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Three discrete classes of *Arabidopsis* cyclins are expressed during different intervals of the cell cycle

(development/meristem)

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ABSTRACT **We have isolated cDNAs encoding four additional mitotic-like cyclins from *Arabidopsis: cyc2aAt, cyc2bAt, cyc3aAt,* and *cyc3bAt.* Examination of amino acid sequences deduced from plant cyclin cDNAs isolated so far showed that they can be grouped into three distinct classes. The members of each plant cyclin family are more related to each other than to any animal or yeast cyclin. Reverse transcription–PCR analysis demonstrated that *cyc2aAt* was expressed in all plant organs, whereas *cyc2bAt* mRNAs were found only in roots; *cyc3aAt* was not expressed in leaves and was barely expressed in flowers. On the other hand, *cyc3bAt* transcripts were observed in all organs. Whole-mount *in situ* hybridizations on roots showed that the cyclin mRNAs were confined to parts of the roots with mitotic activity. Furthermore, results of whole-mount *in situ* hybridizations on roots treated with either oryzalin or hydroxyurea suggest that the different cyclin classes have distinct functions in the cell cycle.**

The control of cell cycle progression is mainly exerted at two transition points: late in G_1, before DNA replication, and at the G_2/M boundary. Cell cycle progression is dependent on the activation of a series of heteromeric protein kinase complexes, known as cyclin-dependent kinases (Cdks; for recent reviews, see refs. 1–4).

In animals, distinct but related Cdks conduct the G_1-to-S and G_2-to-M transitions (5, 6). Different kinases can be distinguished by the associated regulatory subunit, a group of proteins known as cyclins (7). Homology between the different cyclin classes is mainly restricted to the cyclin box, a region of ≈150 aa (8) that is important for interaction with the catalytic subunit (9). In higher animals, cyclins A and B are required for entry into mitosis (10–13). Cyclin A is also implicated in DNA replication (14, 15). Exit from the quiescent state (G_0) and reentry into the cell cycle might need cyclin D (16), while progression from G_1 to S may require cyclin E (17). In *Saccharomyces cerevisiae*, proteins structurally related to B-type cyclins execute functions both in the S phase and at the G_2/M transition (18–20). Passage through START in budding yeast can be achieved by complexes formed by Cdc28 and at least one of three related proteins: Cln1, Cln2, or Cln3 (21). In *Schizosaccharomyces pombe*, B-type cyclins have been implicated with early and late events of the cell cycle (22, 23). While Cdk activities are regulated by posttranslational modifications (4, 24), cyclin transcription and consequent accumulation of its correspondent gene product also play an important role in kinase activation (25, 26).

Recent evidence has revealed that the basic machinery that drives cell division is similar in plants and animals (for reviews, see refs. 27 and 28). Previously, the isolation of a mitotic cyclin from *Arabidopsis* has been reported (29). Here, we describe the characterization of four more cyclins from *Arabidopsis thaliana*[¶]. Sequence analysis showed that plant mitotic-like cyclins fall into three separate classes. Furthermore, each class seems to be expressed in a different interval of the cell cycle.

MATERIALS AND METHODS

Cloning of *Arabidopsis* Cyclins. Amplification of the cyclin-homologous PCR fragments (*cyc1*, *cyc2*, and *cyc3*) has been reported (29). Initial screenings of an *Arabidopsis* flower cDNA library yielded clones homologous to only one type of probe (*cyc1*). The *cyc2* and *cyc3* PCR products were used to screen an *Arabidopsis* (ecotype Columbia) cell suspension cDNA library in the vector λZAPII (gift of Bernard Lescure, Centre National de la Recherche Scientifique–Institut National de la Recherche Agronomique, Toulouse, France). Plasmids carrying the cDNAs were excised according to the protocol from the manufacturer (Stratagene) and sequenced by the dideoxynucleotide method (30). The *cyc3* PCR product was also used to screen a genomic library prepared from total genomic DNA from *Arabidopsis* ecotype Columbia (gift of Diane Jofuku, University of California, Santa Cruz). A *Hin*dIII fragment of ≈5 kb containing the entire coding sequence was subcloned in pUC19 and sequenced.

