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A Novel *cis*-Acting Element in Promoters of Plant B-Type Cyclin Genes Activates M Phase–Specific Transcription

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Plant B-type cyclin genes are expressed late in the G_2 and M phases of the cell cycle. Previously, we showed that the promoter of a *Catharanthus roseus* B-type cyclin, *CYM*, could direct M phase–specific transcription of a β-glucuronidase reporter gene in synchronously dividing BY2 tobacco cells. In this study, we determined the regulatory elements contained within the *CYM* promoter by using a luciferase reporter gene. Mutational analysis showed that a 9-bp element is essential for M phase–specific promoter activity in synchronized BY2 cells. The *CYM* promoter contains three other sequences similar to this element. A gain-of-function assay demonstrated that when fused to a heterologous promoter, these elements are sufficient for M phase–specific expression; therefore, we named these elements M-specific activators (MSAs). We found MSA-like sequences in B-type cyclin promoters from tobacco, soybean, and Arabidopsis as well as in the promoters of two M phase–specific genes, *NACK1* and *NACK2*, which encode tobacco kinesin-like proteins. Thus, MSA may be a common *cis*-acting promoter element that controls M phase–specific expression of cell cycle–related genes in plants.

INTRODUCTION

During progression through the cell cycle, several sets of genes are expressed at specific time points (reviewed in Müller, 1995). Generally, these genes encode proteins that control cell division or are involved in metabolism that occurs in a periodic fashion. For example, several mammalian G_1 cyclins and cyclin-dependent kinases are induced sequentially during the G_1 phase. Other genes that encode cyclin A, Cdc2, DNA synthetic enzymes, and several transcription factors are expressed in the late G_1 and S phases. Another group of genes, including cyclin B and *cdc25C* phosphatase, is expressed in the late S and G_2 phases of the animal cell cycle.

Mechanisms controlling G_1 and S phase–specific transcription are relatively well studied in animal cells. The E2F/DP family of transcription factors is thought to play important roles in gene regulation during S phase (reviewed in La Trangue, 1994). E2F binding sites are found in promoters of many genes specifically expressed during the S phase and include DNA synthetic enzymes, cyclin A, B-*myb*, and *cdc2* (Müller, 1995). E2F has been shown to form complexes with several proteins, including pRb, p107, Cdk2, and cyclins A and E, and these complexes both repress and activate the transcription (Schulze et al., 1995).

In contrast, the control of G_2-specific expression is poorly understood in higher eukaryotes. Recently, two promoter elements, CDE (for cell cycle–dependent element) and CHR (for cell cycle genes homology region), have been identified, and they are involved in transcriptional repression (Zwicker et al., 1995). It is believed that a repressor protein binds to these elements during the G_1 phase and that subsequent dissociation of the repressor in the late S and G_2 phases activates transcription. Mutated CDE/CHR elements confer constitutively high levels of gene expression throughout the cell cycle (Lucibello et al., 1995). CDE/CHR elements are present in cyclin A, *cdc25C*, and *cdc2* promoters and may be common motifs required for the G_2-specific gene expression in animal cells (Zwicker et al., 1995). However, a different inductive mechanism may also operate in the G_2 and M phases. The mammalian cyclin *B1* promoter contains a binding site for an upstream stimulatory factor, and this binding site is essential for transcriptional activation in the G_2 phase (Cogswell et al., 1995). However, no single element in the cyclin *B1* promoter has been shown to be

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sufficient for G_2-specific activation (Hwang et al., 1995; Piaggio et al., 1995).

Mechanisms regulating cell cycle–dependent expression in animals have been well studied; however, much less is known about these mechanisms in plants. A- and B-type cyclins are the best-characterized cell cycle–dependent proteins in plants (Hirt et al., 1992; Ferreira et al., 1994a; Fobert et al., 1994). In all plant species studied thus far, A-type cyclins are expressed earlier in the cell cycle than are B-type cyclins (Kouchi et al., 1995; Setiady et al., 1995; Fuerst et al., 1996; Reichheld et al., 1996; Ito et al., 1997). Based on sequence similarity, A-type cyclins are further subdivided into CycA1, CycA2, and CycA3, whereas B-type cyclins are divided into CycB1 and CycB2 classes (Kouchi et al., 1995; Renaudin et al., 1996). Transcripts of both classes of B-type cyclins accumulate in G_2 and M phases of the cell cycle (Ferreira et al., 1994a; Fobert et al., 1994; Kouchi et al., 1995; Setiady et al., 1995; Ito et al., 1997). Recently, it has been shown that B-type cyclin promoters from Arabidopsis (Shaul et al., 1996) and *Catharanthus roseus* (Ito et al., 1997) conferred M phase–specific expression to a β-glucuronidase (*GUS*) reporter gene in tobacco BY2 cells.

We have chosen to further characterize the M phase–specific activity of the promoter of the CycB1 cyclin *CYM* from *C. roseus.* Reporter activity was measured during the cell cycle of stably transformed tobacco BY2 cell lines, which had been synchronized by the aphidicolin method. We have identified elements designated as M-specific activators (MSAs) that are necessary and sufficient for activation of the *CYM* promoter. The MSA elements placed in a heterologous context could activate the cauliflower mosaic virus (CaMV) 35S basal promoter specifically during M phase. The MSA consensus sequence resembles the binding sites of c-Myb and v-Myb transcription factors. Sequence analysis showed the presence of MSA-like sequences in promoters of several other genes expressed during G_2 and M phases: these are the B-type cyclins from tobacco, soybean, and Arabidopsis and *NACK1* and *NACK2* that encode plant kinesin-like proteins. Thus, MSA-mediated transcriptional activation may be a general mechanism operating in the G_2 and M phases of the plant cell cycle. In this study, we report on a novel regulatory pathway that activates gene expression specifically in the G_2 and M phases of the cell cycle in higher plants.

