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A Family of Cyclin D Homologs from Plants Differentially Controlled by Growth Regulators and Containing the Conserved Retinoblastoma Protein Interaction Motif

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A new family of three related cyclins has been identified in Arabidopsis by complementation of a yeast strain deficient in G_1 cyclins. Individual members show tissue-specific expression and are conserved in other plant species. They form a distinctive group of plant cyclins, which we named δ-type cyclins to indicate their similarities with mammalian D-type cyclins. The sequence relationships between δ and D cyclins include the N-terminal sequence LXCXE. This motif was originally identified in certain viral oncoproteins and is strongly implicated in binding to the retinoblastoma protein pRb. By analogy to mammalian cyclin D, these plant homologs may mediate growth and phytohormonal signals into the plant cell cycle. In support of this hypothesis, we show that, on restimulation of suspension-cultured cells, cyclin δ3 is rapidly induced by the plant growth regulator cytokinin and cyclin δ2 is induced by carbon source.

INTRODUCTION

Cell cycle progression appears to be regulated in all eukaryotes by major controls operating at the G_1-to-S phase and G_2-to-M phase boundaries. Passage through these control points requires the activation of cyclin-dependent kinases (cdks), whose catalytic activity and substrate specificity are determined by specific regulatory subunits known as cyclins and by interactions with other proteins that regulate the phosphorylation state of the complex (reviewed in Atherton-Fessler et al., 1993; Solomon, 1993). In budding and fission yeasts, both the G_1-to-S and G_2-to-M phase transitions are controlled by a single cdk, encoded by the *cdc2*$^+$ gene in *Schizosaccharomyces pombe* and by *CDC28* in *Saccharomyces cerevisiae.* The association of $p34^{cdc2}$ ($p34^{CDC28}$ in budding yeast) with different cyclin partners distinguishes the two control points (reviewed in Nasmyth, 1993). In mammalian cells, a more complex situation prevails, with at least six related but distinct cdks (cdc2/cdk1, cdk2, cdk3, cdk4, cdk5, and cdk6) having distinct roles, each in conjunction with one or more cognate cyclin partners (Fang and Newport, 1991; Meyerson et al., 1991, 1992; Xiong et al., 1992b; Tsai et al., 1993a; van den Heuvel and Harlow, 1993; Meyerson and Harlow, 1994).

B-type cyclins are the major class involved in the G_2-to-M transition and associate with $p34^{cdc22}$ or its direct homologs (reviewed in Nurse, 1990). Cyclin B is one of two cyclins originally described as accumulating in invertebrate eggs during interphase and rapidly destroyed in mitosis (Evans et al., 1983), and it is a component of Xenopus maturation-promoting factor (Murray et al., 1989). Subsequently, cyclin B homologs have been identified from many eukaryotic species. Cyclin A is also of widespread occurrence in multicellular organisms, and its precise role is unclear, although its peak of abundance suggests a function in S phase (reviewed in Pines, 1993).

The G_1-to-S phase transition is best understood in *S. cerevisiae.* Genetic studies define a point late in G_1 called START. After passing START, cells are committed to enter S phase and to complete a full additional round of division, which will result in two daughter cells again in G_1 phase (Hartwell, 1974; reviewed in Nasmyth, 1993). The products of three *S. cerevisiae* G_1 cyclin genes called *CLN1*, *CLN2*, and *CLN3* are the principal limiting components for passage through START (Richardson et al., 1989; Wittenberg et al., 1990; Tyers et al., 1993). Transcription of *CLN1* and *CLN2* is activated in G_1, and accumulation of their protein products to a critical threshold level by a positive feedback mechanism leads to activation of the $p34^{CDC28}$ kinase and transition through START (Dirick and Nasmyth, 1991). The G_1 cyclins are then degraded as a consequence of PEST motifs in their primary sequence that appear to result in rapid protein turnover (Rogers et al., 1986; Lew et al., 1991; reviewed in Reed, 1991).

The *S. cerevisiae* G_1 cyclins are at least partially redundant, because yeast strains in which two of the three G_1 cyclin genes are deleted and the third placed under the control of a galactose-regulated promoter show a galactose-dependent growth phenotype. Such strains have been used to identify Drosophila and human cDNA clones that rescue this conditional *cln*-

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deficient phenotype on glucose plates when the single yeast *CLN* gene present is repressed (Koff et al., 1991; Lahue et al., 1991; Léopold and O'Farrell, 1991; Lew et al., 1991; Xiong et al., 1991). Human cDNAs encoding three new classes of cyclins, C, D, and E, were identified by this means. Although these cyclins show only limited homology with the yeast CLN proteins, they have proved important for understanding controls that operate in mammalian cells during G_1 and at the restriction point at the G_1-to-S phase boundary (Pardee, 1989; Matsushime et al., 1992; Koff et al., 1992, 1993; Ando et al., 1993; Quelle et al., 1993; Tsai et al., 1993b). Cyclin E may act as a rate-limiting component at the G_1-to-S phase boundary (Ohtsubo and Roberts, 1993; Wimmel et al., 1994), whereas the dependency of cyclin D levels on serum growth factors (Matsushime et al., 1991; Baldin et al., 1993; Sewing et al., 1993) suggests that they may form a link between these signals and cell cycle progression.

Plant cells were used in early studies of cell growth and division to define the discrete phases of the eukaryotic cell cycle (Howard and Pelc, 1953), but there is a paucity of data on molecular cell cycle control in plant systems. Plant cells that cease dividing in vivo due to dormancy, or in vitro due to nutrient starvation, arrest at principal control points in G_1 and G_2 (van't Hof and Kovacs, 1972; Gould et al., 1981; reviewed in van't Hof, 1985); this is in general agreement with the controls operating in other eukaryotic systems. Although mature plant cells may be found with either a G_1 or a G_2 DNA content (Evans and van't Hof, 1974; Gould et al., 1981), the G_1 population generally predominates. The G_1 control point is found to be more stringent in cultured plant cells subjected to nitrogen starvation; these cells arrest exclusively in G_1 phase (Gould et al., 1981). Strong analogies thus exist between the principal control point in G_1 of the plant cell cycle, the START control in yeasts, and the restriction point of mammalian cells.

Antibodies or histone H1 kinase assays have been used to indicate the presence and localization of active cdc2-related kinases in plant cells (John et al., 1989, 1990, 1991; Mineyuki et al., 1991; Chiatante et al., 1993; Colasanti et al., 1993; reviewed in John et al., 1993), and cDNAs encoding functional homologs of cdc2 kinase have been isolated by reduced stringency hybridization or redundant polymerase chain reaction from a number of plant species, including pea (Feiler and Jacobs, 1990), alfalfa (Hirt et al., 1991, 1993), Arabidopsis (Ferreira et al., 1991; Hirayama et al., 1991), soybean (Miao et al., 1993), Antirrhinum (Fobert et al., 1994), and maize (Colasanti et al., 1991). A number of cDNA sequences encoding plant mitotic cyclins with A- or B-type characteristics or having mixed A- and B-type features have also been isolated from various species, including carrot (Hata et al., 1991), soybean (Hata et al., 1991), Arabidopsis (Hemerly et al., 1992; Day and Reddy, 1994), alfalfa (Hirt et al., 1992), Antirrhinum (Fobert et al., 1994), and maize (Renaudin et al., 1994). No cyclins with G_1 characteristics have previously been reported from plants.

Because the understanding of G_1 controls operating in mammalian cells has been significantly advanced by cyclins isolated by yeast complementation, we have adopted a similar approach to isolate plant cyclins. We report here three members of a new class of cyclins (δ types), which all contain a conserved motif known in mammalian cells to bind retinoblastoma (pRb) and related proteins. The only proteins previously reported to contain this motif are the transforming viral proteins adenovirus E1A, human papillomavirus E7 and simian virus 40 (SV40) T antigen, and mammalian D-type cyclins, all of which are known to interact directly with pRb (reviewed in Wiman, 1993). This suggests that pRb homologs, known to be important regulators of cell proliferation and differentiation but previously reported only in mammals, may be present in plants and may perform an analogous function, namely, linking growth and cell cycle control. Intriguingly, just as mammalian D-type cyclins are regulated by growth factors, so was one of the plant cyclins that we isolated. It was induced by kinetin, a phytohormone of the cytokinin group, whereas a second responded to carbon source availability. These observations suggest that δ-type cyclins and potential plant Rb homologs may play key roles in controlling plant cell division and differentiation.

RESULTS

Isolation of Plant Cyclins by Yeast Complementation

Cyclins are a group of proteins with rather weak conservation at the sequence level, but they are functionally conserved to the extent that many cyclins from heterologous species can complement cyclin deficiencies in yeast strains. Complementation of yeast strains deficient in G_1 cyclins has been successfully used to identify human C-, D-, and E-type cyclins and the Drosophila C-type cyclin (Koff et al., 1991; Lahue et al., 1991; Léopold and O'Farrell, 1991; Lew et al., 1991; Xiong et al., 1991); these would not have been found by hybridization with mitotic cyclin probes.

We adopted a similar approach to identify novel plant cyclins. The strain BF305-15d #21 (Xiong et al., 1991) has a galactose-dependent growth phenotype due to deletion of *CLN1* and *CLN2* and placement of *CLN3* under the control of the galactose-inducible promoter of the galactose epimerase (*GAL10*) gene. Cells of this strain grow normally on YEPGR media containing galactose (Figure 1B) but cease division when transferred to glucose media (Figure 1A). We found the reversion frequency of this strain on selective glucose plates to be $<10^{-6}$, in agreement with Xiong et al. (1991). This strain was transformed with an Arabidopsis cDNA library constructed in the expression vector pFL61 (Minet et al., 1992), in which plant cDNAs are cloned between the yeast glucose-inducible phosphoglycerate kinase (*PGK*) promoter and *PGK 3'* transcriptional signals. The pFL61 vector has a *URA3* marker for selection in yeast, and sequences from the yeast 2-μm plasmid provide yeast replication and partition functions.

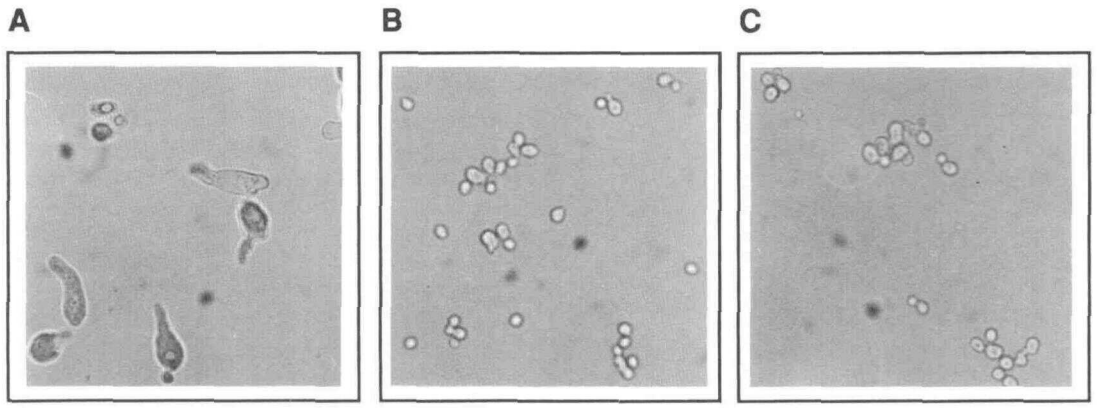

Figure 1. Complementation of G_1 Cyclin-Deficient Yeast Cells with cDNA Clone J3 (Cyclin δ3).

(A) Cells of BF305-15d #21 (*cln1*Δ, *cln2*Δ, and *GAL1-CLN3*) transferred to YPD (glucose-containing) medium do not express any G_1 cyclins and cease dividing. Cells are enlarged and abnormal.

(B) Cells of BF305-15d #21 in YEPGR (galactose–raffinose) medium grow normally, because under these conditions the *GAL1* promoter is induced and *CLN3* is expressed.

(C) Rescue of BF305-15d #21 cells grown in YPD and normal growth when the J3 cDNA is expressed in the vector pFL61. Similar results were obtained with the other cDNA clones shown in Figure 2B.

