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Modulation of Cyclin Transcript Levels in Cultured Cells of *Arabidopsis thaliana*[1]

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Previous studies on the cell cycle of *Arabidopsis thaliana* have been hindered by the lack of synchronous cell culture systems. We have used liquid callus cultures and a cycloheximide-synchronized suspension culture of Arabidopsis to investigate changes in cyclin transcript levels in response to exogenous auxin, cytokinin, and nutrients, and during the cell cycle. *CYCD1* (*δ1*) transcript was virtually undetectable in liquid-cultured callus or suspension-culture cells. CYCD2 (*δ2*) transcript levels were largely unaffected by the readdition of phytohormones or nitrate to the growth medium, and remained constant throughout the cell cycle in suspension-culture cells. *CYCD3* (*δ3*) transcript levels were strongly dependent on nitrate, and were induced at the G1/S transition following phytohormone readdition. In synchronized suspension-culture cells, *CYCD3* transcript accumulated during the S phase, and remained constant thereafter. These results support the hypothesis that D cyclins function as part of the cellular machinery that integrates diverse signals impinging upon commitment to cell division. In synchronized cells transcripts of the mitotic cyclins *CYC1*, *CYC2*, and *CYC3* reached a maximum with peak mitotic index, but *CYC3* transcript levels increased earlier than those of *CYC1* or *CYC2*. The kinetics of accumulation of *CYC* transcript levels support their classification as A-type (*CYC3*) and B-type (*CYC1* and *CYC2*) cyclins, respectively.

Cell division is a process essential for cellular life, enabling proliferation of single-celled organisms and the establishment of a structural and functional division of labor during the growth of multicellular organisms. In complex multicellular organisms cell division is tightly regulated within a developmental program. Higher-plant morphogenesis is intimately associated with regulated cell division and expansion, two closely coordinated processes. Mechanisms controlling cell division have been well conserved throughout evolution, both structurally and functionally. Molecular components of the cell-cycle machinery originally discovered in yeast and animals have been found to exist in higher plants and have been reviewed recently (Doerner, 1994; Francis and Halford, 1995).

A family of Ser/Thr protein kinases, termed cyclin-dependent kinases, and their activating protein cofactors, termed cyclins, play an important role in the regulation of the eukaryotic cell cycle and are well reviewed (King et al., 1994; Nurse, 1994; Sherr, 1994).

In animal and yeast cells oscillations in cyclin abundance during the cell cycle are often mirrored by changes in the abundance of their corresponding mRNAs (Pines and Hunter, 1989, 1990; reviewed by Pines, 1995). Mammalian cyclin A and B transcripts accumulate periodically during the cell cycle, peaking at G2/M (Pines and Hunter, 1989, 1990), and mammalian cyclin E transcripts peak in abundance at the end of G1 (Lew et al., 1991). The correlation between cyclin A and E protein levels and their associated kinase activity in mammalian tissue culture cells suggests that the levels of these cyclins drive their associated kinase activities (Dou et al., 1993). Mammalian D1 cyclin functions during G1, but its transcript level does not show dramatic cell-cycle-related changes in abundance (Matsushime et al., 1991; Sewing et al., 1993). However, cyclin D levels are highly responsive to the presence of serum growth factors, declining rapidly on their withdrawal and increasing rapidly when quiescent cells are reactivated (Matsushime et al., 1991; Ajchenbaum et al., 1993; Ando et al., 1993), suggesting that they play a role in transducing extracellular signals into the cell-cycle control machinery (Ando et al., 1993; Baldin et al., 1993; Sewing et al., 1993; reviewed by Sherr, 1993, 1994).

All *Arabidopsis thaliana* cyclins isolated to date fall into three broad groupings bearing the sequence hallmarks and patterns of expression of D (δ) cyclins (Soni et al., 1995) A or B, which are predominantly expressed during G2/M (Hemerley et al., 1992; Day and Reddy, 1994; Ferreira et al., 1994a). Renaudin et al. (1996) have shown that plant cyclins have sequence characteristics that clearly relate them to animal cyclins, and the nomenclature A, B, and D has been proposed to indicate this.

A. thaliana has become a model organism because of its suitability for classical and molecular genetics (Meyerowitz and Pruitt, 1985), but cell-cycle studies in this species have been impeded because *A. thaliana* suspension cultures have

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proved difficult to synchronize (Ferreira et al., 1994a). Synchrony provides large numbers of cells at defined points of the cell cycle, and as such is a prerequisite for many biochemical techniques used to investigate molecular events during the cell cycle. Investigations into the role of cyclins in *A. thaliana* have to date largely involved populations of cells allowed to accumulate at a particular point of the cell cycle in the presence of toxins (Hemerley et al., 1992; Ferreira et al., 1994a, 1994b). Limited synchrony of liquid-cultured callus cells following release from hydroxyurea arrest can be achieved (Soni et al., 1995), but the agglomeration of cells in such cultures makes synchrony difficult to induce and measure.

Cell cultures providing a high degree of synchrony are generally homogeneous and well dispersed (Darzynkiewicz et al., 1979; Jonak et al., 1993), with a rapid culture doubling time, and are available for a number of species including *Medicago sativa* (alfalfa; Hirt et al., 1992), *Catharanthus roseus* (Madagascan periwinkle; Kodama and Komamine, 1995), and *Nicotiana tabacum* (tobacco; Nagata et al., 1992). In some plant cell cultures, simply subculturing cells into fresh medium is sufficient to induce synchrony (Conia et al., 1990), but this is an exception. Synchrony in plant cell cultures can also be achieved by sequential withdrawal and provision of nutrients or phytohormones, but different cell lines from the same species often display marked differences in their susceptibility to such treatments (Kodama and Komamine, 1995). Despite problems of uncertain specificity, the use of inhibitors to synchronize cultures is common, and has been reviewed (Pardee and Keyomarsi, 1992). We have tested a number of inhibitors for synchronizing *A. thaliana* suspension-culture cells and have developed an effective synchronization procedure using cycloheximide.

We report on the expression of *A. thaliana* cyclins in both reactivated and synchronized cells. The pattern of expression of cyclins observed was in agreement with conclusions drawn by Soni et al. (1995) on *A. thaliana* D-type cyclins, and is consistent with the classification of Cyc1, Cyc2, and Cyc3 cyclins proposed by Renaudin et al. (1996).

MATERIALS AND METHODS

Liquid Culture of Callus

Liquid-grown callus cultures were derived and maintained as described by Soni et al. (1995). Deprivation of phytohormones (2,4-D, kinetin) was as previously described (Soni et al., 1995), with the exception that Suc (3%, w/v) was maintained in the medium throughout the experiment, and samples were taken at the various times indicated. For the nitrate-starvation experiment, callus material was transferred to a medium as described by Murashige and Skoog (1962) with the omission of potassium nitrate and ammonium nitrate, and incubated for 48 h. RNA gel blot analysis and probes were as described (Soni et al., 1995).

Culture of Suspension Cells

A suspension culture of *Arabidopsis thaliana* ecotype Landsberg *erecta* (May and Leaver, 1993) was used as the starting material. The cells were grown in Murashige and Skoog medium (Imperial Laboratories, Andover, Hampshire, UK) containing 3% (w/v) Suc, 0.5 mg/L NAA, and 0.05 mg/L kinetin; pH was adjusted to 5.8 with KOH. The medium was autoclaved with phytohormones present at 121°C for 10 min, followed by rapid cooling. Prior to inoculation, medium was prewarmed to 25°C. Cells in 200 mL of medium were incubated in a 500-mL conical flask and shaken at 120 rpm at 25°C in constant light. Rapidly dividing cells were selected by gradually reducing the interval of subculture from 7 to 3 d; cells were diluted 10-fold at each subculture. For long-term maintenance of the line, cells were diluted 40-fold and subcultured every week. Growth was monitored by determining cell densities.