RESULTS

Analysis of the 462-bp *CYM* Promoter

Our previous work showed that a region of the *CYM* promoter from positions −462 to +102 was sufficient to direct the M phase–specific expression of the *GUS* reporter gene (Ito et al., 1997). In this article, the *CYM* promoter was used to drive a firefly luciferase gene (*LUC*). The constructs were introduced into tobacco BY2 cells by Agrobacterium-mediated transformation. Stably transformed cell lines were synchronized by the aphidicolin method (Nagata et al., 1992). Aphidicolin treatment caused complete arrest of the cell division cycle early in S phase. As shown in Figure 1B, the removal of aphidicolin resulted in synchronous progression through the cell cycle, with a clear peak in the mitotic index (25 to 35%) after 7 or 8 hr.

Cell cycle–dependent changes in the activity of the *CYM* promoter were first studied by measuring *LUC* mRNA levels by RNA gel blot analysis. As shown in Figures 1A and 1B, when *LUC* transcription was mediated by the 462-bp *CYM* promoter, *LUC* transcript levels increased rapidly during M

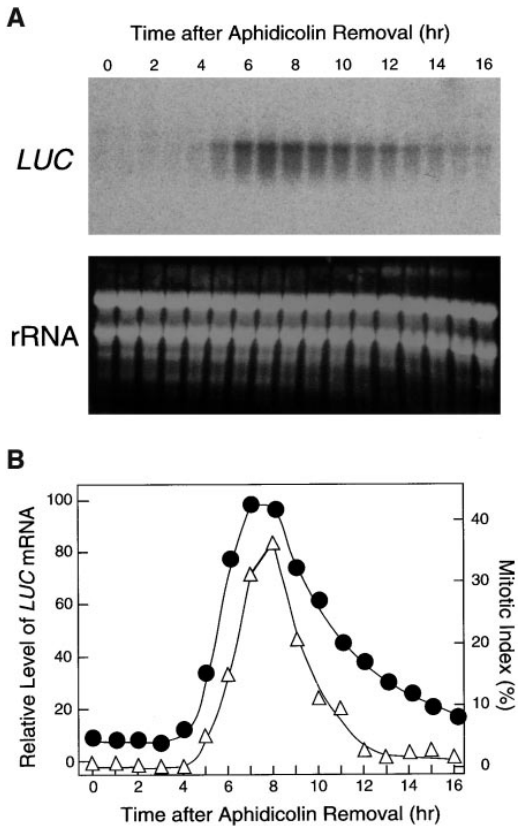

Figure 1. M Phase–Specific Transcription of the *CYM* Promoter–*LUC* Transgene in BY2 Cells.

(A) Tobacco BY2 cells transformed with the *CYM* promoter (positions −462 to +100)–*LUC* transgene were arrested for 24 hr in S phase by aphidicolin. Aphidicolin was removed (point 0), and cells were harvested at 1-hr intervals. Total RNA was isolated and hybridized, with the *LUC* coding region as a probe. The ethidium bromide staining of the rRNA is also shown.

(B) Cell cycle progression was monitored by measuring the mitotic index (triangles). The relative level of *LUC* mRNA was determined by quantifying the radioactivity of each signal on the RNA gel blot (circles).

phase, and a peak was observed 1 hr before the peak of the mitotic index. Subsequently, the levels of *LUC* mRNA fell drastically at the completion of cell division. The observed pattern of expression of *LUC* mRNA closely resembled those of endogenous *NtCYM* (tobacco homolog of *CYM*) mRNA and *GUS* mRNA driven by the *CYM* promoter (Ito et al., 1997).

LUC activity was also assayed to monitor the promoter activity during the cell cycle. As shown in Figure 2A, in cells transformed with the 462-bp *CYM* promoter–*LUC* construct, LUC activity was low for the first 6 hr after aphidicolin removal. A rapid increase in LUC activity was observed 7 hr after aphidicolin removal and coincided with the peak of the mitotic index. LUC activity reached a maximum at 11 hr and remained at constantly high levels for the rest of the experiment. The differences between changes in *LUC* mRNA levels (Figure 1B) and LUC activity (Figure 2A) during the cell cycle suggest that the LUC protein has a low turnover rate in BY2 cells under the applied conditions. Thus, in our experiments, the rate of change in LUC activity depended mainly on de novo LUC biosynthesis and reflected *CYM* promoter activity.

The rate of change in LUC activity is shown in Figure 2C. This curve has a single peak occurring at 7 hr after aphidicolin removal. This peak coincides with the peak in the mitotic index. On the other hand, a CaMV 35S promoter–*LUC* construct produced constantly high levels of LUC activity throughout the cell cycle (Figures 2B and 2C). Therefore, the *CYM* promoter could confer M phase–specific expression to the *LUC* gene in BY2 cells.

To define the minimal region sufficient for M phase–specific activation of the *LUC* gene, 3′ and 5′ deletions were prepared from the 462-bp *CYM* promoter. For each construct, 1000 to 3000 stably transformed BY2 calli were pooled and reintroduced into liquid culture. Cell suspension cultures of transformants were synchronized as described above, and LUC activity was assayed to monitor promoter activity during the cell cycle.

Promoter fragments deleted from the 5′ end were fused directly to the *LUC* reporter gene. As shown in Figures 3A and 3B, 5′ deletions up to position −229 (where position 1 corresponds to the transcription start site located 109 bp upstream of the first ATG codon) did not substantially alter *LUC* reporter activity. Deletions to positions −160 and −80 (5Δ160 and 5Δ80) severely decreased the levels of maximal LUC activity approximately five- and 50-fold, respectively. However, all promoter constructs were still capable of directing the M phase–specific expression.

Promoter fragments deleted from the 3′ end were fused to the CaMV 35S basal promoter–*LUC* construct to provide a transcription start site. Figures 4A and 4B show that 3′ deletions from positions +1 to −40 (3Δ40) were still able to direct M phase–specific expression and maximal activity levels similar to those observed for the wild-type promoter (3Δ1). Deletions to positions −161 and −180 (3Δ161 and 3Δ180) dramatically reduced the levels of maximal LUC activity. However, these constructs were still expressed specifically during M phase. Only the deletion to position −203 (3Δ203) abolished M phase–specific promoter activation.