This library thus contains Arabidopsis cDNAs that can be expressed in yeast, and screening of $>3 \times 10^6$ yeast transformants in 10 separate transformation experiments resulted in the growth of ~70 colonies. Some of the colonies appeared after several weeks. Fourteen plasmid clones were successfully rescued into *Escherichia coli* and shown to recomplement the strain BF305-15d #21 upon retransformation. They also restored the normal appearance of cells under microscopic examination (Figure 1C).

Complementation by these cDNA clones was assessed both by direct selection of transformants on glucose plates and by micromanipulation of individual cells from galactose-grown cultures onto glucose and galactose plates (data not shown). This demonstrated unambiguously that cell proliferation of the transformed yeast cells was occurring under conditions in which the parent strain could not grow. These plant cDNA clones were thus competent to catalyze the G_1-to-S phase transition in yeast in the absence of endogenous yeast G_1 cyclins. Isolated cDNAs were classified on the basis of restriction (Figure 2A) and hybridization (data not shown) analyses into four groups (Figure 2B) of cDNAs, all but one of which is represented by several independent cDNA isolates.

Although human and Drosophila G_1/S cyclins were cloned by functional complementation of conditional *S. cerevisiae* *cln*$^-$ strains, this approach does not always unambiguously identify G_1 cyclins because truncated human cyclin A and B cDNA clones and the Drosophila homolog of the cdc2 kinase can also overcome the block to growth in G_1 cyclin-deficient yeast strains and give rise to a significant background of rescued colonies (Léopold and O'Farrell, 1991; Lew et al., 1991). We therefore used an additional yeast assay to assess the possible identity of the clones by their ability to rescue the temperature sensitive (ts) phenotype of the strain JO221, which carries an *swi4* mutation (Ogas et al., 1991). *swi4* mutants exhibit G_1 arrest at the restrictive temperature because they fail to induce the expression of the *CLN1* and *CLN2* genes (the accumulation of their gene products is required for START) but can be rescued by constitutive high-level expression of yeast G_1 cyclins (Dirick and Nasmyth, 1991; Ogas et al., 1991). Exogenous expression of a cyclin capable of replacing yeast G_1 cyclins would therefore be predicted to suppress the ts phenotype of a *swi4*^{ts} strain, such as JO221. Certain clones of the J1 and J3 groups permitted growth of JO221 at 37°C in both the transformation and micromanipulation assays (Figure 1 and data not shown). The failure of other cDNA clones in the same groups to suppress the phenotype suggests that the expression level of the plant cyclin may be more critical for *swi4* rescue than for direct rescue of total lack of G_1 cyclins. This is supported by sequence analysis of the clone R9 of the J3 group (see the following discussion), which is unable to rescue the *swi4*^{ts} strain. A more extensive upstream region is found in R9, which includes an additional ATG initiation codon not present in the shorter J3 clone. This would be expected to reduce significantly translation of the cyclin in yeast. Similarly, J22, which does not rescue the *swi4*^{ts} defect of JO221, contains upstream ATGs. A higher level of cyclin expression may therefore be necessary to rescue *swi4* mutants; this is consistent with a wider role for SWI4 in yeast than simply inducing G_1 cyclin expression (Epstein and Cross, 1994).

During restriction analysis of the clones, we observed that certain clones (including J3 and J22) were in the opposite orientation in the pFL61 vector compared with other clones in the same groups and, therefore, incorrectly oriented for expression from the yeast *PGK* promoter on the plasmid. These clones

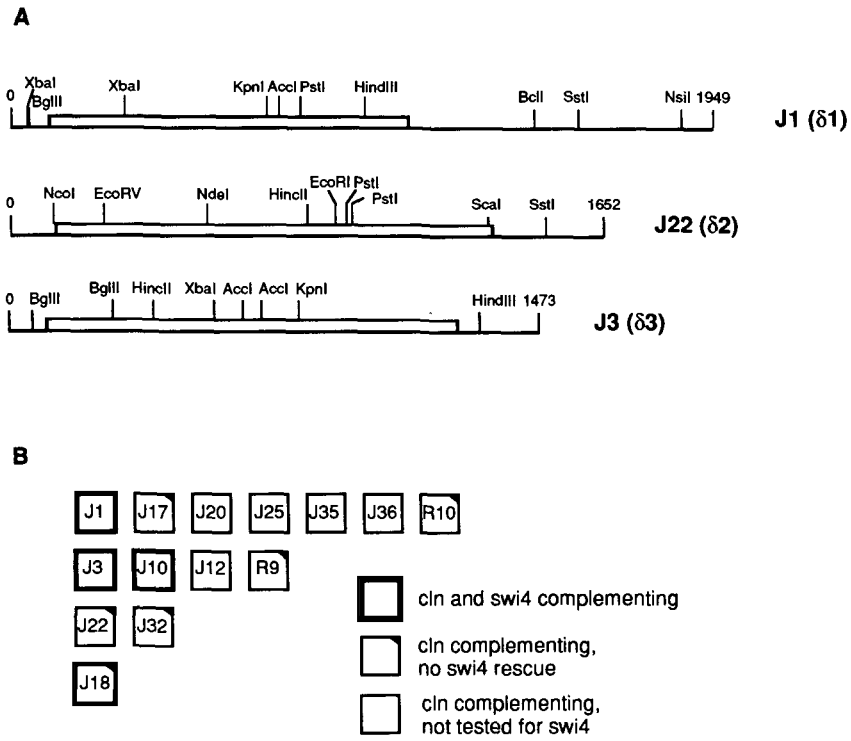

Figure 2. Classification of cDNA Clones.

(A) Plant cDNAs were be excised from the pFL61 vector as NotI fragments, and restriction maps of the NotI inserts of J1, J3, and J22 clones are shown. The coding regions are boxed.

(B) Independent cDNA clones were classified by restriction mapping and hybridization analysis (data not shown). The groups, shown horizontally, are classified according to the lowest numbered clone of that group. All cDNAs were tested for their ability to recomplement the G_1 cyclin deficiency of BF305-15d #21 on glucose plates (cln complementing), as described in Figure 1, and some were tested for complementation of the *swi4*^{ts} mutation of JO221.

rescued the *cln*$^-$ defect at least as efficiently in our assays as correctly oriented clones (J10 and J32). We infer that other promoter sequences present on the plasmid, readthrough from the plasmid *URA3* gene, or upstream plant sequences with yeast promoter activity are responsible for expression of these clones.

Sequence Relationships in the Conserved Cyclin Box

Sequence analysis of clones in all four groups (Figures 3 to 5) showed that the cDNA clones represented by J1, J22, J3, and J18 encode open reading frames whose translation indicated novel proteins with cyclin characteristics. The first three form a family of related cyclins, whose analysis is presented here, whereas J18 represents an additional unrelated cyclin (data not shown). Comparisons of the sequences of J1, J22, and J3 with the OWL data base (compilation of SwissProt, PIR, and GenBank) using FASTA show the greatest relatedness to mammalian G_1 cyclins, particularly D types, with similarities to some fungal G_1 cyclins also scoring higher than mitotic cyclins (Figure 6). Similar results were obtained using the software program BLAST. For a more detailed comparison of our new sequences with other cyclins over their full length, we compiled all of the cyclin sequences available; by using the pairwise comparison software program PILEUP, we found that the three new Arabidopsis cyclins form a new group that is clearly distinct from the previously reported plant mitotic cyclins (data not shown). The exact arrangement of the tree changed when different numbers of sequences were used, but we consistently found that our cyclins formed a separate group always lying near the mammalian G_1 cyclins, particularly the D types. We therefore propose that these cyclins form a new family of plant "D-like" cyclins, which we call δ-type cyclins. This name is chosen to reflect conveniently the sequence similarities and possible analogous functions of plant δ-type cyclins to mammalian D types, without necessarily implying that they are exact homologs. Each of the Arabidopsis δ-type cyclins has ~30% sequence identity with the other two.

Cyclins are characterized by a central domain of higher conservation known as the cyclin box. Alignment of δ-type cyclins with a selection of published plant mitotic cyclins and human

cyclins shows the extent of conservation across this region (Figure 7). Mitotic cyclins generally contain a "destruction box" with variants of the sequence RXXL$(X)_{2-4}$XXN (Nugent et al., 1991), which has been suggested to be involved in degradation of these proteins by the ubiquitin pathway (Glotzer et al., 1991). G_1 cyclins do not have a destruction box but normally contain "PEST" sequences, which are rich in proline, glutamate, serine, and threonine, that are responsible for their rapid turnover (Rogers et al., 1986; reviewed in Rechsteiner, 1990). PEST sequences are found in human cyclins C, D, and E (Lew et al., 1991), *S. cerevisiae* G_1 cyclins (*CLN1*, *CLN2*, and *CLN3*; reviewed in Reed, 1991), and Drosophila cyclin E (Richardson et al., 1993). The program PESTFIND (Rogers et al., 1986) can be used to locate PEST sequences and allocate a "score" that reflects their significance. A positive score is indicative of a likely PEST sequence. Using this program, we determined the locations of PEST sequences within the plant δ-type cyclins (Figures 3 to 5). Cyclin δ1 contains one N-terminal and two C-terminal potential PEST sequences (residues 11 to 60, 253 to 265, and 306 to 316 with PESTFIND scores of +2, +2, and +13, respectively), whereas cyclins δ2 and δ3 each have two potential PEST sequences (δ2, residues 42 to 66, PESTFIND scoring +1, and residues 241 to 266, scoring +1; δ3, residues 28 to 69, scoring +5, and residues 302 to 351, scoring +11).

Rb Interaction Motif

A notable feature of the mammalian D-type cyclins is that they specifically bind to the product of the retinoblastoma susceptibility gene pRb and to the related proteins p105 and p130. This interaction occurs between the "pocket" binding domain on pRb and its homologs and a motif LXCXE at the N terminus of the D-type cyclins (in single-letter code; X being any amino acid). The pocket domain of Rb was originally defined as the binding site of the transforming viral oncoproteins SV40 T antigen, adenovirus E1A, and human papillomavirus E7, and these viral proteins also contain the LXCXE motif. Given the similarity, which was indicated by FASTA, of the new plant δ-type cyclins with mammalian D types, we examined their sequences for this motif. All three δ-type cyclins contain an LXCXE motif near their N termini (Figures 3 to 5 and Figure 8), and in each case, it is preceded by at least one acidic residue (D or E) in a context that is also found in the D-type cyclins and in the

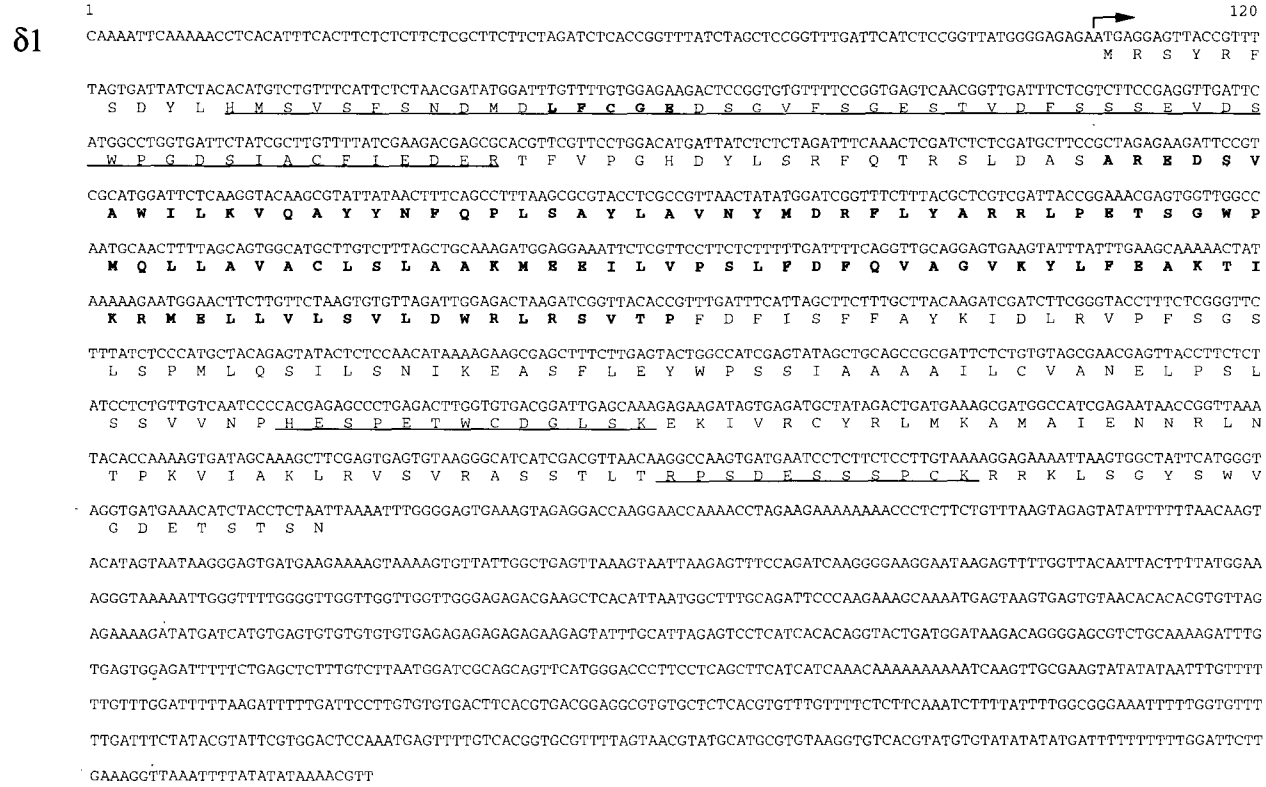

Figure 3. Sequence of the cDNA Encoding Arabidopsis Cyclin δ1.

