The D-Type Alfalfa Cyclin Gene cycMs4 Complements G1 Cyclin-Deficient Yeast and Is Induced in the G1 Phase of the Cell Cycle

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Cyclins are key regulators of the cell cycle in all eukaryotes. In alfalfa, we have previously isolated three D-type cyclins. The closely related cycMs1 and cycMs2 genes are expressed primarily during the G2 and M phases and are most likely mitotic cyclins; expression of the cycMs3 gene is induced in the Go-to-G1 transition, when cells reenter the cell cycle. By complementation of G1 cyclin-deficient yeast cells, a novel alfalfa cyclin, designated cycMs4, was isolated. The predicted amino acid sequence of the cycMs4 gene is most similar to that of the Arabidopsis cyclin 63 gene. CycMs4 and cyclin 63 belong to the class of D-type cyclins and contain PEST-rich regions and a retinoblastoma binding motif. When comparing expression levels in different organs, cycMs4 transcripts were present predominantly in roots. Whereas expression of the cycMs4 gene was cell cycle-regulated in suspension-cultured cells, transcription in roots was observed to depend also on the positional context of the cell. When differentiated G0-arrested leaf cells were induced to resume cell division by treatment with plant hormones, cycMs4 transcription was induced before the onset of DNA synthesis. Whereas this induction was preceded by that of the cycMs3 gene, cycMs2 expression occurred later and at the same time as mitotic activity. These data suggest that cycMs4 plays a role in the G1-to-S transition and provide a model to investigate the plant cell cycle at the molecular level.

INTRODUCTION

In recent years, a refined picture of eukaryotic cell cycle regulation has emerged. At the center of this regulatory network are cyclin-dependent kinases (Cdks). In yeast, a single Cdk provides the functions required for both the G1-to-S and G2-to-M transitions, but in animals and plants, several related kinases have evolved (for reviews, see Nasmyth, 1993; Pines, 1993; Hirt and Heberle-Bors, 1994).

Cdks are not active as monomers (Poon et al., 1993); they become active only when associated with a cyclin regulatory subunit. Cyclins are cell cycle stage-specific activators of Cdks but also bind to other regulatory proteins, such as retinoblastoma (Rb) in animal cells (Dowdy et al., 1993; Kato et al., 1993) and Far1 in yeast (Peter et al., 1993). Cyclin association also appears to be involved in the alteration of Cdk substrate specificity (Peeper et al., 1993), availability for upstream regulators, such as wee1* (Booher et al., 1993), and intracellular localization of the Cdk complex (Maridor et al., 1993). The stage specificity of cyclins is ensured mainly by their oscillating appearance in specific cell cycle stages. This is accomplished by regulation of the expression (Amon et al., 1993) and specific degradation (Glotzer et al., 1991; Tyers et al., 1992) of the respective cyclins.

Cyclins can be grouped according to sequence similarities. A-type cyclins act in S and G2 phases, B-type cyclins act at the G2-to-M phase transition, and D- and E-type cyclins function at the G1-to-S phase transition (for reviews, see Pines, 1993; Sherr, 1993).

To date, many cyclins have been isolated from a variety of plant species (Hata et al., 1991; Hemery et al., 1992; Hirt et al., 1992; Day and Reddy, 1994; Ferreira et al., 1994; Fobert et al., 1994; Renaudin et al., 1994; Meskiene et al., 1995; Soni et al., 1995). All of these sequences can be grouped into four major classes of plant cyclins. Three classes have the highest similarity with mammalian A- and B-type cyclins, and only one class shows homology with G1-type cyclins (Soni et al., 1995). In a functional assay, Arabidopsis, maize, and soybean clones were found to induce maturation after injection into Xenopus oocytes and were therefore supposed to be mitotic cyclins (Hata et al., 1991; Hemery et al., 1992; Renaudin et al., 1994). Expression analysis of alfalfa, Antirrhinum, and Arabidopsis cyclins in plant cells was found to be restricted to cells in certain stages of the cell cycle, indicating phase-specific functions for different cyclins (Hirt et al., 1992; Ferreira et al., 1994; Fobert et al., 1994; Meskiene et al., 1995; Soni et al., 1995).
In contrast to A- and B-type cyclins, which contain a highly conserved central domain (cyclin box), G1 cyclins are not highly conserved. To identify alfalfa cyclins that act early in the cell cycle, we used a yeast selection system based on the functional interaction of an alfalfa cyclin with the yeast Cdc28 protein kinase at the G1-to-S transition. Our approach was based on the fact that yeast cells deficient in all three G1 cyclin genes, CLN1, CLN2, and CLN3, are arrested at a point in the G1 phase called START and cannot divide. We assumed that expression of an alfalfa G1 cyclin in yeast may replace the functions of the three CLN genes in the cell cycle. By using this approach, we isolated a novel alfalfa cyclin, CycMs4 (for cyclin *Medicago sativa*), that has the highest homology with the Arabidopsis cyclin δ3 (Soni et al., 1995). RNA gel blot analysis showed that the cycMs4 gene is expressed in a cell cycle–dependent manner and that the highest transcript levels are found in roots. In situ hybridization analysis of roots indicated that the cycMs4 gene is expressed only in dividing tissues and that the transcription also depends on the positional context of the cell. When differentiated leaf cells were induced to reenter the cell cycle in the G1 phase and resume proliferation, transcription of the cycMs4 gene was induced before onset of DNA replication, which is compatible with a role in the G1-to-S transition of the cell cycle.