Analysis of Cyclin Expression. Total RNA was isolated as described (31). First-strand cDNA was prepared with a Superscript amplification system (GIBCO/BRL). Amplification of cyclin 2-class first-strand cDNA was performed with the oligodeoxynucleotide primers 5′-GAAGAAGTTGAG-ATGGAGGA-3′ (sense) and 5′-ATATTGAATTGCAAA-GTACT-3′ (antisense). For amplification of cyclin 3-class first-strand cDNA, the primers were 5′-GGAGGAGTTT-TGCTTCATTAC-3′ (sense) and 5′-CCACTGGTGT-TGAGCTGCAA-3′ (antisense). The PCR consisted of 25 cycles of 94°C for 1 min (denaturation), 56°C for 30 sec (annealing), and 72°C for 30 sec (extension), with a final extension for 5 min. Similar cycles were employed for the cyclin 3 class, except that the annealing temperature was 60°C. The amplified DNAs were digested with *Pvu* II restriction endonuclease, and the restriction fragments were separated in 1.5% agarose gel, blotted, and hybridized with 5′-AATCTGATAGATAGATTCTTGT-3′ (cyclin 2 samples) or 5′-GATGGACACTCGACCAA-3′ (cyclin 3 samples) probes.

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Abbreviation: Cdk, cyclin-dependent kinase.
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[¶]The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z31400–Z31402 and Z31589).

Whole-mount *in situ* hybridization was performed (32) with modifications (J.d.A.E., M.V.M., and G.E., unpublished work). Antisense mRNAs were used as probes (Riboprobe Gemini II, Promega). For *cyc2aAt* and *cyc2bAt*, subclones containing the 3′ nontranslated regions were used to synthesize the probes. A PCR fragment containing segments of the first and second exons were used to produce the *cyc3aAt* probe. Digestion of the full-length *cyc3bAt* cDNA with *Nco* I generated an antisense probe containing essentially the 3′ nontranslated region.

RESULTS AND DISCUSSION

***Arabidopsis* Has Three Classes of Mitotic-Like Cyclins.** We previously reported the isolation of three different PCR products homologous to cyclins and the characterization of a full-length cDNA corresponding to one of the DNA fragments amplified, *cyc1At* (29). Screening of *Arabidopsis* cDNA and genomic libraries with the other PCR products as probes has yielded four more cyclin cDNAs: *cyc2aAt*, *cyc2bAt*, *cyc3aAt*, and *cyc3bAt*. The longest cDNA corresponding to *cyc2aAt* was 1.6 kb long with a reading frame of 1284 nt, predicted to encode a protein of 428 aa (Fig. 1). A partial cDNA of 1.3 kb corresponding to *cyc2bAt* was initially isolated. Repeated screenings with genomic *cyc2bAt* probes yielded partial cDNA clones containing the 5′ region. The reconstituted sequence of the *cyc2bAt* cDNA contains a reading frame of 1287 bp, expected to encode a protein of 429 aa. The encoded protein products of *cyc2aAt* and *cyc2bAt* are 76% identical (90% in the cyclin box) (Table 1; Fig. 1). In the screening of the cell suspension cDNA library with the *cyc3* PCR probe a highly homologous cDNA, *cyc3bAt*, was isolated. The insert of the *cyc3bAt* cDNA is 1.8 kb long with a 1311-bp open reading frame, predicted to encode a protein of 437 aa. Although repeated screenings with the *cyc3* PCR fragment failed to generate a cDNA clone identical to the probe, a genomic clone containing the whole coding region of *cyc3aAt* could be isolated. Most of the encoded amino acid sequence from *cyc3aAt* could be deduced from comparison of the genomic sequence with the highly homologous *cyc3bAt* cDNA clone. The exception was the region of the junction between the first and the second exon. A fragment encompassing this region was amplified by PCR to solve this problem (data not shown). The predicted Cyc3aAt protein has 445 aa (Fig. 1). The deduced polypeptide and the cyclin box of Cyc3aAt and Cyc3bAt are 71.5% and 82.4% identical, respectively.

