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The D-Type Alfalfa Cyclin Gene *cycMs4* Complements G_1 Cyclin–Deficient Yeast and Is Induced in the G_1 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 B-type cyclins. The closely related *cycMs1* and *cycMs2* genes are expressed primarily during the G_2 and M phases and are most likely mitotic cyclins; expression of the *cycMs3* gene is induced in the G_0-to-G_1 transition, when cells reenter the cell cycle. By complementation of G_1 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 δ3 gene. CycMs4 and cyclin δ3 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 G_0-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 G_1-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 G_1-to-S and G_2-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 G_2 phases, B-type cyclins act at the G_2-to-M phase transition, and D- and E-type cyclins function at the G_1-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; Hemerly 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 G_1-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; Hemerly 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).

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In contrast to A- and B-type cyclins, which contain a highly conserved central domain (cyclin box), G_1 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 G_1-to-S transition. Our approach was based on the fact that yeast cells deficient in all three G_1 cyclin genes, *CLN1*, *CLN2*, and *CLN3*, are arrested at a point in the G_1 phase called START and cannot divide. We assumed that expression of an alfalfa G_1 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 G_1 phase and resume proliferation, transcription of the *cycMs4* gene was induced before onset of DNA replication, which is compatible with a role in the G_1-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 G_1 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 G_1 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 10^6 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 pcycMs4-1 (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 pcycMs4-1 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-

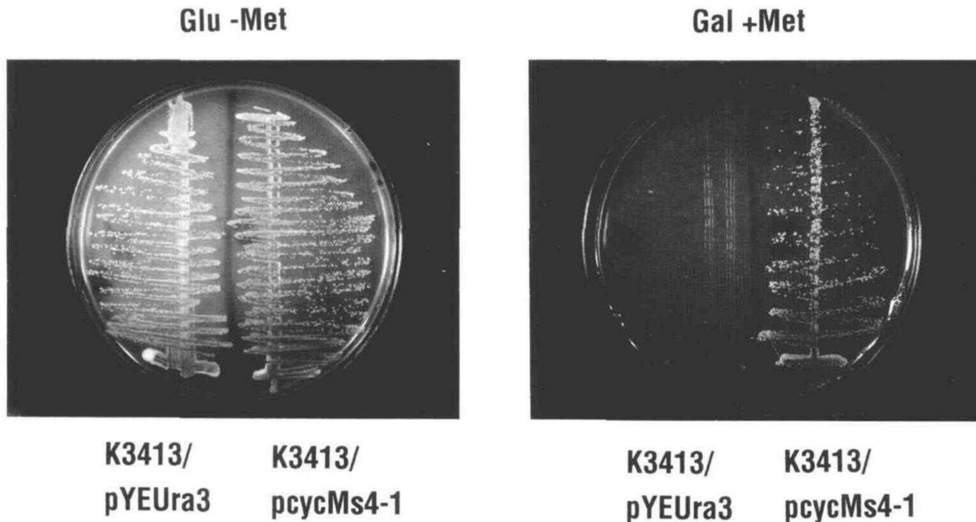

Figure 1. Alfalfa *cycMs4-1* Complements the Function of G_1 Cyclins in Yeast.

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 G_1 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).

est identity with mammalian D-type cyclins (Lew et al., 1991; Xiong et al., 1991). Alignment of CycMs4 and human cyclin D1 revealed 33% identity over the entire length of the proteins. Similar to related Arabidopsis δ-type cyclins, the predicted CycMs4 protein sequence has the typical structural elements of G_1 cyclins, containing a cyclin box in the central region (underlined in Figure 2) and PEST-rich regions in the N- and C-terminal domains. These features are typical for short-lived G_1 cyclins and suggest that a PEST-dependent protein degradation machinery must also exist in plants.