**RESULTS**

Isolation of Alfalfa Genes That Complement CLN1-, CLN2-, and CLN3-Deficient Yeast Cells

Deficiency of the three cyclin genes, CLN1, CLN2, and CLN3, in yeast results in a G1 phase arrest in START. Because this effect is lethal, no colonies can form under these conditions. To select for alfalfa cyclins that can functionally substitute the yeast G1 cyclins, the yeast strain K3413 (Amon et al., 1993) was used, which has deletions of the CLN1, CLN2, and CLN3 genes but is conditionally viable on medium without methionine due to the ectopic expression of CLN2 under the control of the methionine-repressible *MET2* promoter.

K3413 cells were transformed with an alfalfa cDNA expression library that could be selectively expressed in medium containing galactose but not glucose. From 10⁶ yeast transformants, 123 clones were able to grow on medium containing methionine.

Isolation and analysis of the plasmids revealed two types of cDNA inserts. One type of insert, denoted cycMs4-1, potentially encoded a novel alfalfa cyclin and was investigated further. The other inserts encoded a protein with no homology with any known sequence in current data banks. To test whether expression of the cycMs4-1 cDNA conferred the complementing activity, we introduced plasmid pycycMs4-1 (in which the cycMs4-1 cDNA is under control of the galactose-inducible promoter GAL1-10) into the K3413 strain and compared it with K3413 cells that were transformed with the vector. K3413 cells containing the pYEUra3 vector were viable only when CLN2 was expressed under methionine-free conditions, as shown in Figure 1. When CLN2 expression was repressed by addition of methionine, the yeast cells were not able to divide unless expression of cycMs4-1 was induced by galactose (Figure 1), indicating that cycMs4-1 can substitute for the function of CLNs in the yeast cell cycle.

**cycMs4 Encodes a Novel Alfalfa Cyclin**

The pycycMs4-1 plasmid contains a 1000-bp insert. However, the translational start codon of the longest open reading frame of 810 nucleotides is not preceded by an in-frame stop codon. To obtain a full-length cDNA, another cDNA library that was derived from somatic alfalfa embryos was hybridized with a radiolabeled cDNA fragment. The longest clone, termed cycMs4, contained an insert of 1857 bp (Figure 2). The open reading frame of the previously isolated cDNA could be extended by 348 bp. Several in-frame stop codons upstream of the first possible ATG indicate that this clone represents a full-length cDNA sequence. This is supported by RNA gel blot analysis that revealed a total length of ~1800 nucleotides (data not shown). The identified open reading frame potentially encodes a 386–amino acid polypeptide with an estimated molecular mass of 44 kD.

Previously, we isolated cDNA clones encoding three different B-type alfalfa cyclins, namely, CycMs1, CycMs2, and CycMs3 (Hirt et al., 1992; Meskiene et al., 1995). Alignment of the predicted CycMs4 protein sequence with the previously identified alfalfa CycMs1, CycMs2, and CycMs3 proteins over the entire length revealed only 26 to 29% identity with these cyclins. Considering the cyclin box only, identity scores up to 42% were obtained with the CycMs4 protein. Whereas the B-type alfalfa cyclins contain a highly conserved destruction box motif that is responsible for mitotic degradation in animals (Glotzer et al., 1991), no such motif could be found in CycMs4.