The sequence of cDNA J1 encoding cyclin δ1 is shown. The start of the open reading frames is indicated by an arrow, and the potential PEST sequences are underlined (see text). The cyclin box and LXCXE motifs are shown in boldface letters. Cyclin δ1 is a protein of 334 amino acids (predicted molecular mass of 37.7 kD). The EMBL accession number for this sequence is X83369.

Figure 4. Sequence of the cDNA Encoding Arabidopsis Cyclin δ2.

The sequence of cDNA J22 encoding cyclin δ2 is shown. The upstream ATGs present in the J22 cDNA are double underlined. Other features are as given in Figure 3. Cyclin δ2 is a protein of 383 amino acids (predicted molecular mass of 42.9 kD). The EMBL accession number for this sequence is X83370.

viral oncoproteins. This sequence is not present in the other full-length plant cyclin sequences found in the data bases; neither is it present in the cyclin J18, which is not a δ-type cyclin (G.L. Cavet, J.P. Carmichael, R. Soni, and J.A.H. Murray, manuscript in preparation). The presence of the LXCXE motif in all three δ-type cyclins suggests that Rb protein homologs may occur in plants. This provides evidence that pRb homologs could exist outside vertebrates and may also implicate the δ-type cyclins in plant cell proliferation and differentiation control in G_1.

Genomic Arrangement and Conservation between Species

DNA gel blots of Arabidopsis DNA cleaved with a number of restriction enzymes indicate that all three δ-type cyclins are represented by single genes (Figure 9). The band sizes determined by genomic DNA gel blot analysis are larger than would be predicted from the cDNA sequence, suggesting that all three genes appear to contain introns of at least 850 bp in δ1, 550 bp in δ2, and 400 bp in δ3.

We also determined the extent of conservation of these genes in a range of other dicotyledonous plants. We found one to five hybridizing bands in tobacco, Jerusalem artichoke, cauliflower, and Antirrhinum (data not shown). With cyclin δ2 and δ3 probes under reduced-stringency conditions, additional, less strongly hybridizing bands were also observed in Arabidopsis. One additional band is present in δ2, which in some experiments is detectable even at normal hybridization stringency (Figure 9; δ2 panel, HindIII and BglII lanes), and up to three additional bands are seen with δ3. Based on known band sizes from other DNA gel blot experiments (data not shown), we were able to conclude that these additional bands are not due to cross-hybridization between cloned members of the δ-type cyclin family. We concluded that there may be another member of the δ-type cyclin family in Arabidopsis related to δ2 and possibly three other δ3-related genes. Nevertheless, there is a greater degree of conservation between δ-class members in different species than between different δ-class cyclins within Arabidopsis, suggesting that the functions of different δ-type cyclins are nonredundant. This conclusion is also supported by the differences in response of δ-type cyclins to growth stimuli (see following discussion).

Tissue-Specific Expression

The steady state level of mRNA of δ-type cyclins in various tissues of mature Arabidopsis plants showed that cyclin δ3 is represented by a single transcript of ~1.5 to 1.7 kb, which is ~15-fold more abundant in root tissue than in leaves and

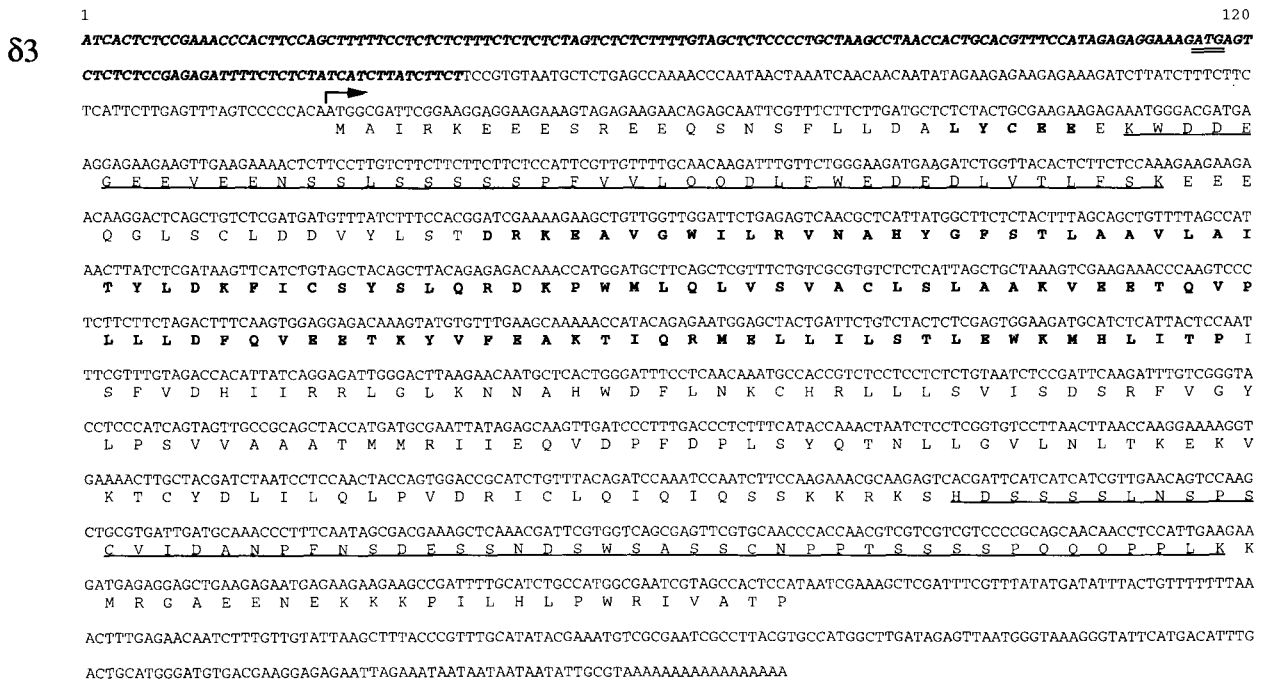

Figure 5. Sequences of the cDNAs Encoding Arabidopsis Cyclin δ3.

The sequences of cDNAs J3 and R9 encoding cyclin δ3 are shown. R9 has a longer 5′ nontranslated region than J3, and this additional sequence is shown in boldface italic letters. R9 contains an upstream ATG (double underlined). Other features are as given in Figure 3. Cyclin δ3 is a protein of 376 amino acids (predicted molecular mass of 42.8 kD). The EMBL accession number for these sequences is X83371.

fivefold more abundant than in flowers (Figure 10; note that the fourth lane contains 12 μg of total root RNA, whereas all other lanes contain 30 μg). The same RNA gel blot was reprobed with cyclin δ1, revealing the presence of three distinct transcripts of approximately 1.5 to 1.7, 2.2 to 2.3, and 3.0 to 3.2 kb with differing abundance in various tissues. There is a particularly striking difference between leaf and flower in the abundance of the longest (>3 kb) transcript. Cyclin δ2 is present as a transcript of 1.5 to 1.7 kb in approximately equal amounts in leaf and root tissue (data not shown). It is interesting to note that, although all three cyclins are expressed in callus-derived suspension-cultured cells, they are relatively less abundant in this dedifferentiated tissue than in regions of the plant undergoing meristematic growth to produce defined cell types. Abundance was found to be very low in the stem, where little cell division occurs.

Timing of Expression

Plant cells can be reversibly arrested at the G_1-to-S phase boundary by inhibition of DNA synthesis with hydroxyurea (HU;

δ1

	initn score
mouse D1	161
rat D1	161
human D1	153
yeast CLB1	131
mouse D2	130
rat D2	130
human D2	127
soybean B S13-7	123

δ2

	initn score
human D1	188
rat D1	188
mouse D1	183
rat D2	168
human D2	168
mouse D2	168
rat E	166
human E	145

δ3

	initn score
human D1	186
rat D2	177
mouse D2	164
human D2	163
mouse D1	147
rat D1	145
S.pombe puc1	110
C. albicans CLN1	102

Figure 6. Sequence Relationships of δ-Type Cyclins.

Results of the FASTA output using cyclin δ sequences and a wordsize of 2 are given. The initn score gives a measure of the overall similarity of the compared sequences.

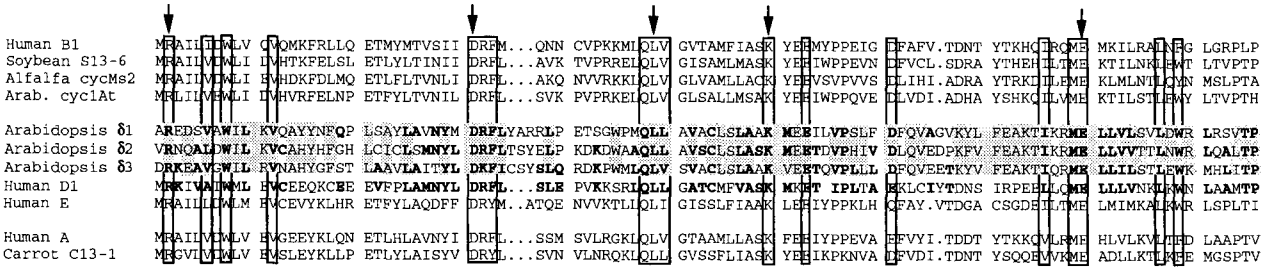

Figure 7. Cyclin Box of δ Cyclins Compared with Representative Plant and Human Cyclins.

Comparison of the cyclin box of δ-type cyclins, human cyclins A, B, D, and E, and representative plant mitotic cyclins is shown. Alfalfa cycMs2 (Hirt et al., 1992), Arabidopsis (Arab.) cyc1At (Hemerly et al., 1992), and soybean S13-6 are classed as B-like cyclins (Renaudin et al., 1994), whereas carrot C13-1 (Hata et al., 1991) is classed as A-like (Renaudin et al., 1994). Identical amino acid residues or those showing conservative substitutions among all three δ types are shaded, conservative residues shared by all cyclins shown are boxed, and conservative residues among any of the δ types and human cyclin D1 are shown in boldface letters. Arrows indicate the five most highly conserved residues of the cyclin box. Conservative substitutions are defined in the groups AVIL, QDEN, PG, HYFW, MC, ST, and KR.