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, severalfold 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

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   1 GAATTCGGCACGAGCTCTTCTGCTACGACTACCTCTCCCTATACTCTCTACTCTTTCTAG
  61 TTCTACTACTTTTCTTTCTTTCTCTGTTCTCTCTCTCTTCTTCATTTCTTCACATTTTCA
 121 CACACAGAGAGAAGACAGAACAAAGAGGAAAAGAGAGAGCGATGGATGTGAGACTCTTCA
 181 GTACTGTTTCCTTCTTTTTATAATGAACAAAGGACCACACACCCTCTTCTTCACTGAAGA

 241 AGATGGCTATCCATCATCATCATCACAATCACCAACAACTTCAACAACACACTTCTTCTC
      M  A  I  H  H  H  H  H  N  H  Q  Q  L  Q  Q  H  T  S  S  L

 301 TTTTTGATGCACTTTACTGTGATGAAGAAGAAAAATGGGAAGATGATGATGAAGGAGAAG
      F  D  A  L  Y  C  D  E  E  E  K  W  E  D  D  D  E  G  E  V

 361 TTGTAGATGAAGGAGCACAAAGTGATGTCACAACAACAAACTATGATATATTGGACTCTA
      V  D  E  G  A  Q  S  D  V  T  T  T  N  Y  D  I  L  D  S  T

 421 CTTCCCTTTTACCTCTGCTTTTGTTAGAACAGAACTTGTTCAATGAAGATGAAGAACTCA
      S  L  L  P  L  L  L  L  E  Q  N  L  F  N  E  D  E  E  L  N

 481 ACACTCTTTTCTCCAAAGAGATAACTCAACAAGAAACATATTACGAGGATCTGAAAAATG
      T  L  F  S  K  E  I  T  Q  Q  E  T  Y  Y  E  D  L  K  N  V

 541 TGATCAACTTTGACTCACTCTCTCAACCTCGTCGTGAAGCTGTTGAATGGATGCTTAAAG
      I  N  F  D  S  L  S  Q  P  R  R  E  A  V  E  W  M  L  K  V

 601 TCAATGCTCATTATGGTTTCTCTGCTCTCACTGCAACACTTGCTGTTAACTATCTTGATA
      N  A  H  Y  G  F  S  A  L  T  A  T  L  A  V  N  Y  L  D  R

 661 GGTTTCTTCTAAGCTTCCATTTCCAAAAAGAGAAACCATGGATGATTCAGCTTGTTGCTG
      F  L  L  S  F  H  F  Q  K  E  K  P  W  M  I  Q  L  V  A  V

 721 TTACTTGCATCTCTTTAGCTGCTAAAGTTGAAGAAACTCAAGTTCCTCTTCTCTTAGACC
      T  C  I  S  L  A  A  K  V  E  E  T  Q  V  P  L  L  L  D  L

 781 TTCAAGTGCAAGATACTAAATATGTGTTTGAGGCAAAGACTATTCAGAGAATGGAGCTAT
      Q  V  Q  D  T  K  Y  V  F  E  A  K  T  I  Q  R  M  E  L  L

 841 TGATTCTGTCAACACTGAAATGGAAGATGCATCCAGTGACAACACACTCTTTTCTAGATC
      I  L  S  T  L  K  W  K  M  H  P  V  T  T  H  S  F  L  D  H

 901 ACATTATAAGAAGGCTTGGATTGAAAACTAATCTTCATTGGGAGTTTCTTAGGCGCTGTG
      I  I  R  R  L  G  L  K  T  N  L  H  W  E  F  L  R  R  C  E

 961 AGAATCTTCTTCTATCTGTACTTTTAGATTCAAGATTTGTTGGTTGTGTTCCTTCTGTGT
      N  L  L  L  S  V  L  L  D  S  R  F  V  G  C  V  P  S  V  L

1021 TGGCCACTGCTACAATGTTGCATGTTATAGACCAGATTGAACAGAGTGATGATAATGGTG
      A  T  A  T  M  L  H  V  I  D  Q  I  E  Q  S  D  D  N  G  V

1081 TGGATTACAAAAATCAGCTTCTTAATGTTCTCAAAATCAGCAAGGAGAAAGTTGATGAAT
      D  Y  K  N  Q  L  L  N  V  L  K  I  S  K  E  K  V  D  E  C

1141 GTTATAATGCGATTCTTCATCTTACAAATGCAAATAATTATGGTCATAAACGAAAATATG
      Y  N  A  I  L  H  L  T  N  A  N  N  Y  G  H  K  R  K  Y  E