**CycMs4 Shows the Highest Similarity with Arabidopsis Cyclin δ3 and Belongs to the Class of D-Type Cyclins**

Sequence comparison of CycMs4 with other plant cyclins showed the highest identity (56%) with the Arabidopsis cyclin δ3 (Soni et al., 1995). Considerably less identity was found between CycMs4 and two other Arabidopsis cyclins (30 to 31%) that had been isolated in the same screen as the Arabidopsis δ3 cyclin (Soni et al., 1995). Identity scores ranging from 23 to 29% were observed when CycMs4 was compared with other plant cyclins. These data indicate that CycMs4 and cyclin δ3 belong to the same subfamily of plant cyclins and suggest that they may perform similar functions. Comparison of the CycMs4 and the three δ-type Arabidopsis cyclin sequences isolated by Soni et al. (1995) with current data banks showed the high-
Glu - Met

K3413/
pYEura3

K3413/
pcycMs4-1

Gal + Met

K3413/
pYEura3

K3413/
pcycMs4-1

Figure 1. Alfalfa cycMs4-1 Complements the Function of G1 Cyclins in Yeast.

Yeast K3413 was transformed with pYEura3 and pcycMs4-1. Under nonselective conditions (Glu - Met, medium containing glucose, but no methionine), K3413 transformants are able to grow because the yeast G1 cyclin CLN2 is expressed. When CLN2 expression is repressed on medium containing methionine (+ Met) and expression of cycMs4-1 is induced by the addition of galactose (Gal), only the transformant K3413/pcycMs4-1 is able to grow (Gal + Met).

The cycMs4 Gene Is Expressed Predominantly in Root Meristem Cells

To study the expression of the cycMs4 gene in different organs, the transcript levels of this gene were compared with those of the histone H3-1 and cycMs2 genes. mRNA was isolated from different alfalfa organs and used in RNA gel blot analysis, using radiolabeled fragments from cycMs4, histone H3-1, and cycMs2, as shown in Figure 3. As a control, the blot was also hybridized with a radiolabeled fragment from the Msc27 gene (Pay et al., 1992), which is expressed at relatively constant levels in all organs and during the cell cycle. The cell cycle phase-regulated histone H3-1 and cycMs2 genes are expressed in plant organs containing dividing cells, such as flower buds and young leaves (Figure 3). Although cycMs4 is also expressed at low levels in these organs, several fold higher transcript levels were observed in roots that had much lower histone H3-1 and cycMs2 transcript levels (Figure 3). These data indicate a tissue-specific regulation of the cycMs4 gene and are not consistent with an expression pattern of genes that are regulated exclusively in a proliferation-dependent manner.

To compare these data with the expression pattern of the cycMs4 gene in tissues of the intact plant, alfalfa root tips were analyzed using in situ hybridization. The specificity of the detection method was controlled by the hybridization of root tip sections with cycMs4 antisense (Figure 4A) and sense (Figure 4B) probes under the same conditions. As shown in Figure 4A, expression of the cycMs4 gene was only detected in actively dividing cells of the root meristem but not in the root cap or elongation zone (data not shown), where cells had exited the cell cycle and underwent differentiation. In situ hybridization of an off-median longitudinal root section with an antisense probe of the S phase-specific histone H3-1 gene is shown in Figure 4C. Expression of the cycMs4 and histone H3-1 gene was found exclusively in the meristematic region, indicating cell proliferation-dependent regulation. The files of cells that originated from the same founder cell and express histone (Figure 4C) are all in S phase, indicating that root meristem cells transit the cell cycle with a certain degree of synchrony. As for the histone H3-1 gene, the presence of cycMs4 transcripts in adjacent cells of a file is consistent with cell cycle phase-specific expression.

To determine in which phase(s) the cycMs4 gene is expressed during the cell cycle, the root tip section of Figure 4A that had been hybridized with an antisense cycMs4 probe was
The predicted amino acid sequence is shown in single-letter code.

The GenBank, EMBL, and DDBJ accession number for cycMs4, comprising the region from nucleotide 84 to 319, respectively. The 5' truncated version of the cDNA and the nucleotide and predicted amino acid sequences is X88864.

Deduced Amino Acid Sequence.

The consensus motif for Rb binding (L-X-C-X-E, where X stands for any amino acid) in the N terminus of CycMs4 is shown in boldface letters. The cyclin box region is underlined. PEST-rich regions with PEST scores of 4.1 and 3.2 are found from amino acid positions 32 to 1201 and 121 to 1444 respectively.

