RESEARCH ARTICLE

Distinct Classes of cdc2-Related Genes Are Differentially Expressed during the Cell Division Cycle in Plants

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cdc2 and several related genes encode the catalytic subunits of cyclin-dependent kinases, which have been implicated in a number of cellular processes, including control of cell division. As a first step in exploring their function in plants, we isolated four cdc2-related genes from Antirrhinum. Two genes, cdc2a and cdc2b, encode proteins that contain a perfectly conserved PSTAIRE motif characteristic of cdc2 homologs, whereas the products of the two remaining genes, cdc2c and cdc2d, appear to represent a new subclass of proteins that have so far only been identified in plants. Transcripts of these novel genes were localized in isolated cells dispersed throughout actively dividing regions of the inflorescence. This localization is consistent with accumulation that is specific to particular phases of the cell cycle. Correlating cell labeling with nuclear condensation and double-labeling experiments using cdc2 and histone H4 as probes indicated that cdc2c transcripts accumulate during S phase as well as during the G2 and M transition, whereas cdc2d expression was specific to the G2 and M phases. All cells labeled with cdc2d also contained cdc2c label, indicating that expression of cdc2d completely overlapped with that of cdc2c. Transcripts of cdc2a and cdc2b were detected in all cells within actively dividing regions, but at levels that were only slightly higher than those observed in nondividing areas. These transcripts did not appear to accumulate in a cell cycle–specific fashion. The genes cdc2a and cdc2b were able to partially complement a yeast cdc2 mutation, although all four genes appeared to interfere with the sizing mechanism of yeast cells. We propose that plants contain at least two classes of cdc2-related genes that differ in structure, expression, and perhaps function.

INTRODUCTION

The timing of crucial events in the eukaryotic cell division cycle is under the control of protein phosphorylation cascades. Genetic studies of cell division in yeast have identified the product of the cdc2 gene (p34^{cdc2}) as a key component of these cascades (reviewed in Forsburg and Nurse, 1991; Reed, 1992). p34^{cdc2} periodically associates with different classes of positive regulators called cyclins to create successive waves of protein kinase activity thought to be capable of regulating cell cycle progression (reviewed in Reed, 1992; Heichman and Roberts, 1994; Pines, 1994). It is now apparent that eukaryotes possess families of cdc2-related genes. Although some of these genes are involved in unrelated processes, such as apoptosis (Lahti et al., 1995), phosphate metabolism (Kaffman et al., 1994), hemopoiesis (Lapidot-Lifson et al., 1992), and neu-

ral tissue differentiation (reviewed in Lew and Wang, 1995), at least six (cdc2, cdk2, cdk3, cdk4, cdk6, and cdk7) participate directly in control of the cell cycle. Along with cdc2, a number of these genes encode proteins that have been demonstrated to interact with cyclins; these are known collectively as cyclin-dependent kinases (Cdks; reviewed in Pines, 1993; Sherr, 1993, 1994; Heichman and Roberts, 1994; Lew and Wang, 1995). The role of different Cdks in cell cycle control has been studied most intensely in vertebrates (reviewed in Heichman and Roberts, 1994; King et al., 1994; Sherr, 1994), whereas a role for Cdks other than p34^{cdc2} in yeast cell cycle control is beginning to emerge (Espinosa et al., 1994; Measday et al., 1994; Buck et al., 1995; Damagnez et al., 1995).

Structurally, at least two classes of cdc2-related genes can be distinguished based on whether they encode proteins that contain a conserved 16–amino acid domain, the so-called PSTAIRE motif. Genes encoding PSTAIRE-containing proteins, which include the fission yeast cdc2 gene itself, can often rescue yeast cdc2 mutants; such genes have been identified in

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all eukaryotes examined, including plants (reviewed in Reed, 1992; Jacobs, 1995). Genes encoding proteins containing only partially conserved PSTAIRE domains share less overall sequence similarity to *cdc2* than do the PSTAIRE genes and generally lack the ability to complement yeast *cdc2* mutants (Simon et al., 1986; Shuttleworth et al., 1990; Hata, 1991; Irie et al., 1991; Imajuku et al., 1992; Meyerson et al., 1992; Xiang et al., 1994), even though some may be implicated in cell division control (Simon et al., 1986; Irie et al., 1991; Meyerson and Harlow, 1994; reviewed in King et al., 1994; Sherr, 1994). Although several such "non-PSTAIRE" *cdc2*-related genes have been identified in yeasts and animals, it is not clear which encode proteins common to all eukaryotes and which encode proteins that may be unique to specific organisms. There have been only two reports of non-PSTAIRE *cdc2* gene isolation from plants: the Arabidopsis *cdc2b* (Imajuku et al., 1992) and the rice *R2* (Hata, 1991) genes. Neither has been character-

ized in detail; consequently, their functions remain to be determined.

