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, cyc Ms1 and cyc Ms2, from alfalfa. The cyc Ms2 gene shows highest similarity to type B cyclins. In contrast, the predicted amino acid sequence of the cyc Ms7 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 cyc Ms1 and cyc Ms2 show maximal expression in the G2 and M phases. Transcripts of cyc Ms2 are found only in late G2 and M phase cells, an expression pattern typical for cyclin B genes, whereas cyc Ms1 appears with the onset of G2. This pattern indicates that alfalfa cyc Ms1 and cyc Ms2 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 p34cdc2 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 cell division in mammalian (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, cyc Ms1 and cyc Ms2, from alfalfa. Comparison of the predicted cyclin protein sequences with 18 other cyclins showed that cyc Ms2 can be classified as a type B cyclin. In contrast, cyc Ms1 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
suspension cultured cells that were induced to form somatic embryos with redundant oligonucleotides encoding the conserved amino acid sequences K-Y-E-M(I)-Y-P for the cyclin A and B type and I-L-V(I)-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 777 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-L). 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
Alignment of the predicted alfalfa cyclin protein sequences with the Swiss-Prot protein data base revealed that the cycMsl gene is equally related to cyclins of the types A and B. Comparison of the amino acid sequences of cycMsl 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.

Cell Cycle Regulation of Alfalfa Cyclin Transcript Levels

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; Ms, Marthasterias glacialis; Ms, Medicago sativa; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Ss, Spisula solidissima; Xi, Xenopus laevis.
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.

... 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 for the *cycMsl* 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. Pay, 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 *cycMsl* and *cycMs2* Transcripts

To test whether the two alfalfa cyclin genes behave differently during the growth cycle of a batch suspension culture, loga-...
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, respectiv-
ely) 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 fac-
tor, 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 con-
trast, 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 cy-
cclins. 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.
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/GCAA/GGAA/GATT/ATAT/CC) and B (ATT/CC/TTIG/ATIGAT/CTGGT/CTIGT) encoding the highly conserved cyclin boxes KY-E-M(I)-Y-P and I-L-V(I)-D-W-L(V) were synthesized. After gel elution from 10% denaturing polyacrylamide gels, 50 ng of each batch of oligonucleotides was 32P-labeled with 50 nCi 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 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 BgIII and separated on 0.7% agarose gels. After blotting, the nylon filters were hybridized to randomly 32P-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 Gyorgyey 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 32P-labeled by random priming (Feinberg and Vogelstein, 1983).

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