Figure 2. Nucleotide Sequence of Alfalfa cycMs4 cDNA and the Deduced Amino Acid Sequence.

The predicted amino acid sequence is shown in single-letter code. The consensus motif for Rb binding (L-X-C-X-E, where X stands for any amino acid) in the N terminus of CycMs4 is shown in boldface letters. The cyclin box region is underlined. PEST-rich regions with PEST scores of 4.1 and 3.2 are found from amino acid positions 32 to 1201 and 121 to 1444, respectively. The 5' truncated version of cycMs4, described in the text as cycMs4-1, comprises the region from nucleotide 471 to 1444. The GenBank, EMBL, and DDBJ accession number for the cycMs4 nucleotide and predicted amino acid sequences is X88864.

Figure 3. Expression of the cycMs4 Gene in Different Alfalfa Plant Organs.

Poly(A)* RNA was extracted from 100 μg of total RNA from alfalfa root, flower bud, flower, stem, young leaf, and suspension-cultured cells and hybridized with radiolabeled probes of the cycMs4, cycMs2, and histone H3-1 genes, with Msc27 used as a control.

Figure 4. Epifluorescent staining of the same section with DAPI shows that the cycMs4 mRNA was found in only a subset of interphase cells and not in mitotic cells (Figure 4F). A similar analysis of cortex cells of the root tip section shown in Figure 4C after hybridization with a histone H3-1 antisense probe is shown in Figure 4E. Epifluorescent staining of the same section with DAPI showed that histone H3-1 expression was confined to a subset of interphase cells (arrowheads in Figure 4E). In summary, these results show that the cycMs4 gene is expressed in only a section of interphase and not during mitosis.

Despite the evidence that the cycMs4 gene is expressed only in a certain period of interphase, the hybridization pattern in root tips was different from that of the histone H3-1 and the alfalfa cyclin cycMs2 gene (Meskiene et al., 1995). Whereas the histone H3-1 and the cycMs2 genes are uniformly expressed in all tissues, cycMs4 transcripts are absent from the stele and are found in highest quantities in the pericycle and endodermis and in the outer cortex (Figure 4A). This position-dependent cycMs4 expression pattern cannot be explained on the basis of cell cycle regulation and must be due to other factors.
Figure 4. In Situ Hybridization of Longitudinal Sections of Alfalfa Root Meristems with cycMs4 and Histone H3-1.

Digoxigenin-labeled antisense fragments of cycMs4 and histone H3-1 and a sense fragment of cycMs4 were hybridized to 10-μm-thick longitudinal sections of young alfalfa root tips and were viewed by bright-field microscopy; counterstaining with DAPI was viewed by epifluorescence. Digoxigenin labeling results in a bluish red color, whereas DAPI-stained nuclei appear light blue. (A) to (E) show hybridization of root tip cells viewed under bright-field conditions. (F) and (G) show epifluorescence microscopy.

(A) Hybridization of a root tip to an antisense probe of cycMs4.
(B) Hybridization of a root tip to a sense probe of cycMs4.
(C) Hybridization of a root tip to an antisense probe of histone H3-1.
(D) High magnification of the root tip hybridization shown in (A).
(E) High magnification of the root tip section shown in (C).
(F) Epifluorescence microscopy of the DAPI-stained nuclei shown in (D).
(G) Epifluorescence microscopy of the DAPI-stained nuclei shown in (E).

Scale bar in (A) = 100 μm for (A) to (C); scale bar in (D) = 10 μm for (D) to (G). Arrowheads are shown for orientation.
The cycMs4 Gene Is Expressed in a Cell Cycle
Phase-Dependent Manner

In situ hybridization analysis of root tips indicated that the expression of the cycMs4 gene depends on the positional context of the cells in the root and on the stage of the cell cycle. To study the transcriptional regulation of the cycMs4 gene in another system, synchronously dividing alfalfa cells were subjected to RNA gel blot analysis at specific stages of the cell cycle. For this purpose, suspension-cultured cells were arrested with aphidicolin that blocks cells at the G1-to-S transition. When aphidicolin was removed, cells entered S phase and proceeded through the cell cycle in a synchronous manner, as shown by flow cytometric analysis in Figure 5A. DNA replication and the percentage of cells in mitosis were monitored by 3H-thymidine incorporation and micrographic analysis of DAPI-stained cells, respectively, as shown in Figure 5C.

