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Alfalfa Cyclins: Differential Expression during the Cell Cycle and in Plant Organs

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Cell division in eukaryotes is mediated by the action of the mitosis promoting factor, which is composed of the CDC2 protein kinase and one of the various mitotic cyclins. We have recently isolated a *cdc2* gene from alfalfa. Here, we report the isolation of two cyclin genes, *cycMs1* and *cycMs2*, from alfalfa. The *cycMs2* gene shows highest similarity to type B cyclins. In contrast, the predicted amino acid sequence of the *cycMs1* gene shows similar homology scores to cyclins of all types (25 to 35%). Both genes are expressed in dividing suspension cultured cells but cease to be expressed when the cells enter stationary phase. In synchronized alfalfa suspension cultured cells, the mRNAs of *cycMs1* and *cycMs2* show maximal expression in the G2 and M phases. Transcripts of *cycMs2* are found only in late G2 and M phase cells, an expression pattern typical for cyclin B genes, whereas *cycMs1* appears with the onset of G2. This pattern indicates that alfalfa *cycMs1* and *cycMs2* belong to different classes of cyclins. In young leaves, expression of both genes is high, whereas in mature leaves no transcripts can be detected, indicating that the two cyclin genes are true cell division markers at the mRNA level. In other organs, a more complex expression pattern of the two cyclin genes was found.

INTRODUCTION

All eukaryotes have been shown to contain a 34-kD protein kinase that plays a central role in the regulation of cell division. The 34-kD protein kinase has been shown to be the product of the cdc2+/CDC28 genes in yeasts and animals (for review, see Nurse, 1990) and in various plant species (Colasanti et al., 1991; Ferreira et al., 1991; Hirt et al., 1991).

The CDC2 kinase is one part of the mitosis promoting factor: its activation involves physical association of $p34^{cdc2}$ with cyclin proteins. In the absence of cyclins, cells cannot enter mitosis and CDC2 kinase activity is absent (Booher and Beach, 1987, 1988; Minshull et al., 1989; Murray and Kirschner, 1989; Murray et al., 1989). At the end of metaphase, cyclins are destroyed and CDC2 kinase activity vanishes (Murray et al., 1989). Degradation of cyclins has been shown to be required for completion of mitosis and might be necessary for inactivation of the CDC2 kinase (Murray et al., 1989).

Cyclins were originally identified in the eggs of marine invertebrates (Evans et al., 1983; Swenson et al., 1986; Standart et al., 1987). On the basis of amino acid homology, cyclins have subsequently been identified in fission yeast (Booher and Beach, 1988; Goebl and Byers, 1988; Hagan et al., 1988; Solomon et al., 1988), Xenopus (Minshull et al., 1989), Drosophila (Lehner and O'Farrell, 1989; Whitfield et al., 1989), humans (Pines and Hunter, 1989), and recently also in carrot, soybean (Hata et al., 1991), and Arabidopsis (Hemerly et al., 1992).

In this study, we describe the isolation of two partial cyclin cDNA clones, *cycMs1* and *cycMs2*, from alfalfa. Comparison of the predicted cyclin protein sequences with 18 other cyclins showed that *cycMs2* can be classified as a type B cyclin. In contrast, *cycMs1* appears to be equally distantly related to the type A, B, and G1 cyclins. RNA gel blot analysis with synchronized alfalfa cell suspension cultures showed cell cycle–dependent expression of both cyclin genes in the G2 and M phases. During the growth cycle of a batch suspension culture, the two genes were expressed during exponential growth phase but not in stationary phase. In the plant, *cycMs1* and *cycMs2* were only found to be expressed in organs with dividing cells.

RESULTS

Isolation and Sequencing of Cyclin cDNAs from Alfalfa

To isolate cyclins of type A and B, we screened 80,000 colonies of an alfalfa (*Medicago sativa*) cDNA library made from

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suspension cultured cells that were induced to form somatic embryos with redundant oligonucleotides encoding the conserved amino acid sequences K-Y-E-E-M(I)-Y-P for the cyclin A and B type and I-L-V(I)-D-W-L-V for the cyclin B class (see Methods for the nucleotide sequences). Positive colonies were isolated and rescreened according to the method of Hanks et al. (1988) at a temperature of 58°C in order to distinguish between false positives with a fit of less than 16 nucleotides. Only one colony was found to hybridize under these conditions to the I-L-V(I)-D-W-L-V oligonucleotide. DNA was prepared from this clone and digested with PstI. Upon gel electrophoresis, a 750-bp insert was observed.