Cell Density

Cell density was measured by hemocytometry after maceration (Brown and Rickless, 1948). Cell suspension (5 mL) was added to an equal volume of 20% (w/v) CrO_3 and left overnight at room temperature. Cells were dispersed by forcing 1 mL of suspended cells seven times through a syringe needle of 0.4-mm diameter. Cell density was then determined using a hemocytometer. Cells in CrO_3 solution could be stored at 4°C for up to 2 weeks with no change in measured cell density. Measurements were performed in triplicate on each of three separate samples for every data point; error bars represent SE.

Mitotic Index

Suspension-culture cells (5 mL) were fixed in an equal volume of 65% (v/v) formic acid. A small quantity of cells was hydrolyzed in 5 N HCl for 10 min at room temperature, and resuspended in aceto-orcein (Sigma). One thousand cells were examined to determine a mitotic index, and the procedure was repeated three times to ensure accuracy. Due to experimental limitations, cells in prophase could not be reliably scored, and mitotic indices, therefore, represent cells in metaphase, anaphase, or telophase.

Thymidine Incorporation

Suspension-culture cells (1 mL) were incubated in 2 μM [*methyl*-^{3}H]thymidine with a specific activity of 5 Ci/mmol (Amersham) for 45 min at 25°C with constant gentle agitation. Labeled cells were washed in fresh medium before being flash-frozen in liquid nitrogen and stored at −70°C. Cells were subsequently incubated in 1 mL of 10% TCA for 2 h on ice, and the acid-soluble label was removed by washing cells on a glass microfiber filter (GF/C, Whatman) with vacuum aspiration of washes. Cells were washed with 2 × 5 mL of 5% TCA at 0°C, followed by 5 mL of water at 100°C. Residual acid-insoluble radioactivity was measured in an aqueous scintillation cocktail. Nonspecific back-

ground incorporation was determined by the labeling of stationary-phase cells, and was subtracted from values obtained with synchronized cells.

Toxins

Stock solutions of toxins were prepared as follows: Cycloheximide (Sigma) was dissolved in 4 mL of absolute ethanol, and then diluted to a final concentration of 5 mg/mL with 16 mL of KOH solution, pH 4.0. Staurosporine (Calbiochem) and quercitin (Sigma) were dissolved in DMSO at 0.146 and 169 mg/mL, respectively. Mimosine (Sigma) was dissolved in 1 M NH_4OH at 25 mg/mL. Toxin solutions were filter-sterilized, diluted, aliquoted, and stored at −20°C. Working concentrations of solvent solutions did not detectably affect cell culture growth. Cells were removed from the inhibitor by washing four times in fresh medium.

Synchronization

For large-scale synchronizations, 800 mL of freshly subcultured cells were incubated in a 2-L flask for 28 h under conditions described above. Cycloheximide was then added to a final concentration of 150 nM. After another 24 h of incubation, cells were rinsed with fresh medium before continued incubation as 200-mL aliquots in 500-mL flasks.

RNA Extraction

Cells were harvested by vacuum filtration of suspensions through cellulose filters with a pore size of 5 μm, then flash-frozen in liquid nitrogen. RNA was extracted using a guanadinium thiocyanate extraction buffer (Wadsworth et al., 1988). RNA pellets were washed twice in 3 M sodium acetate, pH 5.2, to remove contaminants (Logemann et al., 1987).

RNA Gel Blot Analysis

Purified RNA (10 μg) was fractionated on formaldehyde-agarose gels, blotted onto nylon filters (Hybond-N, Amersham), and fixed by UV cross-linking. Probes were prepared by random, hexanucleotide-primed synthesis (Feinberg and Vogelstein, 1984). Labeled fragments were as follows: histone H4 (*Hin*dIII, a gift of Dr. Ben Scheres, University of Utrecht, The Netherlands); Ac16-encoding porphobilinogen deaminase (*Eco*RI, a gift of Dr. A. Smith, University of Cambridge, UK); *cdc2a* (*Bam*HI-*Asp*718, a gift of Dr. Peter Doerner, Salk Institute, La Jolla, CA); *CYC1At*(*Eco*RI), *CYC2aAt*(*Eco*RI-*Xho*I), and *CYC3bAt*(*Eco*RI-*Xho*I), gifts from Dr. Dirk Inzé (Universiteit Gent, Belgium) (Hemerley et al., 1992; Ferreira et al., 1994a); and *CYCD1*, *CYCD2*, and *CYCD3* (δ1, δ2, and δ3) as described (Soni et al., 1995). c7 and c9, two cDNA probes isolated in our laboratory from an *A. thaliana* cDNA library constructed in λYES (Elledge et al., 1991), were used as noncell-cycle-regulated controls. Neither c7 nor c9 had significant homology to functionally characterized sequences present in databases, and were both expressed in suspension-culture cells of *A. thaliana* during the exponential growth phase and the stationary phase (data not shown). Hybridization was carried out in 0.25 M Na_2HPO_4, pH 7.2, 7% SDS, at 65°C overnight. Filters were washed at 65°C in a series of solutions for 20 min in each. The washes all contained 5% SDS, and the salt concentration was reduced from 200, to 100, to 50, to 25 mM Na_2HPO_4, pH 7.2. Filters were analyzed by autoradiography and with a phosphor imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Transcript Levels in Callus Cultures

To investigate transcript levels of D cyclins following release from enforced quiescence, a liquid-cultured callus was deprived of exogenous phytohormones (2,4-D, kinetin) for 48 h and then returned to a complete medium. Following readdition of the phytohormones, samples were removed for RNA analysis at periods up to 79 h (Fig. 1). An increase in *CYCD3* transcript levels was observed after 24 h in parallel with H4 transcript levels, which have an expression strongly correlated with the S phase in tobacco BY-2 cells (Reichheld et al., 1995). *CYCD2* transcript levels were unaffected by reinduction of division, consistent with the fact that they are insensitive to the presence of exogenous phytohormones (Soni et al., 1995). Transcripts of the CDK *cdc2a* remained constant during the experiment, and *CYCD1* cyclin transcripts were not detected (data not shown).

Starvation of nitrate causes plant cells to cease division and accumulate in the G1 phase (Gould et al., 1981). Liquid-cultured callus was transferred to a medium lacking nitrate for 48 h and, following readdition of nitrate, samples were taken for RNA analysis at intervals up to

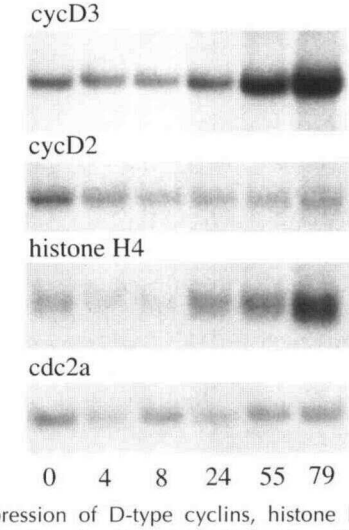

Figure 1. Expression of D-type cyclins, histone H4, and *CDC2a* following activation of quiescent Arabidopsis cells from phytohormone arrest. Liquid-cultured Arabidopsis callus material was deprived of 2,4-D and kinetin for 48 h, resulting in enforced quiescence. At 0 h, 2,4-D and kinetin were added to the growth medium, and samples were taken at the times indicated for RNA preparation. RNA gel blots were hybridized with the probes indicated.