To assess the state of cycMs4 gene expression in the different phases of the cell cycle, mRNA was prepared from the synchronized cells at different time points and hybridized to radiolabeled cycMs4 (Figure 5B). As an internal control for the different cell cycle stages, an S phase–specific histone H3-1 probe (Kapros et al., 1992) and a G2-to-M phase–specific cycMs2 probe (Hirt et al., 1992) were hybridized to the same filter. The results of these experiments are shown in Figure 5B. In G1-to-S–arrested cells, some cycMs4 and histone H3-1 mRNA but no cycMs2 was detected (Figure 5B at 0 hr). As determined from flow cytometry (Figure 5A) and 3H-thymidine incorporation (Figure 5C), cells were in S phase 3 hr after the release from the G1-to-S block. Although no cycMs2 mRNA was detected under these conditions, cycMs4 and histone H3-1 transcript levels increased considerably (Figure 5B at 3 hr). After 9 hr, the strong increase in cycMs2 transcript indicated that cells were in G2 phase (Figure 5B at 9 hr). At this time, cycMs4 and histone H3-1 transcripts had decreased somewhat (Figure 5B at 9 hr). When cells were in mitosis, as indicated by the peak in the mitotic index (Figure 5C) 12 to 15 hr after the release from the aphidicolin block, cycMs4 and histone H3-1 transcript levels declined further (Figure 5B at 12 and 15 hr).

After 24 hr, flow cytometry indicated that cells were predominantly in the G1 phase (Figure 5A), and transcript levels of cycMs4, cycMs2, and histone H3-1 had decreased to a minimum (Figure 5B at 24 hr). When cells entered S phase after 30 hr, as indicated by the increase in 3H-thymidine incorporation (Figure 5C), cycMs4 and histone H3-1 mRNA levels had increased considerably, whereas cycMs2 mRNA remained low (Figure 5B at 30 hr). Considering the limited synchrony of the cells, the fluctuation of cycMs4 transcript levels during the cell cycle indicates a cell cycle phase–dependent expression.

To investigate whether the cycMs4 gene is transcriptionally induced before the onset of the S phase, alfalfa cells were arrested in the G1 phase by phosphate starvation. Flow cytometric analysis indicated that refeeding of phosphate induced entry of the cells into S phase after 6 hr (Figure 6B). Whereas RNA gel blot analysis of these cells showed a concomitant increase of histone H3-1 mRNA with the onset of S phase (Figures 6A and 6B at 6 hr), cycMs4 transcripts had increased 4 hr after the refeeding of phosphate and decreased after 6 hr (Figure 6A). Twenty hours after the refeeding of phosphate, the cells had entered G2 phase, which was indicated by the strong increase of cycMs2 transcripts (Figure 6A) and the increase of cells with a G2 DNA content (Figure 6B). At this time, cycMs4 mRNA levels had increased again (Figure 6A at 20 hr). In agreement with flow cytometry, the increase of cycMs4
mRNA and the persistence of H3-1 transcripts after the majority of cells had passed through S phase might be explained by the occurrence of several populations of cells that entered the cell cycle at different times after the refeeding of phosphate (Figure 6B at 6 and 10 hr).

Altogether, RNA gel blot analysis of synchronized cells showed that cycMs4 transcription is induced shortly before the onset of S phase. After cells have progressed through S phase, cycMs4 gene expression decreases and is absent in mitosis and G1 phase. These results are consistent with the notion that CycMs4 plays a role in the G1-to-S phase transition of the alfalfa cell cycle.

cycMs4 Gene Expression Is Induced before DNA Synthesis in Mitogenically Stimulated Leaf Cells

To investigate cycMs4 gene expression in cells that are reentering the cell cycle in the G1 phase from a quiescent G0 state, pieces of fully differentiated alfalfa leaves were incubated in a medium in the presence of mitogenic concentrations of auxin and cytokinin. Under these conditions, DNA replication and subsequent division occurred after 3 days, as shown in Figure 7A by in situ 3H-thymidine labeling. RNA gel blot analysis over 6 days was performed with radiolabeled fragments of the cycMs4, cycMs3, cycMs2, and histone H3-1 genes and is shown in Figure 7B. Histone H3-1 mRNA was not detected in the leaf pieces before 3 days (Figure 7B, lane 6) and correlated with DNA replication (Figure 7A, day 3). Whereas cycMs3 expression was detected after 1 hr, cycMs4 mRNA was detected only after 12 hr (Figure 7B, lane 4). After maximal expression of cycMs4 after 24 hr (Figure 7B, lane 5), transcript levels decreased somewhat (Figure 7B, lane 6) when cells entered S phase (Figure 7A, day 3). cycMs2 transcript levels became detectable only after 3 days (Figure 7B, lane 6), at the same time as the first mitoses (data not shown). These results show that cycMs4 gene expression is induced before the onset of S phase and is compatible with a function in the G1-to-S phase transition of the cell cycle.