1201 AAGAAATCCCTGGTAGTCCAAGTGGCGTAATTGATGCTGTTTTTAGTTCTGATGGTTCTA
      E  I  P  G  S  P  S  G  V  I  D  A  V  F  S  S  D  G  S  N

1261 ACGATTCGTGGACAGTGGGAGCATCATCATATTCAACCTCAGAGCCTGTGTTTAAGAAGA
      D  S  W  T  V  G  A  S  S  Y  S  T  S  E  P  V  F  K  K  T

1321 CCAAGAATCAAGGACAAAATATGAATTTGTCACCGATTAACAGGGTCATTGTCGGAATTC
      K  N  Q  G  Q  N  M  N  L  S  P  I  N  R  V  I  V  G  I  L

1381 TTGCCACTGCAACCTCTCCTTAAAACCCTCTATCCGTTTTCTGTCCTTTTTATTTAAAAA
      A  T  A  T  S  P

1441 AAAATACCATATAAAAAATTACCCCCAAAAAGATCTATATTTTATTTACTATGGTTATGT
1501 TCATGTTCGTACTAAACTCTAGTTAGTTTAGTAGTCTTTCTTTCTATCTCTTCATTTCCA
1561 ACAATGTCCCAAATTCATTTACATGAATCTCTTGAAGAGGCAGTGGCAAGATGATGATAG
1621 AGGATTAAAGGAATGGTTAATTTCTGATGAGTTAAAGAGGAAAGGACAAAGTTGGCAATG
1681 NAGATTTTATTACTATGAGCAGAAAAGAACCCTATGATATCTGTTTCATTTCAAGGACCT
1741 GTTTTTTTATTTTATTCAATGGTTCTCTTCTAGACCATACCCAATTTGGACATATTTATA
1801 TCATATTTCTATAATAAATTGGGAATAATTTTTGGTCCAAAAAAAAAAAAAAAAAAA
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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 84 and 319 to 357, 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.

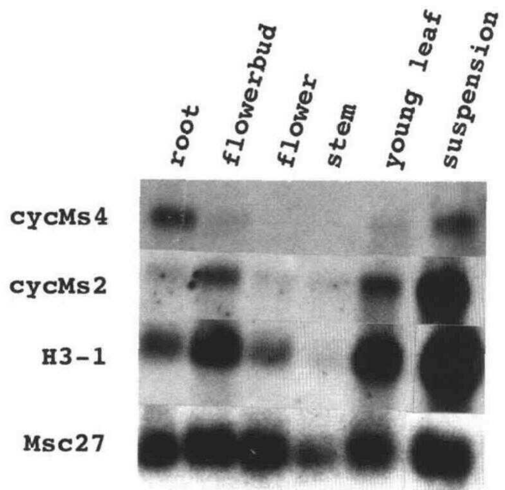

Figure 3. Expression of the *cycMs4* Gene in Different Alfalfa Plant Organs.

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.

analyzed at high magnification. A region of cortex cells of this section is shown in Figure 4D. Although high levels of *cycMs4* mRNA were present in three adjacent cells (indicated by arrowheads in Figure 4D), several cells belonging to the same file did not contain any transcript, indicating that the *cycMs4* gene is not expressed in certain phases of the cell cycle. The epifluorescence microscopy of the same section after staining with 4′,6-diamidino-2-phenylindole (DAPI) shows that *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 4G). 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.

