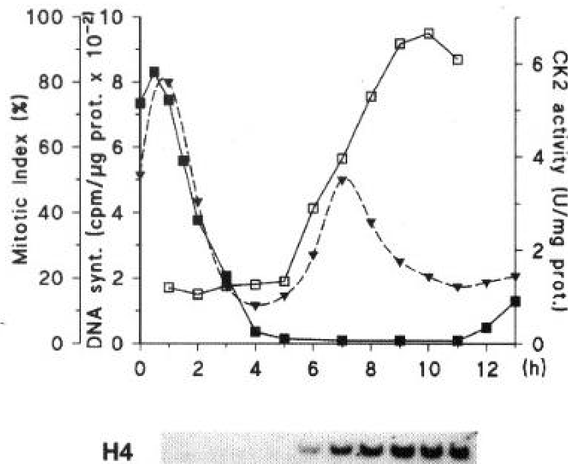


Figure 3. Time course of CK2 activity in synchronously dividing BY-2 cells after release from propyzamide block.
Cells were synchronised by sequential treatment with aphidicolin (3 µg ml^{-1}) and propyzamide (3.6 µM) as described in Experimental procedures. After release from propyzamide, cells were grown synchronously for one complete cycle and analysed for ^{3}H-thymidine incorporation (□), mitotic index (■), and CK2 activity (▼, dashed line). The transcript levels of histone H4 were also determined. CK2 activity was monitored by measuring ^{32}P incorporation into the peptide RRRDDDTDDD using [γ-^{32}P]-ATP as phosphate donor.

the specificity of CK2 activity measurements using β-casein and GTP and to discard putative interferences with other kinases, we compared the data using the above substrate with those using the specific peptide RRRDDDTDDD, that contains the consensus phosphorylation sequence for CK2. Similar results were obtained, and only the data for the specific peptide are shown here. The results presented in Figure 3 confirm those obtained with aphidicolin treatment alone. The oscillatory behaviour of CK2 activity during the cell division cycle gave rise to two sharp peaks of CK2 activity, one concomitant with the peak of mitotic index and the other with histone H4 induction (G1/S).