After mitosis, inactivation of the Cdc2 kinase is mainly achieved by proteolysis of the cyclin moiety. The conserved amino acid sequence motif RXXLXXIXN, known as the mitotic destruction box, is a target for ubiquitin-mediated degradation (37, 38). Sequences that resemble the mitotic destruction box have also been found in yeast B-type cyclins that are connected with earlier events of the cycle (20, 23). Motifs that resemble the destruction box are located near the amino-terminal regions of the *Arabidopsis* cyclin 2 class (positions 37–46 and 39–48 in Cyc2aAt and Cyc2bAt, respectively; Fig. 1). Curiously, both Cyc2aAt and Cyc2bAt contain a conserved region with "PEST" sequences (39), which could also be important for rapid protein degradation (Cyc2aAt, positions 133–162; Cyc2bAt, positions 115–134 and 162–181; Fig. 1). In the cyclin 3 family, sequences that best match the consensus destruction box are not found so close to the usual location in the amino terminus (positions 75–83 and 56–64 in Cyc3aAt and Cyc3bAt, respectively; Fig. 1).

Cyclin B and Cdc25 interact physically, and cyclin B can stimulate Cdc25 phosphatase activity (40). Mutagenesis analysis has demonstrated that the P box, a conserved region in cyclins B which shares limited homology with tyrosine phosphatases, is important for Cdc25 binding and consequent stimulation of phosphatase activity toward Cdc2 (36). Residues identified as involved in the cyclin B–Cdc25 interaction are almost completely conserved in the *Arabidopsis* cyclins (Fig. 1).

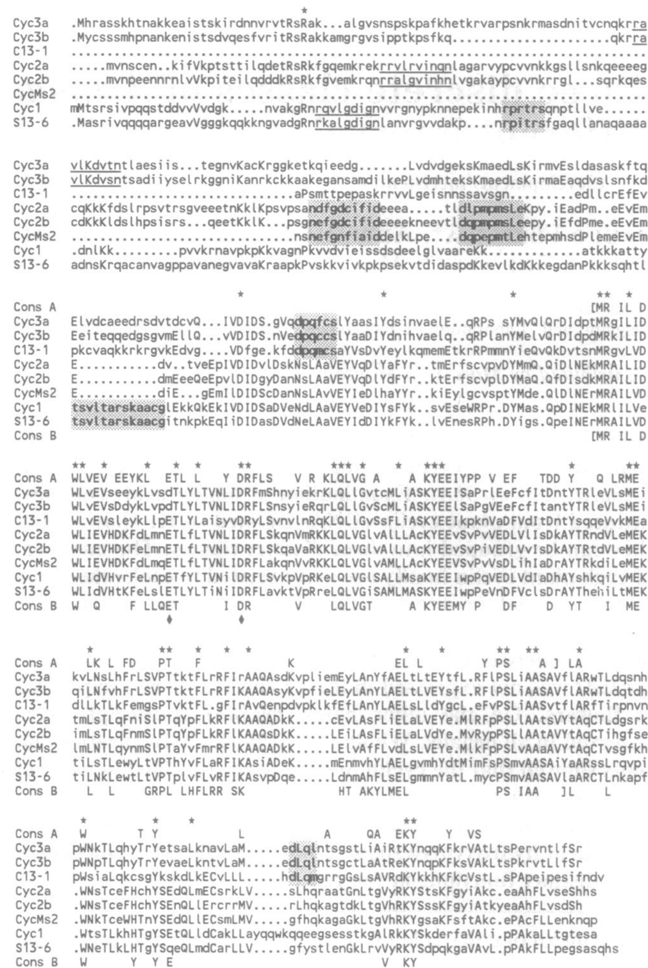

FIG. 1. Amino acid comparison of plant cyclins. The *Arabidopsis* cyclin sequences are aligned with homologous cyclins from carrot (C13-1; ref. 33), alfalfa (CycMs2; ref. 34), and soybean (S13-6; ref. 33). Consensus (Cons) cyclin-box sequences of A-type and B-type cyclins are shown above and below the sequences, respectively (35). Residues identical in at least four sequences are shown in capital letters. Asterisks indicate amino acids conserved in all sequences. Destruction boxes are underlined. Cyclin boxes are indicated by the pair of square brackets. Conserved residues in the P box (36) are indicated by diamonds. Stretches of conserved amino acids outside the cyclin box shared only by members of each class of cyclins are shaded.