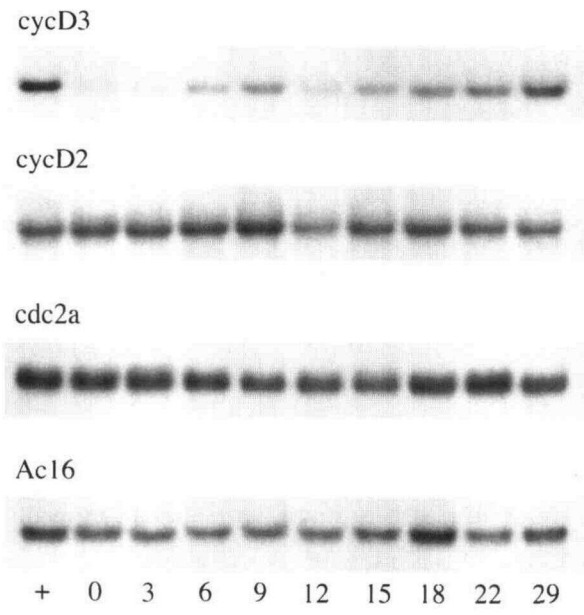

Figure 2. Expression of D-type cyclins, cdc2a, and *Ac16* following activation of quiescent Arabidopsis cells from nitrate starvation. Liquid-cultured Arabidopsis callus material was deprived of nitrate for 48 h, resulting in enforced quiescence. At 0 h, nitrate was replaced in the growth medium, and samples were taken at the times indicated for RNA preparation. RNA gel blots were hybridized with the probes indicated.

29 h (Fig. 2). No change was observed in the expression of *CDC2a*, *CYCD2*, *CYCD1* (not shown), or the control probe encoding proporphobilinogen deaminase (Ac16), but cyclin D3 transcript levels were found to be strongly dependent on nitrate availability.

Suspension Culture

To investigate the timing of cyclin expression during the Arabidopsis cell cycle more precisely, we devised an improved method for synchronizing *A. thaliana* cells. Although partial synchrony can be induced in liquid-grown callus cultures (Soni et al., 1995), the agglomeration of cells in such cultures makes synchrony difficult to induce and measure. A rapidly dividing, finely dispersed cell line appears to be a prerequisite for good synchronization of cell suspensions (Nagata et al., 1992; Jonak et al., 1993). Such a culture of *A. thaliana* was produced by sequential subculturing of a suspension culture at decreasing time intervals. Under conditions favoring rapid cell division, the culture was well dispersed (Fig. 3) and grew with a doubling time of 22 h. Exponentially dividing asynchronous populations displayed a mitotic index of 3.1% (SE 0.09%). A high proportion of the cells appeared densely cytoplasmic and contained approximately 20 chromosomes per cell, which is the tetraploid number for *A. thaliana*.

Toxin Characterization

A variety of toxins were evaluated as suitable inhibitors for synchronizing suspension-culture cells of *A. thaliana*. Because of our interest in control points within G1 and at the G1/S transition, inhibitors capable of leading to arrest within G1 were chosen as candidates. The precise molecular targets of the toxins used are not known, but they have been used to induce synchrony in other systems. Quercitin is a flavone capable of inhibiting a broad range of enzymes, and reversibly arrests mammalian cells in G1 (Yoshida et al., 1992). Cycloheximide inhibits protein synthesis by preventing peptide bond formation. It delays the cell cycle by elongating the prerestriction point of the cell cycle in mammalian cells (Rossow et al., 1979) and the prestart point of the cell cycle in budding yeast (Ko and More, 1990). Its action is due to the inhibition of a specific regulatory step in the cell cycle sensitive to protein synthesis inhibition rather than a general metabolic inhibition (Campisi et al., 1982). Inhibition of protein synthesis in *Catharanthus roseus* cells with either cycloheximide or ansiomycin is more effective in preventing cell-cycle progression during the G1 phase than the S phase, and cycloheximide has been used to arrest an asynchronous population of dividing *Catharanthus* cells during G1 (Ohnishi et al., 1990). Mimosine is the most effective inhibitor of the S phase known (Levenson and Hamlin, 1993), arresting cells in the very late G1 (Hanauskee-Abel et al., 1995) and/or S phase (Gilbert et al., 1995). Staurosporine is a potent, broad-range inhibitor of protein kinases, including $p34^{cdc2}$-type kinases (Gadbois et al., 1992), capable of reversibly arresting mammalian cells in the G1 phase (Crissman et al., 1991).

Aphidicolin and hydroxyurea are both agents that have been used successfully to synchronize plant suspension cultures (Nagata et al., 1992; Savouré et al., 1995). We did not assess these as agents for inducing synchrony, since both act within or at the onset of the S phase as direct or indirect inhibitors of DNA polymerase activity (Levenson and Hamlin, 1993). We were interested in regulatory processes involved in the G1 exit, which operate at points unlikely to provide cell populations with good synchrony at the times of interest.

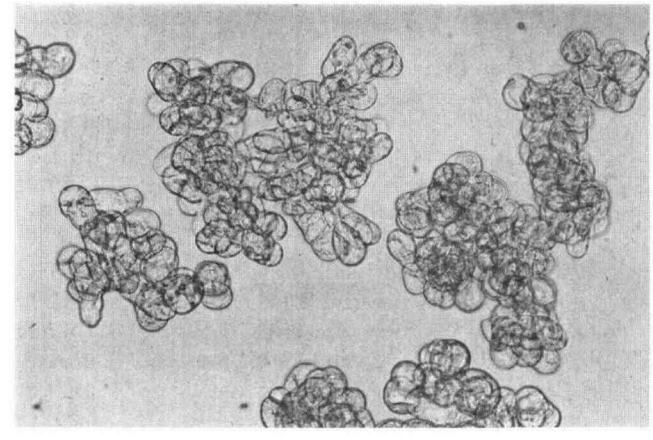

Figure 3. Suspension culture of *A. thaliana* cells. A rapidly dividing line of well-dispersed cells was obtained by sequential subculture at decreasing time intervals.

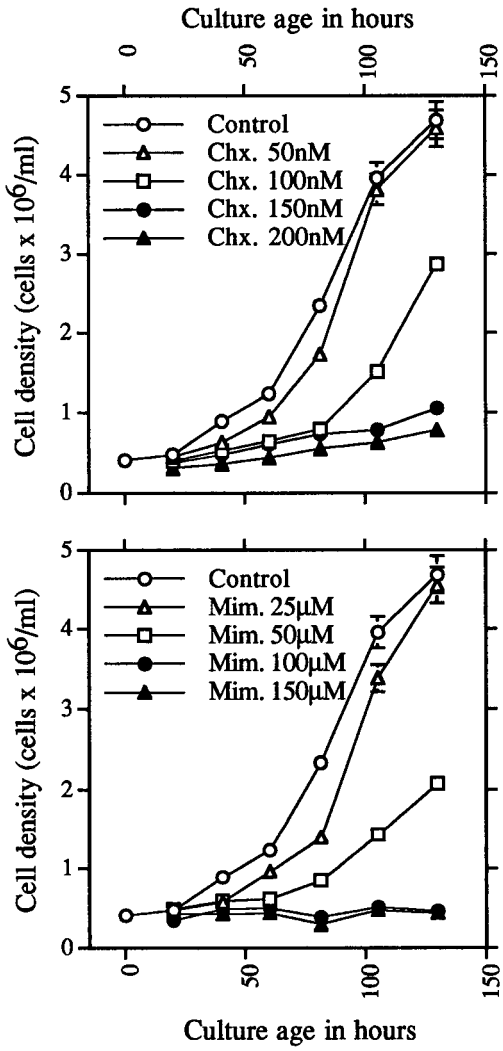

Figure 4. Inhibition of cell division by toxins. Cells were subcultured into fresh medium containing either cycloheximide (Chx.) or mimosine (Mim.). Cell density was determined at intervals following subculture to determine the minimum effective concentration of the toxin.