Here, we characterize four distinct *cdc2*-related genes from Antirrhinum. Two of the genes encode proteins containing only partially conserved PSTAIRE domains, homologs of which have yet to be identified in non-plant eukaryotes. In situ hybridization revealed major differences in the expression patterns of these novel genes compared with those of PSTAIRE-containing *cdc2* genes. Transcripts for the non-PSTAIRE genes appear to accumulate at distinct and different stages of the cell cycle, whereas those of PSTAIRE homologs are present throughout the cell cycle. Although only PSTAIRE-containing proteins were able to partially complement a yeast *cdc2* mutation, all appeared to interfere with the sizing mechanism of these yeast cells. The data reveal that plants contain at least two classes of *cdc2*-related genes that differ in structure, expression, and possibly function.
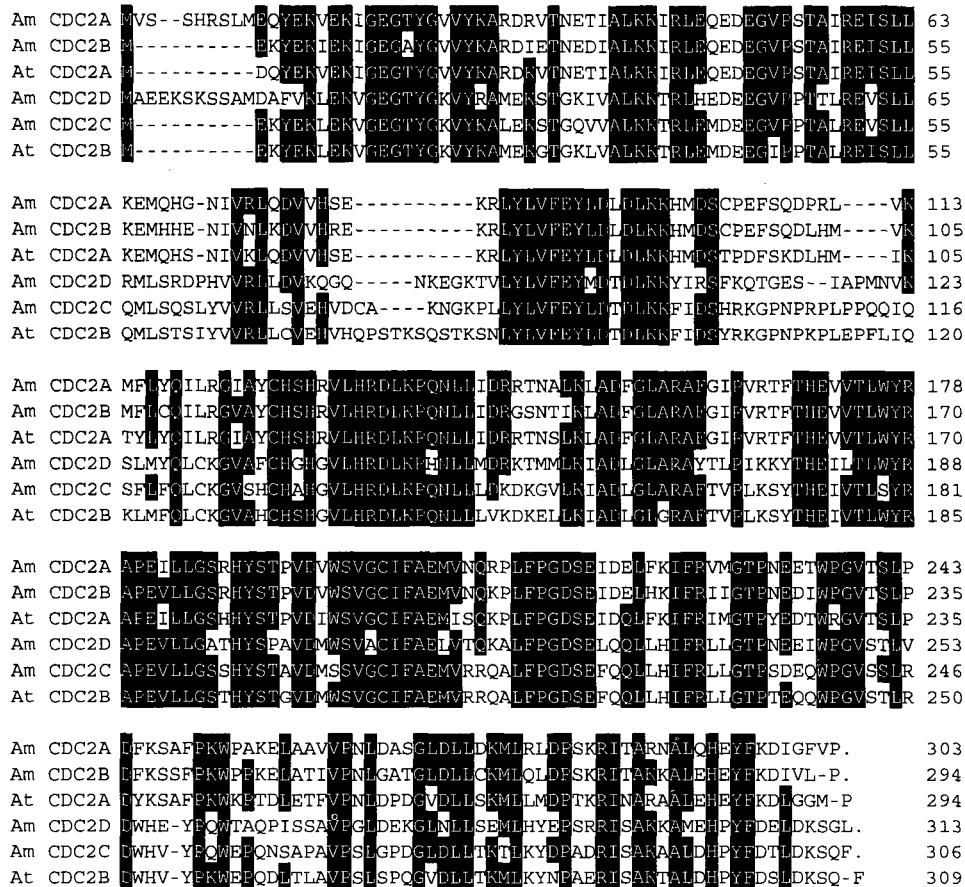

Figure 1. Predicted Amino Acid Sequences of Antirrhinum *cdc2*-Encoded Proteins Illustrating Their Similarities with Those from Arabidopsis.

Dashes indicate gaps introduced to maximize alignment. Amino acids that are identical in three or more of the proteins are blocked. EMBL accession numbers are X97637 (Antirrhinum *cdc2a*), X97638 (Antirrhinum *cdc2b*), X97639 (Antirrhinum *cdc2c*), X97640 (Antirrhinum *cdc2d*), X57839 (Arabidopsis *cdc2a*), and D10851 (Arabidopsis *cdc2b*). Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*.
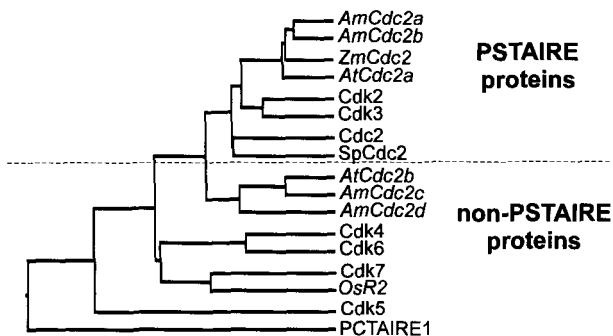
Figure 2. Phylogenetic Tree of the Cdc2 Protein Family.

The tree was constructed with selected sequences from the data bases, using the CLUSTAL program. Sequences from plant sources are italicized. With the exception of Cdk4, which is from mouse, and SpCdc2, which is from yeast, all remaining proteins are from humans. More extensive analyses containing additional Cdc2-related proteins yielded similar groupings of the Antirrhinum proteins. Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Sp, *Schizosaccharomyces pombe*; Zm, *Zea mays*.