CK2 regulation during the tobacco BY-2 growth cycle

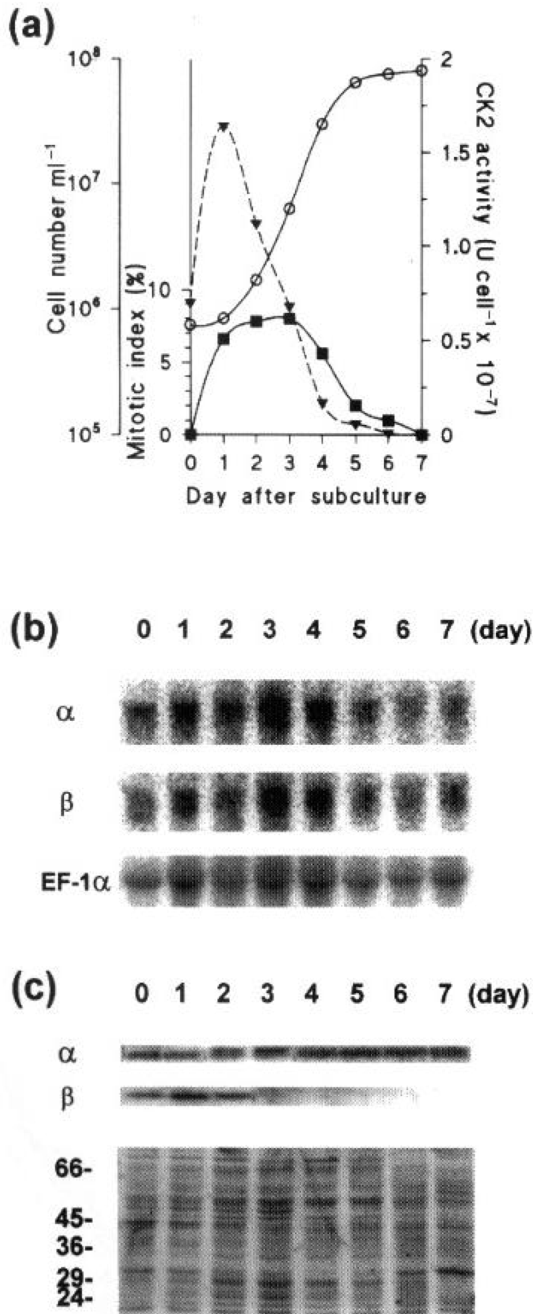
Samples from asynchronously growing BY2 cells were collected every 24 h after subculturing from the stationary phase and CK2 activity and α and β mRNA and protein levels were measured. In this cell line, the cellular proliferation starts very quickly after subculturing, attaining mitotic indexes of 5–8% at days 1–4 after transfer (Figure 4a). Figure 4(a) also shows a rapid rise in CK2 activity (four- to fivefold) during the first 24 h after subculturing, slowly declining afterwards. Similar results were obtained using either β-casein or the CK2 specific peptide as substrate, and, therefore, only the data for the specific peptide are shown. The apparent discrepancy between the high value of CK2 activity at time zero and those at the resting state (day 5 and ahead), suggests a very rapid activation of CK2 following activation of cell division, probably in minutes, that requires further investigation. This time course of CK2 activity does not reflect exactly the transcript levels of CK2α and CK2β genes that increase up to day 3 before decreasing at the end of the proliferation period (Figure 4b). The Western blot analysis revealed that α polypeptide is detected all over the growth curve, including stationary cells (Figure 4c); the presence of two different bands with a slightly different electrophoretic mobility might be due either to a post-translational modification (that we have not been able to identify yet), or to a differential expression of the two different α-type subunits, since our antibodies are not able to discriminate between the α and α′ polypeptides. In contrast, β polypeptide is only detectable during the first 2 days after subculturing (Figure 4c), in spite of the presence of high transcript levels throughout the exponential phase. Moreover, the increase of CK2 activity following cell subculturing correlates well with an increase in the CK2β subunit, suggesting that, as in its animal counterparts, β subunit might represent a major determinant physiological regulator of plant CK2's action. However, other factors besides the presence of the β subunit might arise because CK2 activity still has an appreciable value at day 3, even though the β subunit is undetectable. Indeed, the α subunit has a catalytic activity *in vitro* itself which could explain this finding and, therefore, a post-translational mechanism should be responsible for α subunit inactivation after day 4.

The effect of the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole on cell cycle progression

Halogenated benzimidazoles and benzotriazoles are selective inhibitors of protein kinases CK1 and CK2 (Shugar, 1994). They can traverse cell membranes and, consequently, be used to investigate the *in vivo* inhibition of their target kinases. The 5,6-dichlorobenzimidazole riboside (DRB) is the most frequently used for studies of CK2 inhibition but, recently, a variety of new DRB analogues have been developed with improved discriminating properties between CK1 and CK2. Among them, the 4,5,6,7 tetrabromobenzotriazole shows a Ki of 0.5 µM for yeast CK2 and about 100 µM for yeast CK1, and no activity against other kinases such as protein kinase C and protein kinase A. Hence, the above compound can be considered as a highly specific inhibitor of CK2 activity.

We determined the kinetics of *in vitro* inhibition of *Arabidopsis* CK2 catalytic subunit by the 4,5,6,7 tetrabromobenzotriazole (Figure 5), using crude extracts of a transformed *E. coli* strain expressing the α subunit of *Arabidopsis* CK2. Although most of the recombinant

protein accumulation was found in the insoluble fraction, low levels of CK2 activity could be detected in the supernatant and used for these studies. We accomplished 99% inhibition of the soluble CK2 activity and calculated a Ki value of 0.5 μM, identical to that reported for yeast. Hence, we decided to use this compound for the selective *in vivo* inhibition of CK2 throughout the cell division cycle.