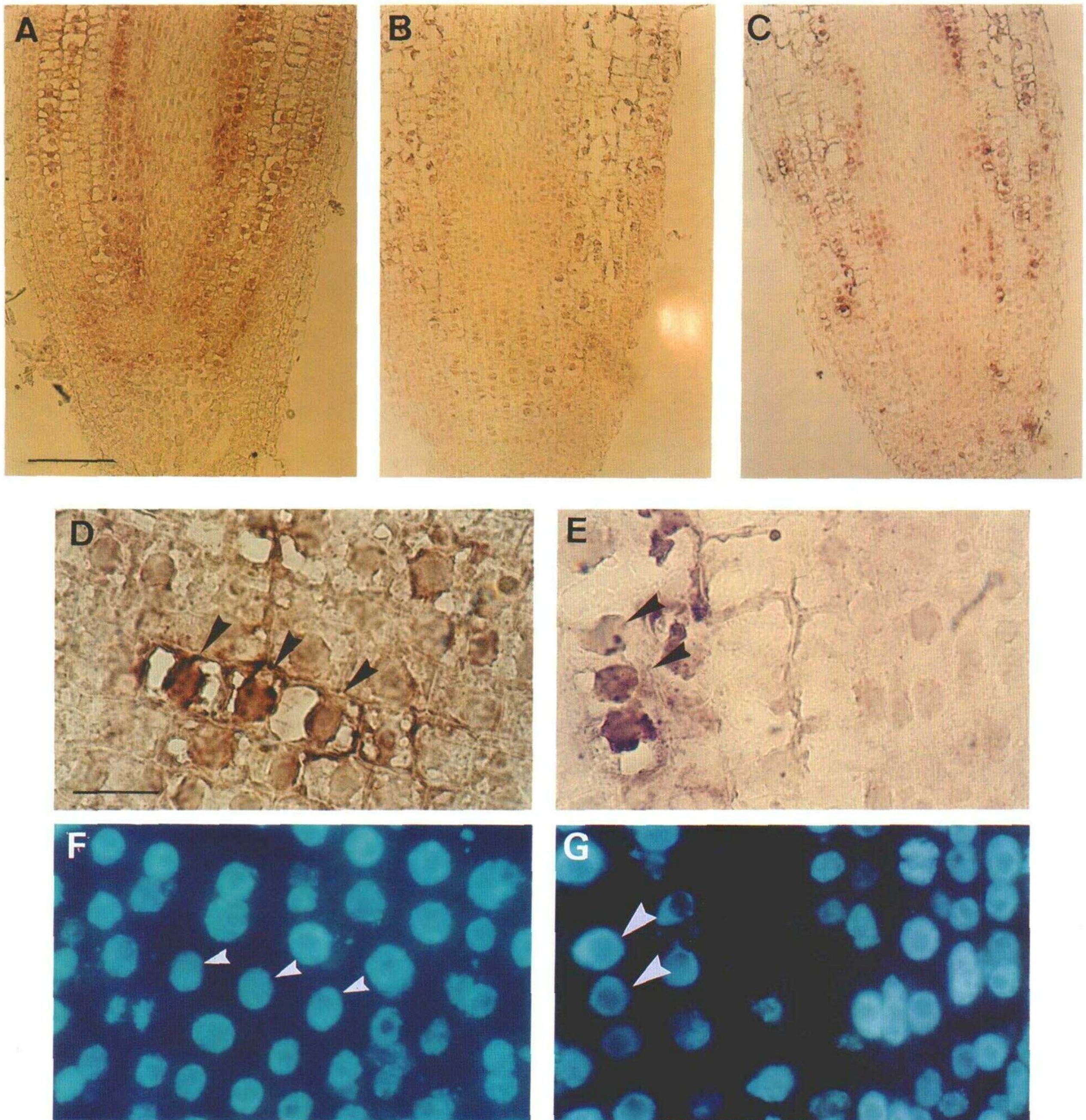

Figure 4. In Situ Hybridization of Longitudinal Sections of Alfalfa Root Meristems with *cycMs4* and Histone H3-1.

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 G_1-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 ^{3}H-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 G_2-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 G_1-to-S-arrested cells, some *cycMs4* and histone H3-1 mRNA but no *cycMs2* transcript was detected (Figure 5B at 0 hr). As determined from flow cytometry (Figure 5A) and ^{3}H-thymidine incorporation (Figure 5C), cells were in S phase 3 hr after the release from the G_1-to-S block. Although no *cycMs2* mRNA was detected under these conditions, *cycMs4* and histone H3-1 transcript levels had increased considerably (Figure 5B at 3 hr). After 9 hr, the strong increase in *cycMs2* transcript indicated that cells were in G_2 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 G_1 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 ^{3}H-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 G_1 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