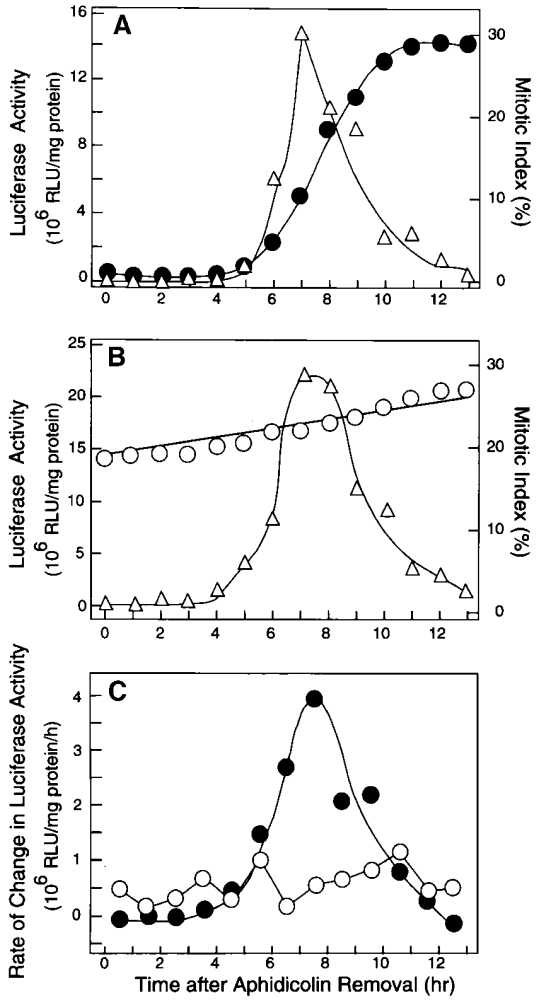

Figure 2. M Phase–Specific Increase of LUC Activity in BY2 Cells Carrying the *CYM* Promoter–*LUC* Transgene.

(A) and **(B)** Tobacco BY2 cells were transformed with *CYM* promoter (positions −462 to +100)–*LUC* **(A)** or CaMV 35S promoter–*LUC* **(B)**. Cells were synchronized by the aphidicolin method and harvested at 1-hr intervals for LUC assays (open circles, CaMV 35S promoter–*LUC*; solid circles, *CYM* promoter–*LUC*). Cell cycle progression was monitored by measurement of the mitotic index (triangles).

(C) The rate of change in LUC activity was calculated at each time point as the difference between activities at two consecutive time points. Open circles, CaMV 35S promoter–*LUC*; solid circles, *CYM* promoter–*LUC*.

h, hr; RLU, relative light unit.

These results led to the following conclusions. First, the *CYM* promoter sequence between positions −462 and −203 does not contain elements required for M phase–

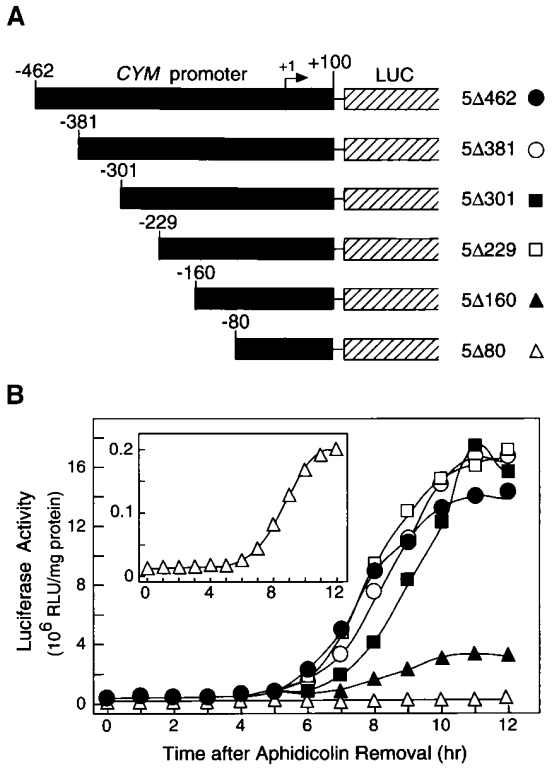

Figure 3. M Phase–Specific Activity of 5′ Deletions of the *CYM* Promoter.

(A) A series of 5′-deleted *CYM* promoter fragments was transcriptionally fused to the *LUC* reporter gene. The numbers above the black bars indicate the distance from the *CYM* transcription start site.

(B) BY2 cells transformed with the promoter–*LUC* constructs were synchronized by the aphidicolin method and harvested at 1-hr intervals for LUC assays. The inset shows LUC activity of 5Δ80 with an expanded scale. Promoter constructs are designated with the symbols shown in **(A)**. RLU, relative light unit.

specific activity. Second, the sequence between positions −203 and −40 contains several elements that regulate the levels of promoter activity. Third, promoter elements crucial for the M phase–specific activity should be present in at least two regions: from positions −80 to −40 and −203 to −182.

Identification of a Regulatory Element Required for M Phase–Specific Expression

Because 80 bp of the *CYM* promoter (5Δ80) was sufficient to confer M phase–specific expression, this sequence was analyzed further. Mutant promoters containing 10-base substitutions in this 80-bp region are shown in Figure 5A. As shown in Figure 5B, several mutations (80mut80, 80mut10, 80mut20, 80mut30, and 80mut40) directed lower levels of LUC activity. However, the expression from these promoters was still M phase specific. Two mutations, 80mut70 and 80mut60, abolished M phase–specific expression. LUC activity directed by the 80mut60 promoter was very low and constant. Expression from the 80mut70 promoter was nearly constant; however, it was higher than that observed for the 80mut60. These data show that an element necessary for M phase–specific promoter activity is located between positions −70 and −50.