Table 1 shows that the cyclin 2 family is slightly more homologous to B-type cyclins than to A-type cyclins. In spite of that, when compared with *Xenopus* A and B1 cyclins in the conserved cyclin box, they are somewhat more similar to the former (45.9% and 46%) than to the latter (44.5% and 44.2%). Whereas the complete cyclin 3 sequences are only marginally more homologous to A-type than to B-type cyclins (Table 1), in the cyclin box they are indeed related to the A type. In this region, Cyc3aAt and Cyc3bAt are 52% and 49.3% identical to the *Xenopus* cyclin A, respectively, and only about 42.5% identical to the frog cyclin B1. Besides, the motif EVXEEYKL, diagnostic of A-type cyclins (35), is perfectly conserved in Cyc3aAt and well conserved in Cyc3bAt (Fig. 1). Nevertheless, the sequence FLRRXSK, diagnostic of type-B cyclins (35), is better conserved in Cyc3bAt than in any other *Arabidopsis* cyclin. When the consensus sequences of A- and

Table 1. Comparison between plant cyclins (% homology)

	2	3	4	5	6	7	8	9	10
1	39.2	36.5	30.9	36.6	55.2	40.5	34.6	33.5	32.4
2		76.4	34.0	33.5	40.0	64.9	30.7	30.5	33.3
3			33.3	33.7	36.0	65.8	31.2	28.8	31.4
4				71.5	30.3	35.8	43.7	34.5	31.3
5					31.9	34.3	42.0	33.9	32.0
6						42.4	33.0	30.8	33.8
7							29.7	33.3	30.9
8								40.2	29.9
9									31.2

Amino acid sequences encoded by *Arabidopsis cyc1At* (no. 1) (ref. 29), *cyc2aAt* (no. 2), *cyc2bAt* (no. 3), *cyc3aAt* (no. 4), and *cyc3bAt* (no. 5) were compared with cyclins from soybean (no. 6) (S13-6; ref. 33), alfalfa (no. 7) (CycMs2; ref. 34), carrot (no. 8) (C13-1; ref. 33), and cyclins A (no. 9) and B1 (no. 10) from *Xenopus* (refs. 11 and 35). Sequence comparison was made with the GAP program of the Genetics Computer Group software package, version 7 (Madison, WI), with default and taking into account that sequences 7, 8, and 10 are shorter than the others.

B-type cyclins are compared with the two cyclin 2 and cyclin 3 families of *Arabidopsis*, the conservation seems to be randomly distributed in the two groups (Fig. 1). Thus, as we observed with cyclin 1, the sequence homology between the *Arabidopsis* sequences and the A- and B-type cyclins is not enough to assign them to any group.

On the other hand, comparison of the amino acid sequence of Cyc2aAt and Cyc2bAt and the alfalfa cyclin sequence CycMs2 (34) reveals ≈65% identity (Table 1). In the cyclin box, the *Arabidopsis* cyclin 2 sequences are 76% and 79% homologous to the alfalfa CycMs2 cyclin. More important, significant homology is found beyond the cyclin box (Fig. 1), an indication that *Arabidopsis* cyclins 2 and alfalfa CycMs2 are possibly functionally equivalent.

The plant sequence most similar to the *Arabidopsis* cyclin 3 class is the carrot C13-1 cyclin (33) (Table 1), which shares 43.7% and 42% identical amino acids with Cyc3aAt and Cyc3bAt, respectively. In the cyclin box, the cyclin 3 class is about 55% homologous to the carrot sequence. Furthermore, they also share unique stretches of identical residues outside of the cyclin box (Fig. 1).

