

Genes regulating the plant cell cycle: Isolation of a mitotic-like cyclin from *Arabidopsis thaliana*

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ABSTRACT A key element of cell cycle control in eukaryotes is the M-phase kinase, composed of p34^{cdc2} and cyclin. To dissect the plant cell cycle, we have previously isolated a *cdc2* gene homolog from *Arabidopsis thaliana*. We have now cloned an *Arabidopsis* cDNA corresponding to cyclins. This gene (*cyc1At*) encodes a protein with a predicted molecular mass of 48.4 kDa and a domain homologous to the cyclin box of mitotic cyclins. However, by sequence comparison the *cyc1At* gene could not be assigned to the A- or B-type group. The mRNA accumulates preferentially in actively dividing cells and when these cells are blocked during the cell cycle, the amount of transcripts decreases dramatically. *cyc1At* mRNA is found mainly in G₂-phase nuclei, suggesting that its expression is periodic in the cell cycle. Microinjection of synthetic *cyc1At* mRNA induced meiotic maturation in *Xenopus* oocytes. *Cyc1At* is encoded by a single gene, but the amplification by the polymerase chain reaction of other fragments homologous to cyclins indicates the presence of a family of cyclins in *Arabidopsis*.

Cell division is an integral part of growth and development. Although the basic mechanism of cell cycle control is apparently conserved in all higher eukaryotes, its regulation can differ according to the different developmental plans and programs of each type of organism. Plants have unique features that distinguish their development from that of the animals. As plant cells cannot move, all root and shoot cells in the embryo have to undergo morphogenesis in the correct position. The morphology and position of plant organs are, therefore, determined only by cell division and expansion. In plants, most of the cell division is confined to specialized regions, the meristems, and, in most cases, they can continuously produce new organs throughout plant life. Even non-dividing cells retain a measure of totipotency that allows them to dedifferentiate and acquire a new function in the plant or even form new plants under appropriate tissue culture conditions. The patterns of cell division and expansion not only are influenced by intrinsic developmental program but also respond to environmental signals such as light and gravity. As a first step in studying the regulation of cell division during plant growth and development, we have isolated plant homologs to mitotic control components of eukaryotes. A key element of cell cycle control is the M-phase kinase, which is composed of p34^{cdc2} and cyclin (1, 2).

Cyclins are proteins first identified in the embryos of marine invertebrates by virtue of their periodic accumulation during the cell cycle. They reach particularly high levels in interphase and undergo rapid proteolysis at mitosis (3). Subsequently, it was demonstrated that they have a critical role in cell cycle regulation (4–6). Several cyclins have been cloned from various organisms. Based on their sequence

similarities and their pattern of expression and action during the cell cycle, they can be divided into three classes: G₁ cyclins and the mitotic B-type and A-type cyclins. Cyclin B has a critical role in the initiation of mitosis. It is synthesized and gradually accumulated between late S and G₂ phases and forms a complex with inactive p34^{cdc2}. This association and the well-defined posttranslational modifications are essential for the kinase activation (7, 8). The role of cyclin A in the cell cycle is less well understood. In several systems cyclin A is expressed in S phase, earlier than cyclin B, and correspondingly, cyclin A-associated kinase activity appears before mitosis (9, 10). Cyclin A can associate with the products of *cdc2* and *CDK2*, a gene closely related to the *cdc2* gene (11). Recent reports suggest that cyclin A complexes may be required in S phase (12), but the phenotype of a *Drosophila* cyclin A null mutant indicates that cyclin A is also important in the G₂/M phase transition (13). The G₁ cyclins best characterized are the CLNs from *Saccharomyces cerevisiae*. They bind p34^{cdc2} in G₁ to form an active kinase complex that is necessary for G₁/S transition (start) (14). Recently, a CLN-related cyclin has been cloned from *Schizosaccharomyces pombe* (15). Several putative G₁ cyclins have been isolated such as the mammalian D- and E-type cyclins (16, 17) and the *Schizosaccharomyces pombe* *cig1*⁺ cyclin (18). They seem to act in G₁ phase although they are more similar in sequence to the mitotic cyclins than to the CLNs. On the other hand, the putative G₁ cyclins of the C type isolated from humans and *Drosophila* are distantly related to all other cyclins, and their point of action in the cell cycle has still to be determined (19, 20).