Of the above inhibitors several proved unsuitable as synchronizing agents. Staurosporine did not inhibit cell division when present at concentrations of up to 312.5 nM, two orders of magnitude higher than levels effective in animal systems (Abe et al., 1991; Crissman et al., 1991). Quercitin inhibited cell division at a concentration of 500 μM, but cells exposed to 100 or 500 μM turned brown in response, symptomatic of necrosis. Cycloheximide and mimosine inhibited cell division (Fig. 4), without causing cells to display stereotypical symptoms of stress. Cycloheximide or mimosine concentrations of 150 nM and 100 μM, respectively, arrested division in exponentially growing asynchronous cell populations. Resumption of division occurred when cells were restored to toxin-free medium in the case of cycloheximide arrest (Fig. 5), but mimosine-arrested cells did not resume division (data not shown).

Culture Synchronization

Cells subjected to the synchronization regimen described in "Materials and Methods" performed their first division after release in a partially synchronous manner (Fig. 5). Greater than 95% of arrested cells recovered and underwent division within 36 h of release. The rate at which cells incorporated [^{3}H]thymidine into acid-insoluble material was used as an indicator of DNA synthesis, which peaked between 9 and 13 h after release (Fig. 6). The mitotic index of the population peaked 16 h after release with 9.1% (SE 0.05%) of cells in metaphase, anaphase, or telophase (Fig. 6). Examination of cells prior to 12 h after release revealed that very few cells were in mitosis; therefore, no mitotic index was determined for these time points. The shape of both the thymidine incorporation and mitotic index peaks suggested that the majority of the population traversed the cell cycle rapidly in synchrony. However, the population contained a minority of laggard cells that reduced the system's ability to resolve events related to the exit from such phases, as indicated by the trailing edge of the peaks in [^{3}H]thymidine incorporation, and by the mitotic index.

Transcript Patterns in Synchronized Cells

To determine the accumulation of cyclin transcripts during cell division RNA was extracted from suspension- culture cells at various times after the release of cells from toxin-induced arrest of cell division. Transcript levels of mRNAs were determined by northern blotting of total RNA, followed by hybridization of radiolabeled probes to membrane-immobilized RNA. Membranes were analyzed using a phosphor imager (Fig. 7A) and film (Fig. 7B). *CYC1*, *CYC2*, and *CYC3*

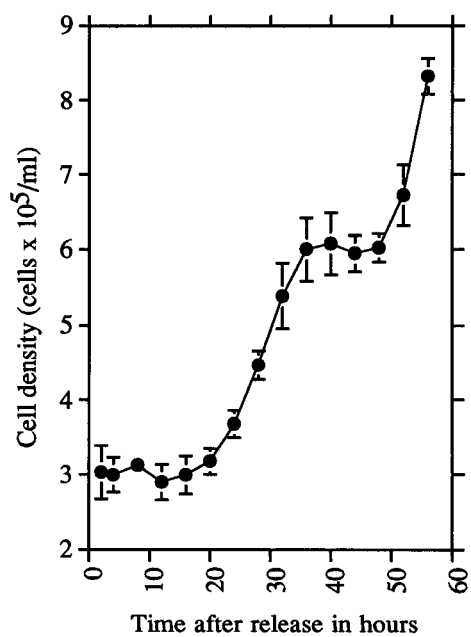

Figure 5. Cell division in synchronized cultures. Cell density was determined following release from cycloheximide-induced arrest.

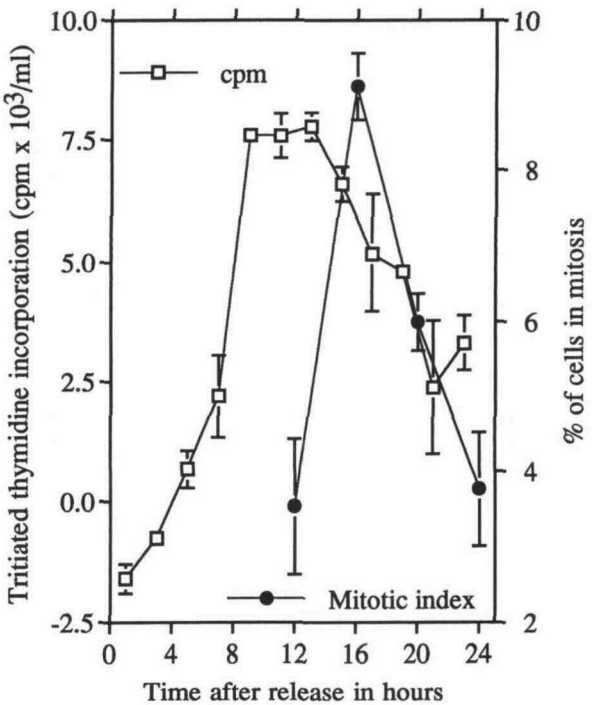

Figure 6. Passage of synchronized cells through S phase and mitosis. The manner in which cells traversed the cell cycle following release from cycloheximide-induced arrest was followed by determining their rate of DNA synthesis and mitotic index.

mRNA levels peaked during mitosis 16 h after release. The kinetics of mRNA accumulation for the different classes of mitotic cyclins were different: levels of *CYC3* increased earlier than those of *CYC1* or *CYC2*, starting close to the beginning of the S phase, as defined by [^{3}H]thymidine incorporation and histone H4 expression.

CYCD1 (δ1) transcript was not detectable at any time, whereas *CYCD2* (δ2) transcript levels remained relatively constant throughout the cell cycle. *CYCD3* (δ3) transcript levels had started to increase by 4 h, slightly before histone H4 and mitotic cyclin transcripts, reaching a maximum during the early S phase and remaining constant thereafter. Levels of all *CYCD* transcripts remained relatively constant from 24 to 56 h after release (data not shown). As a control, the levels of two noncell-cycle-regulated transcripts were followed throughout the experiment and remained constant. Patterns of transcript abundance for histone H4, *CYC1*, *CYC2*, and *CYC3* revealed a marked decay of synchrony subsequent to the first cell cycle (data not shown).

The S phase coincided with elevated histone H4 transcript levels, occurring between 8 and 12 h after release. The degree of induction observed with histone H4 transcripts during the S phase was 60% above basal levels of expression; only slightly lower levels of mRNA accumulation were observed immediately subsequent to mitosis. This was in contrast to the 150% increase observed in the case of *CYCD3*, *CYC2*, and *CYC3* mRNA, and to the 1400% increase observed in *CYC1* mRNA levels upon induction.