A phosphate-starved alfalfa suspension culture (0 hr) was released and grown for 28 hr. Samples were taken at the indicated time points, analyzed by flow cytometry for the DNA content, and used for RNA gel blot analysis.

(A) RNA gel blot analysis of cycMs4, cycMs2, and histone H3-1 genes, with Msc27 used as a control.

(B) Percentage of cells after release from G1 arrest calculated by flow cytometric DNA content determination.

Figure 6. Transcript Analysis of cycMs4 in Suspension-Cultured Alfalfa Cells Synchronized by Phosphate Starvation.
We have recently isolated the cycMs3 cyclin gene from alfalfa. It is induced during the G0-to-G1 reentry into the proliferative cell cycle (Meskiene et al., 1995). To compare the expression pattern of the cycMs4 gene with that of the cycMs3 gene after mitogenic activation of leaf cells, the same RNA gel blot was hybridized with a radiolabeled fragment of the cycMs3 gene (Figure 7B). Whereas an increase of cycMs3 mRNA was observed within 1 hr (Figure 7B, lane 2), cycMs4 transcripts could not be detected before 12 hr after mitogen stimulation (Figure 7B, lane 4). However, compared with lanes 5 to 7, lanes 3 and 4 contain much less RNA, and a very large induction of both genes, cycMs3 and cycMs4, occurred after 12 hr (Figure 7B, lane 4). These data indicate that induction of the cycMs4 gene occurs subsequent to stimulation of cycMs3 expression but before DNA synthesis.

DISCUSSION

Conservation of the basic mechanisms of the eukaryotic cell cycle has enabled the isolation of components that regulate the cell cycle from a wide range of organisms. On the basis of sequence conservation, two mitotic cyclin genes have been isolated from alfalfa (Hirt et al., 1992). To isolate cell cycle regulatory components that potentially act in the early alfalfa cell cycle, a yeast selection system based on the complementation of yeast G1 cyclins was used. Expression of an alfalfa cDNA library in G1 cyclin-deficient yeast cells that are blocked in START led to the isolation of a novel alfalfa cyclin, termed CycMs4. Several lines of evidence indicate that CycMs4 and the closely related Arabidopsis δ cyclins (Soni et al., 1995) are homologs of mammalian D-type cyclins and may be involved in the control of the G1-to-S phase transition of the plant cell cycle.

Sequence comparison of CycMs4 with protein data banks revealed the highest similarity with the Arabidopsis cyclin 63, which was also isolated by functional selection of G1 cyclin-deficient yeast cells (Soni et al., 1995). In contrast, two other δ-type Arabidopsis cyclins showed similar identities with CycMs4 as other nonrelated plant cyclins, indicating that CycMs4 and cyclin 63 are highly conserved during evolution and may perform similar functions in the two plant species. Both alfalfa CycMs4 and the δ-type Arabidopsis cyclins are most similar to mammalian D-type cyclins and also contain PEST-rich regions. Such sequences are responsible for protein instability (Rogers et al., 1986) and are present in most G1 cyclins (Tyers et al., 1992). These features suggest that CycMs4 and δ-type cyclins should have a short half-life and that plants may also have a PEST-dependent protein degradation machinery.