We have reported (21) the isolation and characterization of the *cdc2* homologous from *Arabidopsis*. We have now cloned other polymerase chain reaction (PCR) fragments homologous to cyclins. Here, we report the isolation and characterization of one cyclin cDNA clone, *cyc1At*.[¶] *cyc1At* mRNA is detected mainly in actively dividing cells and in G₂ nuclei.

MATERIALS AND METHODS

Cloning *Arabidopsis* Cyclin cDNAs. First-strand cDNA from total RNA extracted from *Arabidopsis thaliana* ecotype C24 stems was synthesized by using the SuperScript preamplification system kit from GIBCO/BRL. One-fourth of the reaction mixture was used for the first PCR amplification in a 100- μ l PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, all four deoxynucleotide triphosphates (each at 0.2 mM), 1 μ g of each primer, and 2.5 units of *Taq* polymerase. The oligonucleotides used as primers were primer A (5'-GCAGGATCCATGAGRGCATYCTYATYGAYTGG-3'), primer B (5'-GCAGAATTCATYGCITCIAARTAYGAR-3'), and primer

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[¶]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80190).

C (5'-GCAGGATCCRAGYTCIAYIARRTACTTIGC-3'), where R is A or G and Y is C or T. The underlined sequences denote restriction sites *Bam*HI, *Eco*RI, and *Bam*HI, respectively. The first PCR was carried out using the primers A and C. Forty-five cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min with a final extension of 10 min were performed. A fragment of expected size, 380 base pairs, was eluted from the gel and used as DNA template for a second PCR carried out under the same conditions but using primers B and C. The product was digested with *Bam*HI and *Eco*RI and subcloned in pUC18. Twenty clones were sequenced by the dideoxynucleotide method of Sanger et al. (22). Three classes of clones containing homology with cyclins were obtained. They were used as probes to screen ≈400,000 clones from an *Arabidopsis* flower cDNA library in vector λgt10 (gift of Dulce de Oliveira, Universidade Federal de Rio de Janeiro, Rio de Janeiro, Brazil), according to the protocol from Amersham.

Plant Material. Cell suspension cultures of *Arabidopsis* ecotype Columbia (gift of Imre Somssich and Dierk Scheel, Max-Planck-Institut für Züchtungsforschung, Cologne, F.R.G.) were maintained in liquid medium containing Murashige and Skoog salts (Sigma, M5524), sucrose (30 g/liter), myo-inositol (0.1 g/liter), nicotinic acid (0.5 mg/liter), pyridoxine hydrochloride (0.5 mg/liter), thiamine hydrochloride (0.1 mg/liter), and 2,4-dichlorophenoxyacetic acid (1 mg/liter). The cultures were kept in 200-ml Erlenmeyer flasks in the dark at 26°C rotating at 110 rpm. Every 7 days, one-fourth of each culture was diluted with 3 vol of fresh medium.

***Arabidopsis* cycl1At mRNA Analysis.** Northern blot analysis with total RNA isolated from roots, stems, leaves, and flowers of *Arabidopsis* ecotype C24 and the cell suspension was done as described (21). The histone H4 gene from *Arabidopsis*, used as a probe, was a gift of Ben Scheres (University Utrecht, Utrecht, The Netherlands). Nuclei from the cell suspension cultivated for 4 days in fresh medium were isolated, sorted by flow cytometry, blotted, and hybridized as described (23). The 25S rRNA gene from *Arabidopsis* used as a probe was a gift of P. Gruendler (Universität Wien, Vienna) (24).

***cycl1At* mRNA Expression in *Xenopus* Oocytes.** *cycl1At* cDNA was cloned in the *Eco*RI site of pBluescriptII KS⁺. Plasmid DNA was linearized with *Sal* I and *Kpn* I. Capped

sense mRNA of *cycl1At* was transcribed *in vitro* with T7 RNA polymerase and the Riboprobe Gemini II core system kit (Promega) according to the manufacturer's recommendations. Stage VI *Xenopus* oocytes were dissected manually and incubated in Barth's saline (25). The oocytes were microinjected with 25–50 nl of T7 *cycl1At* RNA or water and incubated in Barth's saline at 20°C. Some oocytes were stimulated hormonally by incubation with progesterone (5 μg/ml). After 12 hr, they were fixed with 5% (wt/vol) trichloroacetic acid and dissected to detect the presence or absence of nuclear envelope.

