Planta (1996) 200: 2–12

Planta
© Springer-Verlag 1996

Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34^{cdc2}-like H1 histone kinase

Kerong Zhang[1], David S. Letham[1,2], Peter C.L. John[1,2]

[1] Plant Cell Biology Group, Research School of Biological Sciences, The Australian National University, ACT 2601, Australia
[2] Cooperative Research Centre for Plant Science, GPO Box 475, Canberra, ACT 2601, Australia

Received: 27 October 1995/Accepted: 8 January 1996

Abstract. In excised pith parenchyma from *Nicotiana tabacum* L. cv. Wisconsin Havana 38, auxin (naphthalene-1-acetic acid) together with cytokinin (6-benzylaminopurine) induced a greater than 40-fold increase in a p34^{cdc2}-like protein, recoverable in the p13^{suc1}-binding fraction, that had high H1 histone kinase activity, but enzyme induced without cytokinin was inactive. In suspension-cultured *N. plumbaginifolia* Viv., cytokinin (kinetin) was stringently required only in late G2 phase of the cell division cycle (cdc) and cells lacking kinetin arrested in G2 phase with inactive p34^{cdc2}-like H1 histone kinase. Control of the Cdc2 kinase by inhibitory tyrosine phosphorylation was indicated by high phosphotyrosine in the inactive enzyme of arrested pith and suspension cells. Yeast cdc25 phosphatase, which is specific for removal of phosphate from tyrosine at the active site of p34^{cdc2} enzyme, was expressed in bacteria and caused extensive in-vitro activation of p13^{suc1}-purified enzyme from pith and suspension cells cultured without cytokinin. Cytokinin stimulated the removal of phosphate, activation of the enzyme and rapid synchronous entry into mitosis. Therefore, plants can control cell division by tyrosine phosphorylation of Cdc2 but differ from somatic animal cells in coupling this mitotic control to hormonal signals.

Key words: Cell division proteins (cdc2, cdc25, cdk) – *Nicotiana* (p34^{cdc2}-like protein) – Phosphotyrosine

Abbreviations: BAP = 6-benzylaminopurine; BrdUrd = 5-bromo-2-deoxyuridine; cdc = cell division cycle; Cdc25 = cdc phosphoprotein phosphatase; CKI = cyclin dependent kinase inhibitor; 2,4-D = 2,4-dichlorophenoxyacetic acid; DAPI = 4′,6′ diamidino-2-phenylindole; GST-cdc25 = glutathione sulfur transferase-truncated cdc25 fusion; MS = Murashige and Skoog (1962); NAA = naphthalene-1-acetic acid; p34^{cdc2} = 34-kDa product of the *cdc2* gene

Correspondence to: P.C.L. John; FAX: 61 (6) 249331;
Tel: 61 (6) 2494584; E-mail: john@rsbs-central.anu.edu.au

Introduction

Cell cycle control can be exercised by interaction of the key cell division cycle (cdc) catalyst p34^{cdc2} (the 34-kDa product of the *cdc2* gene) with different cyclin subunits that direct its protein kinase activity to specific substrates (Peeper et al. 1993), by cyclin dependent kinase inhibitor (CKI) proteins (Pines 1995) and by enzymes that control its enzyme activity through phosphorylation (Gould and Nurse 1987; Millar et al. 1991), so providing a likely universal mitotic control (Nurse 1990).

In plants we have noted changes in the level of p34^{cdc2}-like protein that are consistent with an hypothesis (John et al. 1990; Gorst et al. 1991) that depressed levels of this key catalyst act to enforce the cessation of cell division in large actively growing cells that are differentiating but might otherwise be competent to divide. This control may be supplemented by restricted synthesis of the cyclin subunits with which p34^{cdc2} forms complexes since cyclin mRNA is restricted to meristems (e.g. Fobert et al. 1994) and also, as we show here, potentially by inhibitory phosphorylation. However, a low level of p34^{cdc2} can provide more secure control than these alternatives since a missing cyclin can sometimes be substituted, as illustrated by the capacity of diverse cyclins to substitute for G1 cyclins in yeast (Sherr 1993) and by the function of the mitotic cyclin A as a G1 cyclin in human cells (Resnitzky et al. 1995). Furthermore, cyclin proteins are present in non-cycling cells since the kinase-cyclin motif is used to control processes separate from the cell cycle (Kaffman et al. 1994). Moreover, absence of the appropriate cyclin in the continued presence of p34^{cdc2} can disturb cycle progression instead of arresting it, since absence of functional cyclin B leads to repeated rounds of DNA replication (Hayles et al. 1994). Finally, the present paper indicates that tyrosine phosphorylation can inactivate plant p34^{cdc2}-like protein kinase; however, this mechanism may also be insecure as a long-term control because phosphatases that are not specific for cell cycle control can activate p34^{cdc2} and support proliferation (Gould et al. 1990). Therefore, we continue to interpret low levels of the key catalyst p34^{cdc2} as significant for ensuring full quiescence of division and, consistent with this, now report a more than

40-fold increase in the enzyme prior to resumption of division in tobacco pith.

The present study reveals separate contributions of auxin and cytokinin to accumulation and catalytic activation of $p34^{cdc2}$. Classic studies in Skoog's laboratory, making use of the quiescence and probable lack of endogenous cytokinin synthesis in tobacco stem pith, revealed a requirement for auxin together with cytokinin to stimulate resumption of division (Das et al. 1956). Similarly, in excised secondary phloem of carrot, exogenous cytokinin interacts synergistically with auxin and *myo*-inositol to induce cell division (Letham 1966). We now report that auxin stimulates accumulation of $p34^{cdc2}$-like protein in excised tobacco pith but that cytokinin is necessary for activation of its enzyme activity.

The molecular basis of $p34^{cdc2}$ activation in plants needs to be understood to account for the mechanism of cell cycle progression. Evidence from other eukaryotes indicates inactivation of $p34^{cdc2}$-like enzymes after activation at late G1 phase and mitotic initiation (reviewed by Nasmyth 1993; Nurse 1994). At the G1 control point, activation is induced in yeast by decline of CKI and availability of G1 cyclin subunits (Nasmyth 1993) and similar plant cyclins have been detected (Meskiene et al. 1995; Soni et al. 1995). Control is different at the mitotic control point, where mitotic cyclins are usually present in excess and activation of the $p34^{cdc2}$/cyclin B complex is controlled by removal of an inhibitory phosphate from tyrosine at the active site of $p34^{cdc2}$ by the specific phosphoprotein phosphatase Cdc25 (Gould and Nurse 1989). Phosphorylation of plant $p34^{cdc2}$ has been detected previously by radioactive labelling (John et al. 1989) and we now report that an inhibitory phosphorylation of tyrosine in the enzyme links division to hormone stimulation.

Materials and methods

Pith culture. Plants of *Nicotiana tabacum* L. cv. Wisconsin Havana 38 were grown with a 10-h 30°C day, alternating with 25°C night, under high mineral nutrition and light intensity, for about four months. Stem segments were surface-sterilised by immersion for 20 min in sodium hypochlorite (25 $g \cdot l^{-1}$ available Cl), rinsed twice in sterile water, and then all xylem and internal phloem tissue was excised and 10-mm-long cylindrical segments of central pith tissue were split longitudinally into four quadrant blocks and placed on solid MS (Murashige and Skoog 1962) media (Gorst et al. 1991): (1) "MS 0", free of both naphthalene-1-acetic acid (NAA) and 6-benzylaminopurine (BAP); (2) "NAA-only", containing 5.4 μM NAA; (3) "BAP-only", containing 0.56 μM BAP and (4) "complete MS", containing 5.4 μM NAA and 0.56 μM BAP. Pith was incubated at 25°C with 8 h light and 16 h darkness. Synthesis of DNA was detected by incorporation of 100 μM 5-bromo-2′-deoxyuridine (BrdUrd) into pieces (5 × 2 × 2 mm) of pith shaken in liquid MS for 15 h and examined for nuclear labelling as described by Gorst et al. (1991).

Suspension culture. Cells of *N. plumbaginifolia* Viv. were maintained in CSV medium containing 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.23 μM kinetin as described by Zhang et al. (1992). To investigate hormone dependence, cells were depleted in medium without added hormone by sedimenting at 2400·*g* for 2 min and rinsing twice by centrifugation, then resuspended to 1×10^6 $cells \cdot ml^{-1}$ and cultured for 4 d. The suspension was diluted to one-sixth in the same hormone-free medium, but without centrifugation. The arrested cells were then pretreated with hormone for 2 d then supplemented with any hormone they lacked to give complete medium, and sampled.

Nuclear DNA and cell number in suspension culture. The amount of nuclear DNA was determined by propidium iodide staining of fixed cells and measurement of UV-induced fluorescence, as described by Zhang et al. (1992). Cell numbers were estimated by counting, in a haemocytometer, cells fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), partially digested with 1% cellulysin cellulase from *Trichoderma viride* (Calbiochem, La Jolla, Cal., USA) and stained with 2 $\mu g \cdot ml^{-1}$ diamidino-2-phenylindole (DAPI).