The decision of mammalian cells to proceed through G1 phase and enter S phase is regulated through the activation of the D-type associated cyclin-dependent protein kinases (Matsushime et al., 1994; Meyerson and Harlow, 1994). The tumor suppressor Rb protein inhibits the activity of E2F-type transcription factors and thereby prevents induction of S phase-specific genes (for review, see Nevins, 1992). In proliferating cells, cyclin D-dependent Cdk activity appears in G1, resulting in Rb phosphorylation (Matsushime et al., 1994; Meyerson and Harlow, 1994). Phosphorylated Rb is unable to form complexes with the E2F-type transcription factors and allows synthesis of genes involved in initiating DNA synthesis. D-type cyclins can physically interact with Rb via a conserved domain (Dowdy et al., 1993; Kato et al., 1993). The consensus sequence motif for Rb binding that is found in D-type cyclins, L-X-C-X-E (where X is any amino acid), is also present in CycMs4 and all three Arabidopsis δ-type cyclins (Soni et al., 1995). To our knowledge, however, no Rb or E2F homologs have been identified in plants. Overall, the similarity and the presence of all the hallmarks of mammalian D-type cyclins in the CycMs4 and the three Arabidopsis δ-type cyclins suggest that the G1-to-S transition of plants may be regulated by a mechanism similar to that operating in the cell cycle of mammalian cells.

The expression of cyclins is bound to the proliferative state of the cell. RNA gel blot analysis of synchronized alfalfa cells showed a cell cycle phase-dependent expression of the cycMs4 gene, indicating that expression of the cycMs4 gene is induced shortly before onset of S phase, peaks at the G1-to-S transition, and decreases in later stages. These results were confirmed by in situ hybridization analysis of root tips, showing that cycMs4 gene expression is cell cycle phase dependent and is terminated before onset of mitosis. A similar cell cycle regulation of the Arabidopsis cyclin 63 gene has been reported (Soni et al., 1995), suggesting a function of CycMs4 and cyclin 63 in the decision of a plant cell to enter the S phase of the cell cycle.

When the transcript levels of the cycMs4 gene in different organs were compared with those of other genes expressed in a cell cycle phase-specific manner, the cycMs4 gene was found to be expressed predominantly in roots. These results confirm the RNA gel blot analysis of the cyclin 63 gene in Arabidopsis organs by Soni et al. (1995), underscoring the similarities between CycMs4 and cyclin 63. These authors also found a cytokinin dependence of cyclin 63 gene expression in suspension-cultured Arabidopsis cells. Because root tips are considered to be the sites of cytokinin production, we speculate that the preferential expression of the cycMs4 gene in roots may be connected to the cytokinin dependence. Interestingly, in situ hybridization of alfalfa root tips showed a nonhomogeneous expression of the cycMs4 gene in different meristematic cell layers, possibly reflecting different concentrations of cytokinin in these cells.

Many mammalian in vitro-cultured cell types arrest in the G1 phase under growth factor-limiting conditions. Upon re-addition of growth factor, cells resume cell division and D-type cyclin genes are induced in the G1 phase of the cell cycle (Matsushime et al., 1991; Baldin et al., 1993). The appearance of D-type cyclin mRNA in the G1 phase correlates with the accumulation of the proteins and the activation of cyclin D-dependent protein kinases (Matsushime et al., 1994;
Meyerson and Harlow, 1994). When differentiated G_{0}/G_{1}-arrested leaf cells were induced to reenter the cell cycle by treatment with plant hormones, cycMs4 gene expression was induced before DNA replication became detectable. Although one may argue that the plant and the mammalian cell culture systems are not comparable, the early cycMs4 gene induction argues for a role similar to that defined for mammalian D-type cyclins. Overall, the presence of D-type homologous proteins in alfalfa and Arabidopsis and their similar transcriptional regulation suggest that the G_{1}-to-S phase transition of plant cells may be controlled by mechanisms similar to those operating in mammals.

Induction of D-type cyclin gene expression is a delayed early response of mammalian cells to growth factors and is preceded by unknown earlier events (Schneider et al., 1991; Sherr, 1993). In the G_{1} phase of yeast, nutritional state and size of the cells are monitored by the constitutively expressed CLN3 cyclin (Tyters et al., 1993). Under appropriate conditions to enter a new cell cycle, activation of the Cln3-Cdc28 kinase appears to be necessary for cell cycle phase-specific expression of the CLN1 and CLN2 genes, which then trigger expression of genes necessary for DNA replication (Nasmyth, 1993). In plants, an analogous activation mechanism might be acting. The finding that expression of the cycMs3 gene is induced before the cycMs4 gene during the G_{0}/G_{1} reentry of alfalfa leaf cells into the cell cycle suggests a model for how such a mechanism might work. Central to this model is the observation that, in contrast to other alfalfa cyclin genes, few cycMs3 transcripts are present in mature leaf cells before mitotic stimulation. Similar to the situation of Cln3-Cdc28 in yeast, mitogen stimulation would activate a CycMs3-Cdk complex, resulting in subsequent synthesis of other G_{1} cyclins such as CycMs4. A CycMs4-Cdk kinase would then phosphorylate the alfalfa Rb homolog, setting free E2F-type transcription factors, which might then induce expression of genes required for S phase, such as DNA polymerases and histones. Although this model is probably far too simple, soon all of the necessary tools to test this hypothesis will be available.