RESULTS

Isolation of *Arabidopsis* cDNA Homologous to Cyclins. The various cyclin sequences have a conserved region, the cyclin box. Based on three regions of this box, three sets of oligonucleotides were synthesized for use in the PCRs. We were able to amplify three fragments (*cycl1*, *cycl2*, and *cycl3*) showing homology with cyclins. They were used as probes to isolate cDNA clones from a flower-derived cDNA library. One positive phage clone was obtained after screening ≈400,000 plaques with the *cycl1* PCR fragment. The cDNA insert was 1.6 kilobases in length and contained a 1284-base-pair open reading frame expected to encode a protein of 428 amino acids with a predicted molecular mass of ≈48.4 kDa (Fig. 1). Southern blot analysis showed that the corresponding gene, denoted *cycl1At*, is unique in the *Arabidopsis* genome (data not shown).

Sequence comparison between the *cycl1At*-encoded protein and other cyclins inside the conserved region revealed extensive homology with other representatives of the cyclin family. We believe that *Cycl1At* is most likely to be a mitotic cyclin based on its pattern of expression (see below) and its high homology in the conserved region with the mitotic cyclins compared with the CLNs (G₁ cyclins). Furthermore, *Cycl1At* protein contained a mitotic destruction motif (26) near the amino terminus of the protein and lacked PEST (Pro-Glu-Ser-Thr) sequences (27) near the carboxyl terminus, typical of the CLNs (Fig. 1). However, it is less clear whether it is an A- or B-type cyclin. Within the cyclin box, *Cycl1At* protein is 47.3% identical to *Xenopus* cyclin B2 (5) and 46.3% identical to *Xenopus* cyclin A1 (9), and these

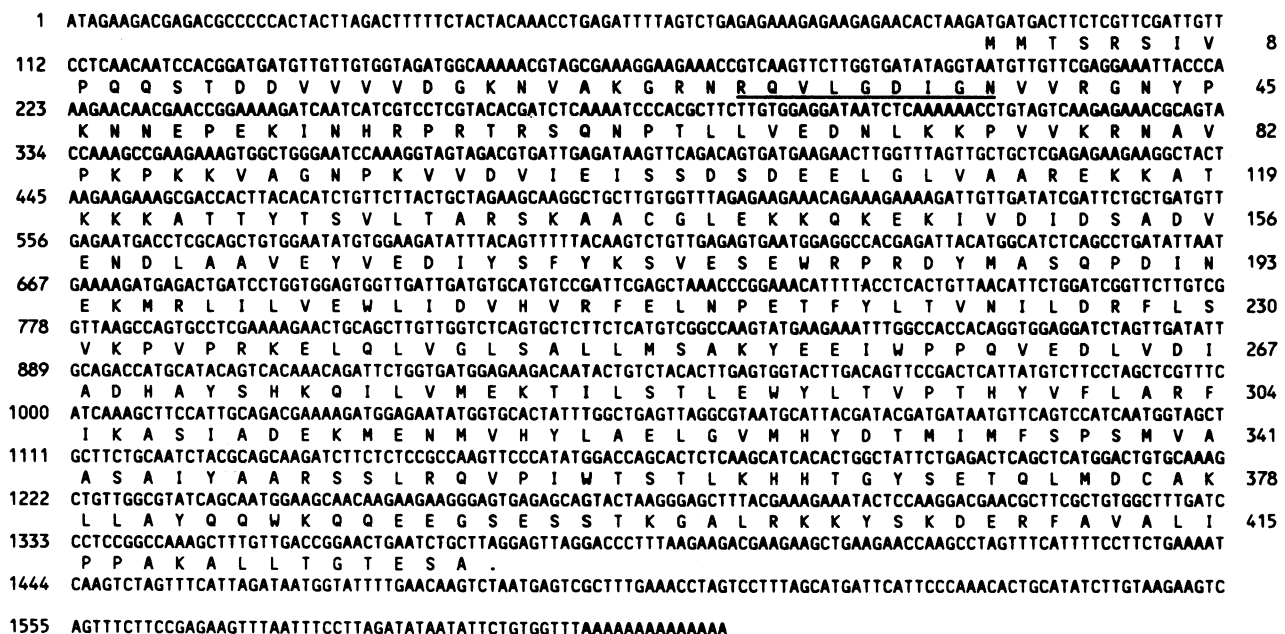


FIG. 1. Nucleotide and deduced amino acid sequences of the *cycl1At* cDNA. Underlined amino acids represent the region homologous to the mitotic destruction box.