Extraction and assay of protein. Cells and tissue were frozen and ground in liquid nitrogen and stored at −80°C. Suspension-culture samples were extracted by vigorously mixing 0.1 g of cell powder with 100 μl of RIPA buffer pH 7.4 as described by John et al. (1989). A soluble protein fraction was obtained as supernatant by centrifugation at 10 000·*g* for 5 min, in which protein was measured by Coomassie dye reagent (Bio-Rad, Richmond, Cal., USA).

Tobacco pith tissue contained less protein, therefore 0.6 g powder was extracted with 4 ml of modified RIPA buffer (Mes-RIPA) in which 25 mM 2-(N-morpholino)ethanesulfonic acid (Mes) was substituted for Tris-HCl. The extract was centrifuged at 10 000·*g* for 10 min and 4 ml of supernatant then mixed with 20 ml −20°C acetone to precipitate protein which was collected by centrifugation and redissolved in 0.3 ml Mes-RIPA buffer. For accurate protein assay of pith extracts, purification was necessary. Protein was purified by SDS-PAGE, transferred to 0.1-μm nitrocellulose (Schleicher & Schuell, Dassel, Germany) as in Western blotting, and then quantified by staining with Ponceau S. The assay was calibrated by electrophoresing and transferring in parallel a mixture of proteins in the size range 14–197 kDa. Calibrating proteins were disolved together, at 250 μg each per ml, in SDS sample buffer that contained the following proteins all from Sigma; transferrin (human), β-glucosidase (almond), phosphitin (egg vitellin), β-lactoglobulin (bovine milk), α-amylase (hog pancreas), ovalbumin (hen), bovine serum albumin, trypsin inhibitor (soybean), cytochrome *c* (horse heart). Typically, 7 calibrating lanes containing 15–144 μg protein were run and blotted with 13 unknowns. The nitrocellulose was stained for 30 min with 0.4% Ponceau S (Sigma) in 3% (w/v) trichloroacetic acid and destained in changes of 0.1% acetic acid until the background was white (about 10 min), then nitrocellulose from individual lanes was placed in vials containing 2 ml of 50 μM Tris pH 9.3 and shaken for 10 min to elute the stain for measurement by absorbance at 518 nm. Protein was deduced from the absorbance curve of calibrating proteins on the same piece of nitrocellulose.

Western blot measurement of $p34^{cdc2}$-like protein and phosphotyrosine. Quantitative Western blotting for $p34^{cdc2}$ was done with polyclonal rabbit antibody specific for the perfectly conserved cdc 2 sequence EGVPSTAIREISLLKE (PSTAIR; Nurse 1990) and ^{125}I-labelled second antibody, as characterised previously (John et al. 1989, 1990, 1991; Gorst et al. 1991). Amounts of $p34^{cdc2}$-like protein were compared in samples on the same nitrocellulose after exposure in a PhosphorImager. Phosphotyrosine was estimated similarly, substituting 1% bovine serum albumin (BSA) as blocking agent and detection by anti-phosphotyrosine mouse monoclonal IgG2a (PY-20) (Santa Cruz Biotechnology, S.C., USA), then rabbit anti mouse IgG antibody (Chemicon, Temecula, Cal., USA), and finally [^{125}I]-anti-rabbit IgG antibody produced in mouse (Amersham).

Purification and activity assay of $p34^{cdc2}$-like protein kinase. Affinity purification with the yeast mitotic protein $p13^{suc1}$ bound to agarose was used to derive $p34^{cdc2}$-like protein (Brizuela et al. 1987), essentially as described previously (John et al. 1991). Protein was

extracted in RIPA, or in NDE buffer [50 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 5 mM NaF, 2 mM dithiothrietol (DTT), 5 μM leupeptin, 5 μM pepstatin, 2 μg·ml^{-1} aprotinin, 0.5 mM sodium orthovanadate, 10 μM ammonium molybdate, 10 mg·ml^{-1} of sodium 4-nitrophenylphosphate with 0.5 mM phenylmethylsulfonylfluoride added immediately before use]. Samples containing 250 μg of soluble protein were mixed with 30 μl of 50% (v/v) agarose (Sepharose 4B; Pharmacia, Uppsala, Sweden) in NDE and rotated at 4°C for 1 h, then spun at 14 000·g for 5 min and the pellet discarded. The supernatant was transferred to a new tube and 30 μl of p13-beads containing 8 mg protein·ml^{-1} beads (John et al. 1991) added and rotated at 4°C for 3 h, spun at 5000·g and the supernatant discarded. The beads were washed twice for 2 min with detergent buffer (HDW; Hepler et al. 1994) and finally washed with 400 μl of HBK (25 mM Hepes, 1 mM EGTA, 5 mM $MgCl_2$, 160 mM KCl and 1 mM DTT). Proteins bound to p13^{suc1} were then eluted in 50 μl of p13^{suc1} in solution at 0.5 mg·ml^{-1} protein in HMD (Hepler et al. 1994).

Assay of p34^{cdc2}-like H1 histone kinase activity was done in 50 μl of pH 7.3 buffer which contained 25 mM β-glycerophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 25 mM Hepes, 5 μg of H1 histone (Boehringer, Mannheim, Germany), 2 μM ATP and 18.5 kBq [γ-^{32}P]ATP. The reaction at 30°C was started by addition of enzyme and stopped by transfer to ice or by addition of 10 μl 1% acetic acid. Reaction mixture (20 μl) was transferred to a 10 mm × 20 mm piece of P81 phosphocellulose, immediately immersed in 75 mM phosphoric acid, rinsed and radioactivity counted by scintillation. The remaining reaction solution was separated by SDS-PAGE in a 0.75 mm 12% gel, stained with Coomassie G-250, dried and scanned in a PhosphorImager.

Preparation of Cdc25. The plasmid pGEX25-BD was kindly provided by Dr. Paul Russell of The Scripps Research Institute, La Jolla, Cal., USA and used to express a fusion protein of glutathione sulfur transferase (GST)-truncated *Schizosaccharomyces pombe* cdc25 by the method of Millar et al. (1991). Purified GST-cdc25 at 1 mg·ml^{-1} was dialysed against 25 mM 3-(N-morpholino)propanesulfonic acid (Mops), 10 mM DTT (pH 7.2) and used to treat p34^{cdc2} purified on p13-beads, from pith and suspension culture.

Dephosphorylation of p34^{cdc2}-like protein kinase with GST-cdc25. p13-beads were loaded with p34^{cdc2}-like protein as for activity assay, washed three times with HDW buffer (Hepler et al. 1994) and then further washed three times with phosphatase buffer (PTB) containing 25 mM Mops (pH 7.2), 5 mM EDTA, 2 mM DTT and 2 mM spermidine, or when specified with HBK buffer. The p34^{cdc2}-like protein kinase on p13-beads was incubated with GST-cdc25 phosphatase in PTB buffer containing 0.1% BSA at 30°C. After incubation the beads were washed twice with HBK and assayed for p34^{cdc2} protein kinase activity at 30°C for 10 min. The kinase reaction was stopped with SDS-PAGE sample buffer containing SDS and histone separated by electrophoresis.

Results

Auxin induced p34^{cdc2}-like protein but activation required cytokinin. Excised tobacco stem pith was incubated on agar MS medium for 4 d to allow wound response to subside (Simard 1971) before division behaviour was observed. Auxin presence alone (NAA 5.4 μM) could stimulate cell enlargement but no cycle activity could be detected by BrdUrd incorporation into nuclear DNA. Active cell proliferation was observed in the combined presence of auxin with cytokinin (5.4 μM NAA with 0.56 μM BAP) and a 15-h pulse label with BrdUrd resulted in incorporation into essentially all nuclei. Neither cell enlargement nor division was observed on medium with only BAP or with no hormone. The level of p34^{cdc2}-like protein detected by PSTAIR antibody was low relative to other proteins in freshly excised (division-quiescent) stem pith from either upper or lower stem regions and increased only slightly during 14 d incubation on medium without hormone, presumably due to some wound response (Fig. 1). However, strong induction of p34^{cdc2} relative to other proteins, to more than 40 times the level when first excised, was observed in medium with auxin alone. Cytokinin by itself was not able to induce the protein above levels reached without hormone, nor could cytokinin increase the induction by auxin, although cytokinin was required for cell proliferation. The same response was observed whether pith was derived from the lower stem, where cells had been in prolonged quiescence, or from the upper stem where they had been more recently in division.