RESULTS

A Family of *cdc2*-Related Genes Is Expressed in Antirrhinum Inflorescences

Using the Arabidopsis *cdc2a* cDNA as probe, we isolated 15 clones from a cDNA library prepared from inflorescence apices of Antirrhinum (Fobert et al., 1994). Restriction enzyme analysis and sequencing revealed that this collection of clones contained four distinct *cdc2*-related genes, which were named *cdc2a*, *cdc2b*, *cdc2c*, and *cdc2d*. Complete sequencing and conceptual translation of the longest cDNAs in each class revealed that clones for *cdc2a* and *cdc2c* potentially encode entire proteins but that the longest available clones for two others (*cdc2b* and *cdc2d*) are truncated at the 5' end. A cDNA containing the presumptive full-length coding sequence of *cdc2b* was isolated by screening a new library with the truncated cDNA as probe. For *cdc2d*, the 5' end of the coding region was obtained by rapid amplification of cDNA ends (RACE). The coding sequences of *cdc2b*, *cdc2c*, and *cdc2d* all appear to be full length because the initiation codons are preceded by in-frame stop codons.

The predicted amino acid sequences of the Antirrhinum proteins are presented in Figure 1. The proteins are of similar size (predicted molecular masses of 34 or 35 kD), and all contain domains characteristic of protein kinases (Hanks, 1987). Specific amino acid residues known to be important for the regulation of vertebrate p34^{cdc2} activity via phosphorylation and dephosphorylation (i.e., Thr-14, Tyr-15, Thr-161, and Ser-277) are conserved in the Antirrhinum proteins, with the exception of a nonconservative substitution of Thr-14 by Ala in

p34^{cdc2b}. This change was identified in two independent cDNA clones and thus does not appear to result from an error in reverse transcription. The characteristic PSTAIRE domain is perfectly conserved in p35^{cdc2a} and p34^{cdc2b} but only partially conserved in p34^{cdc2c} (PPTALRE) and p35^{cdc2d} (PPTTLRE). PAIRWISE (Genetics Computer Group, Madison, WI) and CLUSTAL (DNASTAR Inc., Madison, WI) analyses revealed that Antirrhinum p35^{cdc2a} and p34^{cdc2b} are more similar to other PSTAIRE-containing proteins from plant and animal sources than they are to the non-PSTAIRE Antirrhinum homologs (Figure 2 and data not shown). The latter proteins, along with the product of the Arabidopsis *cdc2b* gene, form a distinct subgroup among the non-PSTAIRE Cdc2-related proteins (Figure 2). The Antirrhinum p34^{cdc2c} and the Arabidopsis p35^{cdc2b} appear to be cognate homologs. Overall, the two proteins share 82% amino acid identity and deviate by only one conservative substitution within the 16–amino acid PSTAIRE domain. The only other *cdc2*-related cDNA characterized from plants is the *R2* gene of rice (Hata, 1991), which encodes a protein distantly related to the Antirrhinum proteins (Figure 2).

Members of the *cdc2* Gene Family Display Differential Patterns of Expression

The histological distribution of *cdc2*-related RNA was studied by in situ hybridization, using digoxigenin-labeled antisense RNA probes against wild-type inflorescence apices. To compare the expression patterns of various members of the gene family with each other, consecutive sections from the same apex were hybridized with probes from different genes. Consecutive sections were also hybridized to sense and antisense probes from the same gene. Sense strand probes consistently yielded no hybridization signal (data not shown).

The Antirrhinum inflorescence contains small leaf-like structures (bracts) arranged in a spiral manner. Flowers develop in the axil of bracts, with the oldest flowers at the base and the youngest at the top of the inflorescence. In longitudinal sections, the inflorescence meristem is seen at the apex and has bract primordia on its periphery. Floral meristems were observed in the axils of bracts; their early development has been detailed elsewhere (Carpenter et al., 1995). Because of the spiral arrangement of flowers and their subtending bracts, not all stages of development were seen in each section.

Transcripts of *cdc2a* and *cdc2b* Are Expressed throughout Dividing Regions of the Apex

