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Cloning of four cyclins from maize indicates that higher plants have three structurally distinct groups of mitotic cyclins

(cell division/cdc2/*Zea mays*)

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ABSTRACT **While a large number of cyclins have been described in animals and yeasts, very limited information is available regarding cyclins in plants. We describe here the isolation of cDNA clones encoding four putative mitotic cyclins from maize. All four cyclins were able to induce maturation of *Xenopus* oocytes, demonstrating that they can act as mitotic cyclins in this system. Northern analysis showed that all four cyclins were expressed only in actively dividing tissues and organs, with a stronger correlation between expression and mitotic activity than is observed with cdc2. The deduced protein sequences suggest that the four maize cyclins belong to the cyclin A and B families identified from animal and yeast studies but that they cannot be described easily as either A-type or B-type cyclins. However, comparison with previously cloned plant cyclins shows that cyclins in higher plants form three distinct structural groups that have been conserved in both monocotyledonous and dicotyledonous species and that cyclins from all three groups are present within a single plant species.**

Studies on the mechanism of cell division control in eukaryotic cells (1) have shown that protein kinases encoded by homologs of the *Schizosaccharomyces pombe cdc2* gene (2), in association with proteins known as cyclins (3), play a key role in driving the cell cycle. Cyclins from the six structural types so far identified are presumed to form part of a kinase complex in association with $p34^{cdc2}$ or closely related protein kinases of the cdk family (2, 4, 5). The large diversity of cyclins, in addition to that of their cdk counterparts, is believed to account for the specific phosphorylation of different sets of substrates at successive transitions of the cell cycle (2, 5).

Significant differences exist between plants and animals in the regulation of cell division during development (6–9), but much less is known about cell cycle regulators in plants (8, 9). Homologs of the *cdc2* gene have been found in plants (10–14), and two putative mitotic cyclins have been cloned, from soybean (15) and from *Arabidopsis* (16). Partial cDNA clones believed to encode cyclins have also been isolated from carrot (15) and alfalfa (17). In this report, we show that several putative mitotic cyclins capable of inducing maturation in *Xenopus* oocytes are present in the same plant species (maize). These findings are used to identify three distinct structural groups of plant mitotic cyclins, which have been evolutionarily conserved between monocotyledonous and dicotyledonous plants.¶

MATERIALS AND METHODS

PCR Amplification. Single-stranded cDNAs were prepared from 30 ng of poly(A)$^+$ RNA from apical meristem tissue of the maize inbred strains B73 and W22 (11). Degenerate oligonucleotides used in PCRs with the maize cDNAs as templates were provided by I. Fitch and B. Futcher (18) and corresponded to amino acid sequences M(R/S)AI(L/F)(V/I/M)DW and KYEE(I/M)Y(A/P/S/T)P from the conserved region of the mitotic cyclin box. The thermocycle conditions were 95°C for 1 min, 45°C for 2 min, and 75°C for 2 min. The amplified DNA was cloned in the *Sma* I site of plasmid pUC119. Inserts from the clones were used for screening an immature ear cDNA library in the Stratagene vector λZAPII (gift of B. Veit and S. Hake, U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA). The dideoxy method was used for DNA sequencing (19, 20).

Biological Activity of Cyclin RNAs in *Xenopus* Oocytes. pBluescript plasmids excised from the λZAPII phage were linearized by cutting with the appropriate restriction enzymes, and sense or antisense capped RNAs were synthesized *in vitro* by T7 or T3 RNA polymerase (20). Oocytes were surgically removed from *Xenopus laevis* females previously anesthetized with 0.1% MS 222 (Sandoz) in water. Defolliculated, fully grown stage VI oocytes were isolated by dispase/collagenase treatment and resuspended in medium A (21). Oocytes were injected with 50 ng of RNA in 50 nl of diethyl pyrocarbonate-treated water and further incubated for 18 hr at 18°C in medium A. Alternatively, oocytes were incubated continuously in the presence of 1 μM progesterone. Meiotic maturation was assessed by the appearance of a white spot surrounded by a pigmented ring at the animal pole of the oocytes. Germinal-vesicle breakdown (GVBD) was ascertained at the end of the incubation period by dissecting oocytes after 10 min of incubation in 10% (wt/vol) trichloroacetic acid. Histone H1 kinase assays were performed *in vitro* on samples of three microinjected oocytes (22).