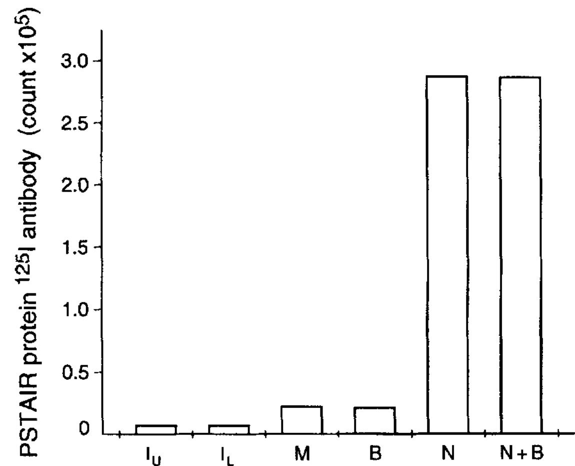


Fig. 1. Levels of p34^{cdc2}-like protein in stem pith of *Nicotiana tabacum.* Initial levels in the upper stem 10–15 cm below apex (l_U) and the lower stem 10–15 cm above base (l_L), at the time of excision from the intact plant, are compared with the level established after 14 d incubation on MS medium without added hormone (*M*) or on MS medium with 0.56 μM BAP (*B*), with 5.4 μM NAA (*N*), or with 5.4 μM NAA and 0.56 μM BAP (*N* + *B*). About 30 segments comprised each sample and for treatments M, B, N and N + B these were taken equally from upper and lower regions of stem. Equal loadings of 70 μg extracted total protein, after concentration by acetone precipitation, were separated on an SDS 10–15% linear gradient acrylamide gel. Proteins transferred onto a single piece of nitrocellulose were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by [^{125}I]anti-rabbit IgG. The image obtained by exposure in a PhosphorImager was analysed to quantify the levels of p34^{cdc2}-like protein, which are shown above

To investigate whether induced p34^{cdc2}-like protein was enzymically active it was affinity-purified using the yeast mitotic protein p13^{suc1} covalently coupled to agarose before assay (Brizuella et al. 1987). Our earlier investigation (John et al. 1991) showed that other enzymes can contribute activity under cdc2 enzyme assay conditions unless the enzyme is purified in this way. A linear recovery of H1 histone kinase activity from tissue treated with auxin plus cytokinin was obtained up to 0.2 g fresh weight, using 30 μl of beads containing 8 mg p13^{suc1}·ml^{-1} beads (Fig. 2). To compare amounts of enzyme, 0.1-g samples of ground tissue (250 μg protein from proliferating

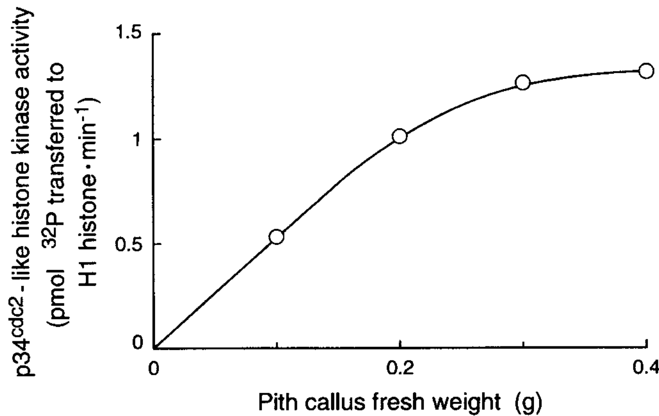


Fig. 2. Relation between the activity of $p34^{cdc2}$-like protein recovered on $p13^{suc1}$-beads and the fresh weight of *N. tabacum* pith callus taken for extraction. The $p34^{cdc2}$-like protein kinase in 0.1 g, 0.2 g, 0.3 g and 0.4 g of pith callus cultured with both 5.4 μM of NAA and 0.56 μM of BAP was purified by binding to 30 μl of $p13^{suc1}$-beads and was eluted with 50 μl of 0.5 $mg \cdot ml^{-1}$ of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was assayed using histone H1 as substrate and quantified by counting 20 μl of reaction mixture on P81 paper. Amounts of extract allowing linear recovery of $p34^{cdc2}$ and not exceeding 250 μg protein were employed in comparisons of activity present in different tissue samples, as in Fig. 3

cells) were employed with 30 μl of beads. Only the combined presence of 5.4 μM NAA with 0.56 μM BAP resulted in $p34^{cdc2}$ activity above the basal level with no hormone (Fig. 3a, b). The protein induced by NAA alone (Fig. 1) was not catalytically active (Fig. 3). The activity induced by auxin with cytokinin was close to 4 pmol ^{32}P transferred to histone $H1 \cdot g$ fresh weight pith callus extracted$^{-1} \cdot min^{-1}$ (1.5 pmol ^{32}P transferred to histone $H1 \cdot mg^{-1}$ total soluble pith protein$\cdot min^{-1}$). Individual plants of tobacco Wisconsin 38 were consistent in responsiveness of their pith (Fig. 3c, d) and the specific requirement for cytokinin with auxin was maintained over a 20-d period (Fig. 3e, f).

To investigate more precisely where in the cell cycle requirement for cytokinin is most stringent we used suspension-cultured cells of *N. plumbaginifolia* for easier determination of cycle phase and cell number and we also investigated the biochemical state of $p34^{cdc2}$-like protein.

Cell proliferation in suspension culture. A long-established suspension culture of *N. plumbaginifolia* (Zhang et al.

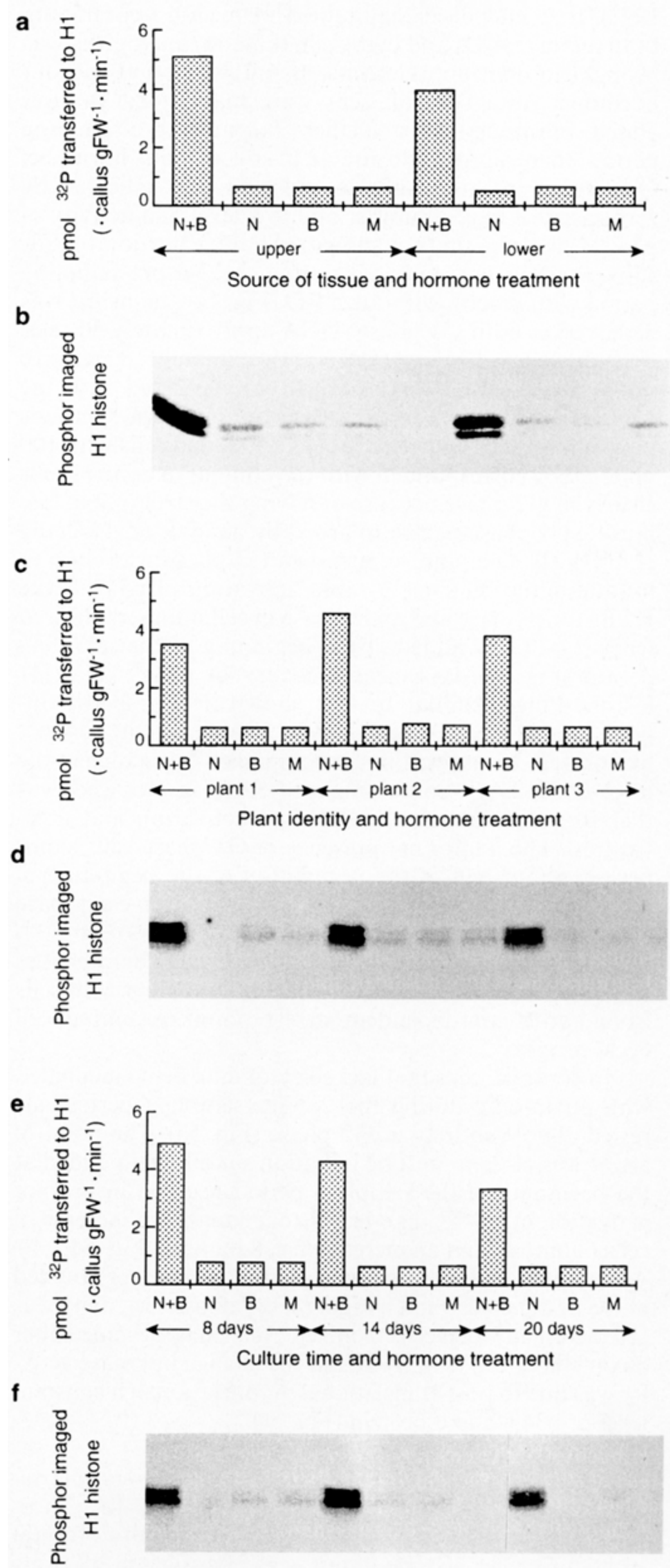


Fig. 3a–f. Activity of $p34^{cdc2}$-like protein kinase induced by auxin and cytokinin in pith taken from different regions of stem (**a, b**), from different individual *N. tabacum* plants (**c, d**), and measured at different times of culture (**e, f**). In **a, b** pith was taken from 0–15 cm below the stem apex (*upper*) and from 0–15 cm above the stem base (*lower*). Throughout, tissue was cultured on MS medium with 5.4 μM NAA and 0.56 μM of BAP (*N + B*), with 5.4 μM NAA-only (*N*), with 0.56 μM BAP-only (*B*), or with medium without hormone (*M*). After 14 d incubation, as shown in **a–d**, the $p34^{cdc2}$-like protein kinase in 0.1 g fresh weight of pith was purified with 30 μl of $p13^{suc1}$ beads and eluted with 50 μl of 0.5 $mg \cdot ml^{-1}$ free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as substrate and ^{32}P transferred to histone was measured in 20 μl of reaction mixture on P81 paper by scintillation counting, as shown in **a**. The radioactive histone was also separated on a 12% gel by SDS-PAGE and exposed in a PhosphorImager as shown in **b**. In **c, d** pith was taken from 0–15 cm above the stem base of three separate plants. In **e, f** pith from a single plant was sampled after culture for 8, 14 and 20 d