A concentration of 35 μM tetrabromobenzotriazole was used for the *in vivo* studies to ensure complete inhibition of the intracellular CK2, a value that is far below the reported Ki value for CK1, which therefore would not be affected. The inhibitor was added at different phases of the cell cycle in three independent experiments, using cells synchronised either with aphidicolin alone or with aphidicolin plus propyzamide, in order to ensure that the effects seen with the inhibitor were not dependent on either the point of cell arrest or on the drug used, even though a control sample was always run in parallel in the same conditions. Identification of the different phases of the cell cycle was based on the determination of DNA synthesis and mitotic index which allowed us to position the peaks of S- and M-phase during one complete cycle. G1 and G2 phases were identified as the gaps between the end of S-phase and the beginning of M-phase, in the first case, and between the end of M-phase and the beginning of the second S-phase, in the second case. Visual screening of the cells, after staining with 4′, 6-diamino-2-phenylindole (DAPI), allowed us to determine when an M-phase was starting or had concluded in the majority of the cells by counting mitotic figures and formation of cell plate. Moreover, the histone H4 transcript level was used as a molecular marker to better delimit G1, G1/S and S-phases. The cells were also systematically stained with fluorescein diacetate (FDA) during the whole experiment to monitor cellular viability. The results obtained were similar using either single-or double-synchronisation protocol.

Regardless of the phase of the cell cycle where the inhibitor was added, CK2 activity was completely inhibited in cell extracts (results not shown). Moreover, in all cases, the inhibitor blocked cell cycle progression, although the cells exhibited different arrest phenotypes. When the inhibitor was added in early S (just after washing off the aphidicolin, hour 0 in Figure 2) cells could not accomplish DNA synthesis (thymidine incorporation was below detection) and they died within a maximum of 8 h showing a

Figure 4. Time course of CK2 activity and expression throughout the growth curve of BY-2 cells.
Stationary BY-2 cells were stimulated to re-enter the proliferative state by dilution into fresh medium (1 : 100) and samples were collected every 24 h. (a) Cell number (○), mitotic index (■) and CK2 activity (▼, dashed line). (b) Northern analysis showing mRNA levels for CK2α and β subunits (same probes and conditions as in Figure 2), and for the loading control (EF-1α). (c) Western blot analysis showing protein levels for CK2α and β subunits. Protein samples (15 μg) were subjected to 10% PAGE-SDS electrophoresis in duplicate and the membranes separately developed with the corresponding α or β antibodies in the same conditions as in Figure 1. A membrane stained with Coomassie-blue is also shown as a control of protein loading and transfer, and the position of the markers is indicated in kDa. CK2 activity was monitored by measuring ^{32}P incorporation into the peptide RRRDDDTDDD using [γ-^{32}P]-ATP as phosphate donor.

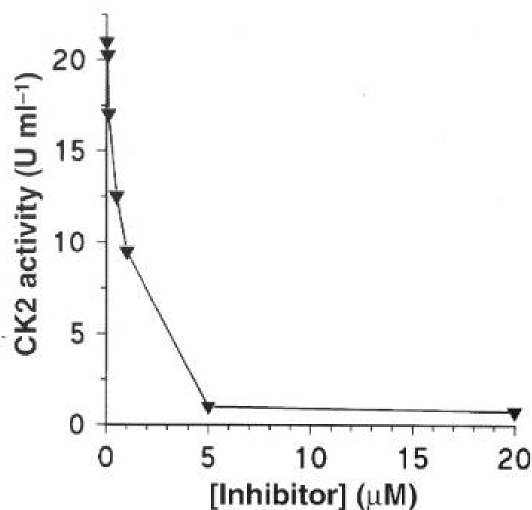


Figure 5. Inhibition of CK2 enzymatic activity by 4,5,6,7-tetrabromobenzotriazole.
Crude extracts of *E. coli* BL21 strain expressing the *Arabidopsis atcka2* gene were incubated with increasing concentrations of 4,5,6,7-tetrabromobenzotriazole and the remaining CK2 activity (▼) measured. CK2 activity was measured using β-casein as substrate and [γ-^{32}P]-GTP as phosphate donor.