Analysis of RNA Expression. Total RNAs were extracted from each type of plant material and poly(A)$^+$ RNAs were isolated by fractionating total RNAs on oligo(dT)-cellulose (11). Northern blot analysis was performed (20) under high-stringency conditions [65°C in 6× standard saline citrate (SSC) for hybridizations, and 65°C in 0.2× SSC for washes].

RESULTS

Isolation of Cyclin-Homologous Sequences from Maize. A 192-bp DNA sequence was amplified by PCR from maize

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Abbreviation: GVBD, germinal-vesicle breakdown.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U10076–U10079).

meristem cDNA by using degenerate oligonucleotides corresponding to conserved sequences of mitotic cyclins (18). Twenty-one cloned fragments were grouped into four types of sequences, all of which exhibited homologies to A and B cyclins when translated (data not shown). These are referred to as types Ia, Ib, II, and III PCR-amplified DNA, for reasons elaborated in the *Discussion*. The DNA sequence identity within clones of the same type was 93% or higher, and between different types varied from 47% to 72%. Each type of sequence was amplified from two different maize inbred lines, B73 and W22, except for type III DNA, for which only one clone was obtained (from W22).

Cloning of Cyclin cDNAs. Types Ia, Ib, II, and III PCR-amplified DNAs were used as probes to screen a cDNA library prepared from immature ears of maize inbred B73. Each probe yielded 50–70 hybridizing clones from 10^6 clones screened. The sizes of the longest cDNAs obtained were 1.5 kb for the Ia probe, 1.65 kb for Ib, and 1.6 kb for II and III. These cDNAs have been designated IaZm, IbZm, IIZm, and IIIZm (Zm, *Zea mays*). The deduced amino acid sequences of the maize cyclins are presented in Fig. 1. cDNAs Ib and III contain complete coding sequences for the corresponding cyclins, since in both cases it is possible to identify start codons with flanking sequences that have good homology to the consensus sequence of AACAATGGC for plant initiation codons (24). cDNAs Ia and II do not include suitable start codons at their 5′ ends; therefore the sequences of these two cyclins are assumed to be incomplete.

The four maize cyclins are more homologous with A and B cyclins than with any other family of cyclins. In the highly conserved "cyclin box" (25), which encompasses ≈170 amino acids of the C-terminal region (see Fig. 1), they show 35–45% amino acid identity with the A and B cyclins from animals and yeasts (Table 1). The homologies of the maize cyclins with each other are of the same order as the homologies of the maize cyclins with the other plant cyclins described so far (Table 1).

Functional Activity of Maize Cyclins in *Xenopus* Oocytes. The ability of the maize cyclins to drive the G_2/M transition was assessed by monitoring their effect on *X. laevis* oocyte meiotic divisions, as has been demonstrated with other cyclins (15, 16, 26, 27). Stage VI oocytes, arrested at G_2/M of meiosis I, were injected with 50 ng of sense or antisense maize cyclin RNA or with water. Only the oocytes injected with sense RNAs underwent meiotic maturation (Table 2). The efficiency of RNA injection to induce maturation was lower than that of the control progesterone treatment, with cyclin IbZm the most efficient, and cyclin IaZm the least efficient. Oocytes injected with cyclin IaZm sense RNA and treated with progesterone always matured, showing that damage from the microinjection was not the problem (Table 2).

Extracts from the oocytes which had matured after mRNA injection had an elevated histone H1 kinase activity (Fig. 2, lanes 1–4) comparable to that in oocytes matured after progesterone treatment (lane 7). Control stage VI oocytes and those injected with antisense RNA (lanes 5 and 6) did not display histone H1 kinase activity. It is concluded that the maize cyclins activated the M-phase-promoting factor, as did the control treatment with progesterone.