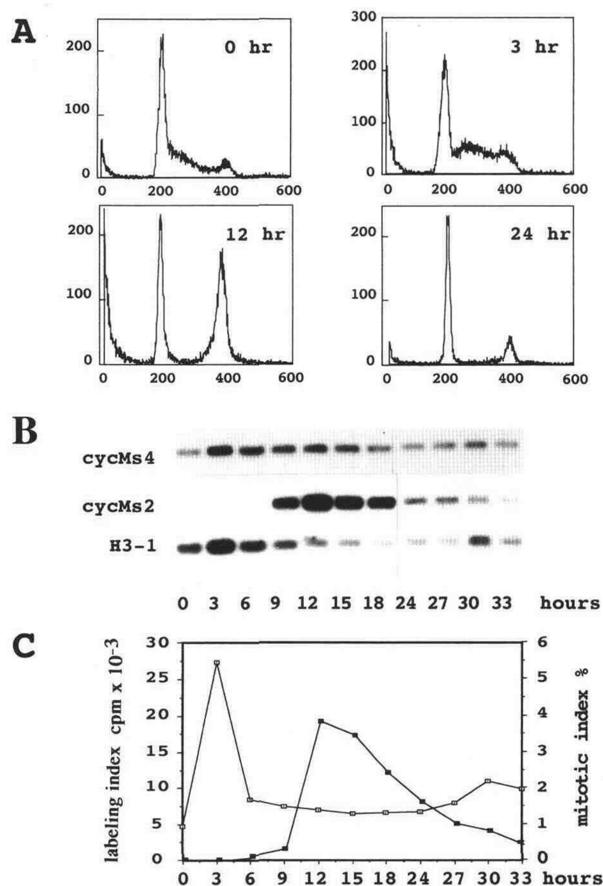

Figure 5. Cell Cycle Phase-Dependent Transcription of *cycMs4*.

Aphidicolin-arrested cells (0 hr) were released and grown synchronously for one complete cell cycle (33 hr). Samples were taken at the indicated time intervals, analyzed for DNA replication and for mitotic index and flow cytometric DNA content, and used for RNA gel blot analysis.

(A) Flow cytometric DNA content determination. Relative DNA fluorescence is indicated on the x-axis. The values 200 and 400 represent cells with a G_1 phase and G_2 phase DNA content, respectively. Intermediate values represent cells in S phase. The number of nuclei is indicated on the y-axis.

(B) RNA gel blot analysis of *cycMs4*, *cycMs2*, and histone H3-1 genes in synchronously growing cells. One microgram of poly(A)$^+$ RNA was loaded in each lane.

(C) ^{3}H-thymidine incorporation analysis of synchronized cell culture (open squares) and mitotic index determination (closed squares).

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 G_2 phase, which was indicated by the strong increase of *cycMs2* transcripts (Figure 6A) and the increase of cells with a G_2 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 G_1 phase. These results are consistent with the notion that CycMs4 plays a role in the G_1-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 G_1 phase from a quiescent G_0 state, pieces of fully differentiated alfalfa leaves were incubated in a medium in the presence of mitogenic concentrations of auxin

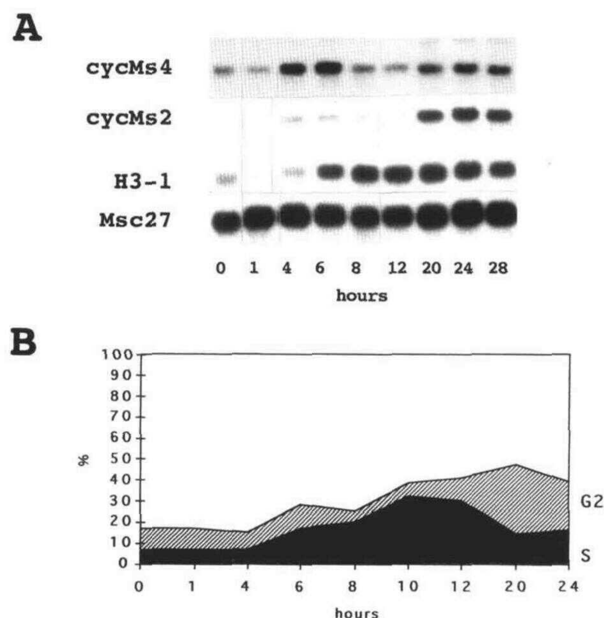

Figure 6. Transcript Analysis of *cycMs4* in Suspension-Cultured Alfalfa Cells Synchronized by Phosphate Starvation.