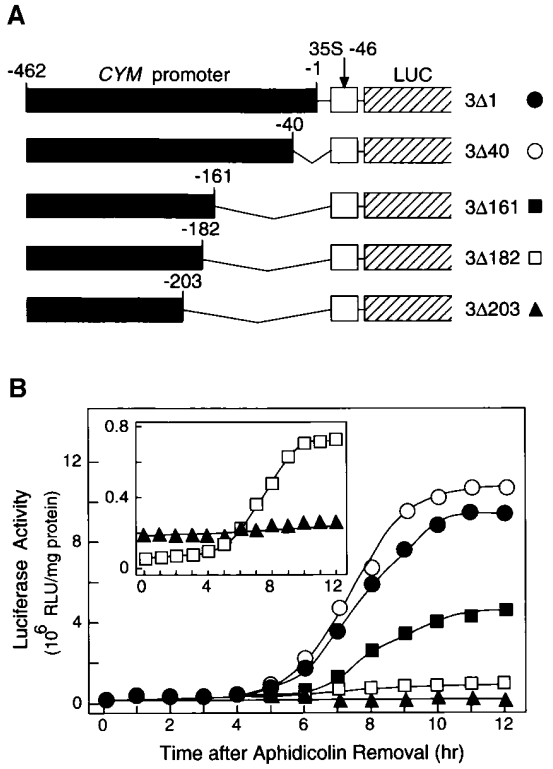

Figure 4. M Phase–Specific Activity of 3′ Deletions of the *CYM* Promoter.

(A) A series of 3′-deleted *CYM* promoter fragments was fused upstream of the CaMV 35S basal promoter (positions −46 to +1). The numbers above the black bars indicate the distance from the *CYM* transcription start site.

(B) BY2 cells transformed with the promoter–*LUC* constructs were synchronized by the aphidicolin method and harvested at 1-hr intervals for LUC assays. The inset shows LUC activity of 3Δ182 and 3Δ203 with an expanded scale. Promoter constructs are designated with the symbols shown in **(A)**. RLU, relative light unit.

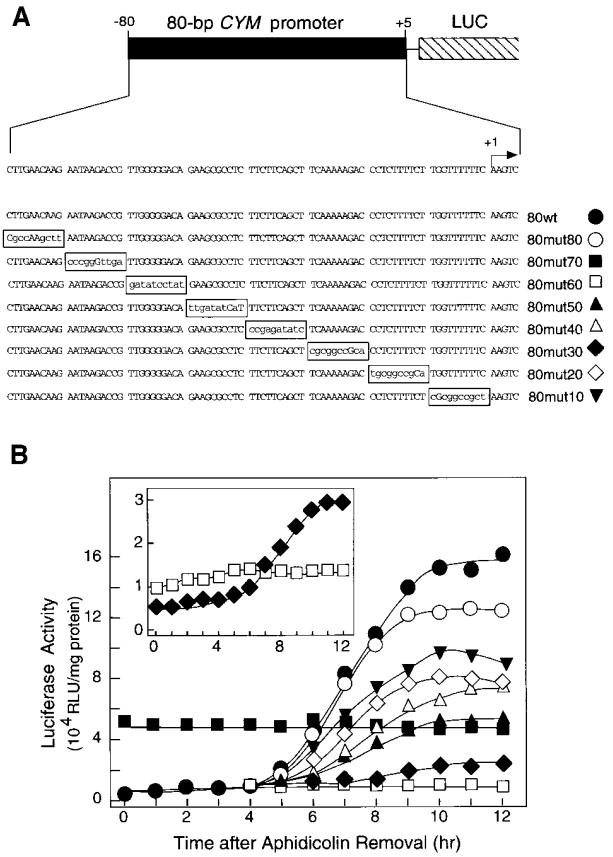

Figure 5. M Phase–Specific Activity of the 80-bp *CYM* Promoter Mutated with 10-Base Substitutions.

(A) Sequences of the 80-bp *CYM* promoter and mutant promoters containing 10-base substitutions. The substituted 10-bp regions are boxed, and lowercase letters represent the bases that are mutated.
(B) LUC activity from BY2 cells transformed with the constructs shown in **(A)**. Cell division was synchronized by the aphidicolin method, and samples were harvested at 1-hr intervals for LUC assays. The inset shows LUC activity of 80mut30 and 80mut60 with an expanded scale. Promoter constructs are designated with the symbols shown in **(A)**. RLU, relative light unit.

The location of the element required for M phase–specific activation was more finely mapped by introducing two-base substitutions in the interval from −70 to −50 bp of the 80-bp *CYM* promoter (Figure 6A). Figure 6B shows that any mutation from position −66 to position −58 (80mut58 to 80mut66) abolished M phase–specific activation. Substitutions at positions −68 and −57 greatly reduced the maximal levels of activity but still activated expression during M phase. Other mutations did not change the M phase–specific pattern of *LUC* expression and only altered expression levels mildly. Thus, we conclude that the sequence between positions −66 and −58, AGACCGTTG, is a regulatory element essential for M phase–specific promoter activity.

Repeat Sequences in the *CYM* Promoter Are Sufficient for M Phase–Specific Promoter Activation

The *CYM* promoter contains three other regions that share high identity with the element AGACCGTTG identified

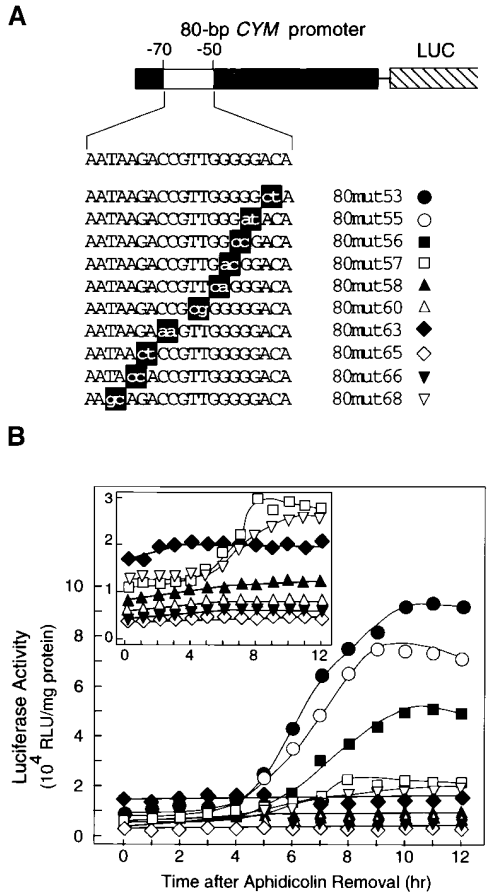

Figure 6. M Phase–Specific Activity of the 80-bp *CYM* Promoter Mutated by Two-Base Substitutions.