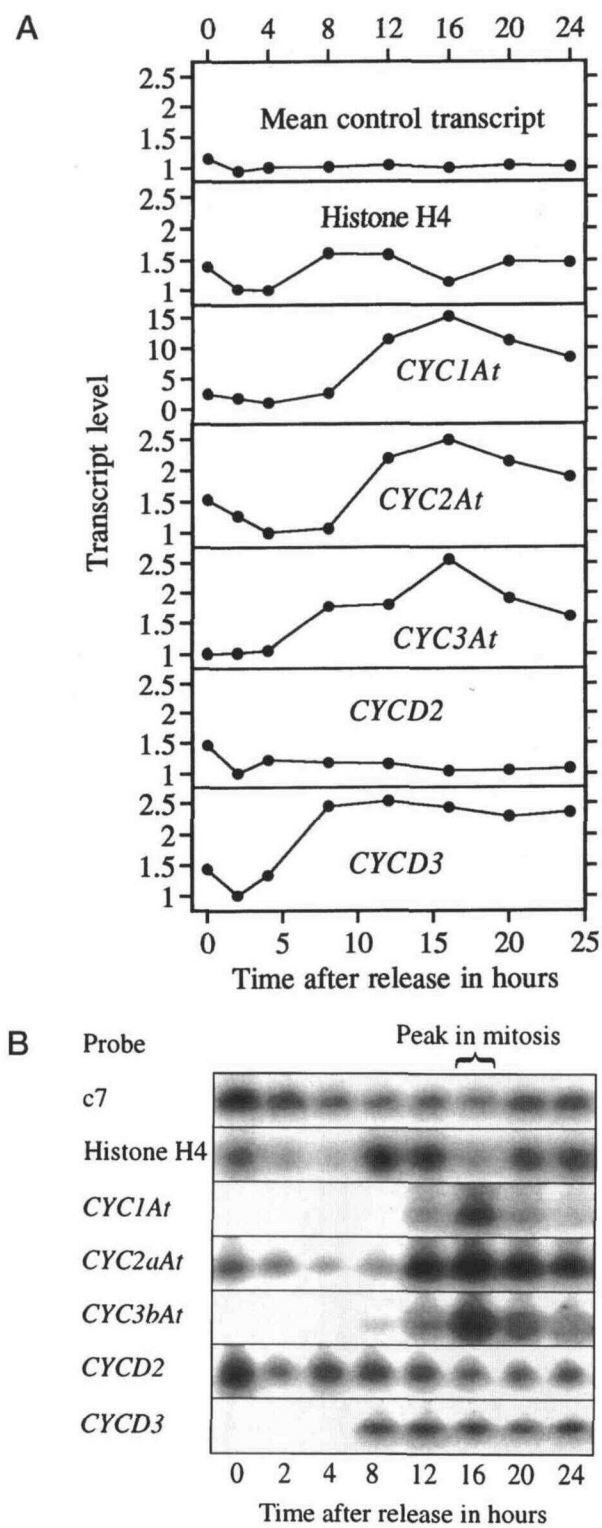

Figure 7. Transcript levels in synchronized cells. A, Phosphor imager quantification of transcript in synchronized cells. Values are expressed as factors of minimum values for each data set. These data are presented to indicate the patterns of each transcript's abundance (as opposed to absolute values). B, Levels of control (c7 and c9), histone H4, and cyclin transcripts as detected by autoradiography of northern blots. Ten micrograms of total RNA, extracted from synchronized cells, was run per lane.

DISCUSSION

We have investigated the transcript levels of *A. thaliana* cyclins in cells deprived and resupplied with exogenous phytohormones or nitrate and during the cell cycle in synchronized cells. Liquid-cultured callus tissue was used to investigate cyclin expression in response to nutrient and phytohormone deprivation, but these cultures proved unsuitable for detailed investigation of transcript levels throughout the cell cycle. Therefore, we developed a procedure for synchronizing suspension-cultured cells of *A. thaliana*.

Synchronization of an *A. thaliana* Cell-Suspension Culture

An important prerequisite for plant cell synchronization is fast growth (Nagata et al., 1992), as long culture doubling times are associated with a low percentage of actively dividing cells (Wang et al., 1982). Although the 22-h doubling time achieved here for *A. thaliana* is substantially longer than the approximately 8.5 h reported for seedling meristem cells (Van't Hof et al., 1978), increase in cycle length is a common feature of suspension-cultured plant cells (Bayliss, 1985). The rate of growth and the well-dispersed nature of this *A. thaliana* cell line was probably a major factor in its successful synchronization.

Toxins were assessed for their ability to synchronize cells in the culture with minimal secondary effects. Several of the toxins proved unsuitable as primary synchronizing agents. Staurosporine did not inhibit the growth of cells, even at concentrations far higher than those found to be effective in animal systems (Abe et al., 1991; Crissman et al., 1991). Tobacco BY-2 cells also have a high resistance to staurosporine inhibition of G2 processes (Katsuta and Shibaoka, 1992), and it is possible that plant cell G1 processes are also poorly inhibited by staurosporine. *A. thaliana* cells proved more resistant to inhibition of cell division by quercitin than were mammalian cells (Yoshida et al., 1992), and phenolic production symptomatic of stress was observed at concentrations below those effective at inhibiting cell division. Mimosine irreversibly arrested cell division at concentrations similar to those effective in animal systems. Irreversible arrest appears to be a property of mimosine when applied to asynchronous populations in both hamster (Mosca et al., 1992) and *A. thaliana* (Perennes et al., 1993) cells.

Cycloheximide was found to be capable of inducing synchrony in suspension-cultured *A. thaliana* cells. Cycloheximide delays cell-cycle progress by extending the pre-restriction point (R) G1 in mammalian cells (Rossow et al., 1979) or pre-START G1 in budding yeast cells (Ko and More, 1990), and has been used to arrest an asynchronous population of dividing *C. roseus* cells in G1 (Ohnishi et al., 1990). Inhibition of cell-cycle progress is due to a sensitive regulatory step, not to general metabolic inhibition (Campisi et al., 1982).

The steady-state levels of many unstable transcripts are increased in the presence of cycloheximide (Cleveland and Yen, 1989), including plant "small auxin-up RNAs" (Franco et al., 1990). Cycloheximide results in increased transcript levels as a result of diverse transcript-specific mechanisms (Pachter et al., 1987; Koeller et al., 1991; Wisdom and Lee, 1991; Stordeur et al., 1995), and also disrupts pre-rRNA processing in HeLa cells (Hadjilova et al., 1993). Although the concentration of cycloheximide used in our synchronization was at least one order of magnitude lower than that used to demonstrate the above effects, we cannot exclude the possibility that the cycloheximide treatment is responsible for an artifactually high starting level of the cyclin transcripts investigated. The small initial decrease observed in the case of most transcripts may be due to such an effect, but most of our inferences are drawn from an increase in levels subsequent to release.

Release from the cycloheximide block was followed by a peak in thymidine incorporation and histone H4 expression associated with the S phase (Reichheld et al., 1995); a peak in mitotic index; and an abrupt increase in cell number with a subsequent plateau phase in which there was no change in cell number. We observed a lag between the peak of mitotic index and cytokinesis (measured as cell number increase), which we ascribe to the method used for determining cell density, since this may fail to detect nascent cell walls (Yeoman and Evans, 1967). Greater than 95% of cells participated in the synchronous division, as determined by cell-density measurements during the plateau phase. Similar levels of participation have been reported in the case of subculture-induced synchrony in *Datura innoxia* (Conia et al., 1990), but the procedures described here provide better participation than the 70 to 80% recovery of *C. roseus* cells subjected to auxin or phosphate synchronization (Kodama and Komamine, 1995), and the approximately 70% participation of alfalfa cells released from hydroxyurea inhibition (Savouré et al., 1995). The peak mitotic index observed during the first division was determined to be only 9%, but this figure is artificially low due to our exclusion of prophase cells from the measurement, and is comparable with auxin- or phosphate-synchronized *C. roseus* cells (Kodama and Komamine, 1995), although low in comparison with aphidicolin-synchronized tobacco BY-2 cells or alfalfa A2 cells, which exhibit peak mitotic indices of approximately 70% (Nagata et al., 1992) and approximately 30% (Savouré et al., 1995), respectively.