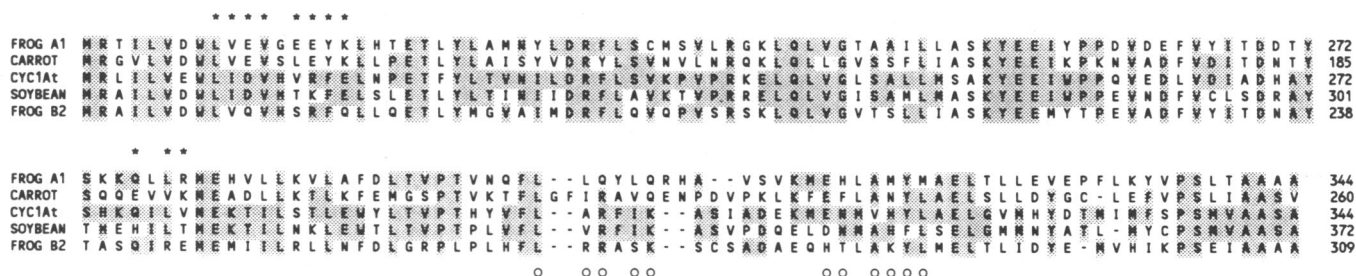


FIG. 2. Amino acid sequence comparison of residues in the cyclin box. The sequence of Cyc1At from *Arabidopsis* is aligned with *Xenopus* cyclin A1, C13-1 cyclin from carrot, S13-6 cyclin from soybean, and *Xenopus* cyclin B2 sharing 46.3%, 43%, 67.6%, and 47.3% of homology with each of them, respectively. Identical matches to the Cyc1At sequence are shaded. Amino acids indicated with an asterisk in the *Xenopus* A1 sequence and with an open circle in the *Xenopus* B2 sequence represent conserved motifs in all A-type and B-type cyclins, respectively.

similarities are maintained, with small variations, in comparison to cyclins of other species (Fig. 2). The conserved amino acid motifs characteristic of either B-type (LRR-SK and HT-AKYL) or A-type cyclins (LVEV-EEYK and Q-LR) are not completely represented in the Cyc1At protein sequence. On the other hand, the mitotic destruction motif is typical of B-type cyclins. Sequence comparison of total Cyc1At protein with the two plant cyclins recently reported (28) shows 30.5% of homology with carrot C13-1 cyclin and 56.3% with soybean S13-6 cyclin. Within the cyclin box, Cyc1At protein has 43% homology with the carrot cyclin and 67.6% homology with the soybean cyclin (Fig. 2).

cyc1At mRNA Expression. Northern blot analysis was used to study the expression of *cyc1At* mRNA in various *Arabidopsis* tissues. As shown in Fig. 3A, *cyc1At* transcripts of ≈ 1.6 kilobases were present in all organs in a very low amount, except in actively dividing cell suspensions, where they were found in much higher levels. To verify whether drugs blocking cell division affect *cyc1At* expression, we treated the cell suspension with hydroxyurea and colchicine. When the cell suspension cultures were treated with 10 mM hydroxyurea to block DNA synthesis in early S phase, the level of *cyc1At* transcripts decreased dramatically. The same change in amount of transcripts was observed after treatment

with 0.05% colchicine, which arrests cells in metaphase (Fig. 3B). A decrease in expression after hydroxyurea or colchicine treatment is also found for a histone H4 gene from *Arabidopsis*. On the other hand, the *Arabidopsis* phosphatase 1A.A1 gene was constitutively expressed under the same conditions (P.F., A.H., M.V.M., and D.I., unpublished data) (Fig. 3B). These data strongly indicate that *cyc1At* gene is expressed preferentially in dividing cells.