Multiple sequence analysis shows that the plant cyclins isolated so far can be grouped in three classes. The first group is composed of the soybean S13-6 clone (33) and the *Arabidopsis* Cyc1At (29). A second group is constituted by the cyclin 2 class from *Arabidopsis* and the CycMs2 cyclin from alfalfa. Finally, a third group is formed by the *Arabidopsis* cyclin 3 class and the carrot C13-1 cyclin. So far, the isolation of A-type cyclins has not been reported in the yeasts. Therefore, some of the functions executed by A-type cyclins in higher eukaryotes are likely to be executed in yeast by B-type cyclins. Thus, although computer simulations have grouped the first two classes with B-type cyclins, and the third class with A-type cyclins, it is conceivable that plant cyclins have evolved separately. Similar conclusions were reached by analysis of several maize cyclins (41). After this work was completed the cloning of two mitotic cyclins from *Antirrhinum majus* was reported (42). The cDNAs encode proteins that are highly homologous to each other and fall in the first cyclin class.

Reverse Transcription–PCR Analysis of Cyclin Expression. Because of the high degree of homology between the members of each cyclin family, analysis of their expression can be done only with gene-specific probes. In addition, the steady-state levels of the cyclin mRNAs are very low, and conventional Northern blot procedures require very long exposure times (data not shown). Reverse transcription–PCR affords a solution to these problems. Primers that specifically amplify the members of only one class of cyclins were designed. First-strand cDNAs were prepared from total RNA from samples under study and were used as templates. After the PCR, the amplified DNAs were cut with *Pvu* II (*cyc2bAt* and *cyc3bAt* have unique *Pvu* II sites that are not present in *cyc2aAt* and *cyc3aAt*) and the restriction fragments were separated in agarose gels. Southern blots were hybridized with probes that detect both *cyc2aAt* and *cyc2bAt* or *cyc3aAt* and *cyc3bAt*.

cyc2aAt mRNAs were present in similar amounts in all examined tissues, whereas signals corresponding to *cyc2bAt* mRNAs were detected only in samples from roots (Fig. 2*A*). While *cyc3bAt* was expressed in all tissues, a more intense signal was detected in roots. *cyc3aAt* was not expressed in leaves, and weak signals were noticed in samples from flowers and siliques. Comparable amounts of *cyc3aAt* and *cyc3bAt* mRNAs were present in roots and stems.

In most plant cell cultures, cell division is dependent on the presence of plant hormone(s). Therefore, we examined the effect of hormone depletion on cyclin expression (Fig. 2*B*). *Arabidopsis* cell suspensions subcultured in medium lacking the hormone α-naphthaleneacetic acid still synthesize DNA, and there is very little change in the ratio between cells in G_1 and G_2 (data not shown). The removal of α-naphthaleneacetic acid did not affect the levels of *cdc2a* transcripts (43). Similarly, the amount of cyclin 1 transcript was not significantly reduced. Surprisingly, cyclin 2 mRNA levels drastically decreased. Furthermore, α-naphthaleneacetic acid-free medium reduced the cyclin 3 transcripts to barely detectable levels. The sharp reduction in transcript levels from the cyclin 2 and 3 classes remains unexplained.

A positive correlation between the amount of plant cyclin transcripts and cell proliferation has been observed (29, 33, 34). To induce nutrient starvation and consequently reduce the ratio of cell division, a 5-day-old *Arabidopsis* cell culture was refreshed in complete medium containing 1/10th the normal sucrose concentration. After 2 days, total RNA from the treated and control cultures was prepared. Measurements of radioactive precursor incorporation into DNA verified that