Expression of *cdc2a* and *cdc2b* was found to be very similar. As shown in Figure 3, transcripts of *cdc2a* were detected predominantly within regions of the inflorescence known to sustain high rates of cell division. Starting at the apex of the inflorescence and proceeding to the base, these regions included the inflorescence meristem, bract primordia, and developing bracts (Figures 3A and 3B); then, in all stages of floral meristem
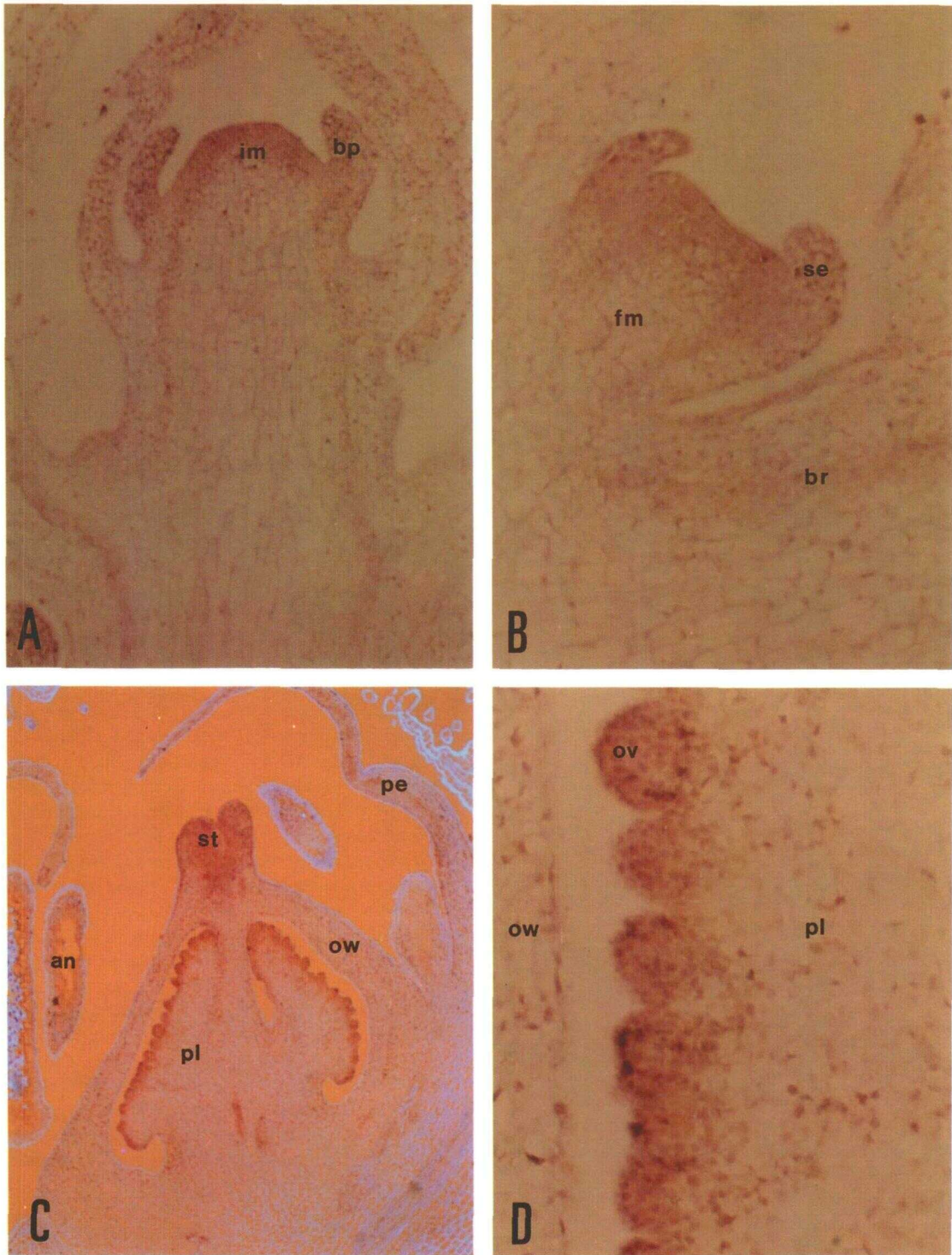
Figure 3. RNA in Situ Hybridization of Wild-Type Inflorescence Apices Probed with *cdc2a*.

Sections were probed with digoxigenin-labeled antisense RNA. A hybridization signal gives a brown-purple label. Epifluorescence was used to reveal calcofluor white–stained cell walls in **(C)**. an, anther; bp, bract primordium; br, bract; fm, floral meristem; im, inflorescence meristem; ov, ovule; ow, ovary wall; pe, petal; pl, placenta; se, sepal; st, style.

(A) Inflorescence apex.

(B) Floral meristem at the floritypic stage.

(C) Central carpels of an older floral meristem.

(D) Developing ovules within carpels.

development observed, including floral meristem, floral organ primordia and developing floral organs (Figures 3B and 3C; data not shown), developing male and female reproductive tissues, such as anthers, microsporocytes, placenta, and ovules, and vascular tissue (Figures 3A, 3C, and 3D; data not shown). Within these regions, transcripts of both cdc2a and cdc2b were detected in most if not all cells. Weak hybridization signals were routinely observed in older bracts and sepals, in postmeiotic anthers, and in the large cells comprising the bulk of the stem axis. Overall, the intensities of cdc2a and cdc2b labeling observed in meristems and organ primordia were consistently weaker than those obtained for other genes that we have analyzed, such as histone, mitotic cyclins (Fobert et al. [1994]), floricaula, deficiens (def), plena, and fimbriata (Bradley et al., 1993; Simon et al., 1994). Use of probes from different regions of the transcripts did not increase the intensity of labeling. Strong labeling for these transcripts was observed only in specific reproductive structures such as the developing anther sac (data not shown) and carpels (Figures 3C and 3D). Transcripts of cdc2a and cdc2b were also detected in the vegetative shoot apical and axillary meristems, leaf primordia, and the root apex (data not shown). Their expression during seed development was not assessed.

Transcripts of cdc2c and cdc2d Show Distinct Cell Cycle–Dependent Expression

Figure 4 shows that in contrast to those of cdc2a and cdc2b, transcripts of cdc2c and cdc2d accumulated only in isolated, dispersed groups of one or a few cells (for an overview of cdc2c expression, see Fobert et al. [1994]). The proportion of cells labeled with cdc2c was considerably higher than when cdc2d was used as probe. For example, in young floral meristems, 30% of the cells were labeled with the cdc2c probe, whereas only 12% of cells were labeled with the cdc2d probe. Nevertheless, the relative frequencies of cells labeled throughout the inflorescence were similar with these two probes. The highest frequencies were in actively dividing regions, and the lowest were in slowly or nondividing regions. Figures 5A to 5C show a comparison of the expression patterns of cdc2c and cdc2d with cyclin1, a mitotic-like cyclin that also accumulates in isolated groups of cells (Fobert et al., 1994). Qualitatively, the cell-specific labeling observed for cdc2c and cdc2d was not as well defined as that of cyclin1, probably because of the presence of cells expressing low levels of the cdc2-related transcripts. This was particularly evident with cdc2c. Expression of cdc2c and cdc2d transcripts, unlike those of cdc2a and cdc2b, did not appear to increase in reproductive structures, but their patterns of expression became more homogenous in anther sacs during microsporogenesis (Fobert et al., 1994).