Because the insert contains GC tails at both ends, it was impossible to perform sequence analysis with this clone. A 400-bp BamHI fragment was subcloned into the vector pTZ19U and sequenced from both sides. Sequencing was also possible after removal of a 5′ 200-bp XbaI fragment from the original clone. The complete sequence is shown in Figure 1A. The sequence at position 751 to 771 shows a fit of 18 out of the 20 nucleotides to the redundant oligonucleotide probe of the cyclin B class (see above). However, at the amino acid level, only five of the encoded seven amino acids fit to the highly conserved cyclin B box (the encoded sequence reads I-L-V-D-C-L-L instead of I-L-V-D-W-L-V). The clone contains a nontranslated region of 132 nucleotides before the first ATG, which is followed by a putative sequence of 211 amino acids. However, there is no stop codon or any indication of a poly(A) tail in the clone. This is surprising because the library was constructed by oligo(dT) priming of the poly(A)$^+$ RNA. Rescreening of 250,000 colonies of the same library and 1,000,000 of another cDNA library, made from alfalfa somatic embryos, resulted in the isolation of two colonies that were found to be identical to the above described clone. Screening of a third cDNA library made from suspension cultured alfalfa cells that were induced to form somatic embryos resulted in the isolation of one clone of 1.4 kb. The cDNA sequence is shown in Figure 1B and was found to be very different from that of *cycMs1*. A 5′ truncated open reading frame of 984 nucleotides was identified and appears to encode a different cyclin that is designated *cycMs2*. Interestingly, in contrast to *cycMs1*, the *cycMs2* clone contains a cyclin B box (nucleotide position 313 to 333) which fits much better to the animal sequences. Rescreening the different libraries yielded no other hybridizing colony than the one indicated. These results indicate high instability and/or very low abundance of these types of mRNAs.

To determine the copy number of the *cycMs1* and *cycMs2* genes in alfalfa, DNA gel blot analysis was performed with genomic DNAs from *M. sativa*. Hybridization with a ^{32}P-labeled *cycMs1* probe, as shown in Figure 2A, revealed the presence of two bands in HindIII-digested total DNA (Figure 2A, lane 1) but only one band in BglII-digested DNA (Figure 2A, lane 2). When the same DNAs were probed with a radiolabeled *cycMs2* probe, as shown in Figure 2B, the HindIII digest revealed hybridization to one band (Figure 2B, lane 1) but to four bands in BglII-digested DNA (Figure 2B, lane 2). Because alfalfa is an autotetraploid species, these data indicate the occurrence of one copy per haploid genome for both cyclin genes.

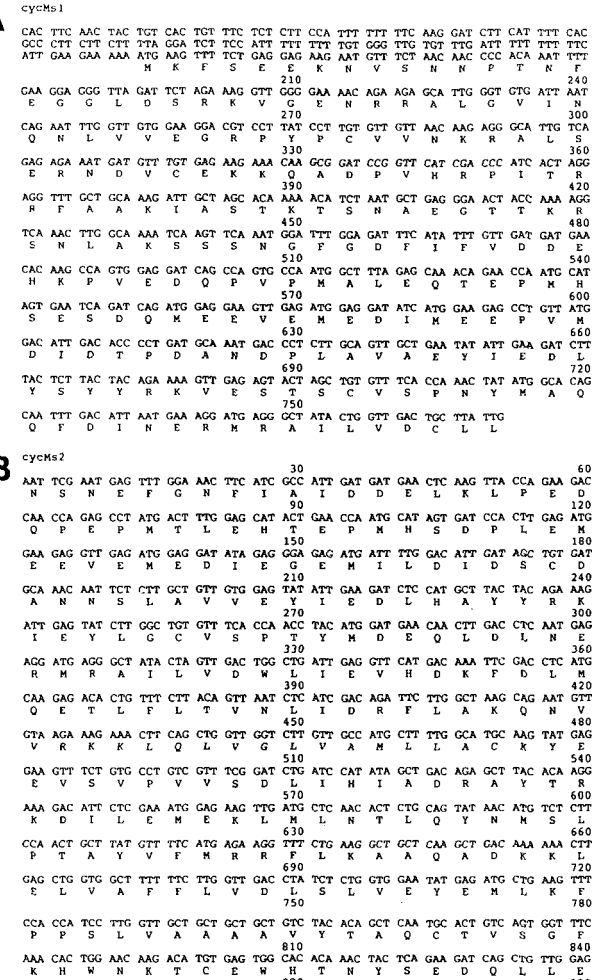

Figure 1. Nucleotide Sequences of Alfalfa *cycMs1* and *cycMs2* cDNAs and Their Predicted Amino Acid Sequences.