Conia et al., 1990), which inhibits the enzyme ribonucleotide reductase. To investigate the timing of expression in the cell cycle, suspension cultures of Arabidopsis cells derived from callus tissue (see Methods) were treated with HU for 48 hr. The HU block was released by placing washed cells in fresh media without HU. The resumption of S phase was monitored by measuring the incorporation of ^{3}H-thymidine into DNA (see Methods) and by following the expression of histone H4, which exhibits substantially increased expression in S phase (Lepetit et al., 1992; Minami et al., 1993). After a lag phase, the incorporation of ^{3}H-thymidine started to rise strongly at 12 hr and peaked within the 12- to 15-hr time interval (Figure 11A; note that incorporation is measured over a 3-hr period indicated by the width of the bars). Gel blots of RNA extracted from cells at the time points indicated (Figure 11B) show that expression of histone H4 is reduced after 48 hr of HU block (0 hr release) compared with the same cells before imposition of the block

Ad5	E1a	EVI**D**L**T**C**H**E**A**GFPPSDDE
SV40	T-Ag	E E**N**L**F**C**S**E**E**M PSSDDE
HPV-16	E7	ETT**D**L**Y**C**Y**E**Q**LNDSSEEE
Arab. cyclin δ1		NDM**D**L**F**C**G**E**D**SGVFSGES
Arab. cyclin δ2		MA**E**N**L**A**C**G**E**TASHGSLTT
Arab. cyclin δ3		LL**D**A**L**Y**C**E**E**EKWDDEGEE
human cyclin D1		M**E**HQ**L**L**C**C**E**VETIRRAYP
human cyclin D2		M**E**L**L**C**H**E**V**PDVRRAVR
human cyclin D3		M**E**L**L**C**C**E**G**TRHAPRAG

Figure 8. LXCXE Motifs Present in Known pRb-Interacting Proteins and δ Cyclins.

The LXCXE motif, present near the N termini of mammalian D-type cyclins and in the viral oncoproteins adenovirus (Ad5) E1A, SV40 T antigen (T-Ag), and human papillomavirus type 16 (HPV-16) E7, is also present in Arabidopsis (Arab.) δ-type cyclins. The motif is preceded by an acidic residue (D or E; shown in boldface type).

(−48 hr C). Histone H4 expression increased markedly in the RNA sample taken at 12 hr, which was marginally before the peak of DNA synthesis. The same blot was reprobed without stripping with the Ac16 control probe derived from an Arabidopsis cDNA for porphobilinogen deaminase. This allowed accurate quantitation and normalization of the histone signal because the histone and Ac16 signals are well separated.

An identical blot was probed with cyclin δ3, which showed a reduction in expression after the HU block and a dramatic increase in steady state mRNA levels at the 9-hr time point (Figure 11B). This high level of cyclin δ3 mRNA continued until the end of the experiment at 18 hr.

In a second parallel experiment, an aliquot of the same starting culture of cells was treated with 0.05% colchicine to block cells in mitosis. After 48 hr, RNA was prepared from these cells, and the results are shown in the RNA gel blots in Figure 11B (lanes labeled COL). The expression levels after 48 hr of colchicine block may be compared with the cells before blocking (lanes labeled −48 hr C). Cyclin δ3 is expressed at very low levels in colchicine-blocked cells, as is histone H4; this latter finding is in agreement with the results reported by Hemerly et al. (1992). The level of the Ac16 control probe remains unaffected. Probing a blot that was equivalent to the one shown in Figure 11B with an Arabidopsis mitotic *cycAt1* probe (Hemerly et al., 1992) showed that no significant induction of this mitotic cyclin occurred after release from the HU block during the time frame of the experiment (data not shown). The cells in this experiment therefore did not progress to the G_2/M boundary.

These results show that cyclin δ3 is expressed in a cell division–dependent manner and, after release of an HU block, that high levels of cyclin δ3 transcript accumulate slightly before peak steady state levels of histone H4 are reached. This suggests that cyclin δ3 is not a mitotic cyclin; this conclusion is presented in more detail in the following discussion.

When identical blots of HU-blocked and HU-released cells were probed with cyclins δ1 and δ2, no significant changes

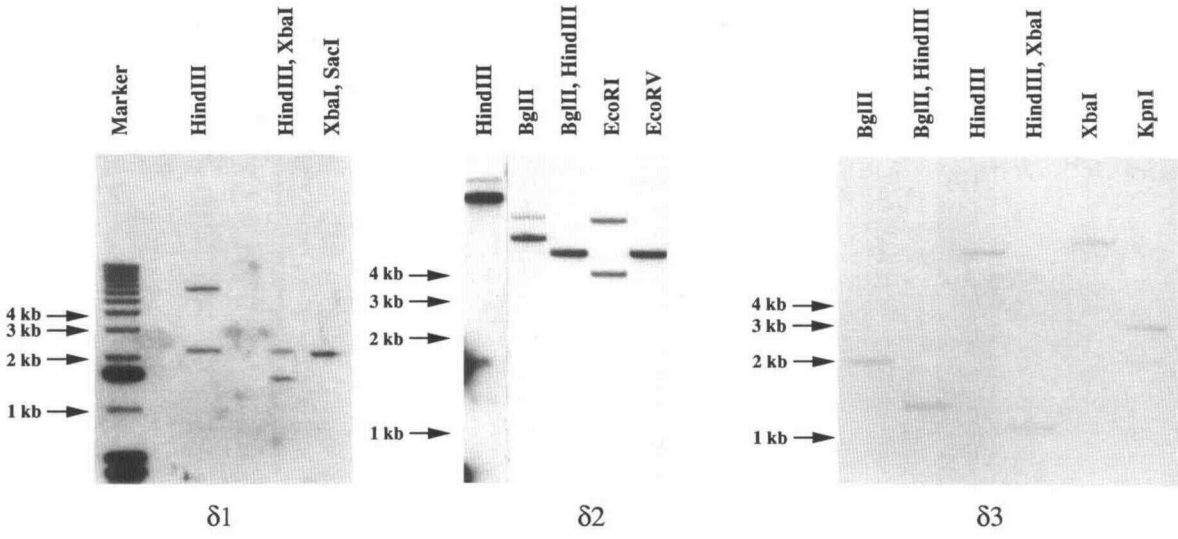

Figure 9. DNA Gel Blot Analysis of Arabidopsis δ-Type Cyclins.

DNA gel blots are shown of Arabidopsis genomic DNA digested with the enzymes indicated and hybridized to probes from cDNAs encoding δ1 (XbaI-SstI), δ2 (NcoI-ScaI), and δ3 (HindIII-XbaI). In this particular experiment, an extra band is observable with the δ2 probe at normal stringency (65°C). Molecular length markers are given at left in kilobases.

in expression were observed after the release of the block. Transcript levels remained low for the duration of the experiment. The colchicine block also resulted in low or undetectable transcript levels. Taken together, these results indicate that cyclins δ1 and δ2 may have their principal expression during G_1 phase before the point at which HU blocks cell cycle progression, whereas δ3 may be expressed later or over a longer period extending into S phase.

To confirm the timing of cyclin δ3 expression, actively growing suspension-cultured cells were resuspended in medium lacking nitrate. Nitrate starvation is known to lead to accumulation of cells in the G_1 phase of the cell cycle (Gould et al., 1981; De la Torre et al., 1989). After 48 hr without nitrate, the abundance of cyclin δ3 mRNA decreased to an almost undetectable level. Readdition of nitrate led to reaccumulation of cyclin δ3 transcript slightly before the peak of DNA synthesis (data not shown). In contrast, the levels of *cdc2a* and cyclin δ2 mRNAs were unaffected by nitrate starvation and readdition.

Induction by Carbon Source and Phytohormones

The sequence similarities between δ-type cyclins and mammalian D types suggested that the plant cyclins might also have a functional role in responding to growth signals. Suspension cultures provide a source of material whose environment can be readily manipulated to study the effects of hormonal and nutritional signals on gene expression and the cell cycle (King, 1977; Gould et al., 1981; Gould, 1984; Bayliss, 1985; Nagata et al., 1992). We used suspension cultures derived from callus tissue growing out from seed germinated in liquid media (see

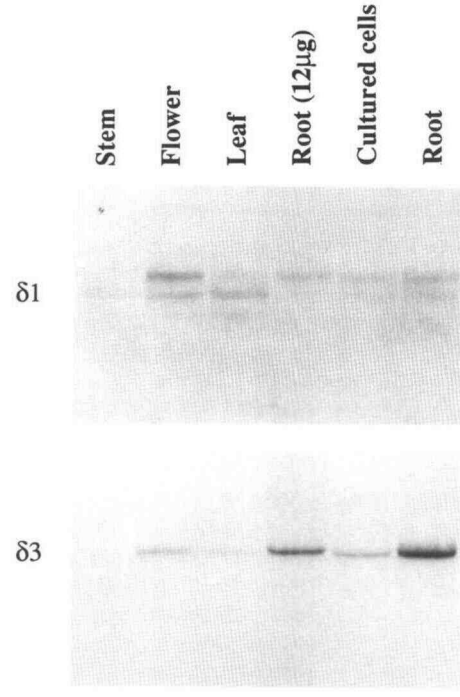

Figure 10. Expression of δ-Type Cyclins in Arabidopsis Tissues.

A gel blot of RNA extracted from the Arabidopsis tissues indicated and from suspension-cultured cells is shown. Total RNA was prepared from these tissues, which were harvested complete from young plants. Thirty micrograms of RNA was loaded in each lane, except in the fourth lane, which contains 12 μg of root RNA. The blot was probed with the HindIII-XbaI fragment of cyclin δ3, stripped, and reprobed with the XbaI-SstI fragment of cyclin δ1.

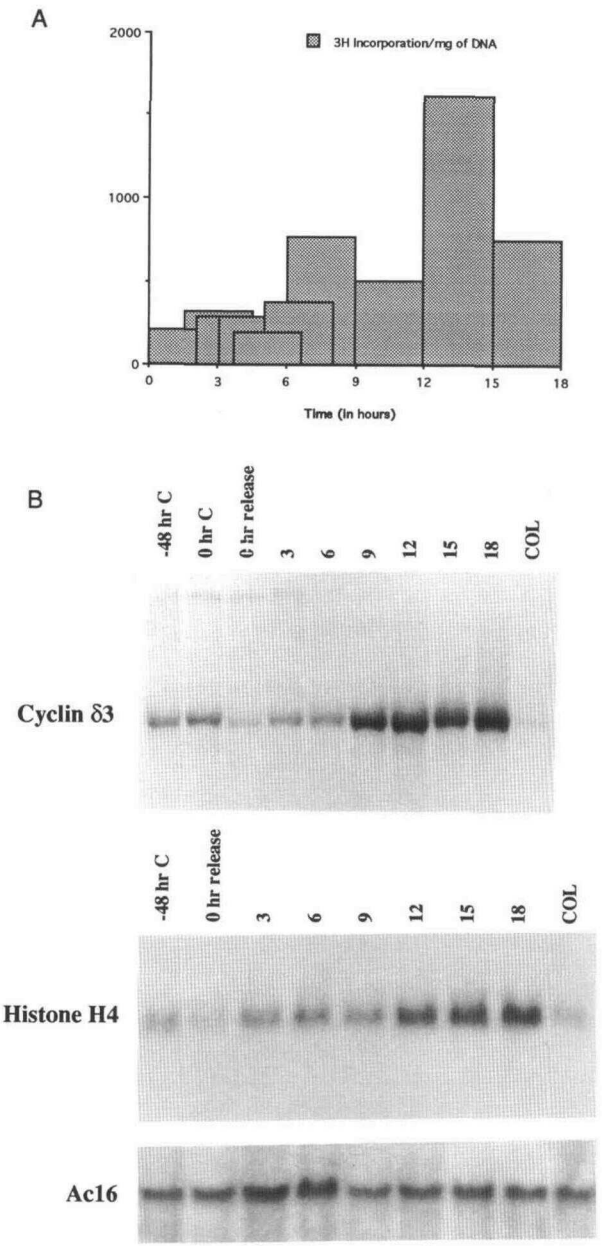

Figure 11. Cell Cycle Expression of Cyclin δ3.

HU block–release of suspension-cultured cells was as described in Methods.

(A) Incorporation of ^{3}H-thymidine into DNA was determined after incubation of aliquots of cells for 3 hr with ^{3}H-thymidine. Incorporation was determined with duplicate samples that were corrected for background incorporation before release and is expressed per milligram of total DNA extracted from each aliquot. Bars overlap when the incorporation period was longer than the time interval between samples.