Counterstaining sections with the DNA-specific dye 4',6-diamidino-2-phenylindole allowed us to relate the condensation state of nuclei to the expression of transcripts. Figure 4 shows that both cdc2c and cdc2d were expressed in cells containing mitotic nuclei. Almost all metaphase cells contained
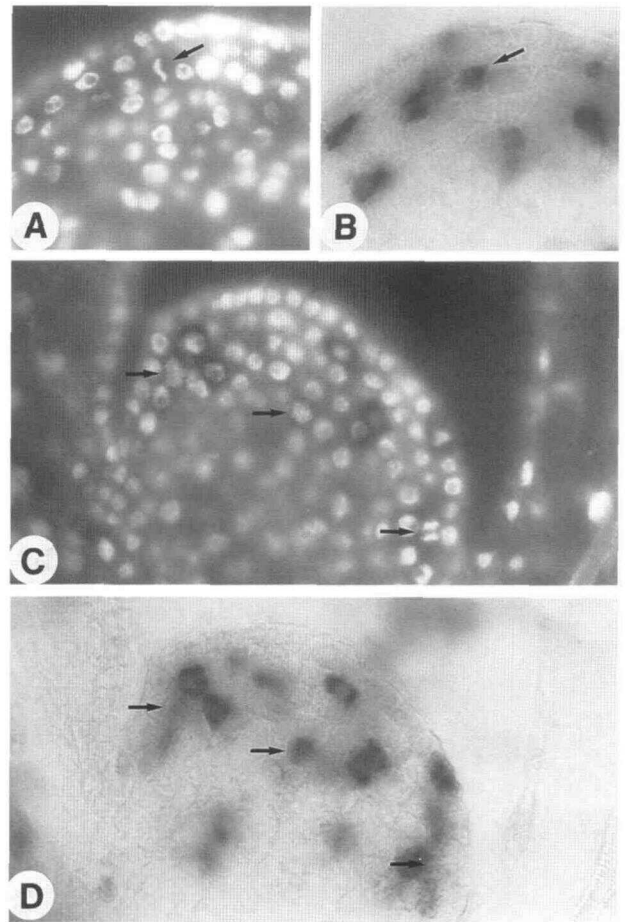

Figure 4. Correlation of Gene Expression with Mitosis.

In situ hybridization of meristems hybridized with antisense RNA probes, counterstained with 4',6-diamidino-2-phenylindole to reveal nuclei and viewed using epifluorescence in **(A)** and **(C)** or bright-field microscopy in **(B)** and **(D)**. Labeled mitotic cells are marked with arrows.
(A) and **(B)** cdc2d.
(C) and **(D)** cdc2c.

transcripts of these genes (>95% for cdc2c, and 99% for cdc2d), whereas only a small proportion of interphase cells were stained (~30% for cdc2c, and ~5 to 10% for cdc2d). A weak or no signal was observed in anaphase and telophase cells.

Transcripts of cdc2c and cdc2d Are Expressed in Overlapping Periods of the Cell Cycle