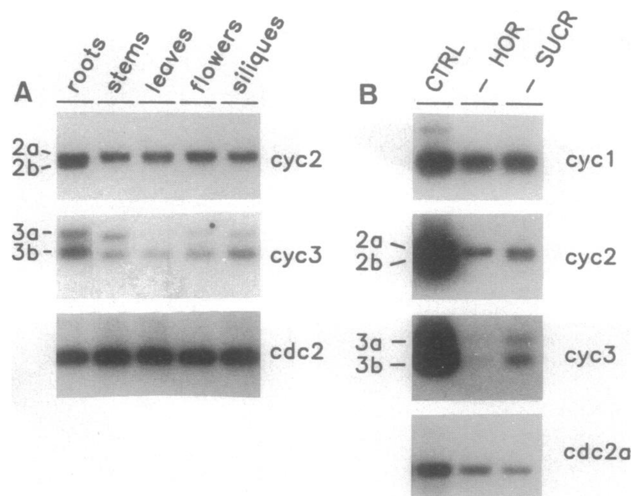

FIG. 2. Expression of *cyc2aAt*, *cyc2bAt*, *cyc3aAt*, and *cyc3bAt* genes from *Arabidopsis*. (*A*) First-strand cDNA of total RNA from roots, stems, leaves, flowers, and siliques was amplified by PCR using oligonucleotide primers which annealed specifically to members of each cyclin class. Amplified DNAs were digested with *Pvu* II, and the restriction fragments were separated in a 1.5% agarose gel, blotted, and hybridized with oligonucleotide probes. (*B*) After growing for 2 days in fresh medium, *Arabidopsis* cell suspensions were subcultured in medium without hormone (– HOR) and with 0.02% sucrose (– SUCR). Total RNA from these samples and untreated cultures (CTRL) was prepared as described (31).

under these conditions the cells stopped dividing (data not shown). Northern analysis showed that sucrose starvation provoked a significant decrease in *cdc2a* transcripts (43). Likewise, cyclin mRNA decreased drastically when the division rate was reduced by sucrose starvation (Fig. 2*B*). When compared with the control cultures, *cyc1At* mRNA was much less decreased than the cyclin 2 and cyclin 3 mRNAs.

Similarly, transcripts of the two alfalfa cyclins have distinct kinetics in starved cell cultures (34). Discrete cyclin classes execute different functions during the cell cycle (2, 3). Thus, it is possible that the pattern of cyclin transcripts reflects the sensitivity to sucrose starvation of the cell cycle events in which they are involved.

Cyclin Expression Is Restricted to Dividing Cells. An important question is how the cell cycle control genes are spatially and temporally regulated in the meristems. Expression analysis of the cyclin 2 and 3 classes in cell suspensions already indicated a positive correlation with cell proliferation. In addition, in plant tissues mRNA levels from these cyclins are not very abundant, suggesting that their expression might be limited to dividing cells. Root apices from *Arabidopsis* are relatively easier to examine than shoot apical meristems, which are very small and difficult to access. Thus, in an initial attempt to examine the patterns of expression of cyclin 2 and cyclin 3 classes during plant development, whole-mount *in situ* hybridization was performed on roots of *in vitro*-grown *Arabidopsis* plants. The *cyc1At* gene, for which a detailed investigation of the expression has been done with transgenic plants carrying a promoter–β-glucuronidase reporter gene construct (P.F., unpublished data), was used as a control.

In roots of 4-day-old seedlings, transcripts from the cyclin 2 family were restricted to the root apical meristem. As the plant developed, signals persisted in the root tip, albeit the level of expression seemed to decrease (Fig. 3*E*). Strong signals were detected during the formation of lateral roots, even before the emergence of the primordia (Fig. 3*F*). Similar results were obtained for the *cyc3bAt* cDNA (Fig. 3 *I* and *J*). Further experiments showed that *cyc3aAt* and *cyc3bAt* were similarly expressed (data not shown). Within the sensitivity of the technique, no cellular preferences were observed between the different members of each class. Cyclin transcripts were not detected in the parenchyma. Likewise, in the pericycle, signals were noticed only in connection with the formation of lateral roots (data not shown). Cyclin expression seems to be confined to tissues with mitotic activity. These results contrast with the observation of *cdc2a* expression in some nonproliferative tissues (43, 44). In the embryonic cycles of *Xenopus* oocytes, cyclin accumulation is rate limiting for the activation of $p34^{cdc2}$ (12). In meristems, cell division must be strictly regulated both in rate and in orientation. The localization of cyclin transcripts in meristematic zones suggests that transcriptional regulation of cyclins could be one of the mechanisms controlling the rates of cell division during plant development.