(A) *cycMs1*. The EMBL accession number is X68740.
(B) *cycMs2*. The EMBL accession number is X68741.

Therefore, we conclude that the *cycMs1* and *cycMs2* genes are both present as single copy genes in alfalfa.

The *cycMs2* Gene Encodes a Member of the Type B Cyclins, but *cycMs1* Cannot Be Classified into Any Cyclin Class

Alignment of the predicted protein sequences from *cycMs1* and *cycMs2* revealed 65% identity over 111 amino acids.

Alignment of the predicted alfalfa cyclin protein sequences with the Swiss-Prot protein data base revealed that the *cycMs1* gene is equally related to cyclins of the types A and B. Comparison of the amino acid sequences of *cycMs1* to the recently reported cyclins from carrot, soybean, and Arabidopsis (Hata et al., 1991; Hemerly et al., 1992) resulted in identity scores similar to the yeast and animal cyclins (25% to maximally 35%). In contrast, the same analysis with the *cycMs2* open reading frame showed clear evidence that this protein belongs to the type B class of cyclins. Identity scores of 35 to 39% were obtained to animal and yeast type B cyclins, whereas alignment with the soybean and Arabidopsis cyclins resulted in identities of 50.5 and 48%, respectively.

Using the CLUSTAL computer program (Higgins and Sharp, 1988), a generic tree was constructed with the predicted protein sequences from 20 cyclins from various sources, as shown in Figure 3. The carrot cyclin turned out to belong to the type A. The *cycMs2* gene was found to belong to the branches of the type B cyclins and was most similar to the soybean cyclin, which is also classified as a type B cyclin (Hata et al., 1991). The Arabidopsis cyclin, which was reported to be of the non-A, non-B type (Hemerly et al., 1992), belongs clearly to the type B cyclin class. The *cycMs1* sequence could not be fitted to any of the classes of eukaryotic cyclins and is shown as a separate branch.

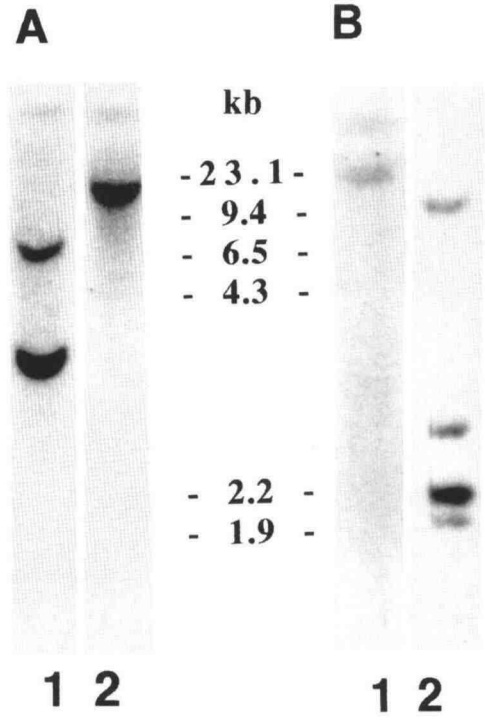

Figure 2. Genomic DNA Gel Blot Analysis of *cycMs1* and *cycMs2*.

Ten micrograms total DNA of *M. sativa* was digested with HindIII (lanes 1) or BglII (lanes 2).

(A) Nylon filters were hybridized with radiolabeled *cycMs1* probe.

(B) Nylon filters were hybridized with radiolabeled *cycMs2* probe.

