1} Timentin and 80 μg ml^{-1} hygromycin, with the Timentin being omitted after the first subculture. Cultures were subjected to at least four rounds of subculturing before being used in synchrony experiments.

Synchronisation, mitotic index, cell size and microdensitometry

Each of the transformed cell lines was synchronised with aphidicolin using the exact protocol of Nagata *et al.* (1992). A 24 h exposure to, and subsequent removal of, this inhibitor of DNA replication will cause the vast majority of cycling cells to accumulate in late G1 at the boundary with the S phase. However, a minority of cells, trapped at the end of S phase will be the first to arrive at mitosis ahead of the bulk of cells previously held at G1/S. Hence, we measured the duration of G2 from zero to the initial rise of the mitotic index.

Following release from aphidicolin, and using Hoechst staining (Sigma-Aldrich) (Herbert *et al.*, 2001), the mitotic index was measured at hourly intervals for 24–26 h. In a separate experiment, LVS (Sigma-Aldrich) was added at a final concentration of 10 μm immediately following the removal of aphidicolin. Cells were sampled at hourly intervals for 10–12 h.

For each experiment a minimum of 200 cells per slide were scored on random transects across the width of the coverslip encompassing three replicate slides per sampling time per experiment. Images of cells in mitosis (prophase, metaphase or anaphase but not half-telophases) initially observed with an Olympus BH2 (UV, λ = 420 nm) were downloaded into a PC and the mitotic cell areas measured by image analysis using Sigmascan® (Jandel Scientific, San Rafel, CA, USA).

Following the removal of aphidicolin, samples were also taken every 2 h and fixed in 3:1 absolute ethanol:glacial acetic acid and stained by the Feulgen reaction (Powell *et al.*, 1988). The major modification was that in between each of the steps of the procedure – fixation, hydrolysis (5 M HCl for 25 min at 25°C), 2× rinse in ice-cold distilled water (5°C) and 2 h in Feulgen's reagent (25°C) – the cells were spun down gently (2000 ***g*** for 3 min), supernatant was removed and the next reagent added. Cells were then maintained in 45% (v/v) acetic acid. About 20 μl of cells were added to microscope slides and nuclear densities were determined using an M85A Scanning Microdensitometer (Vickers, York, UK) at 560 nm.

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from tobacco BY-2 cells using TRI reagent (Sigma-Aldrich) and residual genomic DNA was removed by DNase treatment (Ambion Inc., Austin, TX, USA). Ribonucleic acid (5 μg) was treated with Superscript II reverse transcriptase (GibcoBRL, Paisley, UK). Complementary DNA was subjected to PCR with specific primers to *Spcdc25:* P2, 5′-GCGCGTCGACCATT-AACGTCTGGGGAAGC-3′ and P7, 5′-TTAGGTCCCCTTCTCCGATG-3′ amplifying a fragment of 718 bp, and to 18S rRNA: PUV, 5′-TTCCATGCTAATGTATTCAGAG-3′ and PUV4, 5′-ATGGTGGTG-ACGGGTGAC-3′ amplifying a fragment of 459 bp. Thermocycle conditions were: 35 cycles at 94°C (30 sec), 55°C (45 sec) and 72°C (1 min 20 sec). Separate cDNA reactions omitting reverse transcriptase were PCR amplified to confirm the absence of contaminating genomic DNA.

Expression of histone H4 was determined by semiquantitative RT-PCR using limited PCR cycles (30 cycles), essentially as described in Sorrell *et al.* (2002, 2005). Histone H4-specific primers H4F, 5′-GGCACAGGAAGGTTCTGAGGGATAACA-3′, and H4R, 5′-TAACCGCCGAAACCGTAGAGAGTCC-3′, amplifying a fragment of 320 bp were used. The optimal PCR cycle was determined by quantifying the PCR product for alternate cycles from cycle 24 to 38 of the PCR reaction, and choosing a cycle within the linear response of the curve (data not shown). Internal standards of cDNA dilutions were included with the samples to ensure linear amplification of the target at the cycle number used. Three replicates of the PCR reactions were performed to ensure consistent data and PCR products were quantified from ethidium bromide-stained agarose gels using GeneGenius (Syngene, Cambridge, UK) software.

*Tobacco cells transformed with 35SCaMV::*Spcdc25

Plants of *N. tabacum* cv. *Samsun* were grown in plastic boxes and leaf discs were transformed with a 35S CaMV::*Spcdc25* construct using *Agrobacterium*-mediated transformation as described in Bell *et al.* (1993). Primary transgenics known to be expressing *Spcdc25* by RT-PCR were selfed and seed was collected. One batch of this transgenic seed was sent from Cardiff University to the laboratory at the Department of Plant Physiology, Charles University, Prague. Seeds were germinated under standard *in vitro* conditions and internodes from 6-week-old plants were transferred to modified Murashige and Skoog (1962) liquid medium supplemented with 1 mg l^{-1} NAA and 1 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), and were cultivated on a rotary shaker in a 16 h photoperiod at 25°C.

Protein assays

Proteins were extracted from 5 ml of synchronised cultures essentially as described in Cockcroft *et al.* (2000). Equal amounts of protein were loaded onto gels, and loading was verified by Coomassie staining of replicate gels. Transfer to membranes was checked by Ponceau staining. For CDK assays immunoprecipitations were carried out using antisera raised to CDKA and CDKB1 (kindly provided by Drs Jim Murray and Marget Menges, Institute of Biology, Cambridge University) as described in Sorrell *et al.* (2001). Histone H1 protein kinase assays were as described in Cockcroft *et al.* (2000) using 2 μl of antiserum. Incorporation was assayed by quantification of autoradiographs using GeneGenius (Syngene, Cambridge, UK). For Western blots, an affinity-purified Spcdc25 antiserum was kindly provided by Dr Sergio Moreno, Centro de Investigación del cancer, Salamanca, Spain.

Cytokinin measurements

Frozen cell samples were ground in liquid nitrogen and transferred in Bieleski buffer (Bieleski, 1964). The deuterated standards were added to the extracts and incubated overnight at −20°C. Afterwards the cytokinins were purified on a combination of DEAE-Sephadex and RP-C18 cartridges. The purified extracts were loaded onto immunoaffinity columns containing monoclonal antibodies against cytokinins. After the immunoaffinity purification procedure, the cytokinins were analysed by HPLC linked to a Quattro II mass spectrometer equipped with an electrospray interface (Prinsen *et al.*, 1995). Analysis of the chromatograms obtained was done using MASSLYNX software (Micromass) and the cytokinin concentrations were calculated according to the principle of isotope dilution.

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