Cell Cycle Regulation of Cyclin Expression. In somatic cells of yeast and higher animals, the oscillations in the amounts

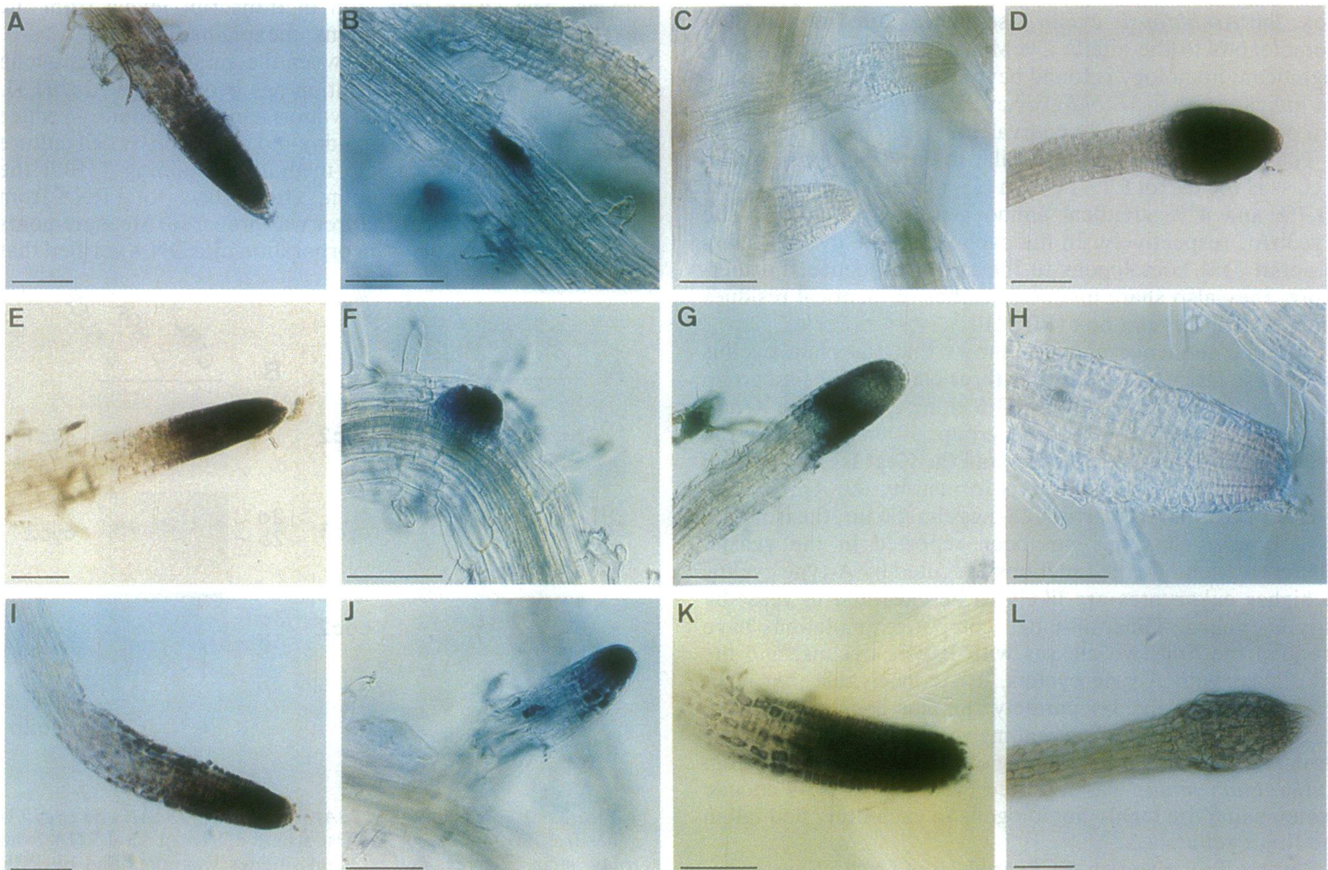

FIG. 3. Whole-mount *in situ* hybridizations of *Arabidopsis* roots. Antisense RNA probes were labeled with digoxigenin. Dark stains represent RNA·RNA hybrids detected by alkaline phosphatase-labeled antibodies. *A–D*, *E–H*, and *I–L* show hybridization with the antisense *cyc1At*, *cyc2aAt*, and *cyc3bAt* probes respectively. (*A*, *E*, and *I*) Roots of 2-week-old plants. (*B*, *F*, and *J*) Lateral roots at different stages. Although distinct phases are shown, all probes hybridized throughout lateral root formation. (*C*, *G*, and *K*) Roots of 2-week-old plants incubated for 2 days in semisolid medium containing 100 mM hydroxyurea. (*D*, *H*, and *L*) Roots of 2-week-old plants incubated for 2 days in semisolid medium containing 30 μM oryzalin. All micrographs were taken with bright-field optics, except *H*, which was taken with Nomarski optics. (Bars = 100 μm.)

of cyclin transcripts during the cell cycle are generally followed by a similar fluctuation in the accumulation of the corresponding gene product. Thus, in most cases, an insight into the role of cyclins can be obtained by determining their expression interval.

Because *Arabidopsis* cell suspensions are difficult to synchronize, drugs that arrest cell division at different stages were used in combination with whole-mount *in situ* hybridization. Roots of *in vitro*-grown *Arabidopsis* plants were treated with either hydroxyurea, which blocks DNA replication, or oryzalin, which arrests cells at the G_2/M boundary. As expected, *cyc1At* transcripts were absent in hydroxyurea-treated roots and were very abundant in root tips of oryzalin-treated plants (Fig. 3 *C* and *D*). An opposite pattern was observed when treated roots were hybridized with the *cyc2aAt* (Fig. 3 *G* and *H*) and *cyc2bAt* (data not shown) cDNAs. Yet the signal in hydroxyurea-treated roots was lower than in untreated roots. These results suggest that the *Arabidopsis* cyclin 2 class function is restricted to events of the cell cycle between S phase and metaphase.