Expression of the Maize Cyclins. The expression of cyclins in the developing plant was studied by Northern blot analysis (Fig. 3). Single transcripts were detected, of sizes 2.1 kb for cyclins IaZm and IIZm and 1.9 kb for cyclins IbZm and IIIZm. The four maize cyclin transcripts were the most highly expressed in apical meristems and immature ears. Expression was at lower levels in developing embryos at 30 days after pollination and was undetectable in tissues with no mitotic activity—i.e., mature leaf and the endosperm at 30 days after pollination (Fig. 3). The abundance of each of the four maize cyclin transcripts relative to each other was roughly constant in every organ. When the same blots were rehybridized with the maize *cdc2* gene (11), some differences were observed (Fig. 3). As in the case of the cyclins, apical meristems and immature ears had the highest level of *cdc2* mRNA. However, in contrast to cyclin mRNA, *cdc2* mRNA was present at an equally high level in the embryo and at low but detectable levels in the endosperm and in mature leaf. Hybridization of the same samples with a probe for *Hcf106*, a gene preferentially expressed in green tissues (28), demonstrated the presence of intact RNA in the lanes (Fig. 3).

DISCUSSION

Previously two functional plant cyclins that are structurally related have been cloned, from soybean (15) and from *Arabidopsis* (16). In addition, cyclin homologous cDNAs have been isolated from carrot (15) and alfalfa (17), but the putative cyclins encoded by them have lower homologies to the cyclins from soybean and *Arabidopsis* and to each other (15, 17). It is not known whether the partial cDNA clones from carrot (15) and alfalfa (17) represent functional homologs of mitotic cyclins and, if so, whether they represent species differences or different structural groups of plant cyclins. Since none of the previously described plant cyclins can be unambiguously classified as A type or B type on the basis of their sequence (15–17), the question of the number and types of mitotic cyclins found in higher plants is of considerable interest. In this study, we have undertaken an extensive search for mitotic cyclins within a single plant species (maize). We have isolated four classes of cDNAs from maize encoding putative mitotic cyclins, which we have called IaZm, IbZm, IIZm, and IIIZm.

We have demonstrated that the maize cyclins are capable of driving the G_2/M transition, since they were able to promote *in vivo* resumption of meiosis in *Xenopus* oocytes (Table 2), and of activating the M-phase-promoting factor, as shown by histone H1 kinase activity (Fig. 2). The partial efficiency of maize cyclins to induce meiotic maturation is more likely to be due to inefficient translation in the oocytes or to inefficient functioning compared with animal A and B cyclins, than to incomplete cDNAs, since cyclins with large N-terminal deletions are functional in *Xenopus* oocytes (29).

The four maize cyclin genes displayed a similar differential expression pattern during plant development and showed a much tighter association with mitotic activity than expression of the *cdc2* gene (Fig. 3). For example, mature leaf tissue, which consists of fully differentiated nondividing cells with no detectable $p13^{suc1}$-precipitable histone H1 kinase activity (11), has detectable *cdc2* mRNA but not cyclin mRNA (Fig. 3 and ref. 11). Since basal levels of *cdc2* transcripts have been widely observed in tissues with no or low mitotic activity (11, 12, 30, 31), the control of proliferation in fully differentiated plant cells may be effected, in part, by turning off transcription of cyclins.

The different cyclins expressed in maize, a monocot, show homologies to individual cyclins described in the dicots soybean, carrot, *Arabidopsis*, and alfalfa, as well as to animal and yeast cyclins of the A and B families (Table 1). Like the A- and B-type cyclins from animals and the B-type cyclins from yeast, all plant cyclins have sequences resembling the RXALG(N/D/E)IXN motif (Fig. 1), called the destruction box, which results in cyclin proteolysis during anaphase (26). The R residue, which is essential (26, 32), is conserved in all the plant cyclin destruction boxes (Fig. 1). The presence of a mitotic cyclin destruction box suggests that these plant cyclins undergo cell cycle-controlled proteolysis at anaphase and are therefore mitotic cyclins.