morphology of S-phase arrested cells (Figure 6b). When added in G2-phase (hour 4 in Figure 2) no mitotic figures were detected during the few hours following the addition of inhibitor (Figure 6d), strongly suggesting that cells were not able to enter mitosis. Moreover, staining with FDA indicated a 100% lethality in a maximum of 2 h. In contrast, when the inhibitor was added in G1-phase (hour 10 in Figure 2), cells could initiate DNA synthesis on a normal timing and a similar slope as those of the control, for the following two hours. However, after hour 3, thymidine incorporation values dropped down below detection, indicating that the process of DNA replication was aborted (results not shown). Analysis of these cells under the microscope revealed a surprising nuclear morphology: the chromatin had condensed and looked like a prophase (Figure 6f,h,i), although with the particularity that the nuclear membrane did not break down. Furthermore, cells remained viable as revealed by FDA staining (results not shown), in contrast to what was observed by adding the inhibitor in S and G2. After 24 h, the cells were still alive and the nucleus morphology remained the same or became even more dramatic. This result reveals that the addition of the inhibitor in G1 and the concomitant prevention of the following CK2 activity peak at G1/S, leads to a premature condensation of chromatine in the still replicating nucleus, strongly suggesting a defect in the G2/M checkpoint that normally prevents the entry into prophase until DNA replication is completed.

Determination of the free polyamines content in BY-2 cells

Polyamines, particularly spermine and spermidine, are allosteric activators of CK2 versus a number of substrates (Valero *et al.*, 1995) and might have a central role in targeting the enzyme to the nucleus (Shore *et al.*, 1997). On the other hand, the involvement of polyamines in the regulation of growth and development in microorganisms, animals and plants is widely accepted (Serafini-Fracassini, 1991). In order to investigate the polyamine's content throughout the cell cycle of BY-2 cells, we determined free spermine, spermidine and putrescine concentrations at different times in synchronised cells and during the growth curve. In cells synchronised with aphidicolin (Figure 7a), the content of free polyamines was already high when released from the block and stayed at a high level throughout the S phase. A drop of free polyamines occurred afterwards, with lower levels at G2, followed by an increase at M-phase, declining slowly in late M and starting to rise again in G1/S. These results were confirmed in cells synchronised with aphidicolin plus propyzamide (Figure 7b), showing peaks of free putrescine and spermidine roughly at M-and S-phases. The amounts of spermine in this experiment were below the level of detection.

Determination of free polyamines during the growth cycle of BY-2 cells showed an increase in putrescine, spermine and spermidine content after transferring the cells to a fresh medium. Days 1 and 2 showed the maximal values of free spermidine and spermine, whose concentrations increased an average of 2.0- and 1.4-fold, respectively (Figure 7c), although this is probably an underestimation of the real figures considering the high values obtained at time zero in comparison to the resting state (day 5 and ahead). This suggests, as in the case of CK2 activity, a very rapid activation of the synthesis of free polyamines following activation of cell division, interestingly paralleled by the appearance of β subunit. An increase in putrescine levels occurred slightly earlier, reaching its maximum concentration at day 1 after subculturing.