Signals detected with the *cyc3aAt* (data not shown) and *cyc3bAt* (Fig. 3*K*) probes in hydroxyurea-treated roots were stronger than in untreated roots (Fig. 3*I*). In contrast, transcripts hybridizing with the probes were barely visible in oryzalin-treated roots (Fig. 3*L*). Thus, the *Arabidopsis* cyclin 3 class might function between late G_1 and metaphase. Hybridizations with nuclei from *Arabidopsis* cell suspensions separated by flow cytometry on the basis of DNA content, using the *Arabidopsis* cyclins as probes, confirmed the results obtained by whole-mount *in situ* hybridization (data not shown).

After each mitosis a eukaryotic cell must decide whether to leave the cell cycle and either become quiescent or differentiate or to commence a new division cycle. In plants, the decision whether to stay in the cycle or differentiate can be controlled either in G_1 or in G_2 (45). Another particular plant feature is the capacity of most nucleated cells to dedifferentiate and reenter the cell cycle. Furthermore, dedifferentiation is to some extent independent of the ploidy, because cells in either G_1 or G_2 have the ability to restart cell division (46). Cyclins are important for targeting the Cdks to different subcellular localizations (47, 48). In addition, the substrate specificity of the catalytic subunit is probably determined by the cyclin moiety (49, 50). Therefore each cyclin–Cdk complex may perform a certain number of tasks needed to ensure cell cycle progression. The availability of distinct classes of plant cyclins, functioning at different periods of the cell cycle, will help to answer the question of how proliferation is controlled during plant development.

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