The plant cyclins show significant homologies to both A and B cyclins within the cyclin box (Fig. 1) but do not appear

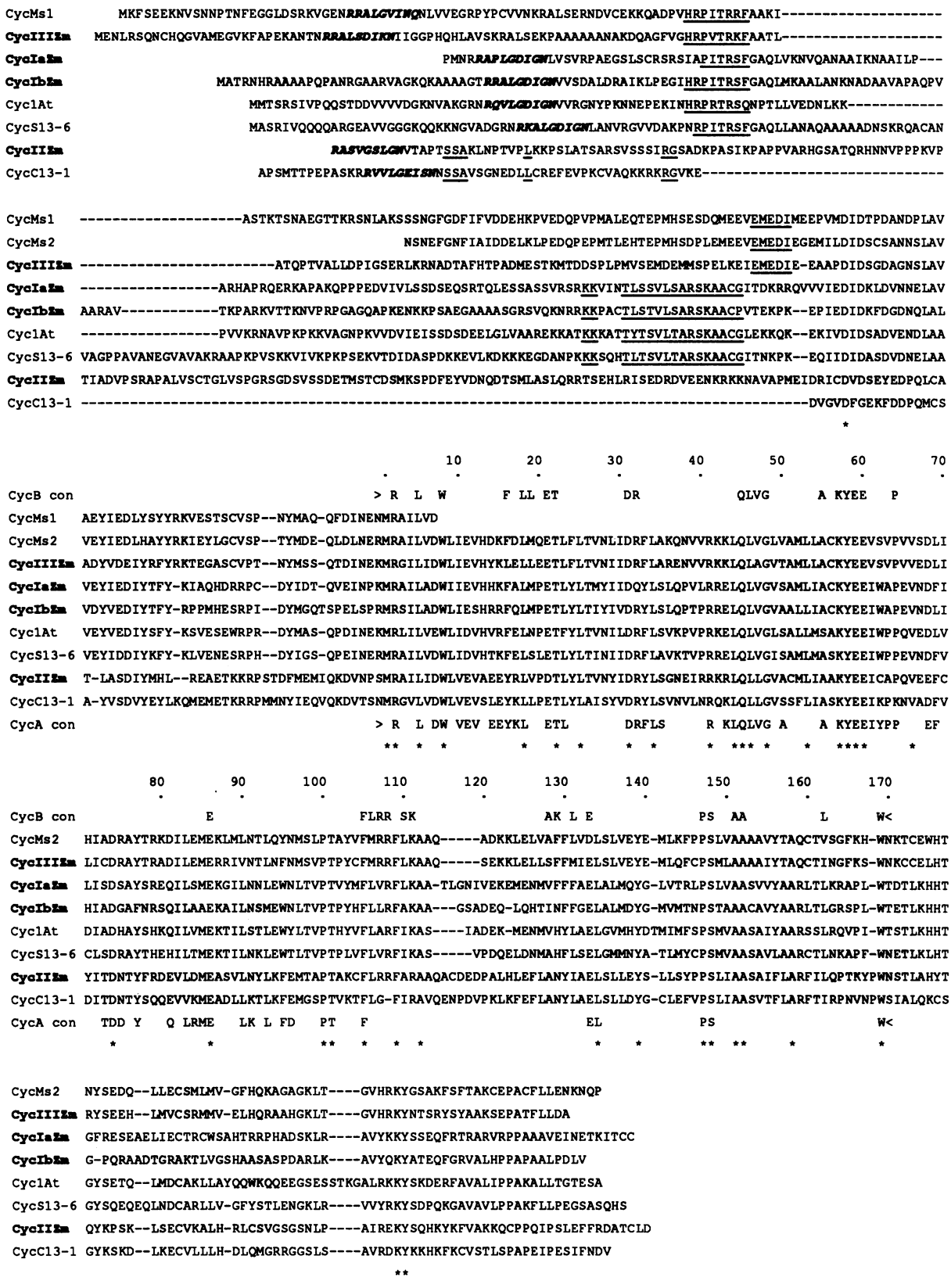

FIG. 1. The deduced amino acid sequences of cyclins IaZm, IbZm, IIZm, and IIIZm aligned with one another and with cycMs1 and cycMs2 from alfalfa (17), cyc1At from *Arabidopsis* (16), S13-6 from soybean (15), and C13-1 from carrot (15). The putative destruction box is in bold and italicized. Residues conserved in one or several groups of plant cyclins in the region outside the cyclin box have been aligned and underlined. Residues identical in all plant cyclins are indicated by asterisks below the sequences. The program MULTALIN (23) was used for the alignment, with subsequent adjustments performed manually. Dashes indicate positions of spaces required to maximally align the sequences. CycMs2, cyclin IaZm, cyclin IIZm, and cyclin C13-1 are incomplete open reading frames from cDNAs truncated at their 5′ ends, and the cycMs1 sequence is truncated at its C-terminal end. The cyclin B and cyclin A consensus sequences in the cyclin box (bordered by the symbols > and <), are shown above and below the plant cyclins, respectively. The numbers above the cyclin B consensus sequence refer to positions of the amino acids within the cyclin box.

Table 1. Comparison of the sequences of maize and other cyclins

Cyclin	% identity			
	IaZm	IbZm	IIZm	IIIZm
IaZm	—			
IbZm	67	—		
IIZm	44	41	—	
IIIZm	50	47	44	—
S13-6 soybean	60	54	41	50
cyc1At Arabidopsis	56	54	41	47
cycMs2 alfalfa	51	49	44	74
C13-1 carrot	41	36	58	39
cyclin A Hs	38	36	47	43
cyclin B Hs	39	37	41	41

The data are percent identities over about 170 amino acids in the cyclin box (see Fig. 1). Cyclins S13-6 and S13-1 are from soybean (*Glycine max*) and carrot (*Daucus carota*), respectively (15); cyc1At is from *Arabidopsis thaliana* (16); cycMs2 is from alfalfa (*Medicago sativa*; ref. 17); cyclin A Hs is human cyclin A (25); and cyclin B Hs is human cyclin B1 (25).