Discussion

There is much evidence that the protein kinase CK2 is one of the components of the protein kinase network controlling cell division in animal cells (Litchfield and Luscher, 1993). However, the involvement of CK2 in cell division in plants has not received much attention to date. The aim of our work was first to determine whether we could detect cyclic variations of CK2 activity throughout the cell cycle in plant cells and, hence, estimate which steps, if any, required the presence of a functional CK2. Furthermore, we were interested in studying the regulation of the

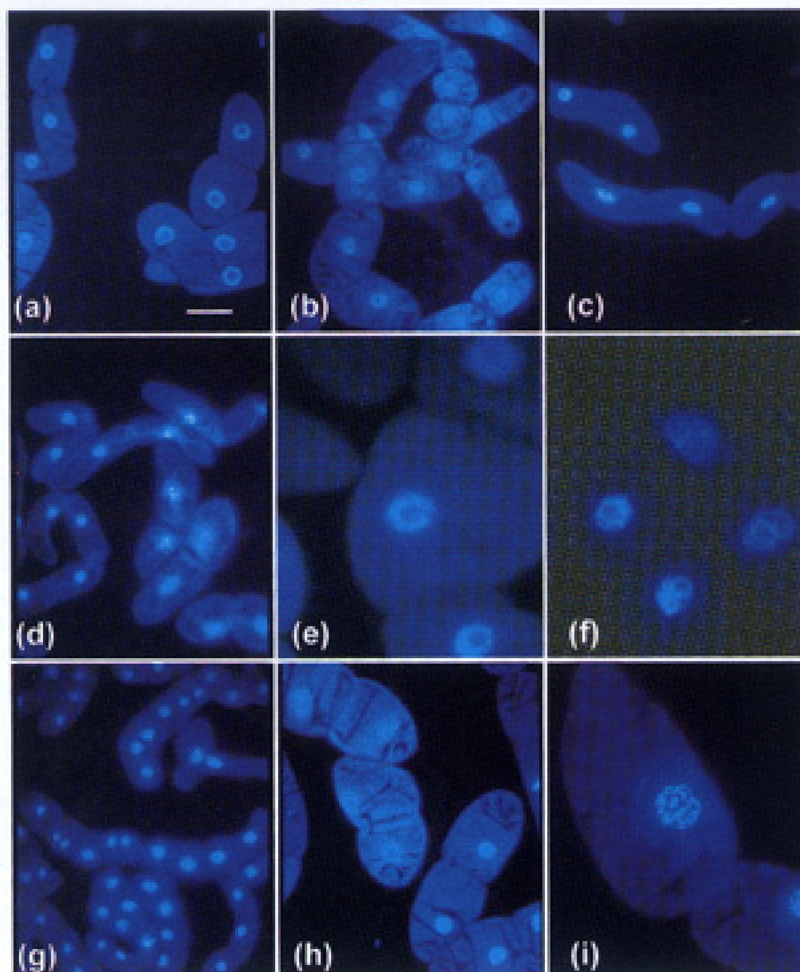


Figure 6. Effect of 4,5,6,7-tetrabromobenzotriazole on BY-2 cells at different points of the cell cycle.
BY-2 cells were synchronised with either aphidicolin alone or combined with propyzamide, as described in Experimental procedures. After removing the drug, the 4,5,6,7-tetrabromobenzotriazole (35 μM) was added at different points of the cell cycle that were positioned relative to the S- and M-phase peaks by using DNA synthesis measurements, mitotic index and pattern of histone H4 expression. Similar results were obtained with either one of the two synchronisation protocols and, therefore, only those from the aphidicolin treatment alone are shown for simplification. The inhibitor was added in S-phase (0 h), G2 (4 h) and G1 (10 h), after release from the aphidicolin block (see Figure 2). Pictures show DAPI-stained cells after 2–3 h in the presence of the inhibitor added in S-phase (b), G2 (d) and G1 (f), respectively. Untreated cells at the same times as above are shown in (a) (S-phase), (c) (G2-phase), and (e) (G1-phase). (h) and (i) present different magnifications of cells after 24 h in the presence of inhibitor added in G1, and (g) the corresponding untreated cells grown for 24 h after G1. The white bar corresponds to 20 μm in (e,f,i), 50 μm in (a,b,h), and 200 μm in (c,d,g).

enzyme during this process and therefore we determined mRNA and protein levels of the two types of subunits that build up its structure, α and β. Although our previous studies to characterise CK2 in plants had been made in *Arabidopsis* (Espunya and Martínez, 1997), the lack of a suitable synchronizable *Arabidopsis* cell line led us to switch to the tobacco BY-2 cells that are highly synchronizable.