1992), that had been maintained in medium containing both auxin (2,4-D) and cytokinin (kinetin) analogues, was brought to division quiescence by an 8-d period without hormone. As a control, cells were maintained without added hormone for a further 2 d in a pre-sampling period, then sampled during 42 h, still without hormone. Quiescence in this control population was indicated by zero increase in cell number and low level and activity of $p34^{cdc2}$-like protein (not shown). Another portion of 8-d-starved cells was incubated during the 2-d pre-sampling period with kinetin without 2,4-D (Fig. 4a) and arrest was observed in both G1 phase (DNA approximately 50 relative units) and G2 phase (DNA approximately 100 relative units). Cells with 50 units DNA corresponded with the content of cells held in G1 phase by the DNA-synthesis inhibitor aphidicolin (Zhang et al. 1992) and cells with 100 units DNA corresponded with the content of control cells clearly in G2 phase because of having the structure of late mitotic prophase or due to arrest by okadaic acid (Zhang et al. 1992). The point of arrest in G2 phase was close to mitotic initiation since a rapid activation of $p34^{cdc2}$-like H1 histone kinase and an increase in cell number followed when 2,4-D was added at the beginning of the sampling period (Fig. 4b, d). Linear recovery of $p34^{cdc2}$-like H1 histone kinase similar to that shown in Fig. 2 was obtained on p13-beads (not shown). The change in $p34^{cdc2}$-like protein kinase was not accompanied by change in the level of PSTAIR-containing protein (Fig. 4c) and was therefore due to post-translational activation and inactivation. The additional presence of G1 phase cells, comprising about half of the population at the beginning of the sampling period, was reflected in a later wave of kinase activation and cell division, which followed at 24–30 h (Fig. 4d, b). The two periods of division together resulted in a doubling of cell number, which is consistent with cells from two auxin-dependent arrest points resuming cell cycle progress.

In contrast, cells that lacked cytokinin, being incubated with auxin-only during the 2-d pre-sampling period, arrested almost entirely in G2 phase (Fig. 5a). The point of arrest was close to mitotic initiation since kinetin added at the beginning of the sampling period resulted in prompt activation of $p34^{cdc2}$-like H1 histone kinase, an increase of cells in mitosis and an increase in cell number (Fig. 5d, e, b). All cells initiated mitotic activity since cell number doubled at the first increase and a higher level of kinase activity was attained than when cells of mixed cycle phase resumed after auxin starvation (Fig. 4). Again the change in kinase activity was due to post-translational modification of a constant level of $p34^{cdc2}$-like protein (Fig. 5c).

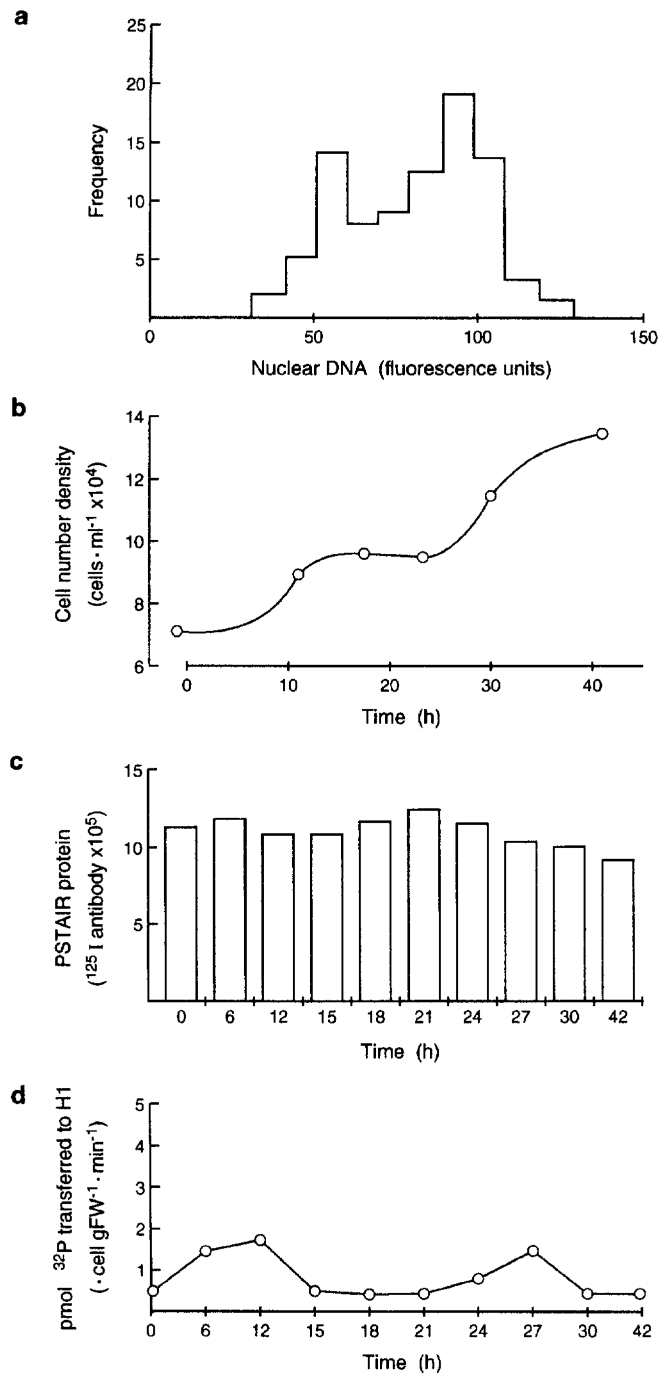
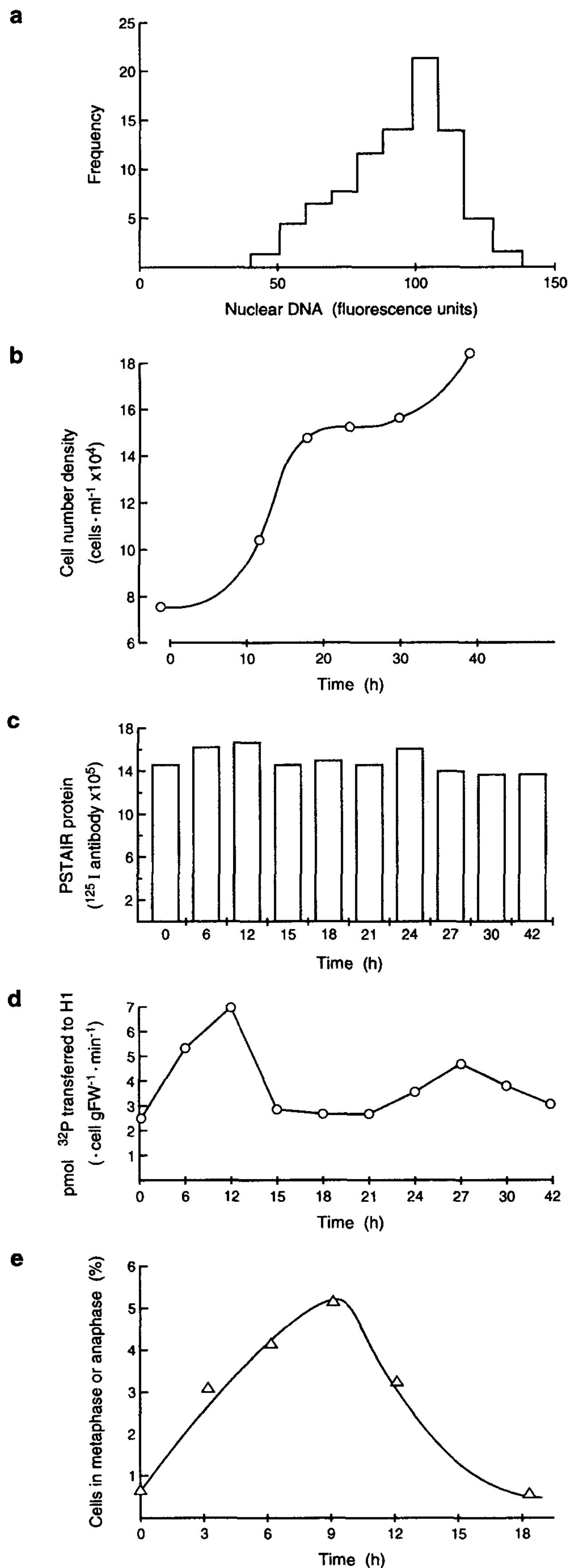