Nevertheless, the synchrony that we observed with *A. thaliana* was the best obtained to date for this species, and is sufficient to enable the study of biochemical events in specific phases of the cell cycle. The system provides useful synchrony from the point of arrest in the G1 phase through mitosis. Potential avenues exist for improving the usefulness of the system, including continued subculture to improve culture homogeneity and the use of inhibitors acting later in the cell cycle. The task of identifying suitable inhibitors should be facilitated by the ability to feed cells from a cycloheximide block into other inhibitors. In both plant (Perennes et al., 1993) and animal (Tobey et al., 1990; Mosca et al., 1992) systems the use of such a strategy is sometimes required to achieve a high proportion of reversibly arrested cells.

Expression of Plant D Cyclins

CYCD1 transcript was present at very low levels in callus and suspension-culture cells, as has been observed previously for callus cells (Soni et al., 1995). *CYCD2* transcript levels appeared to be insensitive to changes in environmental levels of phytohormones and nitrate, despite their sensitivity to Suc levels (Soni et al., 1995). They were also relatively constant throughout the cell cycle in suspension-culture cells synchronized with cycloheximide. This contrasts with the repression of *CYCD2* transcript levels in hydroxyurea-treated cells (Soni et al., 1995), but correlates with the relatively constant levels of mammalian D1 cyclin mRNA maintained throughout the cell cycle (Matsushime et al., 1991; Sewing et al., 1993).

In both liquid-cultured callus cells restimulated with phytohormones and in synchronized suspension-culture cells, *CYCD3* transcript levels increased in parallel with or slightly in advance of histone H4 transcripts, in which expression is closely correlated with the S phase (Reichheld et al., 1995). *CYCD3* transcript levels also increase in advance of those for histone H4 in cells released from a hydroxyurea block (Soni et al., 1995). These observations from a variety of experimental treatments suggest a role for *CYCD3* at G1/S in the cell cycle, as is the case for mammalian D cyclins (Sherr, 1994). Unlike the reduction observed with hydroxyurea (Soni et al., 1995), *CYCD3* transcript levels were elevated slightly on arrest with cycloheximide; however, in light of the ability of cycloheximide to raise steady-state transcript levels this evidence is inconclusive. The uniformly high levels of Arabidopsis cyclin *CYCD3* transcript observed after the S phase during the subsequent mitosis correlates with the relatively constant levels of mammalian D1 cyclin mRNA maintained throughout the cell cycle (Matsushime et al., 1991; Sewing et al., 1993). However, the decay in synchrony with which suspension-culture cells traversed the cell cycle after mitosis precluded accurate quantification of *CYCD3* transcript levels during early G1. Arabidopsis cyclin D3 transcript levels were strongly dependent on the provision of nitrate and exogenous phytohormones, which is consistent with the observed induction of *CYCD3* expression by cytokinins (Soni et al., 1995) and the sensitivity of mammalian cyclin D levels to the presence of serum growth factors (Ando et al., 1993; Sewing et al., 1993).

The *CYCMs4* gene identified in alfalfa is structurally similar to that of *CYCD3* (Dahl et al., 1995); its pattern of expression is also similar. Our tentative observations that *CYCD3* expression precedes the S phase are supported by the increase in *CYCMs4* transcript levels before the increase in histone H3-1 transcript levels in both phosphate-synchronized alfalfa cell-suspension cultures and cells of stimulated leaves to resume division (Dahl et al., 1995). The low levels of *CYCD3* expression observed during hydroxyurea arrest (Soni et al., 1995) are paralleled by reduced *CYCMs4* transcript levels during aphidicolin arrest (Dahl et al., 1995), suggesting that a reduction of *D3*-type cyclin transcript is a feature common to inhibitors such as hydroxyurea and aphidicolin, which block the S phase by preventing chain elongation (Levenson and Hamlin, 1993). This is in contrast to the expression of histone genes in tobacco BY-2 cells, in which hydroxyurea blocks cells with low levels of histone expression and aphidicolin blocks cells with high levels (Reichheld et al., 1995).

The resultant pattern of G1/S to M phase expression for *CYCD3* and *CYCMs4* correlates well with the role mammalian D-type cyclins have in the direction of cyclin-dependent kinase phosphorylation of the retinoblastoma protein Rb (Sherr, 1995). This protein becomes phosphorylated during the late G1 and S phases, remaining so until early G1 (Hamel et al., 1992). In this way *CYCD3* and *CYCMs4* expression patterns support arguments (Dahl et al., 1995; Soni et al., 1995) that in plants D-type cyclins perform a similar role in phosphorylating Rb-related proteins.

Together, these results support a model in which plant D cyclins form part of the cellular machinery integrating diverse signals that impinge upon the commitment to cell division, as opposed to the machinery that deterministically executes that decision (Murray, 1994; Soni et al., 1995). The difference between transcript levels of *CYCD2* and *CYCD3* in cycloheximide-treated suspension-culture cells, compared with hydroxyurea and the colchicine-treated liquid-cultured callus, underlines the caution that must be exercised when interpreting the results of experiments involving the treatment of cells with inhibitors, in which mechanisms of action and secondary effects are often poorly understood.

Expression of *CYC1*, *CYC2*, and *CYC3* in Synchronized Cells

Release of the synchronized culture from cycloheximide was followed by the transcript levels of *CYC1*, *CYC2*, and *CYC3*, reaching a maximum 16 h after release, coincident with the peak in mitotic index. These results are compatible with a role for these cyclins in G2/M. However, notably different kinetics of accumulation were observed, suggesting functional differences.

CYC3 mRNA accumulation started within 8 h after release, coincident with histone H4 expression, but later than cyclin D3 mRNA, accumulation of which started after 2 to 4 h and reached a maximum at 8 h. Therefore, it appears to be the first mitotic cyclin required during the cell cycle, with a role during the S phase. Further accumulation occurred during G2/M, followed by a decline as the mitotic index decreased. *CYC1* and *CYC2* were observed to accumulate with similar kinetics starting within 12 h after release, although the relative induction of *CYC1* was much greater than *CYC2*. Both were found to decline after 16 h, following the peak in mitotic index. We ascribe the failure of cyclin transcript levels to return to basal levels subsequent to mitosis to a decay in synchrony, due to a proportion of the population lagging in their progress through the cell cycle.