Our data demonstrate that in actively dividing synchronised cells, CK2 activity oscillates cyclically, peaking at G1/S or early S and at M-phase, although gene expression of both α and β subunits is constitutive with undetectable variations in mRNA and protein levels. Therefore, a post-translational regulation should be responsible for the cyclic switching the CK2 activity on and off. *In vivo* inhibition of CK2 activity with the specific inhibitor 4,5,6,7-tetrabromobenzotriazole corroborates the requirement of CK2 to progress through the cell cycle. The effects observed by delivering the inhibitor in synchronised cells indicate that functional CK2 is necessary at various points during the cell cycle that correlate well with the peaks of activity detected. Especially interesting is the effect observed by adding the inhibitor in G1 that inhibits the CK2 activity peak in G1/S, and affects the cells later in the cell cycle (for their cell arrest morphology does not correspond to G1 or S but to prophase-like). This suggests that a signal which is normally generated at G1/S, i.e. the phosphorylation of some regulatory protein, initiates a signalling cascade finally leading to the activation of targets necessary for the G2/M checkpoint. This event appears to occur during a very narrow window in the cell cycle because inhibition of CK2 slightly later (early S) does not affect the G2/M checkpoint, neither does its inhibition in G2. One of the suggested roles for CK2 at G1/S might be as a part of a signalling system that regulates by phosphorylation the nuclear import of proteins. CK2 phosphorylation sites are found contiguous to nuclear localisation sequences in a variety of proteins and there are evidences of intracellular redistribution of some of them following phosphorylation by CK2 (Kakinoki *et al.*,