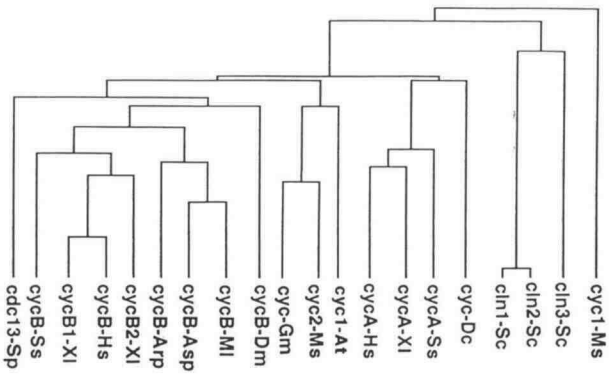

Figure 3. Phylogenetic Tree of the Cyclin Family.

The tree was constructed by the CLUSTAL program (Higgins and Sharp, 1988). Arp, *Arbacia punctulata*; Asp, *Asterina pectinifera*; At, *Arabidopsis thaliana*; Dc, *Daucus carota*; Dm, *Drosophila melanogaster*; Gm, *Glycine max*; Hs, *Homo sapiens*; Ml, *Marthasterias glacialis*; Ms, *Medicago sativa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Ss, *Spisula solidissima*; Xl, *Xenopus laevis*. Lengths of vertical lines reflect divergence between members.

Cell Cycle Regulation of Alfalfa Cyclin Transcript Levels

The various cyclins identified in animals and yeasts not only differ in primary sequence but also in their expression kinetics during the cell cycle. Therefore, a suspension culture of the closely related alfalfa species *Medicago varia* that largely consists of single cells was synchronized by aphidicolin. Synchrony was monitored by flow cytometric analysis of cells stained with 4,6-diamidino-2-2-phenylindole, as shown in Figure 4. Treatment of the cells with aphidicolin for 24 hr resulted in 90% G1 arrest (Figure 4C). After release from the aphidicolin block, approximately 60% of the cells were found in the process of DNA synthesis after 5 hr (Figure 4D). After 10 hr, cells were found to be in G2 phase (Figure 4F), whereas mitosis was observed after 16 hr (Figure 4H). After 24 hr, more than 80% of the cells were found to be in G1 phase again (Figure 4I). As a control, asynchronous and oryzalin-arrested cells were analyzed. In asynchronously growing cells, approximately 85% of the cells were found to be in G1 phase (Figure 4A). When cells were mitotically arrested (Figure 4B), 75% were arrested in metaphase (determined microscopically, data not shown).

RNA was prepared from the synchronized cells at the time points shown in Figure 4. Under stringent hybridization conditions, RNA gel blot analysis with the alfalfa *cycMs1* fragment, as shown in Figure 5A, revealed a single transcript. During G1/S and over the whole of S phase, very little transcript was observed (Figure 5A, lanes 3 to 5). The transcript was mainly present in cells in G2 and M phase (Figure 5A, lanes 6 to 8), but highest levels were found in M phase cells (Figure 5A, lane 8). Transcript levels strongly decreased when cells entered G1 phase (Figure 5A, lane 9). The mRNA detected in cells in G1 phase most likely is from cells that did not pass into G1 phase

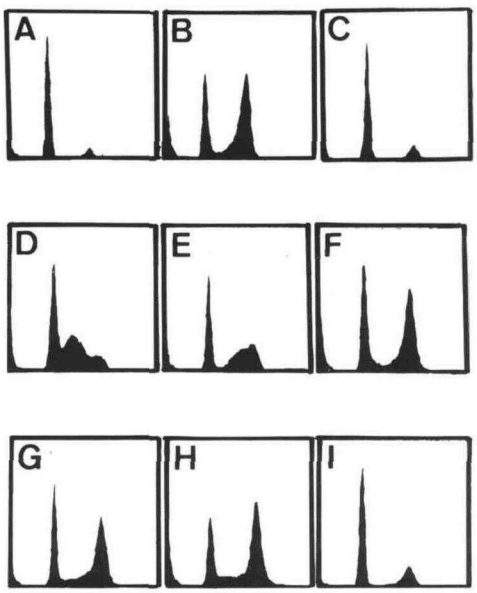

Figure 4. Synchronization of an *M. varia* Suspension Culture.