In contrast, colchicine-arrested *A. thaliana* suspension-culture cells had low levels of *CYC1At* transcript (Hemerley et al., 1992). However, the use of the more stringent inhibitor oryzalin on root tip cells resulted in cells express-

ing *CYC1At* at high levels (Ferreira et al., 1994b). The reason for this difference is probably the ability of oryzalin to arrest cells in metaphase for longer than colchicine (Sree Ramulu et al., 1991). The low levels of *CYC2* and *CYC3* transcripts in oryzalin-arrested root cells (Ferreira et al., 1994a) contrasts with the pattern of expression we report and with that of human mitotic cyclin transcripts in cells arrested at metaphase with the microtubule inhibitor nocodazole when elevated levels of both cyclin A and B transcripts are present (Pines and Hunter, 1990). The interpretation of oryzalin-arrested root-cell transcript levels is complicated by the length of time of inhibition. Similar treatment of potato suspension-cultured cells results in a significant proportion of cells forming micronuclei during arrest, and a high frequency of polyploid cells upon release (Sree Ramulu et al., 1991). The biochemical state of the *A. thaliana* cells during prolonged oryzalin arrest may therefore resemble more closely early G1 than metaphase. Human cyclin A transcripts are less stable than cyclin B transcripts in G1 (Pines and Hunter, 1990), and a similar difference between *A. thaliana* cyclins could explain the levels of mitotic cyclin transcript in oryzalin-arrested root cells while allowing for a peak at G2/M. The increase in *CYC3* transcript levels that we have observed starts in the S phase, significantly earlier than *CYC1* or *CYC2*. This is in agreement with high transcript levels of *CYC3* and low levels of *CYC1* and *CYC2* transcripts in hydroxyurea- arrested cells (Hemerley et al., 1992; Ferreira et al., 1994a).

Renaudin et al. (1996) have proposed a new nomenclature for plant cyclins, reflecting their relationship to animal cyclins. Three classes of plant cyclin A and two classes of plant cyclin B are defined, in contrast to the single cyclin A and three B cyclins found in vertebrates. The Arabidopsis cyclin *CYC1* falls into the B1 group, *CYC2* into the B2 group, and *CYC3* into the A2 group. These classifications allow us to relate the expression observed to that reported in other plant species. Setiady et al. (1995) examined expression of tobacco cyclins in synchronized BY-2 cells. The A-type cyclin genes *NtCYC25* (class A1) and *NtCYC27* (class A2) are induced within 3 h of an aphidicolin block, well before *NtCYC29*, a class B1 cyclin gene. This is consistent with our data that show earlier induction of *CYC3* (A2) than *CYC1* (B1) or *CYC2* (B2). Kouchi et al. (1995) used double in situ hybridization analysis on soybean shoot apices to show that a B1 cyclin (*CYC5Gm*) is expressed in the G2-to-M phases, whereas an A1 cyclin (*CYC3Gm*) is expressed from the late S-to-M phases. Kouchi et al. (1995) also report that the class A3 cyclin (*CYC1Gm*) is expressed only for a short period during the S phase, a pattern of expression observed only with this class of cyclin. These data and those we report here are therefore consistent with those for mammalian cyclins, in that cyclin A is expressed from the S-to-M phase, and B cyclins start to accumulate later, during G2 (Pines and Hunter, 1989, 1990).

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LITERATURE CITED

Abe K, Yoshida M, Usui T, Horinouchi S, Beppu T (1991) Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp Cell Res **192:** 122–127

Ajchenbaum F, Ando K, DeCaprio JA, Griffin JD (1993) Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T-lymphocytes. J Biol Chem **268:** 4113–4119

Ando K, Ajchenbaum-Cymbalista F, Griffin JD (1993) Regulation of G1/S transition by cyclins D2 and D3 in hematopoietic cells. Proc Natl Acad Sci USA **90:** 9571–9575

Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev **7:** 812–821

Bayliss MW (1985) Regulation of the cell division cycle in cultured plant cells. *In* JA Bryant, D Francis, eds, The Cell Division Cycle in Plants. Cambridge University Press, Cambridge, UK, pp 157–177

Brown R, Rickless P (1948) A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and of nutrients. Proc R Soc Lond Ser B Biol Sci **136:** 110–125

Campisi J, Medrano EE, Morreo G, Pardee AB (1982) Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells. Proc Natl Acad Sci USA **79:** 436–440

Cleveland DW, Yen TJ (1989) Multiple determinants of eukaryotic mRNA stability. New Biologist **1:** 121–126

Conia J, Alexander RG, Wilder ME, Richards KR, Rice ME, Jackson PJ (1990) Reversible accumulation of plant suspension cultures in G1 phase and subsequent synchronous traverse of the cell cycle. Plant Physiol **94:** 1568–1574

Crissman HA, Gadbois DM, Tobey RA, Bradbury EM (1991) Transformed mammalian cells are deficient in kinase-mediated control of progression through the G1 phase of the cell cycle. Proc Natl Acad Sci USA **88:** 7580–7584

Dahl M, Meskiene I, Bögre L, Ha DTC, Swoboda I, Hubmann R, Hirt H, Herberle-Bors E (1995) The D-type alfalfa cyclin gene *cycMs4* complements G1 cyclin-deficient yeast and is induced in the G1 phase of the cell cycle. Plant Cell **7:** 1847–1857

Darzynkiewicz Z, Evenson DP, Staiano-Coico L, Sharpless TK, Melamed ML (1979) Correlation between cell cycle duration and RNA content. Cell Physiol **100:** 425–438

Day IS, Reddy ASN (1994) Cloning a family of cyclins from *Arabidopsis thaliana*. Biochim Biophys Acta **1218:** 115–118

Doerner PW (1994) Cell cycle regulation in plants. Plant Physiol **106:** 823–827

Dou Q-P, Levin AH, Zhao S, Pardee AB (1993) Cyclin E and cyclin A as candidates for the restriction point protein. Cancer Res **53:** 1493–1497

Elledge SJ, Mulligan JT, Ramer SW, Spottswood M, Davis RW (1991) λYES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc Natl Acad Sci USA **88:** 1713–1735

Feinberg AP, Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **137:** 266–267

Ferreira P, Hemerley A, De Almeida Engler J, Bergounioux C, Burssens S, Van Montagu M, Engler G, Inzé D (1994a) Three discrete classes of *Arabidopsis* cyclins are expressed during different intervals of the cell cycle. Proc Natl Acad Sci USA **91:** 11313–11317

Ferreira PGC, Hemerly AS, Almeida Engler J, Van Montagu M, Engler G, Inzé D (1994b) Developmental expression of the Arabidopsis cyclin gene cyc1At. Plant Cell **6:** 1763–1774

Francis D, Halford NG (1995) The plant cell cycle. Physiol Plant **93:** 365–374

Franco AR, Gee MA, Guilfoyle TJ (1990) Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. J Biol Chem **265:** 15845–15849

Gadbois DM, Hamaguchi JR, Swank RA, Bradbury EM (1992) Staurosporine is a potent inhibitor of p34cdc2 and p34cdc2-like kinases. Biochem Biophys Res Commun **184:** 80–85

Gilbert GM, Nielson A, Miyazawa H, DePamphlis ML, Burnham WC (1995) Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide reductase. J Biol Chem **270:** 9597–9606

Gould AR, Everett NP, Wang TL, Street HE (1981) Studies on the control of the cell cycle in cultured plant cells. I. Effects of nutrient limitation and nutrient starvation. Protoplasma **106:** 1–13

Hadjilova KV, Nocoloso M, Mazan S, Hadjilov AA, Bachellerie J (1993) Alternative pre-rRNA processing in human cells and their alteration by cycloheximide inhibition of protein synthesis. Eur J Biochem **212:** 211–215

Hamel PA, Gallie BL, Phillips RA (1992) The retinoblastoma protein and cell cycle regulation. Trends Genet **8:** 180–185