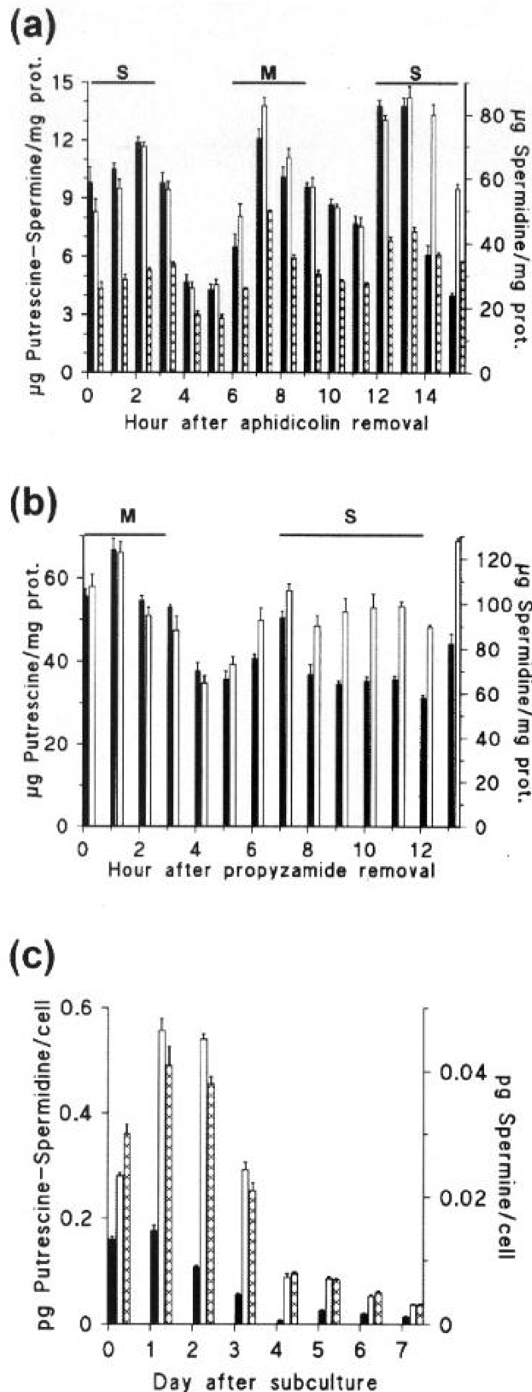


Figure 7. Time courses of free polyamines content in BY-2 cells.
Free putrescine (black bars), spermidine (open bars) and spermine (crossed bars) were determined at 1 h time intervals in synchronised cells after release from the aphidicolin (a) or propyzamide block (b), and at 24 h time intervals throughout the growth curve (c). Synchronisation experiments and the growth curve were performed in the same conditions as those of Figures 2, 3 and 4, respectively. Values shown are the means of two independent experiments. Error bars indicate standard deviations.

1997; Litchfield and Luscher, 1993; Meisner and Czech, 1991). Two proteins with a crucial function in cell cycle control, $p34^{cdc2}$ and p53, are phosphorylated by CK2 specifically at G1/S in animal cells (Koenig *et al.*, 1997; Russo *et al.*, 1992), although no data are available about whether it has an effect on their intracellular localisation. $p34^{cdc2}$ is a key element in the G2/M checkpoint, and p53 is required for two checkpoints in the cell cycle, the G1/S and the G2/M. Moreover, phosphorylation of p53 by CK2 might be required for its growth suppressor activity (Milne *et al.*, 1992), although this finding has been challenged (Fuchs *et al.*, 1995). It is difficult to extrapolate these results to plant cells because a plant p53 homologue has not been cloned to date and the $p34^{cdc2}$ homologues reported from higher plants do not contain the consensus sequence for CK2 phosphorylation. Other candidates in plant cells might be the mitotic cyclins CycB1 that, unlike their animal counterparts, present putative nuclear localisation signals in the N-terminal part of their polypeptide chains, with consensus phosphorylation sites for CK2 in the vicinity (Renaudin *et al.*, 1998).

Regarding M-phase, a promising candidate to be a target of CK2 is topoisomerase II, that is a major structural component of the metaphase chromosome scaffold and essential for chromatid segregation in anaphase. *S. cerevisiae* topoisomerase II is an excellent substrate for CK2 *in vitro* and is phosphorylated *in vivo* and hyperphosphorylated during mitosis, both in yeast and human cells (Ishida *et al.*, 1996), although again no data are available in plant systems.

A differential compartmentalisation of CK2 at various stages of the cell cycle, as has been reported in mammals (Pepperkok *et al.*, 1994), might account for its interaction with different substrates and hence for differential functions at particular points of the cycle.

Previous studies using genetic approaches or microinjection of specific antibodies have reported that protein kinase CK2 is required at specific points of the cell cycle in mammals and yeast. However, this is the first example in the literature of a detailed analysis of the evolution of CK2 activity during the cell cycle progression and of a correlation of these data with mRNA and protein levels. Moreover, this is the first study ever done in plant cells on CK2 and cell division cycle. In *S. cerevisiae*, temperature-sensitive mutants for CK2 have revealed that CK2 is required for cell progression during G1, at a point lying between that of the α-factor arrest (Start point) and the onset of S-phase (Hanna *et al.*, 1995). The authors indicate that their data do not allow them to discriminate between Start itself and the initiation of DNA synthesis (the G1/S transition) and that additional points earlier in G1 cannot be excluded. In mammalian cells, microinjection of antibodies against the β subunit of CK2 arrest the cells at G0/G1, early G1 and G1/S (Pepperkok *et al.*, 1994) and, also, an