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 G_1 arrest calculated by flow cytometric DNA content determination.

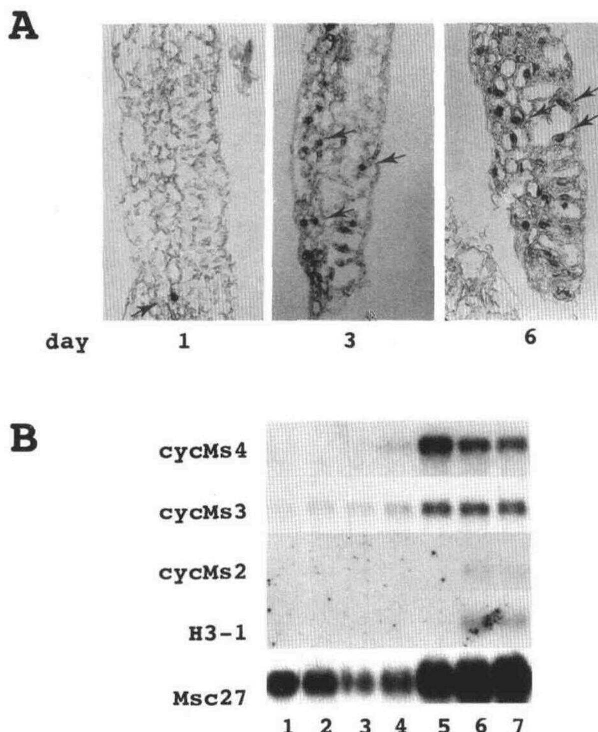

Figure 7. *cycMs4* Transcription Is Induced before DNA Replication in Mitogen-Stimulated Differentiated Alfalfa Leaf Cells.

Alfalfa leaf pieces were incubated in Murashige and Skoog medium containing 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.2 mg/L kinetin. Samples for RNA gel blot analysis were taken at the indicated time intervals.

(A) Autoradiographs of ^{3}H-thymidine pulse-labeled leaf pieces. Arrows indicate cells in S phase.

(B) RNA gel blot analysis of *cycMs4*, *cycMs3*, *cycMs2*, and histone H3-1 genes, with *Msc27* used as a control. Lanes 1 to 7 correspond to 0, 1, 4, 12 hr and 1, 3, and 6 days.

and cytokinin. Under these conditions, DNA replication and subsequent division occurred after 3 days, as shown in Figure 7A by in situ ^{3}H-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 G_1-to-S phase transition of the cell cycle.

We have recently isolated the *cycMs3* cyclin gene from alfalfa. It is induced during the G_0-to-G_1 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 G_1 cyclins was used. Expression of an alfalfa cDNA library in G_1 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 G_1-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 δ3, which was also isolated by functional selection of G_1 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 δ3 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 G_1 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 G_1 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 kinase activity appears in G_1, resulting in pRb 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 G_1-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 G_1-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 δ3 gene has been reported (Soni et al., 1995), suggesting a function of CycMs4 and cyclin δ3 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 δ3 gene in Arabidopsis organs by Soni et al. (1995), underscoring the similarities between CycMs4 and cyclin δ3. These authors also found a cytokinin dependence of cyclin δ3 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 G_1 phase under growth factor–limiting conditions. Upon readdition of growth factor, cells resume cell division and D-type cyclin genes are induced in the G_1 phase of the cell cycle (Matsushime et al., 1991; Baldin et al., 1993). The appearance of D-type cyclin mRNA in the G_1 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 (Tyers 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-to-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 all other alfalfa cyclin genes, few *cycMs3* transcripts are present in mature leaf cells before mitogenic stimulation. Similar to the situtation 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. Amon 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 *cycMs4-1* 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 identitied by using the PCGENE program PESTFIND (Intelligenetics, Mountain View, CA).

Plant Cell Culture, Synchronization, Flow Cytometry, and ^{3}H-Thymidine Incorporation

A suspension culture of alfalfa (*M. sativa* spp. *varia* cv Rambler, line A2) was used (Bögre 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 ^{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-Xho1 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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