Fig. 4a–d. Effect of 2 d treatment of suspension-cultured *N. plumbaginifolia* cells with 0.23 μM kinetin-only, then supplementation with 9 μM 2,4-D at the beginning of the sampling period, on nuclear DNA content at 0 h (**a**), cell number density (**b**), content of $p34^{cdc2}$-like protein (**c**), and $p34^{cdc2}$-like H1 kinase activity (**d**). Cells were derived from the same population previously given 8 d hormone depletion and used for the culture described in Fig. 5. After 2 d with kinetin-only the cells were supplemented with 2,4-D and sampled. Estimation of $p34^{cdc2}$-like protein (PSTAIR protein) in 70 μg total protein was performed as in Fig. 1 and measurement of the activity of $p34^{cdc2}$-like protein kinase as in Fig. 3

Tyrosine dephosphorylation of $p34^{cdc2}$-like protein controlled by cytokinin. The possible influence of the phosphorylation of tyrosine residues on the activity of plant $p34^{cdc2}$-like H1 histone kinase was investigated by using the phosphoprotein phosphatase Cdc25, which can activate the late-G2 form of $p34^{cdc2}$ complexed with cyclin B (Nurse 1990; Millar et al. 1991), and by direct measurement of phosphotyrosine present in $p13^{suc1}$-purified $p34^{cdc2}$.

Schizosaccharomyces pombe Cdc25 phosphatase was purified as a GST-cdc25 catalytic domain fusion protein from an overexpressing strain of *Escherichia coli* (Fig. 6a). This enzyme has been shown to specifically remove phosphate from tyrosine in $p34^{cdc2}$ in vitro (Millar et al. 1991).



When this enzyme was used to treat the p34^{cdc2}-like protein kinase purified on p13-beads, from suspension-cultured cells that had been arrested by cytokinin deprivation and were in the process of activating the kinase following an addition of kinetin, the kinase activity increased in vitro by about 40% due to Cdc25 action (Fig. 6b, c). This activation was suppressed markedly by vanadate, a selective inhibitor of phosphoprotein phosphatase (Fig. 6d). Similarly, the activity of p34^{cdc2}-like kinase from pith callus cultured on MS medium containing NAA-only, was increased by 50% after treatment with Cdc25 enzyme. Our preparation of Cdc25 was able to activate yeast p34^{cdc2} from the mutant *cdc25-22* (Gould and Nurse 1989) in which temperature lability of the Cdc25 enzyme resulted in accumulation of phosphate on tyrosine (Fig. 7). However, kinase from pith cultured on MS medium with both NAA and BAP was only moderately activated by Cdc25, indicating that most p34^{cdc2} was not tyrosine-phosphorylated because most cells were cycling and not in late G2 when sampled, consistent with the detection of cell proliferation and active p34^{cdc2}-like protein kinase (Fig. 3).

These conclusions were confirmed by direct quantification of the level of phosphotyrosine in p34^{cdc2}-like protein, using phosphotyrosine-specific antibody (Fig. 8). The level was considerably higher on NAA than on NAA plus BAP. The reliability of the above results was established in two ways. First, tyrosine-phosphorylated p34^{cdc2}, from the *S. pombe* mutant *cdc25-22* transferred to 36°C, was clearly detected and comparable levels of phosphotyrosine were seen in plant p34 from the same amount of callus protein after culture on NAA-only (Fig. 8). Second, authentic phosphotyrosine was found to compete strongly with tyrosine-phosphorylated yeast and plant p34 for binding to the antibody (Fig. 9).

Cytokinin-stimulated activation of p34^{cdc2}-like kinase in G2-phase suspension-cultured cells (Fig. 5) correlated with lowered levels of phosphotyrosine after activation (Fig. 9). Reaction with Cdc25 confirmed that activation involved dephosphorylation of tyrosine. For this test, *N. plumbaginifolia* cells were arrested at the cytokinin control point by 8 d in hormone-free medium and 2 d in 2,4-D-only medium, then kinetin was supplied. When cells were sampled at 3 h, in early prophase (Fig. 5e), they yielded p34^{cdc2}-like kinase which was activated by treatment with Cdc25 enzyme. This activation was specific for the G2

Fig. 5a–e. Effect of 2 d treatment of suspension-cultured *N. plumbaginifolia* cells with 9 μM 2,4-D, then supplementation with 0.23 μM kinetin at the beginning of the sampling period, on nuclear DNA content at 0 h (**a**), cell number density (**b**), content of p34^{cdc2}-like protein (**c**), p34^{cdc2}-like H1 kinase activity (**d**), and percentage of cells that were in metaphase or anaphase during the first 18 h of the sampling period (plotted on extended abscissa scale) (**e**). Cells were derived from the same population previously given 8 d hormone depletion and used for the culture described in Fig. 4. After 2 d with 2,4-D-only the cells were supplemented with kinetin and sampled. Estimation of p34^{cdc2}-like protein (PSTAIR protein) in 70 μg total protein was performed as in Fig. 1, measurement of the activity of p34^{cdc2}-like protein kinase as in Fig. 3 and for detection of metaphase and anaphase nuclei cells were stained with DAPI

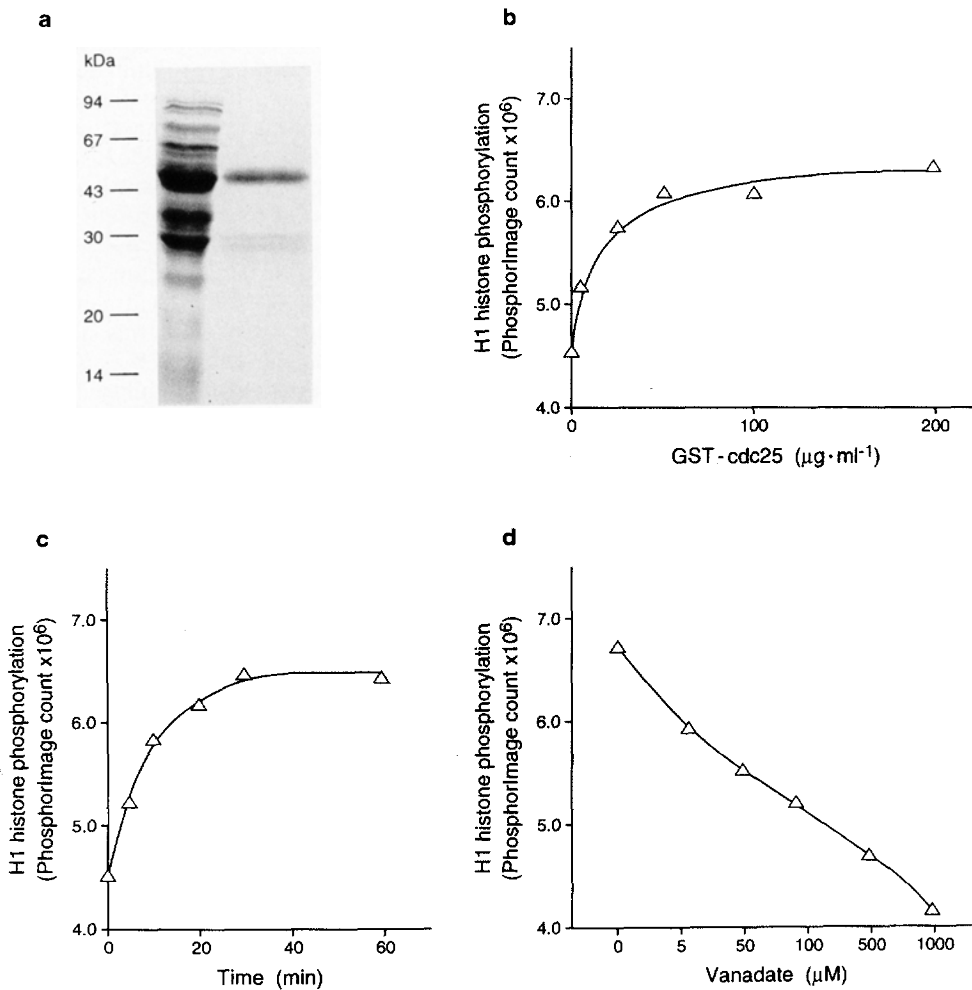


Fig. 6a–d. Purification of GST-cdc25 fusion protein and its effect on activity of p34^{cdc2}-like protein kinase purified from suspension-cultured *N. plumbaginifolia* cells that had been arrested by 2 d cytokinin deprivation and given kinetin 3 h previously (equivalent to cells at 3 h in Fig. 5). **a** GST-cdc25 purified by glutathione affinity chromatography (*right*) from whole extract of *E. coli* cells overexpressing the protein (*left*), both seen after SDS PAGE on 12% gel and staining with Coomassie G-250. **b** Activity of p34^{cdc2}-like kinase after treatment with concentrations of GST-cdc25. **c** Time course of activation by 100 µg·ml^{-1} GST-cdc25. **d** Inhibition by vanadate of the activation of p34^{cdc2}-like kinase by GST-cdc25. In **b, c,** and **d** p34^{cdc2}-like kinase was purified on 60 µl p13^{suc1} beads from 500 µg of total extracted protein for each determination and treated with GST-cdc25 directly on the beads, then activity was measured using H1 histone as substrate

form of p34^{cdc2} since no activation was detectable with enzyme from post-mitotic cells taken at 18 h (Fig. 10a). More precisely, in-vitro activation was specific for the early-prophase form of p34^{cdc2} awaiting mitotic activation and was not seen with enzyme from a cell population in which most cells were completing mitosis since Fig. 10b shows that at 3 h, when cells were mostly in prophase and only 2% were detected in metaphase/anaphase (Fig. 5e), a 70% activation of the kinase was obtained. But by 12 h, when the peak of metaphase anaphase abundance was passing (Fig. 5e) and kinase activity was retained only in late dividing cells that had already activated p34^{cdc2}, a negligible in-vitro activation by Cdc25 occurred.