(B) RNA was extracted from samples at the time points indicated before adding HU (−48 hr C), at the time of release after 48 hr of HU treatment (0 hr release), from control cells grown for 48 hr without HU (0 hr C), and at 3, 6, 9, 12, 15, and 18 hr after release. A duplicate sample of cells was also blocked with colchicine for 48 hr (COL). The probes used are as follows: cyclin δ3, HindIII-XbaI fragment; histone H4, 2-kb BamHI genomic fragment containing the Arabidopsis histone H4 gene from B. Scheres (University of Utrecht, The Netherlands); Ac16, 1.5-kb EcoRI fragment from the Arabidopsis cDNA for porphobilinogen deaminase from A. Smith (University of Cambridge).

Methods). Proliferation of cells in these de novo cultures is dependent on auxin and cytokinin, which can be added to the media as 2,4-D and kinetin. At the start of the experiment, a control sample of cells was used for RNA preparation, and the remainder of the culture was resuspended in media lacking carbon source (sucrose), auxin, and cytokinin, thus forcing the cells into a "quiescent" state. After 48 hr, another control sample was taken for RNA preparation, and the rest of the culture was divided into eight aliquots, which were resuspended in media containing sucrose (indicated by +S in Figure 12) or lacking sucrose (indicated by −S), containing or lacking 2,4-D (indicated by + A and −A, respectively), and containing or lacking kinetin (+C or −C, respectively). Identical aliquots of cells were thus each placed in media containing the eight possible combinations of sucrose, auxin, and cytokinin.

After 4 hr in these various media, RNA was prepared from the cultures. An RNA gel blot probed with cyclin δ3 showed that the level of cyclin δ3 mRNA does not decline significantly after 48 hr of hormone- and sucrose-free media (Figure 12C, compare lanes labeled C and 0 hr), in contrast to the reduction of expression on HU treatment (Figure 11B). Resuspending cells in the same hormone- and sucrose-free media had no effect on cyclin δ3 expression after 4 hr (Figure 12C, lanes labeled −S−A−C), whereas readdition of all three components resulted in levels of cyclin δ3 mRNA increasing fourfold (lane labeled +S+A+C). Surprisingly, however, we found that the addition of cytokinin alone in the absence of carbon source also induced δ3 expression fourfold (Figure 12C, lane labeled −S−A+C), an effect that was more marked when sucrose was also added (lane labeled +S−A+C). This combination showed the highest level of cyclin δ3 mRNA (increase of 10-fold), even though the absence of added 2,4-D does not allow these cells to enter S phase, as shown by the absence of histone H4 induction (data not shown).

We also noted that the induction of cyclin δ3 expression by cytokinin is consistent with its greater abundance in roots, which contain higher concentrations of this hormone. The concomitant addition of auxin antagonized the cytokinin-dependent induction of expression, so that in the presence of sucrose a smaller induction was observed (Figure 12C, lane labeled +S+A+C) and in its absence (lane labeled −S+A+C) no accumulation over basal cyclin δ3 mRNA levels was observed. We note that a small increase in δ3 expression was observed when sucrose alone was added (Figure 12C, lane labeled +S−A−C); we believe that this reflects the synergistic effect of sucrose in conjunction with low levels of endogenous cytokinin. Again, the presence of auxin blocks this induction (Figure 12C, lane labeled +S+A−C), which is consistent with the auxin repression, cytokinin-inducible model we present. The equal loading of lanes was demonstrated with the Ac16 probe; the

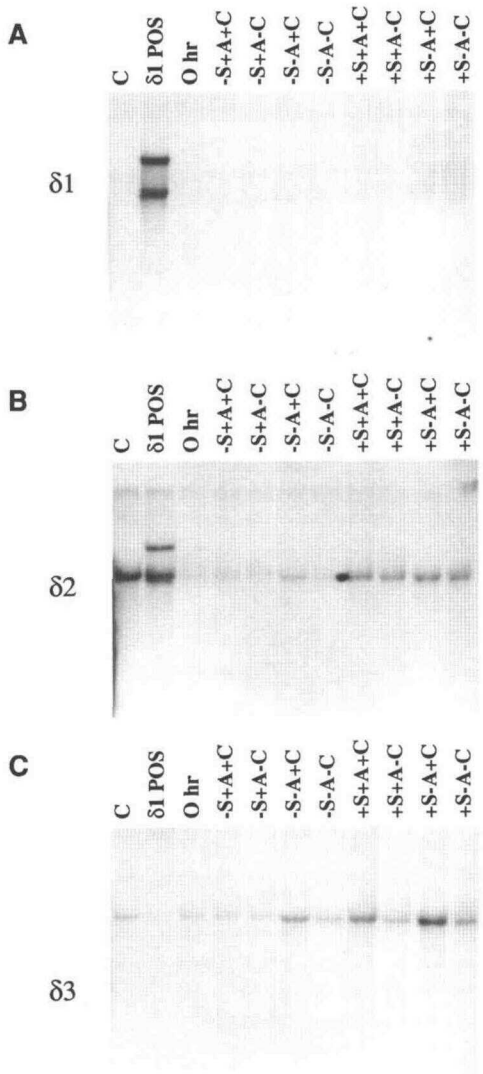

Figure 12. Induction of δ Cyclins by Carbon Source Availability and Plant Growth Regulators.

Shown is the response of δ-type cyclins to carbon source and plant growth regulators after 48 hr in media lacking sucrose, 2,4-D, and kinetin. Lane C contains RNA extracted from cells before withdrawal of sucrose and growth regulators; lane δ1 POS, RNA from cells expressing δ1 (positive control for δ1 blot; note that this signal is still apparent in the blot after rehybridizing to the δ2 probe); 0 hr, RNA after 48 hr of withdrawal; other lanes, RNA samples taken from cells that have been resuspended for 4 hr in media containing (+) or lacking (−) combinations of sucrose (S), 2,4-D (A), and/or kinetin (C) at the concentrations given in Methods. Total RNA (30 μg) was loaded in each lane. Hybridization to the Ac16 probe was used as a further control for loading (data not shown). No significant increase in *cyc1At* expression was seen during the 4 hr of induction (data not shown).

(A) δ1 probe, XbaI-SstI fragment.

(B) δ2 probe, NcoI-ScaI fragment.

(C) δ3 probe, HindIII-XbaI fragment.

histone H4 probe was also used to show that, as expected, during the 4-hr induction, none of the cultures had reached S phase (data not shown).

We next probed an equivalent blot with cyclin δ1 and obtained almost no detectable signal. A new blot was therefore prepared in which a control sample of RNA from cells known to express cyclin δ1 was included (Figure 12A, lane labeled δ1 POS). This control showed that suspension cultures appear to express low levels of this cyclin and that it is not induced by the treatments included in this experiment. The same blot was reprobed without stripping with cyclin δ2, and the two bands shown in the δ1 hybridization positive control lane are still apparent (Figure 12B, lane labeled δ1 POS). In this case, cyclin δ2 expression is apparent in the control cells before treatment but, in contrast to cyclin δ3, is significantly reduced after 2 days in hormone-free and sucrose-free media. The previous expression level was reached within 4 hr of sucrose readdition and independently of the addition of plant hormones (Figure 12B, all four lanes labeled +S).

We concluded that plant δ-type cyclins may be involved in response to nutritional signals and plant hormones.

DISCUSSION

A New Plant Cyclin Family

More than 70 cyclins have been identified by various cloning approaches, including reduced stringency hybridization, polymerase chain reaction, and complementation, resulting in the description of eight major cyclin families (A to H), in addition to various yeast and fungal G_1 cyclins (Tamura et al., 1993; Fisher and Morgan, 1994; for reviews, see Xiong and Beach, 1991; Pines, 1993). We have used complementation of a G_1 cyclin–deficient yeast strain to identify novel plant cyclins. We have characterized four different classes of cDNAs in detail, and sequence analysis shows that three of these form a novel family of cyclins we have named δ-type cyclins. Plant cyclins previously reported can be classed as "A-like" or "B-like" (Renaudin et al., 1994) and are expressed in G_2 and M phase (Hemerly et al., 1992; Hirt et al., 1992; Renaudin et al., 1994). In contrast, we have identified plant "D-like" cyclins that may play an important role in the G_1-to-S phase transition. The presence of conserved homologs in a range of plant species indicates their likely general importance in the control of plant cell division.

The plant cyclins described to date fall into three distinct structural groups (Renaudin et al., 1994). Although most plant cyclins do not contain all of the characteristic diagnostic features expected of animal cyclin classes, two of the plant cyclin groups (I and III) can be classed as broadly B-like and the third (group II) as A-like (Renaudin et al., 1994). We propose the nomenclature cyclin δ for a new class of plant D-like cyclins, a name that incorporates information regarding their sequence and possibly functional homologs in mammals. We further

suggest that this terminology could usefully be extended to other plant cyclin groups to indicate their sequence relationships with the well-known mammalian cyclins, so that the group I and group III B-like cyclins described by Renaudin et al. (1994) would be referred to as cyclins β1 and β2, respectively, and group II A-like cyclins as cyclin α.

Evidence for a G_1/S Role for δ Cyclins

The approach used to identify δ cyclins was equivalent to that which identified members of mammalian and Drosophila C-, D-, and E-type cyclin families (Koff et al., 1991; Lahue et al., 1991; Léopold and O'Farrell, 1991; Lew et al., 1991; Xiong et al., 1991), which have subsequently been shown to have important roles in G_1-to-S control in metazoans (Quelle et al., 1993; Knoblich et al., 1994; Wimmel et al., 1994; reviewed in Sherr, 1993). Despite the use of G_1 cyclin–deficient yeast strains, in two of these screens, truncated mitotic cyclins A and B and the Drosophila *cdc2* homolog gave rise to a substantial background of rescued yeast clones (Léopold and O'Farrell, 1991; Lew et al., 1991). Therefore, the ability to complement a G_1 cyclin–deficient yeast strain does not necessarily identify G_1/S cyclins. However, none of the cyclins we analyzed had the features of B- (mitotic) or A-type cyclins, a number of examples of which have already been cloned from various plant species and can be readily identified from their grouping with other B- and/or A-type cyclins. Preliminary analysis of additional clones from our screen suggests that they are additional representatives of the groups presented here (data not shown). This lack of background from truncated mitotic cyclins could reflect differences between mammalian and plant mitotic cyclins, the high proportion of full-length clones in the library (Minet et al., 1992), or the BF305-15 #21 yeast strain used because Xiong et al. (1991), using the same strain, also did not report a high background from mitotic cyclins.

We present here a number of lines of evidence indicative of a G_1/S role for δ-type cyclins in plants. G_1/S cyclins in fungi and metazoans do not contain the destruction box motif characteristic of mitotic cyclins but are rapidly turned over in the cell due to the presence of PEST motifs (Rogers et al., 1986; reviewed in Reed, 1991). All three δ-type cyclins contain at least two PEST sequences, one copy located near the N terminus, with a second copy (two additional copies for δ1) C-terminal to the cyclin box. The PESTFIND scores for these motifs range from +1 to +13, comparable to the score for PEST sequences in human cyclin C (+12), cyclin D (+1), and cyclin E (+1; Lew et al., 1991) and yeast CLN2 (+12; Hadwiger et al., 1989), whereas Drosophila cyclin E type II contains 11 putative PEST sequences scoring −3 to +24 (Richardson et al., 1993). The presence of PEST motifs in δ-type cyclins is suggestive evidence for a nonmitotic role.

We have also investigated the timing of expression of δ-type cyclins after an HU block, which inhibits DNA synthesis. We observed that suspension cultures treated with HU for 48 hr exhibited substantially reduced expression of histone H4. This is consistent with previous published results on HU treatment of plant cells in which reduced expression of histone H4 in Arabidopsis suspension cultures (Hemerly et al., 1992) and of histone H2A in pea roots (Tanimoto et al., 1993) was reported. Another S phase–specific gene, *cyc07*, of the rose periwinkle (*Catharanthus roseus* syn *Vinca rosea*) also shows significantly reduced expression after HU treatment of cells (Ito et al., 1991). Release of the HU block results in an increase of expression of these S phase–specific genes due to the synchrony induced in the culture. We observed that histone H4 expression increased markedly at 12 hr, slightly before the peak of DNA synthesis (Figure 11B; 12 to 15 hr). This agrees with the model of Tanimoto et al. (1993), who proposed that histone expression starts slightly before DNA synthesis. Cyclin δ3 expression was reduced after HU treatment and then increased significantly 9 hr after release, slightly before the high-level accumulation of histone H4 mRNA. This indicates a role for cyclin δ3 in the onset, resumption, or continuation of S phase. This is supported by experiments in which cells were nitrate starved, which led to arrest in G_1. This resulted in a significant decline in δ3 mRNA levels, which returned on readdition of nitrate concomitantly with the onset of DNA synthesis.