Flow cytometric analysis of cell synchrony is as follows:
(A) Asynchronous cells.
(B) Mitotically arrested cells.
(C) Cell distribution 24 hr after treatment with aphidicolin.
(D) to **(I)** Cell distribution 5, 8, 10, 13, 16, and 24 hr, respectively, after release from the aphidicolin block.
Samples were taken at the indicated time points and analyzed by flow cytometry. The profiles represent the distribution of the cells in different phases: left peak constitutes cells with a 2C value of DNA content, right peak is 4C cells, and the area between the 2C and 4C peaks represents cells with an intermediate DNA content.

but remained in G2 (20% as seen in Figure 4I). When the same set of RNAs was analyzed with a probe against the *cycMs2* gene, a different pattern was obtained (Figure 5B). Although maximal expression occurred again in M phase (Figure 5B, lane 8), *cycMs2* transcript levels appeared later in G2 than those for the *cycMs1* gene. The two cyclin genes attained about equal steady states in M phase (Figures 5A, lane 8, and 5B, lane 8). After rehybridization of these mRNAs with several other genes, only the gene coding for an alfalfa protein phosphatase 2A homolog (*pp2aMs*; M. Pirck, A. Páy, H. Hirt, and E. Heberle-Bors, unpublished data) showed real constitutive expression during the cell cycle (Figure 5C). Therefore, the *pp2aMs* gene was used as a control in all subsequent experiments.

Growth Phase–Dependent Expression of *cycMs1* and *cycMs2* Transcripts

To test whether the two alfalfa cyclin genes behave differently during the growth cycle of a batch suspension culture, logarithmic alfalfa suspension cultured cells (*M. varia*) were diluted into fresh medium and grown for 9 days. After a 2-day lag phase, maximal increase in cell number and packed cell volume was observed during days 3 to 6, as shown in Figure 6. Then, the rate of cell number increase leveled off and the cells became stationary after day 8. Every day during this growth cycle, an aliquot was withdrawn from the culture and analyzed for *cycMs1* and *cycMs2* transcript levels by RNA gel blot hybridization, as shown in Figure 7. During the first 6 days, high levels of both cyclin transcripts were observed (Figures 7A and 7B, lanes 1 to 6, for *cycMs1* and *cycMs2*, respectively). Thereafter, mRNA levels of both genes decreased and were not detected anymore after day 8 (Figures 7A and 7B, lanes 8 to 10, respectively). *cycMs1* transcript levels were consistently higher than *cycMs2* levels. These expression patterns paralleled the growth kinetics during logarithmic and stationary phase but not during lag phase. The *pp2aMs* gene used to demonstrate presence of RNA was expressed throughout the growth cycle, but transcript levels decreased gradually (Figure 7C).

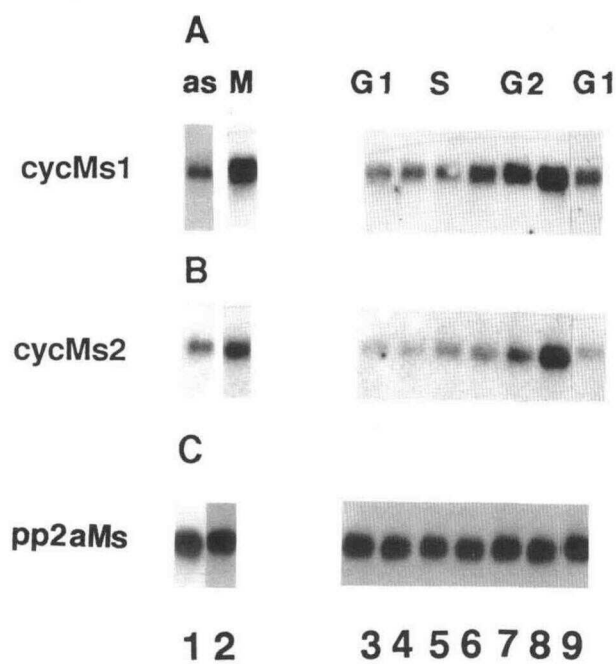

Figure 5. Cell Cycle–Dependent Transcription of Alfalfa *cycMs1* and *cycMs2* Genes.

Lanes 1 to 9 correspond to profiles **(A)** to **(I)** in Figure 4. as, asynchronous suspension culture; M, mitotically arrested cells. RNA was isolated from synchronized *M. varia* suspension cultured cells. Total RNA (100 μg) was poly(A)$^+$ purified and applied to each lane.
(A) Nylon filters were hybridized with *cycMs1*.
(B) Nylon filters were rehybridized with *cycMs2*.
(C) Nylon filters were rehybridized with *pp2aMs* as a control.