Discussion

p34^{cdc2}-like kinase and organ development. From evidence in diverse tissues including wheat leaf (John et al. 1990), carrot cotyledon (Gorst et al. 1991) and pea root, it has been proposed that high levels of p34^{cdc2}-like protein are restricted to tissues in which division is developmentally appropriate (John et al. 1990, 1993a) and that the switch from division to differentiation is enforced by a decline in the level of p34^{cdc2}. The distribution of *cdc2* mRNA and *cdc2* promoter activity is generally consistent with this since high levels have been found restricted to the meristem regions of maize leaf (Colasanti et al. 1991), and to root and shoot meristems of *Arabidopsis* (Martinez et al. 1992; Hemerly et al. 1993), soybean (Miao et al. 1993) and pea (John et al. 1993). The biological significance of a low p34^{cdc2} level is demonstrated by the restoration of the ratio of p34^{cdc2} relative to other proteins to meristem levels when cells are stimulated to resume division (Gorst et al. 1991; John et al. 1993), and is underlined here by the more than 40-fold increase prior to resumption of cell division in tobacco pith (Fig. 1).

A control of the activity of the p34^{cdc2}-like enzyme, not requiring a change in its level, can be detected in the mitotic peak of activity seen in synchronous cells with constant p34^{cdc2} (Figs. 4, 5) and in the more rapid decline in activity than in p34^{cdc2} protein at the margin of the meristem in seedling wheat leaves (John et al. 1993). Although lack of cyclins could control p34^{cdc2} activity in development (see *Introduction*), within the cell cycle mitotic cyclins are usually accumulated to excess before mitosis is initiated, and mitosis is controlled by tyrosine dephosphorylation of the p34^{cdc2} (Gould and Nurse 1989;

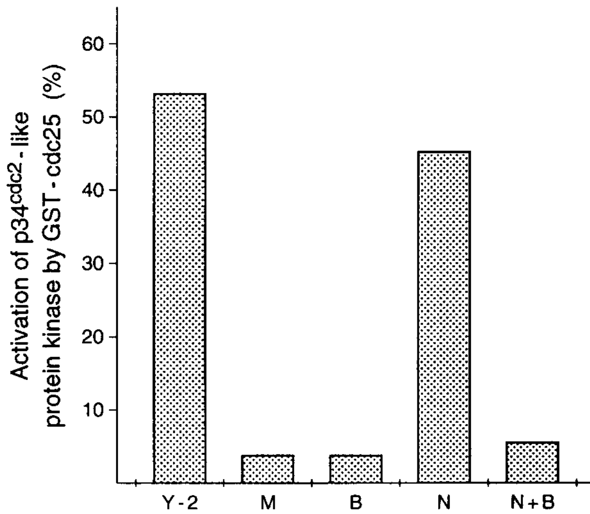


Fig. 7. Activation of $p34^{cdc2}$ kinase from *S. pombe cdc25-22* and from stem pith callus of *N. tabacum*, by GST-cdc25. Yeast cells (*Y-2*) were shifted from culture at 25°C to 36°C for 3 h to arrest them with tyrosine-phosphorylated $p34^{cdc2}$. Pith was cultured for 14 d on: MS medium without added hormone (*M*), or on MS medium with 0.56 μM BAP (*B*), with 5.4 μM NAA (*N*) or with 5.4 μM NAA and 0.56 μM BAP (*N* + *B*). For each determination, $p34^{cdc2}$ from 500 μg of total extracted protein of yeast or plant cells was purified by binding to 60 μl $p13^{suc1}$ beads and reacted with GST-cdc25 at 100 $\mu g \cdot ml^{-1}$ directly on the beads, then activity was measured using H1 histone as substrate

Nurse 1990; Millar et al. 1991). Our data indicate that plant cells share this mitotic control. They could also use it when terminating division during organogenesis or in response to environmental stress.

Requirement for auxin and cytokinin at specific cell-cycle transitions. Early attempts to analyse the roles of auxin and cytokinin in induction of cell division were made in Skoog's laboratory using freshly excised stem pith parenchyma (e.g. Das et al. 1956). By allowing the wound response-to subside prior to analysis Simard (1971) observed that auxin alone, but not cytokinin alone, could stimulate DNA synthesis. Similarly Meyer and Cooke (1979) found that DNA synthesis could be induced by auxin (2,4-D) in protoplasts released from tobacco mesophyll cells but that cytokinin (BAP) was required for completion of the cell cycle. However, reviewing the mass of evidence from stimulation of differentiated cells, both Fosket (1977) and Bayliss (1985) concluded it uncertain that cytokinin plays a particular role in cell cycle regulation. Cells in culture have provided clearer indications of a G2 role for cytokinin. Varieties of soybean have yielded callus in which mitosis was delayed without cytokinin and, although the cells did not arrest in G2 phase, Fosket (1977) concluded that a G2 function was established. However, others have found the role of cytokinin to be less certain (reviewed by Bayliss 1985), perhaps acting as a general modifier of cell physiology that allows division but does not directly stimulate it. Our results show that cytokinin acts specifically at late G2, stimulating $p34^{cdc2}$ activation leading to mitosis (Fig. 5).

The present studies are in accord with the principle control point hypothesis of Van't Hof (1974) but suggest that the hypothesis can now be refined by recognising that auxin is necessary for progress from late G1 phase to S phase and also from G2 phase to mitosis, while cytokinin is stringently required only at a control point in G2 phase (Fig. 5a) at which it stimulates $p34^{cdc2}$-like protein kinase activation (Fig. 5d) and progress through mitosis to cell doubling (Fig. 5e, b). The same arrest point in tobacco pith cells stimulated by auxin without cytokinin is indicated by their having replicated DNA (Simard 1971), and by the presence of a $p34^{cdc2}$-like protein that contains phosphotyrosine (Fig. 8) and can be activated by Cdc25 (Fig. 7). In whole plant tissues, therefore, arrest in G2 phase (Van't Hof 1974) may occur because of a low cytokinin level and in some tissues hormones other than cytokinin could activate the control mechanism.

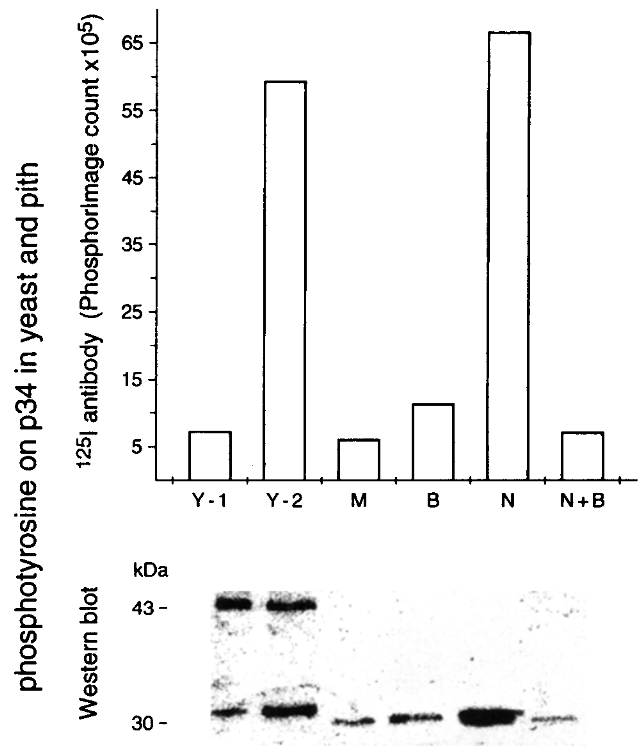


Fig. 8. Levels of phosphotyrosine in $p34^{cdc2}$ from the temperature sensitive mutant *S. pombe cdc25-22* cycling at 25°C (*Y-1*), and the same cells arrested in G2 phase by 3 h at 36°C (*Y-2*), as well as from stem pith of *N. tabacum* after 14 d incubation on MS medium without added hormones (*M*), and an MS medium with 0.56 μM BAP (*B*); with 5.4 μM NAA (*N*); or with 5.4 μM NAA and 0.56 μM BAP (*N* + *B*). For each determination, $p34^{cdc2}$ from 1 mg total extracted protein was purified on 80 μl $p13^{suc1}$ beads, washed with HDW buffer, then 60 μl of 2 × SDS sample buffer was added to the beads and boiled. 60 μl of sample was separated by SDS-PAGE in a 12% gel, transferred to nitrocellulose and probed with anti-phosphotyrosine and iodinated second antibody. The image obtained by exposure in a PhosphorImage was analysed to determine levels of p34 phosphotyrosine, shown above. The phosphorylated protein of 45 kDa detected in yeast is unknown but was unaffected by the inactivation of cdc25 enzyme