cyc1At Gene Is Preferentially Expressed in G₂ Nuclei. To analyze the periodicity of the *cyc1* mRNA expression during the cell cycle, cell suspension nuclei were separated by flow cytometry on the basis of DNA content, blotted, and hybridized with *cyc1At* cDNA probe. The DNA content profile monitored by flow cytometry shows the presence of 51% of 2C nuclei (G₀-G₁ phase), 39% of 4C nuclei (G₂ phase), and 10% of nuclei in S phase (Fig. 4A) (where C is the amount of DNA in a haploid nucleus). To be sure that the cell suspension was diploid, petunia nuclei were used as a control in the first sorting. Filters containing G₀-G₁ and G₂ nuclei were hybridized with *cyc1At* cDNA as a probe. As shown in Fig. 4B, *cyc1At* transcripts were detected mainly in G₂ nuclei, whereas G₀-G₁ nuclei have much less mRNA. The same filters hybridized with the constitutive 25S rRNA gene showed equivalent amount of transcripts in G₀-G₁ and G₂

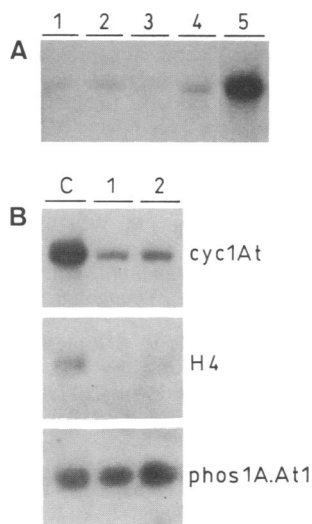


FIG. 3. Expression analysis of *cyc1At* gene in *Arabidopsis*. (A) A Northern blot with 10 μ g of total RNA from roots, stems, leaves, flowers, and the cell suspension (lanes 1-5, respectively) was probed with *cyc1At* cDNA. (B) Cell suspension, after growing 2 days in a fresh medium, was treated with 10 mM hydroxyurea (lane 1) or 0.05% colchicine (lane 2). Total RNA was extracted after 2 days of treatment and total RNA from untreated cells was used as a control (lane C). Northern blot was probed with *cyc1At* (upper blot), H4 (middle blot), and *phos1A.At1* (lower blot) cDNAs.

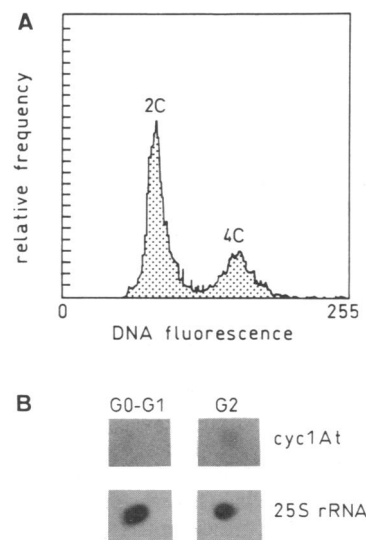


FIG. 4. *cyc1At* mRNA expression in G₀-G₁ and G₂ phase nuclei. (A) Approximately 5×10^3 cell suspension nuclei were analyzed by flow cytometry on the basis of DNA fluorescence. The frequency distribution of the nuclei according to their DNA content, displayed in a linear scale, shows the 2C and 4C peaks corresponding to nuclei in G₀-G₁ and G₂ phases, respectively. (B) Approximately 5×10^4 sorted *Arabidopsis* nuclei in G₀-G₁ and G₂ were blotted and hybridized with the *cyc1At* cDNA (upper blots) and 25S rRNA gene (lower blots) probes.

Table 1. *cycl1At* mRNA induces oocyte maturation

Treatment	RNA		GVBD, %
	injected, ng per oocyte	Oocytes injected, no.	
T7 <i>cycl1At</i> mRNA	40	40	80
T7 <i>cycl1At</i> mRNA	20	38	51
H ₂ O	—	39	2
Progesterone	—	40	60
None	—	40	0

Synthetic *cycl1At* mRNA and water were injected into *Xenopus* oocytes in 25–50 nl. As a control, uninjected oocytes were induced to undergo meiosis by treatment with progesterone (5 µg/ml). The appearance of white spot and absence of nuclear envelope were scored after 12 hr. GVBD, germinal vesicle breakdown.

nuclei. These results suggest that *cycl1At* mRNA abundance is regulated during the cell cycle, being mainly expressed in the G₂ phase.