In contrast, mRNA levels for cyclins δ1 and δ2 were decreased by HU treatment, suggesting that their accumulation depends on cycling cells, and no increase in their levels was observed during the time course of the experiment. No expression was observed in mitotically blocked cells. In view of the other evidence that these cyclins do not belong to a known mitotic family, these results could suggest a role for cyclins δ1 and δ2 in G_1, which has already been passed before cells accumulate at the HU block. In support of a G_1 role for cyclin δ2, we saw no decrease in mRNA levels for this cyclin in nitrate-blocked cells. We note that the decline in cyclin δ levels in cells treated with colchicine or HU is in contrast to the unaltered levels of Arabidopsis *cdc2a* mRNA, which are unaffected by these treatments (Hemerly et al., 1993). Further evidence for the role of δ-type cyclins comes from their sequence relationships to mammalian G_1 cyclins, particularly members of the cyclin D group.

Relationships to Mammalian G_1 Cyclins

δ-Type cyclins are related to mammalian D-type cyclins, with δ2 also showing a weaker similarity with mammalian cyclin E (Figure 6). A number of lines of evidence implicate these mammalian cyclins in important processes in G_1 exit and progression into S phase, including the independent isolation of mammalian cyclin D1 at the breakpoint in chromosome 11 inversions leading to parathyroid adenomas, in which the cyclin D1 gene (also known as the *PRAD1* oncogene) becomes fused to the parathyroid hormone promoter (Motokura et al., 1991). Deregulated expression of cyclin D genes is now implicated in a variety of tumors (Xiong et al., 1992a; reviewed in Sherr, 1993). The importance of D-type cyclins in controlling cellular proliferation is also reinforced by their transcriptional

regulation. Unlike B- and A-type cyclins, their expression does not show strong periodicity in the cell cycle of continuously proliferating cells (Won et al., 1992), but levels of D-type cyclins decline rapidly on withdrawal of growth factors and are directly induced by restimulation with growth factors (Ando et al., 1993; Sewing et al., 1993). It has thus been suggested that D-type cyclins may act primarily as growth factor sensors, feeding information on the external environment of the cell into the cell cycle control system (Sherr, 1993).

Cyclin E, also isolated by complementation of a yeast triple *cln*$^-$ mutant, may be more directly involved than cyclin D in the G_1-to-S phase transition in both mammals and Drosophila (Koff et al., 1991; Lew et al., 1991; Knoblich et al., 1994). In contrast to the relatively constant levels of cyclin D in proliferating cells, cyclin E expression in mammalian cells is strongly cell cycle regulated, peaking eightfold just prior to S phase. In cells restimulated after serum depletion, cyclin E levels peak later than those of cyclin D, consistent with the proposed direct role of D-type cyclins in responding to growth signals.

The analogy of δ-type cyclins with mammalian D types therefore extends beyond a sequence similarity, because we show here that cyclin δ3 is strongly induced by the plant growth regulator cytokinin, whereas cyclin δ2 levels increase when sucrose is resupplied. Cyclins δ2 and δ3 are thus involved in linking plant growth regulators, nutritional status, and cell cycle progression in a manner similar to the involvement of cyclin D in the response of mammalian cells to growth factors. Moreover, the three cyclin D homologs show differential expression in a variety of cell lineages (Ajchenbaum et al., 1993; Ando et al., 1993), suggesting that the interpretation of these signals can be performed in a way appropriate to the cell type in question. We have found that cyclins δ3 and δ1 also show a tissue specificity of expression that is not directly related to the proliferative state of the tissue involved and, in the case of cyclin δ1, differential abundance of the three transcripts. These observations suggest possible functional differences in the responses of different plant tissues to growth signals, and perhaps a complex regulational regime involving the different length transcripts observed in the case of δ1. Further analysis will be required to ascertain the role of these various transcripts and the significance of their differential abundance in different tissues.

We thus propose that the δ-type cyclins represent functional plant homologs of mammalian D-type cyclins in that they undertake equivalent roles in controlling cell cycle progression. The controls and stimuli to which plant cells respond from their cellular environment and from their developmental program are very different from those to which mammalian cells are exposed. We therefore do not believe that a precise one-to-one relationship will be found between plant and mammalian cyclins, but the timing of expression, the tissue specificity of different cyclins, and the response of cyclin δ2 to sucrose and of cyclin δ3 to cytokinin have strong parallels in features of mammalian D- and E-type cyclin expression. As in mammals, the stronger conservation of different subclass members between rather than within species is indicative of nonredundant functions. We have initiated a more precise definition of the role of these plant cyclins using cell culture systems with improved synchrony, combined with the use of biochemical tools for the analysis of cyclin–cdk complexes.

Evidence for a Plant Retinoblastoma Protein Homolog

In addition to their primary kinase partner, cdk4 (Matsushime et al., 1992), mammalian D-type cyclins also interact with the retinoblastoma gene product (pRb) and the pRb-related proteins p107 and p130 (Xiong et al., 1992b; Hannon et al., 1993; Kato et al., 1993). *Rb* is a tumor suppressor gene that appears to have an important role in controlling cell cycle progression (reviewed in Wiman, 1993). The transforming proteins of the DNA tumor viruses SV40 (T antigen), adenovirus (E1A protein), and papillomavirus (E7 protein) bind to pRb and p107 and promote the G_1-to-S phase transition. This interaction is mediated through an N-terminal amino acid sequence LXCXE in the oncoproteins, which binds to a defined pocket domain in pRb and its relatives (Hannon et al., 1993; reviewed in Wiman, 1993). This LXCXE motif is also present in D-type cyclins but is absent from all other cyclins described to date. The functional significance of its presence in cyclin D is indicated by several lines of experimental evidence (Dowdy et al., 1993; Ewen et al., 1993).

A particularly unexpected finding in all three of the Arabidopsis δ-type cyclins was the identification of a conserved LXCXE pRb interaction motif near their N terminus in the position equivalent to the location of this sequence in D-type cyclins. This motif is also present in a correct general context (Figure 8) and is not a general feature of other plant cyclins. Its presence therefore supports our identification of δ-type cyclins as the plant functional equivalents of mammalian cyclin D and suggests that conserved Rb-related proteins could be present in plants.

Regulation of Cell Cycle Genes by Plant Growth Regulators and Carbon Source Availability

Several plant growth regulators are known to have effects on cell division (reviewed in Bayliss, 1985), but the lack of requirement of many cultured cell systems for exogenously added regulators has largely restricted analysis to the effects of auxins and cytokinins. Of these, auxins are the most important, and most culture systems are dependent on exogenously supplied auxin (King, 1976; Everett et al., 1981; Gamburg, 1982).

Hormone induction of genes in plants has been demonstrated by the cloning of a number of genes induced by both auxin (Guilfoyle et al., 1993) and cytokinin (Crowell et al., 1990), but the only cell cycle genes reported to respond to plant growth regulators are histone H3 (Kapros et al., 1992) and *cdc2* homologs, which both show an auxin response. In the case of *cdc2*, available evidence points to the general conclusion that auxin is required for its expression (Hirt et al., 1991; Dudits et al.,

1993; Hemerly et al., 1993; John et al., 1993; Magyar et al., 1993; Miao et al., 1993) and that this induction is probably direct, because in pea root tissue an increase in *cdc2* mRNA levels was observed within 10 min of exposing the tissue to the auxin 3-indolyacetic acid (John et al., 1993). Cytokinin induction of the *cdc2a* gene promoter has also been reported by Hemerly et al. (1993), but this was recorded 72 hr after treatment of intact roots and correlated with increased DNA synthesis. Therefore, this may reflect a general increase in division in responsive cells rather than direct induction by cytokinin. We are not aware of previous data showing phytohormone induction of any cyclin genes in plants.

Despite considerable evidence for the importance of cytokinin in promoting cell division (Wang et al., 1981; reviewed in Bayliss, 1985), most systems used for analysis of cell cycle–dependent gene expression require only exogenously added auxin to resume cell division, probably because endogenous cytokinin synthesis levels are sufficient for continued growth (reviewed in Hanke, 1993). In situ hybridization on root sections shows high levels of *cdc2* expression throughout the pericycle, not limited to the xylem poles, where lateral root formation occurs; this suggests that elevated *cdc2* mRNA levels are by themselves insufficient to promote cell division (Martinez et al., 1992). Further studies by John et al. (1993) using segments of central stem pith from tobacco, a tissue of nondividing cells known from classic experiments to require an exogenous supply of both auxin and cytokinin for the resumption of cell division, showed that *cdc2* expression was induced by treatment with auxin alone but that the protein was catalytically inactive unless cytokinin was also present in the growth medium. Because the normal mechanism of activation of a cdk is by association with its regulatory subunit or cyclin, it is very tempting to speculate that the cytokinin-induced component required for activation of *cdc2* in G_1 cells might be cyclin δ3, which we show here to be specifically induced by cytokinin after only 4 hr of treatment. Other data that suggest a G_2-dependent step for cytokinin in the cell cycle (John et al., 1993; reviewed in Bayliss, 1985) have been derived from rapidly cycling cell systems often after extended periods in culture and may reflect differences between cell cycle controls on rapidly growing cells in culture and the induction of cell division from a quiescent or G_0 state.

We have also found that cyclin δ2 responds to carbon source availability within 4 hr of readdition to starved cells. Hemerly et al. (1993) have previously shown that depletion of sucrose in the growth medium causes a reduction in Arabidopsis *cdc2a* transcripts, but no data are presented on the timing of induction on refeeding. The work presented here thus represents the most complete analysis of the response of any plant cell cycle gene to carbon source and hormonal signals.

Implications for Plant Cell Division

The control and pattern of cell division in plants are very different from those in animals (Steeves and Sussex, 1989). After the early embryo stages, cell division is largely confined to limited meristematic regions of the roots and shoot tips. Much increase in size outside these regions is due to cell expansion, but division can be initiated in local regions to give rise to structures such as lateral root primordia and leaves. It may also be reactivated during a de-differentiation process induced by the application of plant hormones. In the absence of cell migration, the polarity of division and expansion is of considerable importance in defining morphology. Thus, within a conserved framework of eukaryotic cell cycle machinery, we expect important new controls and insights to be present as a result of the developmental programs and environmental responsiveness of plants.

Multiple signals, both developmental and environmental in origin, must impinge on a control point in late G_1 at which cells become committed to enter S phase, analogous to the START control in yeasts and the restriction point in mammalian cells. The importance of controls in plants operating at this point and at the G_2-to-M phase boundary has been recognized for some time in the principal control point hypothesis of cell division (reviewed in van't Hof, 1985). We have proposed that START is also a useful concept in plant cells to indicate a point at which the cdk–kinase complex becomes activated and processes leading to S phase are precipitated (Murray, 1994). START in plants thus represents a point at which multiple regulatory pathways converge to produce a simple output signal.

Our data agree with a model for the activation of cell division in plants in which both auxin and cytokinin are generally required, particularly during reentry into the cell cycle by quiescent tissue arrested in G_1 phase. We propose that auxin is responsible for the induction of the expression of the kinase subunit of the cdk complex, whereas the cytokinin-dependent component is contributed by cyclin δ3. The further developmental and environmental controls on plant cell division will reveal important new understanding of growth and differentiation in plants.