(A) Sequences of the 80-bp *CYM* promoter and mutant promoters containing two-base substitutions. Mutant bases are shown on a black background.
(B) LUC activity from BY2 cells transformed with the constructs shown in **(A)**. Cell division was synchronized by the aphidicolin method, and samples were harvested at 1-hr intervals for LUC assays. The inset shows LUC activity with an expanded scale. Promoter constructs are designated with the symbols shown in **(A)**. RLU, relative light unit.

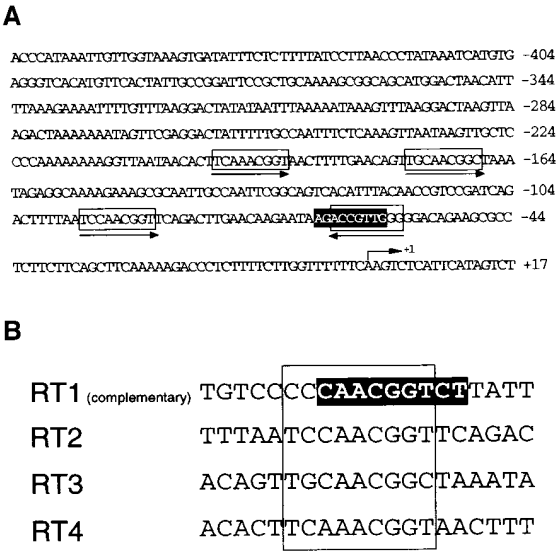

Figure 7. Repeat Sequences Similar to the Nonamer Element Identified as Essential for M Phase–Specific Promoter Activation.

(A) Nucleotide sequence of the 462-bp *CYM* promoter. Four repeat sequences are boxed, and their orientations are shown with arrows. Position +1 indicates the transcription start site of *CYM.*

(B) Alignment of four repeat sequences (RT1, RT2, RT3, and RT4). The boxed regions are the 9-bp sequences that show high identity to each other. For RT1, the complementary sequence is used for comparison.

In **(A)** and **(B)**, the nonamer element identified by mutational analysis is shown on a black background.

above. As shown in Figure 7A, these sequences are located between positions −203 and −80 of the *CYM* promoter. In addition, these three regions are oriented in the opposite direction from the original sequence identified by mutagenesis. Figure 7B shows an alignment of the repeats and the surrounding sequences. All repeat sequences (RTs) contain the fully conserved pentamer AACGG. The region of high similarity is comprised of 9 bp that overlap seven bases from the 5′ end of the element CAACGGTCT and extend 2 bp upstream. The repeat sequences are referred to as follows: RT1, CCCAACGGT; RT2, TCCAACGGT; RT3, TGCAACGGC; and RT4, TCAAACGGT (Figure 7B).

Three repeat sequences, RT1, RT2, and RT4, were tested for ability to confer M phase–specific expression. To minimize the potential problem of inadequate spacing between elements, we prepared each construct from three copies of a given repeat sequence separated by six adenine residues (3 × RT1, 3 × RT3, and 3 × RT4), as shown in Figure 8A. An additional construct was made from three complementary copies of the RT1 sequence (3 × RT1R). The constructs were inserted upstream of the CaMV 35S basal promoter–*LUC* reporter gene. Figure 8B shows that the CaMV 35S basal promoter alone (35S −46) did not produce any detectable expression. In contrast, all four promoters containing the repeat sequences from the *CYM* promoter could activate *LUC* gene expression, specifically in M phase. Both 3 × RT1 and 3 × RT1R constructs conferred high levels of reporter gene activity, indicating that the RT1 element can work in an orientation-independent fashion.

To verify that M phase–specific promoter activation by these RT sequences also occurs in a noninductive system, we analyzed the LUC activity in asynchronously growing BY2 cells transformed with 3 × RT1. The transgenic BY2 cells were first treated with luciferin for in vivo bioluminescent imaging and then stained with Hoechst 33258. Figures 9A and 9B show that detectable bioluminescence was observed only for cells in the M phase. Our data show that the repeat sequences identified in the *CYM* promoter are sufficient to confer M phase–specific activity.

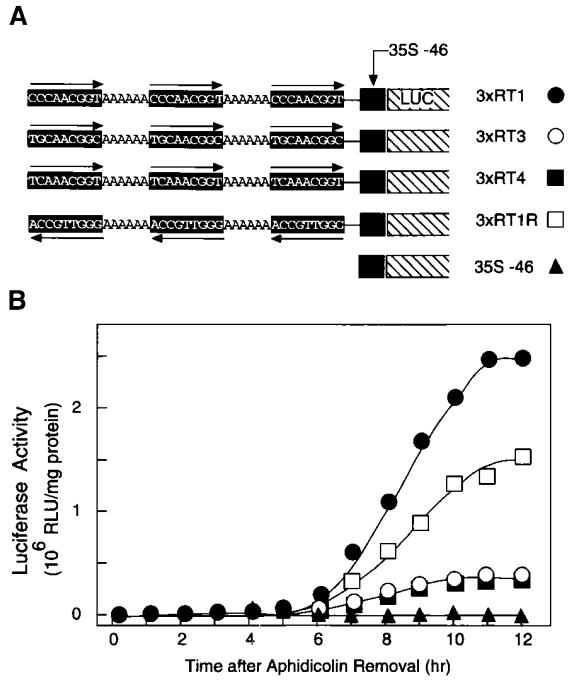

Figure 8. Repeat Sequences of the *CYM* Promoter Can Direct the M Phase–Specific Activation in a Heterologous Context.