***cycl1At* mRNA Can Induce Meiotic Maturation of *Xenopus* Oocytes.** Stage VI *Xenopus* oocytes are arrested in the G₂/M phase transition and are induced to enter meiosis and complete maturation by progesterone. It has been shown that microinjection of cyclin mRNA can also induce oocyte maturation (2, 28, 29). To show the functional capabilities of *cycl1At*, we microinjected T7 *cycl1At* mRNA into G₂-arrested oocytes and assayed for germinal vesicle breakdown, checking for white spot formation and absence of nuclear envelope. As shown in Table 1, *cycl1At* mRNA was able to induce oocyte maturation in a dose-dependent way whereas the controls, uninjected or microinjected only with water, did not change. Progesterone-treated oocytes were induced to enter meiosis but not with very high efficiency probably due to the use of undefolliculated oocytes. These results show that *cycl1At* is functional in a different organism, but it is important to point out that many of the *cycl1At*-activated oocytes were not very stable after several hours of incubation, often showing necrotic changes.

DISCUSSION

Using PCR amplification, we have obtained a DNA fragment homologous to cyclins. Using this fragment as a probe, we have isolated a full-length cDNA clone. Sequence homologies enabled us to identify the cloned *cycl1At* as a cyclin, but not to assign it to a specific cyclin group. In the conserved region, the *Cycl1At* protein shows 67.6% homology with a soybean cyclin that was tentatively classified as a B-type, but we cannot rule out the possibility that both are part of a different plant cyclin family. Furthermore, several cyclins recently isolated seem to act in G₁ phase but have more sequence similarities to the mitotic types than to the CLNs, indicating that the cyclin family may be more complex than previously assumed (16–18). Southern blot analysis shows that *Cycl1At* is encoded by a single gene. However, the PCR amplification of two other fragments homologous to cyclins, with low homology between themselves, indicates that the cyclin family in *Arabidopsis* has at least three classes.

We have shown that the *cycl1At* gene is preferentially expressed in dividing cells. This was most apparent when the high amount of *cycl1At* transcripts in actively dividing cell suspensions was found to decrease dramatically after treatment with drugs that block the cell cycle. Based on this result, the higher expression of *cycl1At* in cell suspensions compared with plant organs was likely due, at least partly, to the dividing state of the cells and not to tissue specificity. The low amount of transcripts actually detected in the different organs could even be restricted to a small number of dividing cells in meristematic regions. We have also shown that *cycl1At* mRNA abundance is regulated during the cell cycle, since

cycl1At transcripts are found mainly in G₂ nuclei. The *cycl1At* mRNA levels in the whole cell during the entire cell cycle still have to be analyzed, but the fact that we detected transcription mainly in G₂ nuclei suggests a periodic pattern of expression, typical for cyclins. The point of action of *Cycl1At* in the cell cycle still has to be determined. Some sequence features and the transcription at G₂ phase indicate the protein may be important during mitosis. The fact that microinjection of *cycl1At* mRNA in stage VI *Xenopus* oocytes can deblock the oocytes, triggering the G₂/M phase transition, shows that *cycl1At* is biologically functional. However, we cannot conclude its mode of action since the control of meiosis in *Xenopus* oocytes is not completely understood. Recently, it has been shown that although injection of cyclin A mRNA can induce oocyte maturation, cyclin A is not required for meiosis and the stored cyclin B polypeptides are enough to induce oocyte maturation without any *de novo* cyclin synthesis (30, 31).

The isolation of related *cdc2* and cyclin genes in *Arabidopsis* reinforces the evidence that the cell cycle controls in plants are very similar to the other eukaryotes. The preferential expression of the *cycl1At* gene in dividing *Arabidopsis* makes it a very good marker of cell division. An important question is whether the individual cyclin species found in plants have different patterns of expression and function during the cell cycle. Because more than one *cdc2*-related gene has been identified in *Arabidopsis* (32), it might also be possible that different cyclins would complex specifically with a particular Cdc2-related protein. It is even possible that some cyclin genes could have a tissue-specific expression. All these questions will be readily answered through further molecular analysis of cyclin during plant development.

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