To define further the expression period of these two genes, we undertook a series of double-labeling experiments in which
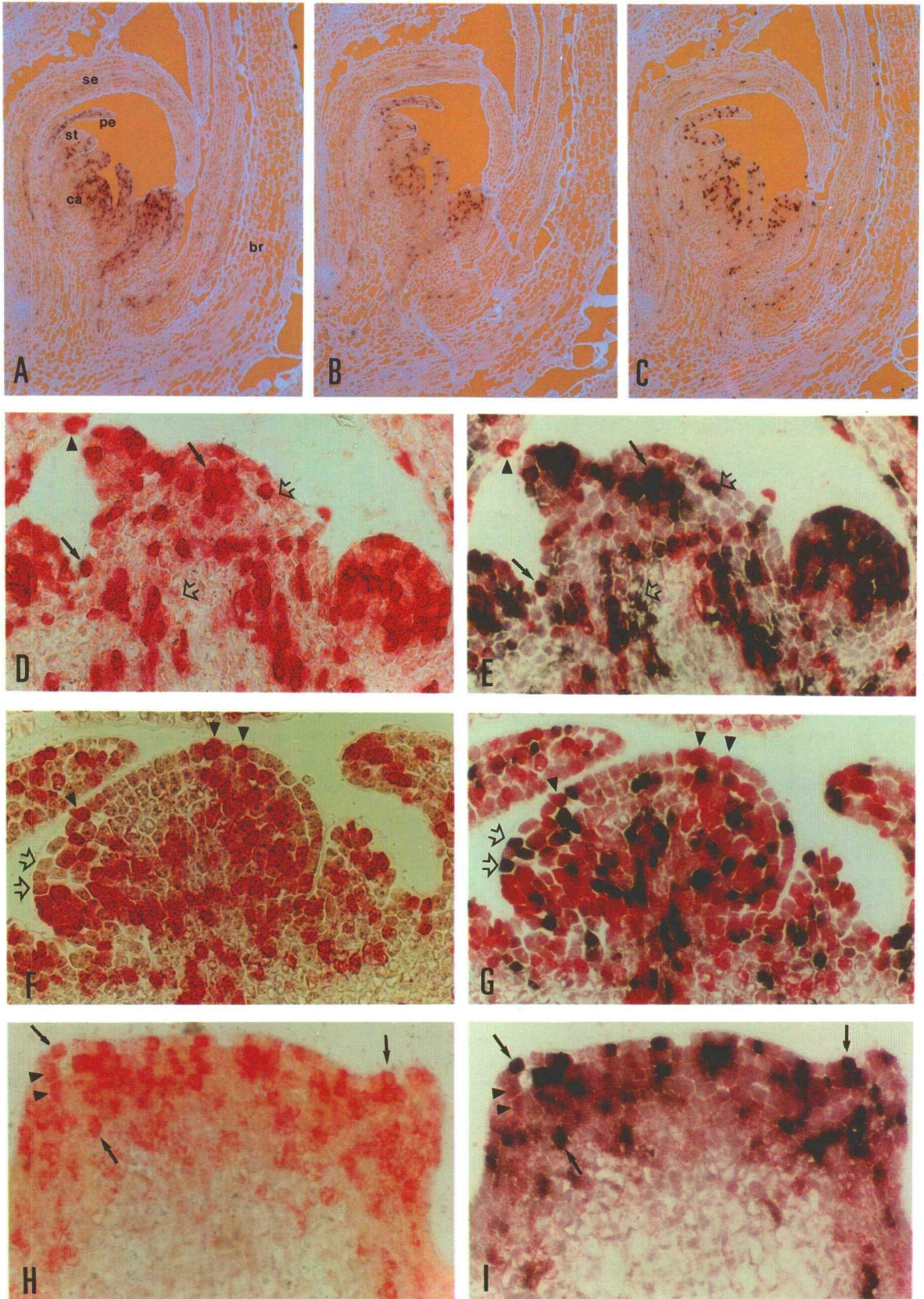
Figure 5. In Situ Hybridization of Wild-Type Inflorescence Apices Probed with Cell Cycle Genes.

individual slides were hybridized with different combinations of cdc2c, cdc2d, and histone H4 probes. In each case, the first gene probe was labeled with fluorescein and developed to produce a red color. The second gene was labeled with digoxigenin and developed to produce a purple color. By photographing sections after the red color development and again after the purple color development, individual cells could be monitored for the presence of more than one transcript. Figures 5D and 5E show that expression of cdc2c extensively overlapped with that of histone: 5% of the cells contained label only for histone, and 22% of the cells were labeled for histone and cdc2c, whereas ~8% were labeled only for cdc2c. Cells with the strongest histone signal usually contained a low level of cdc2c label, suggesting that the expression of these transcripts peak at different times. None of the cells containing cdc2d label contained histone label (Figures 5F and 5G), but all were labeled for cdc2c (Figures 5H and 5I). Because the overall number of cells labeled with cdc2c is higher than the number labeled with cdc2d (30% versus 12%), numerous cells were labeled for cdc2c but not for cdc2d (Figures 5H and 5I).

Antirrhinum cdc2 Genes Are Not Functionally Equivalent When Expressed in Fission Yeast

As a first step in elucidating the possible functions of the Antirrhinum Cdc2-related proteins, we tested their ability to complement a yeast cdc2 mutation. Each cdc2 coding region was placed under the control of the thiamine-repressible nmt1 (for no message in thiamine) promoter (Maundrell, 1990) and transformed into a strain of fision yeast carrying a temperature-sensitive cdc2 mutation (see Methods). Three expression vectors were used (pREP1, pREP41, and pREP81); these vectors differed only in their relative nmt1 promoter strength (Basi et al., 1993). Growth of transformed cells harboring these plasmids alone (without Antirrhinum genes) was indistinguishable from that of nontransformed cells under inductive, noninductive, permissive, and restrictive conditions (data not shown). Micrographs of cells grown under some of these different conditions are shown in Figure 6. Because results obtained with cdc2a were very similar to those obtained with cdc2b and those

obtained with cdc2c were similar to those seen with cdc2d, only data for cdc2b and cdc2c are shown. In addition, data are from transformants containing the weakest promoter construct to show the relevant phenotype.

At the restrictive temperature (35°C), nontransformed mutant cells were more elongated than were the wild-type cells (Figures 6A and 6B) and failed to form colonies when plated onto agar-solidified media (data not shown). The same phenotypes (elongated cells and failure to form colonies) were observed in transformants harboring the cdc2a or cdc2b coding regions when grown under noninductive conditions (Figure 6C) or in transformants harboring the cdc2c or cdc2d coding regions when grown under inductive or noninductive conditions (Figures 6E and 6F). In contrast, when grown under inductive conditions, cells harboring cdc2a or cdc2b were shorter than the mutant cells and resembled those of the wild type (Figure 6D). However, unlike the wild-type control, these cells were unable to form colonies when plated on inductive agar medium; this observation suggests that either complementation was weak or expression of cdc2a and cdc2b had deleterious effects. The latter possibility is supported by the phenotypes of cells grown at the permissive temperature (27°C) under inductive conditions. Transformants harboring the cdc2a or cdc2b coding regions cloned into the pREP81 vector formed very small colonies compared with those grown under noninductive conditions, whereas those cloned into the pREP41 or pREP1 vectors, which contain stronger promoters, failed to produce colonies (data not shown). Microscopic examination of cultures revealed that cells expressing cdc2a and cdc2b were of similar or smaller size than wild-type cells (Figures 6B, 6G, and 6H). This semi-wee phenotype (wee cells divide at a smaller size than do wild-type cells) was more obvious when pREP41 or pREP1 was used as a vector. Expression of cdc2b but not cdc2a from the pREP41 and pREP1 vectors also caused a reduction in colony size in a wild-type (cdc2+) background (data not shown).