(A) The diagram shows constructs containing three copies of the repeat sequences. The repeat sequences (RT1, RT3, and RT4) are shown on a black background. The directions of the motifs are shown by arrows.

(B) BY2 cells transformed with the promoter–*LUC* constructs were synchronized by the aphidicolin method and harvested at 1-hr intervals for LUC assays. Promoter constructs are designated with the symbols shown in **(A)**. RLU, relative light unit.

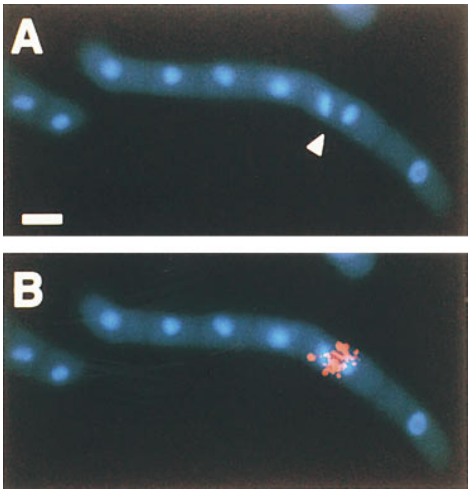

Figure 9. M Phase–Specific Promoter Activation by the Repeat Sequence in Asynchronously Growing BY2 Cells.

Asynchronously growing BY2 cells transformed with 3 × RT1 were first treated with luciferin for bioluminescence imaging and then stained with Hoechst 33258. Each luminescent image and Hoechst fluorescent image was captured with the photon-counting video-intensified microscope camera and analyzed by the image processor. Blue was assigned to Hoechst fluorescence, and red was assigned to luciferase luminescence to produce false-color images.
(A) Hoechst fluorescence. A mitotic cell is indicated by the arrowhead. Bar = 10 μm.
(B) LUC luminescent image superimposed on the image shown in **(A)**.

MSA Elements in Promoters of Other M Phase–Specific Genes from Various Plant Species

Several promoters from genes that are expressed specifically in the M phase were examined to determine whether they contain MSA-like sequences. We cloned promoters of four B-type cyclins from different plant species: tobacco *NtCYM* (Ito et al., 1997), soybean *cyc4Gm* (Kouchi et al., 1995), and Arabidopsis *cyc1bAt* (Day et al., 1996) and *cyc2aAt* (Ferreira et al., 1994a). As shown in Figure 10, these promoters contain three to five repeat sequences that are similar to the MSA identified in the *CYM* promoter. In addition, promoters of two other genes, *NACK1* and *NACK2*, which encode tobacco kinesin-like proteins, contain two MSA-like sequences. In all analyzed promoters, the MSA-like sequences are usually located within the first 200 bp upstream of the transcription start sites.

Comparison of MSA-like promoter motifs has shown that all of them contain a fully conserved central core pentamer, AACGG. It is flanked on both sides by less conserved 3-bp sequences. Figure 10 shows that the MSA consensus sequence (T/C)C(T/C)AACGG(T/C)(T/C)A closely matches the consensus sequences of c-Myb and v-Myb binding sites (Howe and Watson, 1991; Grotewold et al., 1994). This observation suggests that MSA elements may bind some plant transcriptional factors related to Myb.

DISCUSSION

Most eukaryotic cyclins accumulate at specific points in the cell cycle. The oscillation of cyclin levels is regulated, at

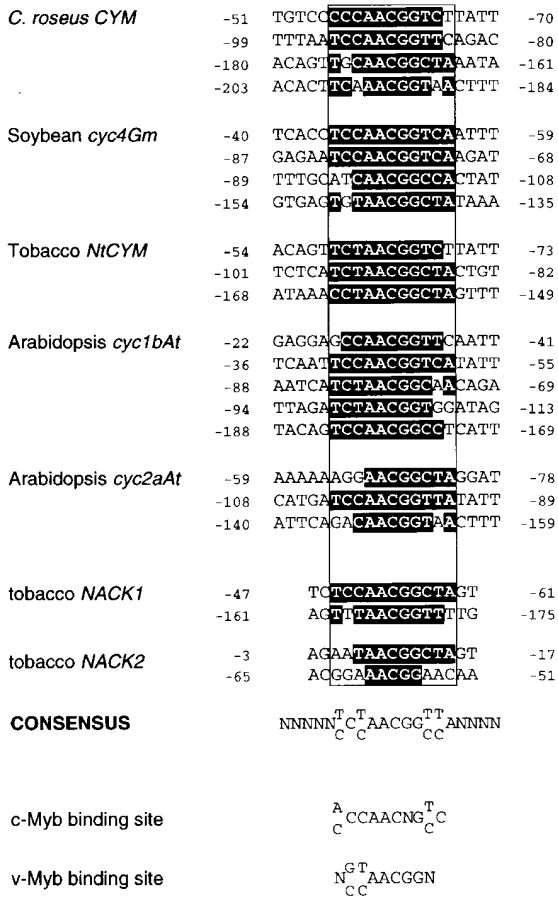

Figure 10. Alignment of MSA-like Sequences Present in the Promoters from Other Plant Species.

Shown is an alignment of MSA-like sequences found in promoters of B-type cyclin genes: *C. roseus CYM*, soybean *cyc4Gm*, tobacco *NtCYM*, Arabidopsis *cyc1bAt* and *cyc2aAt*, and tobacco genes for kinesin-like proteins *NACK1* and *NACK2*. The boxed region indicates the 11-bp sequences that share high homology with each other. Nucleotides identical to those of the 11-bp consensus sequence are shown on a black background. Numbers indicate the nucleotide positions from the transcription start sites, which were determined by primer extension. The binding sequences of c-Myb (Howe and Watson, 1991) and v-Myb (Grotewold et al., 1994) are also shown.

least partially, at the transcriptional level. To understand the mechanism responsible for periodic transcription of cyclin genes, we have analyzed the promoter of a *C. roseus* B-type cyclin, *CYM.* This cyclin gene has been shown to be expressed strictly during the late G_2 and M phases of the cell cycle in *C. roseus* and tobacco BY2 cells (Ito et al., 1997). A 462-bp fragment from the *CYM* promoter can confer M phase–specific expression to reporter genes (Ito et al., 1997; this study).