Tyrosine phosphorylation of $p34^{cdc2}$-like protein at the cytokinin control point. Plant $p34^{cdc2}$ from diverse taxa

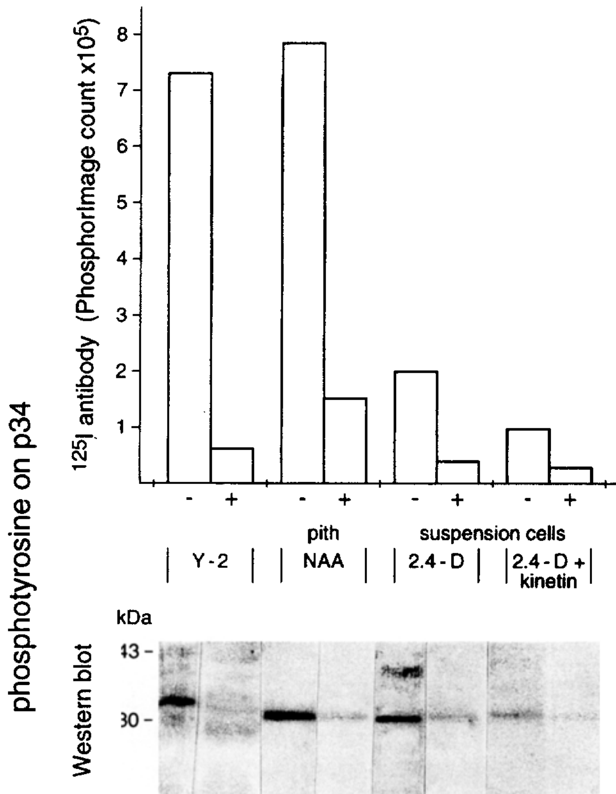


Fig. 9. Effect of pre-competition with phosphorylated tyrosine (+), or pre-incubation without amino acid (−), on binding of anti-phosphotyrosine antibody to $p34^{cdc2}$-like protein in the $p13^{suc1}$-affinity-purified fraction from *S. pombe cdc25-22* cells arrested in G2 phase at 36°C (*Y-2*), from pith callus of *N. tabacum* cultured on NAA-only (*NAA*), and from cells of *N. plumbaginifolia* arrested in 2.4-D-only medium (*2,4-D*), or cells in G2 phase resuming cycle progress after supply of kinetin following arrest in 2,4-D-only medium (*2,4-D + kinetin*). For each determination, $p34^{cdc2}$ from 500 µg total extracted protein was purified on 60 µl $p13^{suc1}$ beads, washed with HDW buffer, then 60 µl of 2 x SDS sample buffer was added to the beads and boiled. 60 µl of sample was separated by SDS-PAGE in a 12% gel, transferred to nitrocellulose and probed with antiphosphotyrosine then iodinated second antibody. For pre-competition, antibody was incubated (+) with, or (−) without, 1 mM phosphorylated tyrosine for 1 h before application to the transferred proteins. The image obtained by exposure in a PhosphorImager was analysed to determine levels of antibody bound to p34, shown above

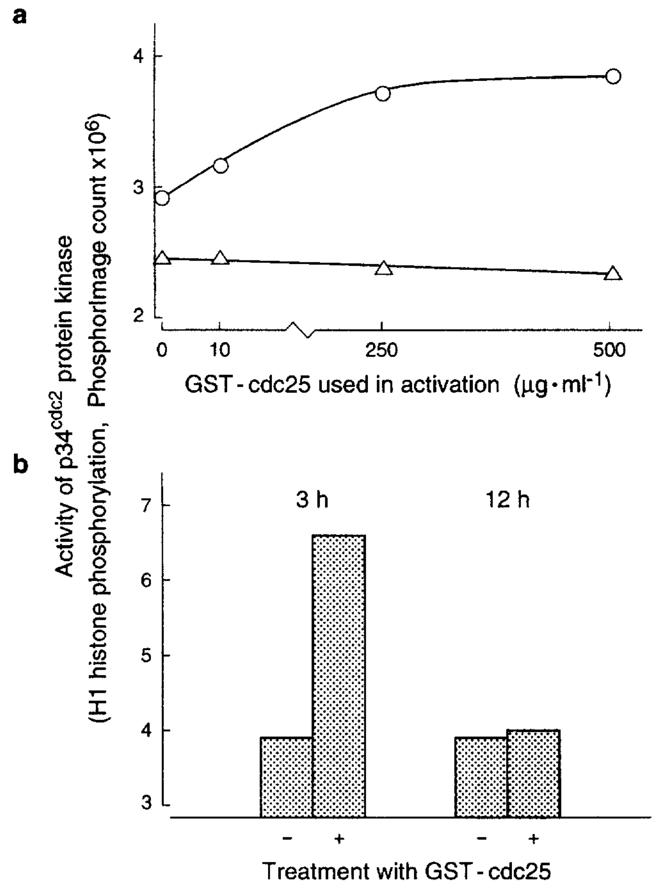


Fig. 10a, b. Activation of $p34^{cdc2}$-like kinase from *N. plumbaginifolia* by GST-cdc25. **a** Enzyme was taken from cells that had been arrested in medium with 2,4-D-only, then stimulated with kinetin and sampled at 3 h (○) when in prophase and at 18 h (△) when in mid interphase (equivalent to 3 h and 18 h in Fig. 5). **b** Enzyme was taken at 3 h and 12 h after kinetin release from a 2.4-D-only block, when cells were respectively in early prophase or had predominantly completed mitosis, then incubated with (+), or without (−), $100\ \mu g \cdot ml^{-1}$ GST-cdc25 for 20 min. For each determination, $p34^{cdc2}$-like kinase was purified with 40 µl $p13^{suc1}$ beads from 300 µg of total extracted protein, reacted with GST-cdc25 directly on the $p13^{suc1}$ beads, and then activity was measured using H1 histone as substrate, as in Fig. 3. Washes prior to reaction with GST-cdc25 were conducted in **a** with PTB and in **b** with HBK

has been deduced from the gene sequence to contain tyrosine at position 15. The capacity of the plant protein to support division in *cdc2/cdc28* mutants of yeasts suggests that it can participate in mitotic control as in other eukaryotes (Colasanti et al. 1991; Hirt et al. 1991) where phosphorylation of Tyr15 is a mojor control element (Gould and Nurse 1989; Nurse 1990), but control of the plant enzyme by phosphotyrosine has not previously been detected. A consistent inverse relationship of phosphotyrosine with enzyme activity was directly indicated by high phosphotyrosine levels (Figs. 8, 9) detected in a $p34^{cdc2}$-like protein of low enzyme activity (Figs. 3, 5) from cells in auxin without cytokinin. The enzyme could be activated by dephosphorylation, in vivo when stimulated by cytokinin (Figs. 3, 5), or in vitro when reacted with GST-cdc25 (Figs. 6, 10).

Three considerations indicate that the enzyme activated by cytokinin in cells arrested at the cytokinin-dependent control point was $p34^{cdc2}$/cyclin B. (i) Arrested cells had a G2-phase DNA content and, from evidence of rapid progress to mitosis (Fig. 5), were in late G2 phase when $p34^{cdc2}$ is predominantly in complex with cyclin B (Nurse 1990; Hayles et al. 1994; Pines 1995). (ii) Monomeric $p34^{cdc2}$ is catalytically inactive (Labbé et al. 1989) whereas the isolated enzyme was active (Figs. 6, 7, 10) when dephosphorylated. (iii) The fission-yeast Cdc25 phosphatase is targeted to the cyclin B-$p34^{cdc2}$ complex and did activate enzyme from arrested cells. These findings indicate that the activity of $p34^{cdc2}$-cyclin B in plants can be suppressed by tyrosine phosphorylation and support the suggestion (John 1984; John et al. 1989) that control points in the plant cell cycle have parallels in other eukaryotes.

Hormonal control at mitosis. Our data indicate a marked difference between plant and animal cells in the points at which hormones stimulate cell cycle progress. In somatic animal cells, hormones stimulate growth in G1 phase (Sherr 1993) and this leads to commitment to DNA synthesis but thereafter hormonal stimulation has not been detected as a requirement for completion of the cell cycle (Pardee et al. 1978; Zetterberg and Larsen 1985). In contrast, we found in plant cells a requirement for auxin (perhaps with other hormones) to initiate progress through G1 phase and DNA synthesis, then in late G2 phase a stringent requirement for a different class of hormone, the cytokinins, to activate $p34^{cdc2}$-like H1 histone kinase and enter mitosis.