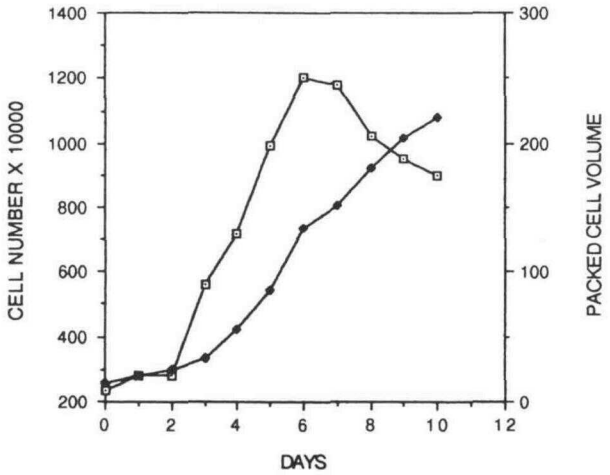

Figure 6. Growth Curve of an *M. varia* Suspension Culture.

Cells from logarithmic phase were diluted into fresh medium and grown for 10 days. Every day, samples were analyzed for cell number (open squares) and packed cell volume (filled squares).

Differential Expression of *cycMs1* and *cycMs2* in Different Alfalfa Organs

To investigate expression of the *cycMs1* and *cycMs2* genes in alfalfa (*M. sativa*) plants, poly(A)$^+$ RNA was extracted from suspension cultured cells, developing young leaves, mature leaves, roots, stems, nodes, and flower buds and was analyzed by RNA gel blot analysis with radiolabeled probes against *cycMs1* and *cycMs2*, as shown in Figures 8A and 8B, lanes 1 to 7, respectively. Transcript levels of *cycMs1* were observed at high levels in suspension cultured cells, developing young leaves, and flower buds (Figure 8A, lanes 1, 2, and 7, respectively) and at moderate levels in nodes (Figure 8A, lane 6) but not in mature leaves, roots, and stems (Figure 8A, lanes 3 to 5, respectively). High levels of *cycMs2* mRNA were detected in suspension cultured cells, developing young leaves, nodes, and flower buds (Figure 8B, lanes 1, 2, 6, and 7, respectively) and at very low levels in roots and stems (Figure 8B, lanes 4 and 5, respectively) but not in mature leaves (Figure 8B, lane 3). As a control, a radiolabeled *pp2aMs* probe was hybridized to the same filter, as shown in Figure 8C. *pp2aMs* transcripts were detected in all organs at relatively high levels except in flower buds (Figure 8C, lane 7). This indicates that the differences in cyclin transcript levels were not due to different amounts of RNA.

DISCUSSION

Recently, we have isolated a cDNA clone for the *cdc2* gene of *M. sativa* (Hirt et al., 1991). Here, we show that cyclins, which together with the CDC2 kinase form the mitosis promoting factor, are also present in this species. Sequence comparison of the predicted alfalfa CYCMs1 protein sequence with the Swiss-Prot data bank revealed identity scores of 25 to 35% to animal, yeast, and other plant cyclins of all classes. In contrast, CYCMs2 showed highest identity to the soybean and Arabidopsis cyclins (50 and 48%, respectively). Construction of a generic tree revealed the alfalfa CYCMs2 and the recently reported soybean and Arabidopsis cyclins to belong to the type B cyclins. In contrast, the carrot cyclin was classified as a type A cyclin, whereas the alfalfa CYCMs1 could not be fitted into either type A, B, or G1 cyclins. This analysis indicates that the alfalfa CYCMs1 belongs to a novel, yet unknown class of cyclins. However, because the *cycMs1* cDNA clone is truncated at the 3′ end, the classification as a novel class may be premature.