more closely related to either A- or B-type cyclins in terms of overall amino acid identity in this region (Table 1 and refs. 15–17). Nor is it possible to assign any plant cyclin to either A or B types by using sequence motifs. None of the plant cyclins has the EVXEEYKL motif (amino acids 11–18 of the cyclin box) found in all A cyclins (25), though cyclin IIZm and cyclin C13-1 of carrot have closely related sequences (Fig. 1). Similarly the FLRRXSK motif that is found in all B cyclins (amino acids 105–111 of the cyclin box) is not conserved in any plant cyclin (Fig. 1), but related sequences are found in all the plant cyclins, including cyclin IIZm. The AK(Y/F)L motif typical of B cyclins (amino acids 128–131 of the cyclin box) is absent from plant cyclins, except for the (Y/F) residue, which is also conserved in A cyclins. Finally, all the plant cyclins have both the cyclin A-specific T^{101} residue and the cyclin B-specific $A^{151}A^{152}$ motif.

When the sequences of plant cyclins are compared with those of cyclins A and B by use of a phylogenic alignment program (4), cyclin IIZm and cyclin C13-1 from carrot appear as members of the cyclin A family, while the six other plant cyclins fall into the large cyclin B family (Fig. 4). In both cases, the plant cyclins constitute a separate group (Fig. 4). We note, however, that this alignment program is based on the first 106 amino acids (M^1 to L^{106}) of the cyclin box and excludes some of the typical cyclin B motifs. Due to the lack of definitive cyclin A and cyclin B motifs, it may be better to refer to these cyclins as "A-like" and "B-like," as has been previously suggested for the carrot and soybean cyclins (15). The partial homologies of the plant cyclins to both A- and B-type consensus motifs suggest the separate evolution of the plant cyclin group. For these reasons, we propose a Roman numbering system to describe the different structural groups of plant cyclins that can be defined by data from this study and other studies (see below).