METHODS

Library Screening and Yeast Techniques

The pFL61 library was constructed by Minet et al. (1992), and a sample was obtained from A. Smith (University of Cambridge). The library was amplified by electroporation into *Escherichia coli* and plating of $>10^7$ *E. coli* colonies. These were washed from plates for plasmid DNA preparation. High-efficiency yeast transformation of BF305-15d #21 was performed according to Schiestl and Gietz (1989), except that cells for transformation were pregrown in media in which the carbon source was 1% galactose (Sigma G-0750, <0.01% glucose), 1% raffinose and transformed cells were allowed to recover in this medium for 2 hr at 30°C before plating on selective glucose drop-out media at a density of 10^4 to 5 × 10^4 transformants per 14-cm plate. Transformation frequency was measured by plating an aliquot on selective galactose plates and was $>10^4$ transformants per microgram of library DNA. In each experiment, 10 aliquots of competent yeast cells were prepared

from 250 mL of starting culture (8×10^6 cells per mL), and each was transformed with 10 μg of library DNA. Transformed colonies were picked into selective glucose and selective galactose liquid media, and plasmids were rescued into *E. coli* as described by Soni and Murray (1992). Routine yeast transformation of plasmid clones into yeast was as described previously (Soni et al., 1993).

BF305-15d #21 (*MATa leu2-3,112 his3-11,15 ura3-52 trp1 ade1 met14 arg5,6 GAL1-CLN3 HIS3::cln1 TRP1::cln2*) was maintained on YEPGR plates containing 1% yeast extract, 2% peptone, 1% galactose, 1% raffinose, and 2% Bactoagar (Difco) at 30°C (Xiong et al., 1991). Selective glucose drop-out plates (Ausubel et al., 1987) were used for screening the pFL61 library and contained 2% glucose, 0.67% yeast nitrogen base without amino acids, and 2% Bactoagar, with tryptophan, leucine, and uracil dropped out. On these plates, the reversion frequency was $<10^{-6}$ but was considerably higher if tryptophan and leucine selection was not maintained for the markers disrupting the *CLN1* and *CLN2* genes. Selective galactose plates contained 1% galactose (<0.1% glucose) and 1% raffinose as carbon source. JO221 (Ogas et al., 1991; *MATa/MATα TRP1/TRP1 swi4Δ/swi4-ts1 BAR1/bar1::LEU2 ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1*) was grown at 25°C. Micromanipulation was performed using a Singer MSM Micromanipulator (Watchet, Somerset, UK) equipped with video camera and printer.

Plasmid Constructions and DNA Sequencing

NotI cDNA inserts were cloned into pBluescript SK+ (Stratagene) for restriction analysis, and subclones were used for dideoxy sequencing according to the manufacturer's instructions (Sequenase kit; Amersham International). Gel reading and contig assembly were performed manually or by using Millipore (Bedford, MA) BioImage DNA Sequence Analysis software. For sequence analysis and data base comparisons, the software programs FASTA and PILEUP from the GCG package (Genetics Computer Group, Madison, WI; Devereux et al., 1984) and the BLAST server were used. The program PESTFIND (Rogers et al., 1986) was obtained from M. Rechsteiner (University of Utah Medical Center, Salt Lake City).

Hybridization Analysis

DNA preparation from suspension-cultured cells and RNA preparation from plant tissues and suspension-cultured cells were as described by Soni and Murray (1994). GeneScreen Plus (Du Pont–New England Nuclear) membranes were used for DNA gel blot analysis according to the manufacturer's instructions at 65°C for normal and 50°C for reduced-stringency hybridization. RNA gel blot analysis was conducted as described previously (Murray et al., 1987). Blots were stripped and reprobed according to manufacturer's instructions.

Cell Culture and Block-Release Experiments

Suspension cultures were established by growing seeds of *Arabidopsis thaliana* ecotype Landsberg *erecta* in liquid Murashige and Skoog media (ICN Biomedicals Inc., Costa Mesa, CA) supplemented with 3% sucrose, 0.55 μg/mL 2,4-D (Sigma), and 0.25 μg/mL kinetin (Sigma) at 24°C with orbital shaking at 100 rpm (50-mm throw). Fresh media was added every 3 days. For the hormone induction experiment, cultures were resuspended in fresh media 48 hr before the start of the experiment. At the start of the experiment, a sample was taken for RNA preparation, and the remaining cells were resuspended in media lacking sucrose, 2,4-D, and kinetin and returned to the shaker. After 48 hr, another sample was taken (0 hr), and the remaining cells were split into eight aliquots with different combinations of sucrose, 2,4-D, and kinetin as described in the text and Figure 12. The cells were disrupted for RNA preparation after 4 hr of additional incubation with shaking. For the hydroxyurea (HU) block–release experiment, cultures were grown as described previously, except at the start of the experiment, they were resuspended in fresh Murashige and Skoog media (containing sucrose, 2,4 D, and kinetin) with the addition of freshly prepared HU at 10 mM final concentration. After 48 hr, the cells were washed twice in fresh media and then resuspended in fresh media. Samples were taken for RNA preparation at the time points indicated in Figure 11. Incorporation of ^{3}H-thymidine was monitored by transferring an aliquot of cells to media containing 0.05 μCi/mL ^{3}H-thymidine (35 Ci/mmol; ICN Biomedicals Inc.). After 3 hr of incorporation, cells were thoroughly disrupted using an Ultra-Turrax T8 (IKA Labortecknik, Staufen, Germany) hand-held cell disrupter at maximum speed for 20 sec. Incorporation was measured as described by Kapros et al. (1992) and corrected according to the OD_{260} of the disrupted cell suspension as a measure of the total nucleic acid content of the cells. The figures plotted therefore represent the incorporation of ^{3}H-thymidine into DNA over the 3-hr periods indicated.

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REFERENCES

Ajchenbaum, F., Ando, K., DeCaprio, J.A., and Griffin, J.D. (1993). Independent regulation of human D-type cyclin gene expression during G_1 phase in primary human T-lymphocytes. J. Biol. Chem. **268,** 4113–4119.

Ando, K., Ajchenbaum-Cymbalista, F., and Griffin, J.D. (1993). Regulation of G_1/S transition by cyclins D2 and D3 in hematopoietic cells. Proc. Natl. Acad. Sci. USA **90,** 9571–9575.

Atherton-Fessler, S., Hannig, G., and Piwnica-Worms, H. (1993). Reversible tyrosine phosphorylation and cell cycle control. Semin. Cell Biol. **4,** 433–442.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1987). Current Protocols in Molecular Biology. (New York: John Wiley and Sons).

Baldin, V., Lukas, J., Marcote, M.J., Pagano, M., and Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. Genes Dev. **7,** 812–821.

Bayliss, M.W. (1985). Regulation of the cell division cycle in cultivated plant cells. In The Cell Division Cycle in Plants, J.A. Bryant and D. Francis, eds (Cambridge: Cambridge University Press), pp. 157–177.

Chiatante, D., Crosti, M., Brusa, P., Trewavas, A.J., and John, P.C.L. (1993). Protein-kinases and $p34^{cdc2}$-like protein in root meristems of *Pisum sativum* during germination. Plant Sci. **89,** 13–21.

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

Colasanti, J., Cho, S.-O., Wick, S., and Sundaresan, V. (1993). Localization of the functional $p34^{cdc2}$ homolog of maize in root tip and stomatal complex cells: Association with predicted division sites. Plant Cell **5,** 1101–1111.

Conia, J., Alexander, R.G., Wilder, M.E., Richards, K.R., Rice, M.E., and Jackson, P.J. (1990). Reversible accumulation of plant suspension cell cultures in G_1 phase and subsequent synchronous traverse of the cell cycle. Plant Physiol. **94,** 1568–1574.

Crowell, D.R., Kadlecek, A.T., John, M.C., and Amasino, R.M. (1990). Cytokinin-induced mRNAs in cultured soybean cells. Proc. Natl. Acad. Sci. USA **87,** 8815–8819.

Day, I.S., and Reddy, A.S.N. (1994). Cloning of a family of cyclins from *Arabidopsis thaliana*. Biochim. Biophys. Acta Gene Struct. Express. **1218,** 115–118.

De la Torre, C., Gonzalez-Fernandez, A., and Gimenez-Martin, G. (1989). Stringency at four regions of the plant cell cycle where proteins regulating its progression are synthesized. J. Cell Sci. **94,** 259–265.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. **12,** 387–395.

Dirick, L., and Nasmyth, K. (1991). Positive feedback in the activation of G_1 cyclins in yeast. Nature **351,** 754–757.

Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A., and Weinberg, R.A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. Cell **73,** 499–511.

Dudits, D., Bögre, L., Bakó, L., Dedeoglu, D., Magyar, Z., Kapros, T., Felföldi, F., and Györgyey, J. (1993). Key components of cell cycle control during auxin-induced cell division. In Molecular and Cell Biology of the Plant Cell Cycle, J.C. Ormrod and D. Francis, eds (Dordrecht: Kluwer), pp. 111–131.

Epstein, C.B., and Cross, F.R. (1994). Genes that can bypass the CLN requirement for *Saccharomyces cerevisiae* cell cycle START. Mol. Cell. Biol. **14,** 2041–2047.

Evans, L.S., and van't Hof, J. (1974). Promotion of cell arrest in G_2 in root and shoot meristems of *Pisum* by a factor from the cotyledons. Exp. Cell Res. **87,** 259–264.

Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: A protin specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell **33,** 389–396.

Everett, E.R., Wang, T.L., Gould, A.R., and Street, H.E. (1981). Studies on the control of the cell cycle in cultured plant cells. II. Effects of 2,4-dichlorophenoxyacetic acid (2,4-D). Protoplasma **106,** 15–22.

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J., and Livingston, D.M. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73,** 487–497.

Fang, F., and Newport, J.W. (1991). Evidence that the G_1-S and G_2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. Cell **66,** 731–742.

Feiler, H.S., and Jacobs, T.W. (1990). Cell division in higher plants: A *cdc2* gene, its 34-kDa product, and histone H1 kinase activity in pea. Proc. Natl. Acad. Sci. USA **87,** 5397–5401.

Ferreira, P.C.G., Hemerly, A.S., Villarroel, R., van Montagu, M., and Inzé, D. (1991). The Arabidopsis functional homolog of the $p34^{cdc2}$ protein kinase. Plant Cell **3,** 531–540.

Fisher, R.P., and Morgan, D.O. (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. Cell **78,** 713–724.

Fobert, P.R., Coen, E.S., Murphy, G.J.P., and Doonan, J.H. (1994). Patterns of cell division revealed by transcriptional regulation of genes during the cell cycle in plants. EMBO J. **13,** 616–624.

Gamburg, K.Z. (1982). Regulation of cell division by auxin in isolated cultures. In Plant Growth Substances, P.F. Wareing, ed (London: Academic Press), pp. 59–67.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature **349,** 132–138.

Gould, A.R. (1984). Control of the cell cycle in cultured plant cells. CRC Crit. Rev. Plant Sci. **1,** 315–344.

Gould, A.R., Everett, N.P., Wang, T.L., and Street, H.E. (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.

Guilfoyle, T.J., Hagen, G., Li, Y., Ulmasov, T., Liu, Z., Strabala, T., and Gee, M. (1993). Auxin-regulated transcription. Aust. J. Plant Physiol. **20,** 489–502.

Hadwiger, J.A., Wittenberg, C., Richardson, H.E., De Barros Lopes, M., and Reed, S.I. (1989). A family of cyclin homologs that control the G_1 phase in yeast. Proc. Natl. Acad. Sci. USA **86,** 6255–6259.

Hanke, D.E. (1993). Cell cycle regulation in cultured cells. In Molecular and Cell Biology of the Plant Cell Cycle, J.C. Ormrod and D. Francis, eds (Dordrecht: Kluwer), pp. 173–178.

Hannon, G.J., Demetrick, D., and Beach, D. (1993). Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev. **7,** 2378–2391.

Hartwell, L.H. (1974). *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. **38,** 164–198.

Hata, S., Kouchi, H., Suzuka, I., and Ishii, T. (1991). Isolation and characterization of cDNA clones for plant cyclins. EMBO J. **10,** 2681–2688.

Hemerly, A., Bergounioux, C., van Montagu, M., Inzé, D., and 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.

Hemerly, A.S., Ferreira, P., de Almeida Engler, J., Van Montagu, M., Engler, G., and Inzé, D. (1993). *cdc2a* expression in Arabidopsis is linked with competence for cell division. Plant Cell **5,** 1711–1723.

Hirayama, T., Imajuku, Y., Anai, T., Matsui, M., and Oka, A. (1991). Identification of two cell-cycle-controlling *cdc2* gene homologs in *Arabidopsis thaliana*. Gene **105,** 159–165.