The cyclin genes in the various eukaryotes do not show much sequence conservation. To distinguish the different cyclins, phase-specific expression during the cell cycle was tested. The present paper presents data on the kinetics of cyclin gene

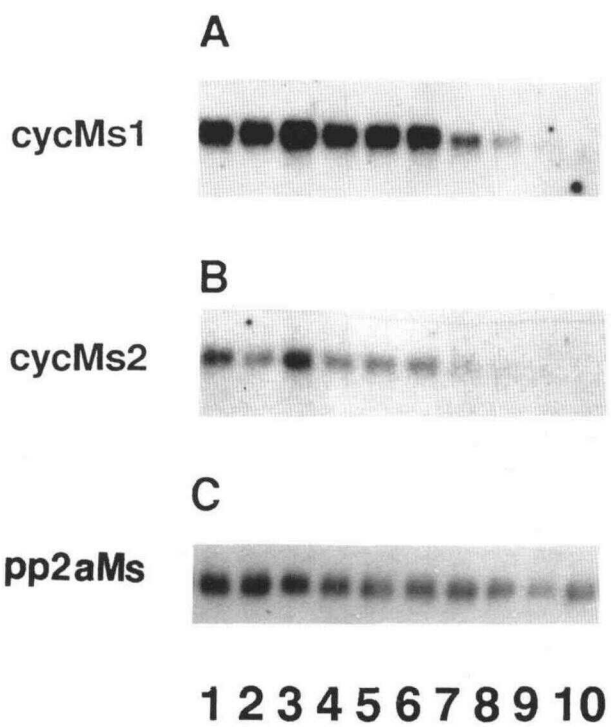

Figure 7. Growth Curve Kinetics of *cycMs1* and *cycMs2* Transcripts in an *M. varia* Suspension Culture.

On the days indicated in Figure 6, poly(A)$^+$ RNA was isolated from equal aliquots.

(A) Nylon filters were hybridized with *cycMs1*.

(B) Nylon filters were rehybridized with *cycMs2*.

(C) Nylon filters were rehybridized with *pp2aMs* as a control.

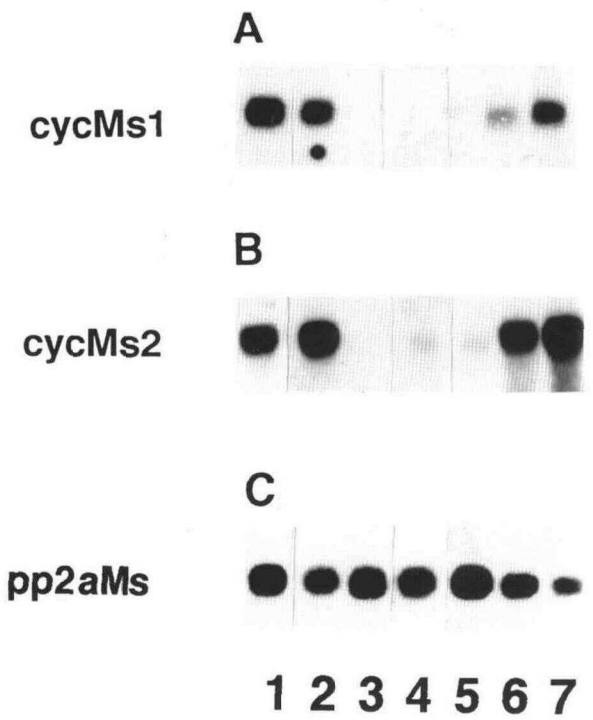

Figure 8. Expression of Alfalfa *cycMs1* and *cycMs2* Genes in Different Plant Organs.

Poly(A)$^+$ RNA was extracted from *M. sativa* suspension cultured cells, developing young leaves, mature leaves, roots, stems, nodes, and flower buds (lanes 1 to 7, respectively).
(A) Nylon filters were hybridized with *cycMs1*.
(B) Nylon filters were rehybridized with *cycMs2*.
(C) Nylon filters were rehybridized with *pp2aMs* as a control.

expression during the cell cycle, and *cycMs1* and *cycMs2* transcripts accumulate with different kinetics. *cycMs2* mRNA appears late in G2 phase and is barely detectable in G1 or S phase. This periodicity is typical for type B cyclins and agrees with its classification by primary sequence comparison. We conclude that CYCMs2 is a type B cyclin.