Table 2. Effect of maize cyclin mRNAs on meiotic maturation of *X. laevis* oocytes

Treatment	Experiment 1		Experiment 2	
	No. of oocytes	GVBD, %	No. of oocytes	GVBD, %
IaZm sense mRNA	22	32	22	0
IbZm sense mRNA	21	71	20	70
IIZm sense mRNA	20	65	13	15
IIIZm sense mRNA	31	48	26	31
IIZm antisense RNA	—	—	15	0
IIIZm antisense RNA	20	0	—	—
Water	15	0	—	—
Progesterone	15	100	25	100
IaZm sense mRNA + progesterone	10	100	14	100

Oocytes were microinjected with 50 nl of RNA (1 ng/nl) or 50 nl of water, or incubated in the presence of 1 μM progesterone. Meiotic maturation was monitored after 18 hr by the disappearance of the germinal vesicle (GVBD). The results of two experiments with oocytes from two animals are presented.

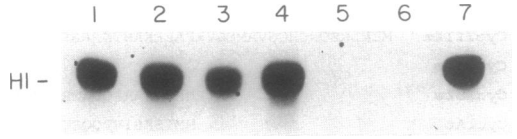

FIG. 2. Histone H1 kinase activity in *Xenopus* oocytes after maize cyclin RNA injection. The data shown are from experiment 1 in Table 2. Extracts were made from three oocytes and purified on $p13^{suc1}$ beads (22). The kinase activity was assayed in the presence of histone H1 and [γ-^{32}P]ATP, and the radioactivity incorporated into histone H1 was detected by autoradiography. Lanes 1–4, injection with sense cyclin IaZm, cyclin IIZm, cyclin IbZm, and cyclin IIIZm mRNAs, respectively; lane 5, injection with cyclin IIIZm antisense RNA; lane 6, control oocytes in prophase (stage VI); lane 7, incubation with 1 μM progesterone.

From Table 1 and Fig. 4, we conclude that the nine plant cyclins for which sequence data are available fall into three groups: I, II, and III. Since at least one cyclin from each group can be found in maize, it is likely that all higher plants have these three types of mitotic cyclins. Group I cyclins and group III cyclins are "B-like," and group II cyclins are "A-like." Although the N-terminal domains of the cyclins are highly variant, consensus motifs typical of the groups of plant cyclins, and absent in animal and yeast cyclins, can be identified in this region, as shown in Fig. 1. Maize has two related homologs of group I cyclins, which we have called cyclin IaZm and cyclin IbZm. As seen in Table 1, they are homologous to cyclin S13-6 reported in soybean (15) and cyc1At in *Arabidopsis* (16). Group I cyclins have a conserved motif, KKXXXTL(S/T)(S/T)VL(S/T)ARSKAACG, located upstream of the cyclin box (Fig. 1). They also share

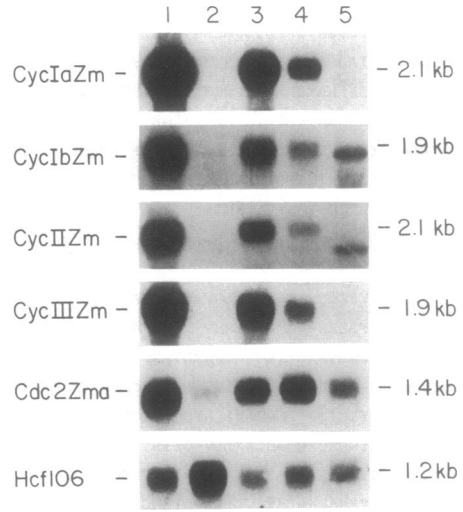

FIG. 3. Expression of cyclin mRNAs in the maize plant. Northern blots with 0.5 μg of poly(A)$^+$ RNA from apical meristems (lane 1), mature leaves (lane 2), young ears (lane 3), embryos at 30 days after pollination (lane 4) and endosperm at 30 days after pollination (lane 5) were hybridized with cyclin IaZm, cyclin IbZm, cyclin IIZm, or cyclin IIIZm cDNAs and then with a 600-bp fragment from cdc2ZmA cDNA (11) or with *Hcf106* cDNA (28) as a control. The hybridization signals in lane 5 for cyclin IbZm and cyclin IIZm are due to contaminating rRNA in this sample; similar signals were obtained with longer exposures for cyclins IaZm and IIIZm.