**METHODS**

**Construction of a cDNA Expression Library**

From 5 μg of poly(A)^+ RNA, which was isolated from suspension-cultured alfalfa (Medicago sativa spp varia cv Rambler, line A2) cells, cDNA was generated with a Stratagene cDNA kit according to the manufacturer's recommendations. The cDNA was ligated into the EcoRI site of the yeast/Escherichia coli shuttle vector λ-Max1 (Clontech, Palo Alto, CA). After in vitro packaging and transformation into E. coli, the phage library was in vivo excised as pYEUra3 plasmids, which were subsequently used for yeast transformation.

**Yeast Techniques**

The yeast strain K3413 (relevant genotype: cln1::HisG cln2::del cln3::LEU2 Yiplac204-MET2-CLN2) was kindly provided by A. Amond and K. Nasmyth (Institute of Molecular Pathology, Vienna, Austria). The strain was used for transformation with the alfalfa cDNA expression library. Yeast transformation was performed according to Gietz et al. (1992). After transformation, cells were plated on uracil-free medium containing 2% glucose, and after 2 days, they were replica plated on uracil-free but galactose-containing medium. After 24 hr, cells were replica-plated onto selective induction plates (2 mM methionine, 2% galactose, without uracil). Growth was assayed for 5 days. Methionine-resistant transformants were propagated on selective induction plates. Standard methods were used for culturing and manipulating yeast.

**Cloning and Sequence Analysis**

Most molecular techniques were performed as described by Sambrook et al. (1989). To isolate a full-length cycMs4 (for cyclin M. sativa) clone, a cDNA library prepared from somatic alfalfa embryos (Hirt et al., 1993) was screened with the cycMs4-7 cDNA fragment. Plasmid pSP72 (Pharmacia) was used for subcloning and a Pharmacia T7 Sequencing Kit for sequence determination. A sequence homology search was performed at the National Center of Biological Information (Bethesda, MD) using the BLAST network service. Sequence comparisons were done with the program GAP from the Genetics Computer Group (Madison, WI). PEST-rich regions were identified by using the PGENE program PESTFIND (Intelligenetics, Mountain View, CA).

**Plant Cell Culture, Synchronization, Flow Cytometry, and \textsuperscript{3}H-Thymidine Incorporation**

A suspension culture of alfalfa (M. sativa spp. varia cv Rambler, line A2) was used (Bögére et al., 1988). Subculturing was performed in 5-day intervals in 1:10 dilutions in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.2 mg/L kinetin.

Synchronization of suspension-cultured alfalfa cells by aphidicolin and phosphate starvation was performed as described by Meskiene et al. (1995).

**RNA Extraction and RNA Gel Blot Analysis**

RNA isolation from 0.3 to 1 g of plant material was performed according to Cathala et al. (1983). Poly(A)^+ RNA was isolated from 100 μg of total RNA with Dynabeads according to the instructions of the manufacturer (Dynal, Oslo, Norway). Formaldehyde–agarose gel electrophoresis and RNA gel blot analysis were performed according to standard protocols (Sambrook et al., 1989). Radiolabeled probes were generated by random primed \textsuperscript{32}P-labeling from the following genes: the entire Msc27 gene (Pay et al., 1992); fragments containing the coding regions of the cycMs2 (Hirt et al., 1992), cycMs3 (Meskiene et al., 1995), or the cycMs4 gene; and fragments containing the 3' nontranslated regions of the histone H3-1 gene (Kapros et al., 1992; S.C. Wu, unpublished results).
In Situ Hybridization
A 442-bp fragment of the 5' coding region of cycMs4 and a 132-bp EcoRI-XhoI fragment of the 3' noncoding region of histone H3-1 cDNA (Kapros et al., 1992; S.C. Wu, unpublished results) were cloned into pBluescript SK+ vectors and used for preparing the hybridization probes. Digoxigenin labeling of sense and antisense probes by in vitro transcription, tissue preparation, and in situ hybridization were performed as described by Bradley et al. (1993) with modifications of Fobert et al. (1994).

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