Detectable transcripts of *cycMs1* were present in all cell cycle phases, increased at the onset of G2, and disappeared at the end of mitosis. This kinetics resembles the pattern of type A cyclins. However, in various organisms, type A cyclins appear in S phase and also disappear earlier during mitosis (Pines and Hunter, 1989; Whitfield et al., 1989; Lehner and O'Farrell, 1990; and Minshull et al., 1990). We could not detect this type of periodicity in our experiments. However, when the same RNAs were probed with *cycMs1* at low stringency, two more transcripts appeared with a strikingly similar kinetics as that reported for type A cyclins. These mRNAs appeared at the onset of S phase, stayed constant over the entire G2 period, and vanished in M phase slightly before *cycMs1* and *cycMs2* transcripts disappeared (data not shown). It appears, therefore, that plants contain typical type A cyclins. Based on sequence comparison and expression kinetics during the cell cycle, CYCMs1 seems to be a member of a novel class of mitotic cyclins.

The two cyclin genes were both maximally expressed in G2 phase of dividing suspension cultured cells, indicating that both are mitotic cyclins. Their different periodicity indicates that they may have different functions.

RNA gel blot analysis showed high transcript levels of *cycMs1* and *cycMs2* in those organs that contained meristematic activity (young leaves, nodes, and flower buds) but not in mature organs. In young leaves expression levels similar to suspension cultured cells were found, whereas in mature leaves no *cycMs1* and *cycMs2* transcripts could be detected. This demonstrates that in plants as well as in animal cells cyclin expression is a marker for cell division activity. Also in stationary phase of batch cultured cells, transcripts of neither cyclin could be detected.

In suspension cultured cells, about equal amounts of both cyclin transcripts were observed. In plant organs, however, mRNA levels of the *cycMs1* and *cycMs2* genes showed considerable variation. This result could reflect the different distribution of the cells in the cell cycle. Alternatively, the two cyclin genes might be expressed in different cells. Future work, particularly in situ hybridization, should help to answer these questions.

METHODS

Library Screening and Sequence Analysis

Two degenerate oligonucleotides A (AAA/GTAT/CGAA/GGAA/GATITAT/CCC) and B (ATT/CC/TTIG/ATIGAT/CTGGT/CTIGT) encoding the highly conserved cyclin boxes K-Y-E-E-M(I)-Y-P and I-L-V(I)-D-W-L(F)-V were synthesized. After gel elution from 10% denaturing polyacrylamide gels, 50 ng of each batch of oligonucleotides was ^{32}P-labeled with 50 μCi γ-ATP and polynucleotide kinase. Approximately 300,000 colonies of a cDNA library from an alfalfa (*Medicago sativa*) cell suspension culture (Hirt et al., 1991) were screened with both probes. Putatively positive clones were rescreened to a minimum fit of 16 nucleotides according to Wood et al. (1985). The only positive clone *cycMs1* was subcloned into pBluescript SK+ (Stratagene) and sequenced from both directions with the T7 polymerase sequencing kit from Pharmacia. Several other *M. sativa* suspension culture cDNA libraries were screened with the randomly primed ^{32}P-labeled *cycMs1* cDNA fragment (Feinberg and Vogelstein, 1983). In one case, a 1.4-kb cDNA clone *cycMs2* was isolated that was then sequenced as described above.

Cell Culture, Synchronization, and Flow Cytometry

A suspension culture of *Medicago varia* cells was grown in MS medium (Murashige and Skoog, 1962), containing 0.2 mg/L kinetin and 1 mg/L 2,4-dichlorophenoxyacetic acid. Mitotic synchronization was achieved by treatment with aphidicolin. Aphidicolin was added at time zero and after 12 hr to a final concentration of 20 μg/mL. Cells were then washed twice with fresh medium and allowed to grow for the indicated times. Flow cytometric analysis was performed as described (Pfosser, 1989).

Genomic DNA Gel Blot Analysis

Ten micrograms of DNA of *M. sativa* was digested with HindIII or BglII and separated on 0.7% agarose gels. After blotting, the nylon filters were hybridized to randomly ^{32}P-labeled fragments of *cycMs1* or *cycMs2*.

RNA Extraction and RNA Gel Blot Analysis

Suspension cultured cells of *M. varia* or plant tissue of *M. sativa* was frozen in liquid nitrogen and extracted by grinding in a mortar as described (Cathala et al., 1983). RNA gel blot analysis was performed according to Györgyey et al. (1991). Probes for hybridization were the coding regions of *cycMs1* and *cycMs2* cDNA clones or the coding region of a phosphoprotein phosphatase 2A cDNA clone from alfalfa as a control. All fragments were ^{32}P-labeled by random priming (Feinberg and Vogelstein, 1983).

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