Hanauskee-Abel HM, Slowinska B, Zagulska S, Wilson RC, Staiano-Coico L, Hanauske AR, McCaffery T, Szabo P (1995) Detection of polysomal mRNAs associated with modulation of hypusine formation at the G1-S boundary: proposal of a role for eI-5A in onset of DNA replication. FEBS Lett **366:** 92–98

Hemerley A, Bergounioux C, Van Montagu M, Inzé D, Ferreira P (1992) Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. Proc Natl Acad Sci USA **89:** 3295–3299

Hirt H, Mink M, Pfosser M, Bögre L, Györgyey J, Jonak C, Gartner A, Dudits D, Herberle-Bors E (1992) Alfalfa cyclins: differential expression during the cell cycle and in plant organs. Plant Cell **4:** 1531–1538

Jonak C, Páy A, Bögre L, Hirt H, Herberle-Bors E (1993) The plant homologue of MAP kinase is expressed in a cell cycle-dependent and organ-specific manner. Plant J **3:** 611–617

Katsuta J, Shibaoka H (1992) Inhibition by kinase inhibitors of the development and the disappearance of the preprophase band of microtubules in tobacco BY-2 cells. J Cell Sci **103:** 397–405

King RW, Jackson PK, Kirschner MW (1994) Mitosis in transition. Cell **79:** 563–571

Ko H, More SA (1990) Kinetic characterisation of a prestart cell division control step in yeast. J Biol Chem **265:** 21652–21663

Kodama H, Komamine A (1995) Cell cycle studies: induction of synchrony in suspension cultures of *Catharanthus roseus* cells. *In* K Lindsey, ed, Plant Tissue Culture Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–30

Koeller DM, Horowitz JA, Casey JL, Klausner RD, Harford JB (1991) Translation and the stability of mRNAs encoding the transferrin receptor and *c-fos*. Proc Natl Acad Sci USA **88:** 7778–7782

Kouchi H, Sekine M, Hata S (1995) Distinct classes of mitotic cyclins are differentially expressed in the soybean shoot apex during the cell cycle. Plant Cell **7:** 1143–1155

Levenson V, Hamlin JL (1993) A general protocol for evaluating the specific effects of DNA replication inhibitors. Nucleic Acids Res **21:** 3997–4004

Lew DJ, Dulic V, Reed SI (1991) Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell **66:** 1197–1206

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Matsushime H, Roussel M, Ashmun R, Sherr C (1991) Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell **65:** 701–713

May M, Leaver C (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. Plant Physiol **103:** 621–627

Meyerowitz EM, Pruitt RE (1985) *Arabidopsis thaliana* and plant molecular genetics. Science **229:** 1214–1218

Mosca PJ, Dijkwel PA, Hamlin JL (1992) The plant amino-acid mimosine may inhibit initiation at origins of replication in Chinese-hamster cells. Mol Cell Biol **12:** 4375–4383

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

Murray JAH (1994) The beginning of START. Plant Mol Biol **26:** 1–3

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. Int Rev Cytol **132:** 1–30

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

Ohnishi N, Kodama H, Satoshi A, Komanine A (1990) Synthesis of protein and mRNA is necessary for transition of suspension-cultured *Catharanthus roseus* cells from the G1 to the S phase of the cell cycle. Physiol Plant **80:** 95–101

Pachter JS, Yen TJ, Cleveland DW (1987) Autoregulation of tubulin expression is achieved through specific degradation of polysomal tubulin mRNAs. Cell **51:** 283–292

Pardee AB, Keyomarsi K (1992) Modification of cell proliferation with inhibitors. Curr Opin Cell Biol **4:** 186–191

Perennes C, Quin L-X, Glab N, Bergounioux C (1993) Petunia p34cdc2 kinase activity in G2/M cells obtained with a reversible cell cycle inhibitor, mimosine. FEBS Lett **333:** 141–145

Pines J (1995) Cyclins and cyclin-dependent kinases: theme and variations. Adv Cancer Res **66:** 181–212

Pines J, Hunter T (1989) Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell **58:** 833–846

Pines J, Hunter T (1990) Human cyclin A is adenovirus E1A-associated protein and behaves differently from cyclin B. Nature **346:** 760–763

Reichheld J-P, Sonobe S, Clément B, Chaubet N, Gigot C (1995) Cell cycle-regulated histone gene expression in synchronised plant cells. Plant J **7:** 245–252

Renaudin JP, Savouré A, Philippe H, VanMontagu M, Inzé D, Rouzé P (1996) Characterization and classification of plant cyclin sequences related to A- and B-type cyclins. *In* D Francis, D Dudits, D Inzé, eds, Plant Cell Division. Portland Press, London (in press)

Rossow PW, Riddle VGH, Pardee AB (1979) Synthesis of a labile, serum-dependent protein in early G1 controls animal cell growth. Proc Natl Acad Sci USA **76:** 4446–4450

Savouré A, Fehér A, Kaló P, Petrovics G, Csanádi G, Szécsi J, Kiss G, Brown S, Kondorosi A, Kondorosi E (1995) Isolation of a full-length cyclin cDNA clone CycIIIMs from *Medicago sativa*: chromosomal mapping and expression. Plant Mol Biol **27:** 1059–1070

Setiady YY, Sekine M, Hariguchi N, Yamamoto T, Kouchi H, Shinmyo A (1995) Tobacco mitotic cyclins: cloning, characterisation, gene expression, and functional assay. Plant J **8:** 949–957

Sewing A, Bürger C, Brüsselbach S, Schalk C, Lucibello FC, Müller R (1993) Human cyclin D encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. J Cell Sci **104:** 545–554

Sherr CJ (1993) Mammalian G1 cyclins. Cell **73:** 1059–1065

Sherr CJ (1994) G1 phase progression: cycling on cue. Cell **79:** 551–555

Sherr CJ (1995) D-type cyclins. Trends Biochem Sci **20:** 187–190

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

Sree Ramulu K, Verhoeven HA, Dijkhuis P (1991) Mitotic blocking, micronucleation, and chromosome doubling by

oryzalin, amoprophos-methyl, and colchicine in potato. Protoplasma **160:** 65–71

Stordeur P, Schandené L, Durez P, Gérard C, Goldman M, Velu T (1995) Spontaneous and cycloheximide-induced interleukin-10 mRNA expression in human mononuclear cells. Mol Immunol **32:** 233–239

Tobey RA, Oishi N, Crissman HA (1990) Cell cycle synchronization: reversible induction of G2 synchrony in cultured rodent and human diploid fibroblasts. Proc Natl Acad Sci USA **87:** 5104–5108

Van't Hof J, Kuniyuki A, Bjerknes CA (1978) The size and number of replicon families of chromosomal DNA of *Arabidopsis thaliana*. Chromosoma **68:** 269–285

Wadsworth GJ, Redinbaugh MG, Scandalios JG (1988) A procedure for small-scale isolation of plant RNA suitable for RNA blot analysis. Anal Biochem **172:** 279–283

Wang AS, Phillips RL, Mi CC (1982) Cell cycle parameters and doubling time of Black Mexican Sweet corn suspension cultures. Maize Genetics Cooperative News Letters **56:** 144–146

Wisdom R, Lee W (1991) The protein coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Genes Dev **5:** 232–243

Yeoman MM, Evans PK (1967) Growth and differentiation of plant tissue cultures. Ann Bot **31:** 323–332

Yoshida M, Yamamoto M, Nikaido T (1992) Quercitin arrests human T-cells in late G1 phase of the cell cycle. Cancer Res **52:** 6676–6681