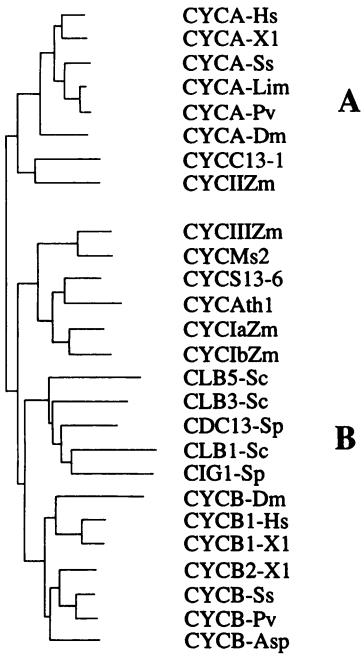

FIG. 4. Evolutionary tree of cyclins A and B from animals, yeasts, and plants constructed as described by Xiong and Beach (4), from a sequence alignment of 106 amino acids in the cyclin box. Length of the horizontal lines reflects divergence. The sequences used were as follows: CYCA-Hs, human cyclin A; CYCA-X1, *Xenopus* cyclin A; CYCA-Ss, clam cyclin A; CYCA-Pv, *Patella vulgata* cyclin A; CYCA-Dm, *Drosophila* cyclin A; CycC13-1, carrot cyclin; CycMs2, alfalfa cyclin; CycS13-6, soybean cyclin; CycAth1, *Arabidopsis* cyclin; CLB5-Sc and CLB3-Sc from budding yeast (*Saccharomyces cerevisiae*); CDC13-Sp from fission yeast (*Sch. pombe*); CLB1-Sc from budding yeast; CIG1-Sp, cyclin from fission yeast; CYCB-Dm, *Drosophila* cyclin B; CYCB1-Hs, human cyclin B1; CYCB1-X1 and CYCB2-X1, *Xenopus* cyclins B1 and B2; CYCB-Ss, clam cyclin B; CYCB-Pv, *P. vulgata* cyclin B; CYCB-Asp, starfish cyclin B. For sources of the cyclins used in the alignment, see ref. 4 and references therein.

with group III cyclins the motif HRPITRSF, located close to the cyclin destruction box (Fig. 1).

Group II of plant cyclins is defined by cyclin IIZm in maize and cyclin C13-1 in carrot (Table 1); they also share several short motifs (Fig. 1). The C13-1 clone was reported to be smaller than the transcript size (15) and appears to have a deletion of 120 amino acids between the destruction box and the cyclin box when compared with cyclin IIZm (Fig. 1). The identification of longer defining motifs outside the cyclin box may become possible with the isolation of complete clones from other species.

The plant cyclins of group III comprise the maize cyclin IIIZm and the alfalfa cycMs2 (17). A second truncated cDNA clone from alfalfa, cycMs1, was proposed to represent a cyclin very different from all other known cyclins (17). We show in Fig. 1 that the cycMs1 and cycMs2 sequences have a 108-amino acid overlap over which they are 64% identical. We propose that cycMs1 is a close homolog of cycMs2 and belongs to group III of plant cyclins. In addition to the HRPITRSF motif that they share with group I cyclins, the three cyclins of group III have a conserved EMEDI motif in the N-terminal region (Fig. 1).

In animal cells, cyclins A and B have different mitotic functions, and cyclin A also plays a role in S phase (5, 33). Mitosis in plant cells has many significant differences with animal and fungal cells (34), and in plant cells $p34^{cdc2}$ is associated with establishment of the division site (35). It should be possible in the future to elucidate the roles played by each of the three types of plant mitotic cyclins in this process, as well as in other problems of cell division in plants, such as the regulation by phytohormones or the activation of cell division in meristematic zones (9).

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