The plant cell cycle may commonly involve a burst of cytokinin accumulation in late G2 phase, precisely when our cells required it, since there is a threefold peak in cytokinin concentration at this time in a cytokinin-autonomous suspension culture of *N. tabacum* L. cv. Xanthi (Nishinari and Syono 1986). In conformity with this, pith cells without cytokinin were also arrested in late G2 phase with $p34^{cdc2}$-like protein of high phosphotyrosine content that could be removed by cytokinin (Fig. 8) or Cdc25 enzyme (Fig. 7). This consensus suggest that in plant cells cytokinin increases at the critical G2/M transition. There may also be requirement for supplementation by hormonal signals from elsewhere, thus retaining hormonal responsiveness.

Conclusion. The auxin-cytokinin interaction is particularly important in development as it controls shoot and root branching. We now suggest one mechanism for the synergistic effect of cytokinin with auxin as being a cytokinin-dependent tyrosine dephosphorylation that activates the late G2 form of the $p34^{cdc2}$/cyclinB enzyme. It is significant that plants use this phosphorylation because in yeast it is regulated by a cascade of kinase and phosphatase enzymes (Nurse 1990; Millar et al. 1991) that inhibit $p34^{cdc2}$ in operation of a division checkpoint that monitors cell growth and the accurate replication and repair of DNA (reviewed by Nurse 1994). Plants can be expected to monitor the same events but additionally we have found a coupling of $p34^{cdc2}$ mitotic phosphorylation to hormone level. This may be unique to plants and provide a means of controlling division during development.

References

Bayliss MW (1985) Regulation of the cell division cycle in cultivated plant cells. In: Bryant JA, Francis D (eds) The cell division cycle in plants. Cambridge University Press, Cambridge, UK, pp 157–177

Brizuela L, Draetta G, Beach D (1987) $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. EMBO J 6: 3507–3514

Colasanti J, Tyres M, Sundaresan V (1991) Isolation and characterisation of cDNA clones encoding a functional $p34^{cdc2}$ homologue from *Zea mays*. Proc Natl Acad Sci 88: 3377–3381

Das NK, Patau K, Skoog F (1956) Initiation of mitosis and cell division by kinetin and indole-acetic acid in excised tobacco pith tissue. Physiol Plant 9: 640–651

Fobert PR, Coen ES, Murphy GJP, Doonan JH (1994) Patterns of cell division revealed by transcriptional regulation of genes during the cell cycle in plants. EMBO J 13: 616–624

Fosket DE (1977) The regulation of the plant cell cycle by cytokinin. In: Rost TL, Gifford EM (eds) Mechanisms and control of cell division. Dowden Hutchinson and Ross Inc, Strousberg, Pensylvania, pp 62–91

Gorst JR, John PCL, Sek FJ (1991) Level of $p34^{cdc2}$-like protein in dividing, differentiating and dedifferentiating cells of carrot. Planta 185: 304–310

Gould KL, Nurse P (1989) Tyrosine phosphorylation of the fission yeast *cdc2*$^{+}$ protein kinase regulates entry into mitosis. Nature 342: 39–45

Gould KL, Moreno S, Tonks NK, Nurse P (1990) Complementation of the mitotic initiator $p80^{cdc25}$ by a human protein tyrosine phosphatase. Science 250: 1573–1576

Hayles J, Fisher D, Woollard A, Nurse P (1994) Temporal order of S phase and mitosis in fission yeast is determined by the state of the $p34^{cdc2}$-mitotic B cyclin complex. Cell 78: 813–822

Henmerley AS, Ferriera P, Engler J de A, Van Montagu M, Engler G, Inzé D (1993) cdc2a expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5: 1711–1723

Hepler PK, Sek FJ, John PCL (1994) Nuclear concentration and mitotic dispersion of the essential cell cycle protein, $p13^{suc1}$, examined in living cells. Proc Natl Acad Sci USA 91: 2176–2180

Hirt H, Pay A, Gyorgyey J, Bako L, Nemeth K, Bogre L, Schweyen RJ, Heberle-Bors E, Dudits D (1991) Complementation of a yeast cell cycle mutant by an alfalfa cDNA encoding a protein kinase homologous to $p34^{cdc2}$. Proc Natl Acad Sci USA 88: 1636–1640

John PCL (1984) Control of the cell division cycle of *Chlamydomonas*. Microbiol Sci 1: 96–101

John PCL, Sek FJ, Lee MG (1989) A homologue of the cell cycle control protein $p34^{cdc2}$ participates in the cell division cycle of *Chlamydomonas* and a similar protein is detectable in higher plants and remote taxa. Plant Cell 1: 1185–1193

John PCL, Sek FJ, Carmichael JP, McCurdy DW (1990) $p34^{cdc2}$ homologue level, cell division, phytohormone responsiveness and cell differentiation in wheat leaves. J Cell Sci 97: 627–630

John PCL, Sek FJ, Hayles J (1991) Association of the plant $p34^{cdc2}$-like protein with $p13^{suc1}$: implications for control of cell division cycles in plants. Protoplasma 161: 70–74

John PCL, Zhang K, Dong C, Diederich L, Wightman F (1993) $p34^{cdc2}$ related proteins in control of cell cycle progression: the switch between division and differentiation in tissue development and stimulation of division by auxin and cytokinin Aust J Plant Phy6siol 20: 503–526

Kaffman A, Herskowitz I, Tjian R, O'Shea EK (1994) Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. Science 263: 1153–1156

Labbé J-C, Capony J-P, Caput D, Cavadore J-C, Derancourt J, Kaghad M, Lelias J-M, Picard A, Dorée M (1989) MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. EMBO J 8: 3053–3058

Letham DS (1966) Regulators of cell divisions in plant tissues II. A cytokinin in plant extracts: isolation and interaction with other growth regulators. Phytochemistry 5: 269–286

Martinez CM, Jorgensen J-E, Lawton MA, Lamb CJ, Doerner PW (1992) Spatial pattern of cdc2 expression in relation to meristem activity and cell proliferation during plant development. Proc Natl Acad Sci USA 89: 7360–7364

Meskiene I, Bogre L, Dahl M, Pirck M, Ha DTC, Swoboda I, Heberle-Bors E, Ammere G, Hirt H (1995) *cycMS3*, a novel B-type alfalfa cyclin gene, is induced in the G0-to-G1 transition of the cell cycle. Plant Cell 7: 759–771

Meyer Y, Cook R (1979) Time course of hormonal control of the first mitosis in tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta 147: 181–185

Miao G-H, Hong Z, Verma DPS (1993) Two functional soybean genes encoding p34^{cdc2} protein kinases are regulated by different plant developmental pathways. Proc Natl Acad Sci USA 90: 943–947

Millar JBA, McGowan GH, Lenaers G, Jones R, Russell P (1991) p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast. EMBO J 10: 4301–4309

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nasmyth K (1993) Control of the yeast cell cycle by Cdc28 protein kinase. Curr Opin Cell Biol 5: 166–179

Nishinari N, Syono K (1986) Induction of cell division synchrony and variation of cytokinin contents through the cell cycle in tobacco cultured cells. Plant Cell Physiol 27: 147–153

Nurse P (1990) Universal control mechanism regulating onset of M-phase. Nature 344: 503–508

Nurse P (1994) Ordering S phase and M phase in the cell cycle. Cell 79: 547–550

Pardee AB, Dubrow R, Hamlin JL, Kletzein RF (1978) Animal cell cycle. Annu Rev Biochem 47: 715–750

Peeper DS, Parker L, Ewen ME, Toebes M, Hall FL, Xu M, Zantema A, van der Eb AJ, Piwnica-Worms H (1993) A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. EMBO J 12: 1947–1993

Pines J (1995) Cyclins and cyclin dependent kinases: a biochemical view. Biochem J 308: 697–711

Resnitzky D, Hengst L, Reed SI (1995) Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27^{Kip1}. Mol Cell Biol 15: 4347–4352

Sherr JC (1993) Mammalian G1 cyclins. Cell 73: 1059–1056

Simard A (1971) Initiation of DNA synthesis by kinetin and experimental factors in tobacco pith tissues in vitro. Can J Bot 49: 1541–1548

Soni R, Carmichael JP, Shah ZH, Murray JAH (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7: 85–103

Van't Hof J (1974) Control of the cell cycle in higher plants. In: Padilla GM, Cameron IL, Zimmerman A (eds) Cell cycle controls. Academic Press, New York, pp 77–85

Zetterberg A, Larsson O (1985) Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. Proc Natl Acad Sci USA 82: 5365–5369

Zhang K, Tsukitani Y, John PCL (1992) Mitotic arrest in tobacco caused by the phosphoprotein phosphatase inhibitor okadaic acid. Plant Cell Physiol 33: 677–688