Expression of cdc2c or cdc2d at the permissive temperature also resulted in a reduction in colony size. This reduction was only observed when the coding regions were fused to the stronger promoters (pREP41 and pREP1 for cdc2c; pREP1 for cdc2d) and was not as severe as that induced by cdc2a or

Figure 5. (continued).

(A) to **(C)** Consecutive serial sections of a floral meristem hybridized with antisense RNA probes for cdc2c **(A)**, cdc2d **(B)**, and cyclin1 **(C)**. br, bract; ca, carpel; pe, petal; se, sepal; st, stamen.

(D) to **(I)** Double labeling of inflorescence apices with a fluorescein (red label) and a digoxigenin (blue-black label) probe. Sections in **(D)**, **(F)**, and **(H)** were photographed after detection of the fluorescein probe but before detection of the digoxigenin probe. Sections in **(E)**, **(G)**, and **(I)** are the same as those in **(D)**, **(F)**, and **(H)**, respectively, photographed after both probes were detected. In **(D)**, histone H4 was detected. In **(E)** histone H4 (red label) and cdc2c (blue-black label) were detected. For **(D)** and **(E)**, the arrowheads mark cells labeled for histone H4 only; black arrows indicate representative cells labeled for both transcripts, and open arrows mark a few cells labeled for cdc2c only. In **(F)**, histone H4 was detected. In **(G)**, histone H4 (red label) and cdc2d (blue-black label) were detected. For **(F)** and **(G)**, arrowheads mark representative cells labeled for histone H4 only, and open arrows mark a few cells labeled for cdc2d only. In **(H)**, cdc2c was detected. In **(I)**, cdc2c (red label) and cdc2d (blue-black label) were detected. For **(H)** and **(I)**, arrowheads mark representative cells labeled for histone cdc2c only, and black arrows mark a few cells that were labeled for both transcripts.
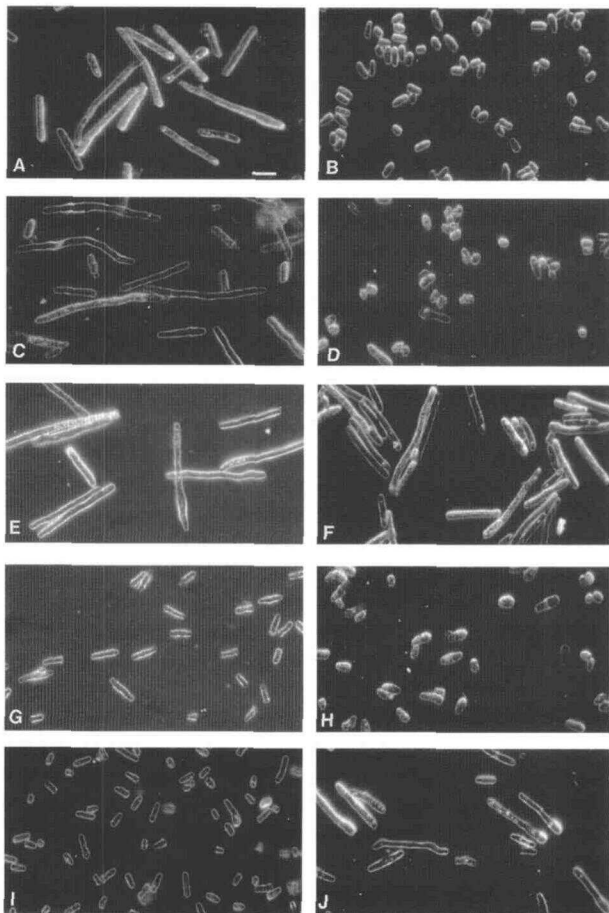
Figure 6. Effect of Antirrhinum *cdc2* Gene Expression on Size and Shape of Yeast Cells.

With the exception of **(B)**, which depicts wild-type cells, all cells shown are of *cdc2-33^{ts}* mutant cells.

(A) Untransformed *cdc2-33^{ts}* at 35°C.

(B) Wild type at 35°C.

(C) and **(D)** Transformant harboring the *cdc2b* coding region cloned into pREP81 grown at 35°C in the presence **(C)** or absence **(D)** of thiamine.

(E) and **(F)** Transformant harboring the *cdc2c* coding region cloned into pREP1 grown at 35°C in the presence **(E)** or absence **(F)** of thiamine.

(G) and **(H)** Transformant harboring the *cdc2b* coding region cloned into pREP81 grown at 27°C in the presence **(G)** or absence **(H)** of thiamine.

(I) and **(J)** Transformant harboring the *cdc2c* coding region cloned into pREP1 grown at 27°C in the presence **(C)** or absence **(D)** of thiamine.