Hirt, H., Páy, A., Györgyey, J., Bakó, L., Németh, K., Bögre, L., Schweyen, R.J., Heberle-Bors, E., and 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.

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

Hirt, H., Páy, A., Bögre, L., Meskiene, I., and Heberle-Bors, E. (1993). *cdc2MsB*, a cognate *cdc2* gene from alfalfa, complements the G_1/S but not the G_2/M transition of budding yeast *cdc28* mutants. Plant J. **4,** 61–69.

Howard, A., and Pelc, S.R. (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity **6** (suppl.), 216–273.

Ito, M., Kodama, H., and Komamine, A. (1991). Identification of a novel S phase-specific gene during the cell cycle in synchronous cultures of *Catharanthus roseus* cells. Plant J. **1,** 141–148.

John, P.C.L., Sek, F.J., and Lee, M.G. (1989). A homolog of the cell cycle control protein p34*cdc2* participates in the division cycle of *Chlamydomonas*, and a similar protein is detectable in higher plants and remote taxa. Plant Cell **1,** 1185–1193.

John, P.C.L., Sek, F.J., Carmichael, J.P., and McCurdy, D.W. (1990). $p34^{cdc2}$ homologue level, cell division, phytohormone responsiveness and cell differentiation in wheat leaves. J. Cell Sci. **97,** 627–630.

John, P.C.L., Sek, F.J., and 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, P.C.L., Zhang, K., Dong, C., Diederich, L., and 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 Physiol. **20,** 503–526.

Kapros, T., Bögre, L., Németh, K., Bakó, L., Györgyey, J., Wu, S.C., and Dudits, D. (1992). Differential expression of histone H3 gene variants during cell cycle and somatic embryogenesis in alfalfa. Plant Physiol. **98,** 621–625.

Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. **7,** 331–342.

King, P.J. (1976). Studies on the growth in culture of plant cells, part 20. Utilization of 2,4-D by steady-state cultures of *Acer pseudoplatanus*. J. Exp. Bot. **27,** 1053–1072.

King, P.J. (1977). Studies on the growth in culture of plant cells. J. Exp. Bot. **28,** 142–155.

Knoblich, J.A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C.F. (1994). Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell **77,** 107–120.

Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Philippe, M., and Roberts, J.M. (1991). Human cyclin E, a new cyclin that interacts with two members of the *CDC2* gene family. Cell **66,** 1217–1228.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R., and Roberts, J.M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. Science **257,** 1689–1694.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J.M., and Massagué, J. (1993). Negative regulation of G_1 in mammalian cells: Inhibition of cyclin E-dependent kinase by TGF-β. Science **260,** 536–539.

Lahue, E.E., Smith, A.V., and Orr-Weaver, T.L. (1991). A novel cyclin gene from Drosophila complements *CLN* function in yeast. Genes Dev. **5,** 2166–2175.

Léopold, P., and O'Farrell, P.H. (1991). An evolutionarily conserved cyclin homolog from Drosophila rescues yeast deficient in G_1 cyclins. Cell **66,** 1207–1216.

Lepetit, M., Ehling, M., Chaubet, N., and Gigot, C. (1992). A plant histone gene promoter can direct both replication-dependent and -independent gene expression in transgenic plants. Mol. Gen. Genet. **231,** 276–285.

Lew, D.J., Dulic, V., and Reed, S.I. (1991). Isolation of three novel human cyclins by rescue of G_1 cyclin (Cln) function in yeast. Cell **66,** 1197–1206.

Magyar, Z., Bakó, L., Bögre, L., Dedeoglu, D., Kapros, T., and Dudits, D. (1993). Active *cdc2* genes and cell cycle phase-specific cdc2-related kinase complexes in hormone-stimulated alfalfa cells. Plant J. **4,** 151–161.

Martinez, M.C., Jorgensen, J.-E., Lawton, M.A., Lamb, C.J., and Doerner, P.W. (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.

Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G_1 phase of the cell cycle. Cell **65,** 701–713.

Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.-Y., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992). Identification and properties of an atypical catalytic subunit ($p34^{PSK\text{-}J3}$/cdk4) for mammalian D type G_1 cyclins. Cell **71,** 323–334.

Meyerson, M., and Harlow, E. (1994). Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14,** 2077–2086.

Meyerson, M., Faha, B., Su, L.-K., Harlow, E., and Tsai, L.-H. (1991). The cyclin-dependent kinase family. Cold Spring Harbor Symp. Quant. Biol. **56,** 177–186.

Meyerson, M., Enders, G.H., Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L.-H. (1992). A family of human cdc2-related protein kinases. EMBO J. **11,** 2909–2917.

Miao, G.-H., Hong, Z., and Verma, D.P.S. (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.

Minami, M., Huh, G.H., Yang, P., and Iwabuchi, M. (1993). Coordinate gene expression of five subclass histones and the putative transcription factors, HBP-1a and HBP-1b, of histone genes in wheat. Plant Mol. Biol. **23,** 429–434.

Minet, M., Dufour, M.-E., and Lacroute, F. (1992). Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. **2,** 417–422.

Mineyuki, Y., Yamashita, M., and Nagahama, Y. (1991). $p34^{cdc2}$ kinase homologue in the preprophase band. Protoplasma **162,** 182–186.

Motokura, T., Bloom, T., Kim, H.G., Jüppner, H., Ruderman, J.V., Kronenberg, H.M., and Arnold, A. (1991). A novel cyclin encoded by a *bcl1*-linked candidate oncogene. Nature **350,** 512–515.

Murray, A.W., Solomon, M.J., and Kirschner, M.W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature **339,** 280–286.

Murray, J.A.H. (1994). Plant cell division: The beginning of START. Plant Mol. Biol. **26,** 1–3.

Murray, J.A.H., Scarpa, M., Rossi, N., and Cesareni, G. (1987). Antagonistic controls regulate copy number of the yeast 2μ plasmid. EMBO J. **6,** 4205–4212.

Nagata, T., Nemoto, Y., and 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.

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

Nugent, J.H.A., Alfa, C.E., Young, T., and Hyams, J.S. (1991). Conserved structural motifs in cyclins identified by sequence analysis. J. Cell Sci. **99,** 669–674.

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

Ogas, J., Andrews, B.J., and Herskowitz, I. (1991). Transcriptional activation of *CLN1*, *CLN2*, and a putative new G_1 cyclin (*HCS26*) by SWI4, a positive regulator of G_1-specific transcription. Cell **66,** 1015–1026.

Ohtsubo, M., and Roberts, J.M. (1993). Cyclin-dependent regulation of G_1 in mammalian fibroblasts. Science **259,** 1908–1912.

Pardee, A.B. (1989). G_1 events and regulation of cell proliferation. Science **246,** 603–608.

Pines, J. (1993). Cyclins and cyclin-dependent kinases: Take your partners. Trends Biochem. Sci. **18,** 195–197.

Quelle, D.E., Ashmun, R.A., Shurtleff, S.A., Kato, J., Bar-Sagi, D., Roussel, M.F., and Sherr, C.J. (1993). Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. Genes Dev. **7,** 1559–1571.

Rechsteiner, M. (1990). PEST sequences are signals for rapid intracellular proteolysis. Semin. Cell Biol. **1,** 433–440.

Reed, S.I. (1991). G_1-specific cyclins: In search of an S-phase-promoting factor. Trends Genet. **7,** 95–99.

Renaudin, J.-P., Colasanti, J., Rime, H., Yuan, Z., and Sundaresan, V. (1994). Cloning of four cyclins from maize indicates that higher plants have three structurally distinct groups of mitotic cyclins. Proc. Natl. Acad. Sci. USA **91,** 7375–7379.

Richardson, H.E., Wittenberg, C., Cross, F., and Reed, S.I. (1989). An essential G_1 function for cyclin-like proteins in yeast. Cell **59,** 1127–1133.

Richardson, H.E., O'Keefe, L.V., Reed, S.I., and Saint, R. (1993). A Drosophila G_1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. Development **119,** 673–690.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. Science **234,** 364–368.

Schiestl, R.H., and Gietz, R.D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16,** 339–346.

Sewing, A., Bürger, C., Brüsselbach, S., Schalk, C., Lucibello, F.C., and Müller, R. (1993). Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. J. Cell Sci. **104,** 545–555.

Sherr, C.J. (1993). Mammalian G_1 cyclins. Cell **73,** 1059–1065.

Solomon, M.J. (1993). Activation of the various cyclin/cdc2 protein kinases. Curr. Opin. Cell Biol. **5,** 180–186.

Soni, R., and Murray, J.A.H. (1992). A rapid and inexpensive method for isolation of shuttle vector DNA from yeast for the transformation of *E. coli*. Nucl. Acids Res. **20,** 5852.

Soni, R., and Murray, J.A.H. (1994). Isolation of intact RNA and DNA from plant tissues. Anal. Biochem. **218,** 474–476.

Soni, R., Carmichael, J.P., and Murray, J.A.H. (1993). Parameters affecting lithium acetate-mediated transformation of *Saccharomyces cerevisiae* and development of a rapid and simplified procedure. Curr. Genet. **24,** 455–459.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development. (Cambridge: Cambridge University Press).

Tamura, K., Kanaoka, Y., Jinno, S., Nagata, A., Ogiso, Y., Shimizu, K., Hayakawa, T., Nojima, H., and Okayama, H. (1993). Cyclin G: A new mammalian cyclin with homology to fission yeast Cig1. Oncogene **8,** 2113–2118.

Tanimoto, E.Y., Rost, T.L., and Comai, L. (1993). Histone H2A expression during S phase: Histological co-localisation of H2A mRNA and DNA synthesis in pea root tips. In Molecular and Cell Biology of the Plant Cell Cycle, J.C. Ormrod and D. Francis, eds (Dordrecht: Kluwer), pp. 85–96.

Tsai, L.-H., Takahashi, T., Caviness, V.S., Jr., and Harlow, E. (1993a). Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development **119,** 1029–1040.

Tsai, L.-H., Lees, E., Faha, B., Harlow, E., and Riabowol, K. (1993b). The cdk2 kinase is required for the G_1-to-S transition in mammalian cells. Oncogene **8,** 1593–1602.

Tyers, M., Tokiwa, G., and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G_1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. **12,** 1955–1968.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science **262,** 2050–2054.

van't Hof, J. (1985). Control points within the cell cycle. In The Cell Division Cycle in Plants, J.A. Bryant and D. Francis, eds (Cambridge: Cambridge University Press), pp. 1–13.

van't Hof, J., and Kovacs, C.J. (1972). Mitotic cycle regulation in the meristem of cultured roots: The principal control point hypothesis. In The Dynamics of Meristem Cell Populations, M.W. Miller and C.C. Keuhnert, eds (New York: Plenum), pp. 15–32.

Wang, T.L., Everett, N.P., Gould, A.R., and Street, H.P. (1981). Studies on the control of the cell cycle in cultured plant cells. III. The effects of cytokinin. Protoplasma **106,** 23–35.

Wiman, K.G. (1993). The retinoblastoma gene: Role in cell cycle control and cell differentiation. FASEB J. **7,** 841–845.

Wimmel, A., Lucibello, F.C., Sewing, A., Adolph, S., and Müller, R. (1994). Inducible acceleration of G_1 progression through tetracycline-regulated expression of human cyclin E. Oncogene **9,** 995–997.

Wittenberg, C., Sugimoto, K., and Reed, S.I. (1990). G_1-specific cyclins of *S. cerevisiae*: Cell cycle periodicity, regulation by mating pheromone, and association with the $p34^{cdc28}$ protein kinase. Cell **62,** 225–237.

Won, K.-A., Xiong, Y., Beach, D., and Gilman, M.Z. (1992). Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA **89,** 9910–9914.

Xiong, Y., and Beach, D. (1991). Population explosion in the cyclin family. Curr. Biol. **1,** 362–364.

Xiong, Y., Connolly, T., Futcher, B., and Beach, D. (1991). Human D-type cyclin. Cell **65,** 691–699.

Xiong, Y., Menninger, J., Beach, D., and Ward, D.C. (1992a). Molecular cloning and chromosomal mapping of *CCND* genes encoding human D-type cyclins. Genomics **13,** 575–584.

Xiong, Y., Zhang, H., and Beach, D. (1992b). D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell **71,** 505–514.