Deletion analysis of the *CYM* promoter identified a region (−203 to −40) necessary for M phase–specific expression. This promoter region could direct the levels of *LUC* expression similar to those observed for the full-length *CYM* promoter. A smaller region of the *CYM* promoter (−80 to +5) was sufficient for M phase–specific expression, although the expression levels were much lower for this construct than for those observed with the full-length promoter. A 9-bp element located between positions −66 and −58 was identified. When mutated, it completely abolished M phase–specific promoter activation. Three additional repeat sequences sharing high identity with the identified element were found in the *CYM* promoter. In a heterologous promoter context, these repeat sequences could function in an orientation-independent fashion to direct M phase–specific expression of the *LUC* reporter gene. Taken together, our data indicate that the identified MSA elements are necessary and sufficient for M phase–specific expression in cultured tobacco cells.

Transcription of several B-type cyclins, including a member of the CycB2 class from Arabidopsis, *cyc2aAt* (Ferreira et al., 1994a), and two members of the CycB1 class, soybean *cyc4Gm* (Kouchi et al., 1995) and tobacco *NtCYM* (Ito et al., 1997), has been shown to be induced during the G_2 and M phases. Phase-specific expression of the Arabidopsis CycB1 cyclin *cyc1bAt* (Day et al., 1996) has not been examined; however, it has high sequence homology to M phase–specific *cyc1At* (Ferreira et al., 1994a). The promoters of these four cyclin genes carry three to five MSA-like elements. Thus, both CycB1 and CycB2 classes of plant cyclins are likely to be regulated by the same MSA-mediated mechanism.

The genes encoding A-type cyclins from plants have been shown to be expressed earlier than the B-type cyclin genes (Reichheld et al., 1996; Ito et al., 1997). The promoter of a *C. roseus* A-type cyclin, *CYS* (Ito et al., 1997), does not contain MSA-like sequences. Also, we could not find MSA-like elements in the published promoter sequences of two plant cyclin-dependent kinases: one is the constitutively expressed Arabidopsis *cdc2aAt* gene (Imajuku et al., 1992; Chung and Parish, 1995), and the other is *cdc2bAt* (Imajuku et al., 1992), which is expressed during the S and G_2 phases (Segers et al., 1996). Thus, among various plant cyclins and cyclin-dependent kinases, the MSA-mediated regulatory pathway seems to be restricted to the B-type cyclins. On the other hand, we have found MSA-like motifs in the promoters of *NACK1* and *NACK2* genes. These genes encode plant kinesin-like proteins that are expressed in a fashion similar to B-type cyclins (R. Nishihama and Y. Machida, unpublished results). We speculate that the MSA-mediated mechanism may function in a narrow window in late G_2 and M phases to regulate a group of genes specifically expressed at this timing.

MSA-like sequences could not be found in the promoters of mammalian B1 cyclins (Cogswell et al., 1995; Hwang et al., 1995; Piaggio et al., 1995) and *cdc25C* (Lucibello et al., 1995). At the same time, plant cyclin promoters do not contain motifs similar to animal regulatory elements. Thus, higher plants may have a unique mechanism of M phase–specific regulation of gene expression.

It has been suggested that abundance of B-type cyclin in plants may be a limiting factor that determines the rate of cell division during developmental processes (Ferreira et al., 1994b; Doerner et al., 1996). Ectopic expression of the Arabidopsis CycB1 cyclin *cyc1At* stimulates organized growth of roots (Doerner et al., 1996). We have shown that MSA elements are sufficient for specific expression in root tips of transgenic tobacco plants (M. Ito and H. Kodama, unpublished results). Thus, transcriptional regulation of cyclin gene expression mediated by MSA elements may play an important role in controlling growth of established meristems.

The MSA consensus sequence is similar to the consensus binding sites of c-Myb and v-Myb factors from animals (Howe and Watson, 1991; Grotewold et al., 1994). There is some evidence that Myb-related proteins can participate in the control of cell division in plants. Recent studies have shown that an Arabidopsis Myb-related protein, AtCDC5, may be a specific regulator of gene expression in the G_2 and M phases (Hirayama and Shinozaki, 1996). This protein shares similarity with *Schizosaccharomyces pombe* Myb-related protein $Cdc5^+$, which is essential for progression through the G_2 and M phases (Ohi et al., 1994). However, the DNA sequence recognized by the recombinant AtCDC5 protein, CTCAGCG, differs from the consensus MSA element. In our work, gel shift assays performed to show in vitro binding of some nuclear factors to the MSA elements were not successful. We speculate that the putative MSA binding factor(s) might be present only in cells in the M phase, when the nuclear envelope is lost. Thus, the factor may be absent in extract prepared from the isolated nuclei.

In mammalian cells, G_2 phase–specific transcription is regulated by a complex mechanism with elements that are thought to be involved in activation (Ohno et al., 1996) and repression (Lucibello et al., 1995). Our data indicate that the MSA-mediated regulatory pathway alone is sufficient to induce the cyclin expression observed during the G_2 and M phases of the plant cell cycle. We are currently in the process of identifying the protein(s) that interacts with MSA elements. Cloning of the corresponding cDNA will provide the means to determine the mechanism that controls M phase–specific gene expression in plants.

METHODS

Plasmid Construction

The two binary vectors pBI-LUC (Nishiuchi et al., 1995) and pBICL46 were used. pBI-LUC contains a cauliflower mosaic virus (CaMV) 35S promoter–luciferase (*LUC*) fusion gene originated from pDO432 (Ow et al., 1986). For cloning of the modified *CYM* promoter fragments, the CaMV 35S promoter was removed by HindIII-BamHI digestion, and the promoter fragments were cloned into the HindIII-BamHI interval of pBI-LUC. A fragment of the CaMV 35S basal promoter (−46 to +1) was synthesized by polymerase chain reaction (PCR), using pDO432 as a template with a set of linker/primers (HindIII-SalI 5′ and BamHI 3′). The resulting PCR fragment was then used to replace the full-length CaMV 35S promoter in pBI-LUC with the 35S basal promoter. Plasmid pBICL46 contains HindIII and SalI sites upstream of the CaMV 35S basal promoter–*LUC* fusion. Modified *CYM* promoter fragments were cloned into the HindIII-SalI sites.

A series of 5′ deletion derivatives of the *CYM* promoter was obtained by PCR, using the *CYM* genomic fragment as a template (Ito et al., 1997). Specific linker/primers (28-mers) were used to generate restriction sites (HindIII 5′ and BamHI 3′) for directional cloning upstream of the *LUC* reporter gene in pBI-LUC. 3′ end deletions of the *CYM* promoter were generated in a similar fashion by using HindIII 5′- and SalI 3′-specific linker/primers and cloned upstream of the CaMV 35S basal promoter of pBICL46. Site-directed mutagenesis of the *CYM* promoter (−80 to +5) was done by PCR, using 85-mer synthetic DNAs with 10- or two-base substitutions as templates. The mutagenized promoters were cloned as HindIII-BamHI fragments into pBI-LUC. Constructs containing repeat sequences (RTs) were prepared by annealing complementary oligonucleotides that carry HindIII 5′ and SalI 3′ linkers. Then they were ligated into the HindIII-SalI site of pBICL46. All plasmid constructs were sequenced.

Plant Material

Tobacco (*Nicotiana tabacum*) BY2 cells were maintained at 27°C in a medium that contained Murashige and Skoog salt base (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.2 mg/mL KH_2PO_4, 0.1 mg/mL myoinositol, 1.0 mg/L thiamine, and 9×10^{-7} M 2,4-dichlorophenoxyacetic acid. Plasmid constructs were introduced into *Agrobacterium tumefaciens* LBA4404 and used to transform tobacco BY2 cells, essentially as described by An (1987). Approximately 1000 to 3000 kanamycin-resistant colonies were pooled, reintroduced into suspension cultures, and maintained in the same way as BY2 cells. To synchronize cell division of transgenic tobacco BY2 cells, 10 mL of 7-day-old cells was diluted 10 times with fresh medium containing aphidicolin (final concentration of 5 μg/mL), cultured for 24 hr, washed with 1 liter of fresh medium, and finally suspended in 100 mL of medium. The mitotic index was determined by counting cells stained with Hoechst 33258 (Sigma) in the presence of 0.5% Triton X-100.

RNA Extraction and Gel Blot Hybridization

Extraction of total RNA was performed as previously described (Ito et al., 1997). RNA was subjected to electrophoresis on 1% agarose gels that contained 2% formaldehyde by a modified version of the procedure of Lehrach et al. (1977). RNA was then blotted onto nylon membranes (Biodyne A; Pall, Port Washington, NY). The entire open reading frame of the *LUC* gene (BamHI DNA fragment of pBI-LUC) was labeled with ^{32}P, using the Megaprime DNA Labelling System (Amersham). Hybridization and washings were performed as described previously (Ito et al., 1997). Signals were detected using the Fuji Imaging Analyzer (model BAS2000; Fuji Photofilm, Tokyo, Japan).

LUC Assay

Protein extracts were obtained by homogenizing BY2 cells in LUC extraction buffer (50 mM sodium phosphate, 2 mM DTT, and 0.1% Triton X-100). LUC activity assays were performed using a PicaGene assay kit (Toyo Co. Inc., Tokyo, Japan), according to the manufacturer's instructions. Measurements of LUC activity were performed with a luminometer (model LB9501; Berthold, Bad Wildbad, Germany).

For imaging of in vivo LUC bioluminescence, 2 mL of 3-day-old BY2 cell suspension was treated with 5 mM luciferin (Sigma) for 30 min to diminish the activity of accumulated LUC. BY2 cells were then washed with 100 mL of fresh medium, resuspended in 2 mL of the medium, and cultured for 8 to 10 hr. Cells were placed on glass slides coated with poly-L-lysine (Sigma). They were first treated with 5 mM luciferin to detect luminescence and then stained with Hoechst 33258 (Sigma). Each luminescent image and Hoechst fluorescent image was captured with the photon-counting video-intensified microscope camera connected to the microscope and analyzed by the image processor ARGUS-20 (Hamamatsu Phototonics, Hamamatsu, Japan). When producing the false-color images, blue was assigned to the Hoechst fluorescence, and red was assigned to LUC luminescence.

Isolation of Promoter Sequences by Inverse PCR

Total genomic DNA of *Arabidopsis thaliana* ecotype Columbia and tobacco BY2 cells was isolated by the method of Murray and Thompson (1980). Soybean (*Glycine max* cv Akisengoku) genomic DNA was kindly supplied by H. Kouchi (National Institute of Agrobiological Resources, Tsukuba, Japan). Genomic DNA was cut with several restriction enzymes, ligated with a DNA ligation kit (Takara, Kyoto, Japan), and used as a template for PCR with specific primer sets (25-mers). Nucleotide sequences of the primers were designed using published cDNA sequences. Amplified fragments were directly cloned into pGEM-T vector (Promega) and sequenced. Fidelity of the determined sequences was confirmed by sequencing DNA fragments obtained by independently performed PCR reactions.

Transcription start sites of *NtCYM*, *cyc4Gm*, *cyc1bAt*, *cyc2aAt*, *NACK1*, and *NACK2* were determined by primer extension, using poly(A)$^+$ RNA extracted from soybean (cv Akisengoku), tobacco BY2, and Arabidopsis T87 cell lines (Axelos et al., 1992), as described previously (Ito et al., 1997).

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