Bar in **(A)** = 10 μm for **(A)** to **(J)**.

cdc2b. In contrast to expression of *cdc2a* or *cdc2b*, which appeared to reduce cell length, expression of *cdc2c* or *cdc2d* resulted in cells that were more elongated than the wild-type cells (compare Figures 6I and 6J) and resembled mutant cells

grown at the restrictive temperature (Figure 6A). Expression of *cdc2c* in wild-type *cdc2^+* cells did not appear to affect colony formation or size, although microscopic examination revealed that the cultures did contain a small fraction of cells that were more elongated than were the wild-type cells (data not shown). Therefore, induction of plant *cdc2* gene expression in yeast strongly interfered with the sizing mechanism of these cells at both the permissive and restrictive temperatures, with individual genes inducing distinct and sometime opposite phenotypes. Only *cdc2a* and *cdc2b* appeared to be able to partially complement the cell size defect of the *cdc2* mutant studied.

DISCUSSION

We show that at least two classes of *cdc2*-related genes are expressed in Antirrhinum inflorescences. This classification is based on sequence comparison, histological distribution of transcripts, and phenotypes induced when genes are expressed in a yeast *cdc2* mutant. The first class of genes, represented by *cdc2a* and *cdc2b*, encodes proteins that contain perfectly conserved PSTAIRE domains and are capable of partially complementing a yeast *cdc2* mutation. Transcripts from these genes accumulate at low levels in all cells within actively dividing regions of the inflorescence. The second class of genes is represented by *cdc2c* and *cdc2d*. These encode proteins that lack perfectly conserved PSTAIRE domains and failed to complement the yeast *cdc2* mutation. Transcripts of these genes accumulate only in discrete groups of cells within the inflorescence meristem. Although the precise functions encoded by members of the Antirrhinum *cdc2* gene family remain to be determined, the data suggest that proteins from the different classes are likely to have distinct roles.

Proteins encoded by the *cdc2c* and *cdc2d* genes are clearly related to p34^{cdc2} but represent a new subgroup within the family. These novel genes have so far been identified only in Antirrhinum and Arabidopsis, even though detailed searches for *cdc2*-related genes have been undertaken in mammalian cells (for example, Meyerson et al., 1992). In contrast, *cdc2*-related genes encoding PSTAIRE-containing proteins appear to be ubiquitous among eukaryotes (Reed, 1992; Jacobs, 1995). One possible explanation for this is that the novel Antirrhinum genes encode plant-specific functions. Several aspects of plant morphogenesis and cell division are unique to these organisms (Steeves and Sussex, 1989; Staiger and Doonan, 1993), and it is conceivable that they may be controlled by distinct groups of Cdks and cyclins. Evidence for plant-specific subclasses of cyclins already exists (Ferreira et al., 1994a; Renaudin et al., 1994; Meskiene et al., 1995; Soni et al., 1995). The only other non-PSTAIRE *cdc2* plant gene, the rice *R2* gene, does not appear to be specific to plants. It shows sequence similarity to yeast and mammalian Cdk-activating kinases, suggesting that it may be a plant Cdk-activating kinase. It will be of interest to determine whether plants possess homologs of

other classes of vertebrate Cdks or whether these are specific to animals or vertebrates.

The histological distribution of transcripts revealed that members of the Antirrhinum *cdc2* gene family are differentially expressed during the cell division cycle. Transcripts from *cdc2c* and *cdc2d* accumulate only in small groups of isolated cells dispersed throughout actively dividing regions of the inflorescence, the vegetative shoot, and roots. This hybridization pattern is similar to that reported for transcripts of mitotic cyclins and histone H4 and has been proposed to reflect gene expression specific to particular phases of the cell cycle (Fobert et al., 1994). Isolated cells are labeled because cell division is poorly synchronized, and thus neighboring cells are unlikely to be at the same phase of the cycle. According to this view, transcripts of *cdc2c* and *cdc2d* are expressed at particular phases of the cell cycle because they are detected in isolated cells. Counterstaining with a DNA-specific dye to correlate cell labeling with mitosis suggests that the two genes are likely to be expressed during G2 and early M phases, because transcripts from both are detected in most (>90%) of the cells in early mitosis but only in a small fraction of cells late in mitosis or in interphase ($\sim$30% for *cdc2c* and 10 to 12% for *cdc2d*). Coexpression of these transcripts is further supported by results of double-labeling in situ hybridization experiments revealing that all cells containing *cdc2d* label are also labeled for *cdc2c*. However, the expression phase of *cdc2c* is much wider than that of *cdc2d*: approximately three times more cells hybridize to the *cdc2c* probe, including a large proportion that also hybridize with histone H4. In contrast, expression of *cdc2d* and histone H4 do not overlap. Because accumulation of the histone transcript is likely to occur during S phase, the simplest explanation for these results is that the initial expression of *cdc2c* occurs earlier in the cell cycle than that of *cdc2d*, possibly in mid-to-late S phase. Taken together, the data are consistent either with *cdc2c* expression spanning mid-S phase to early M phase or with the possibility that this gene may be expressed more than once during the cycle, but not continuously.

The cell-specific expression pattern of *cdc2a* and *cdc2b* transcripts is strikingly different from those described above. Instead of accumulating in groups of isolated cells, transcripts are detected in all cells within actively dividing regions. Given that meristematic regions contain populations of poorly synchronized cells capable of being at any of the various stages of the cell cycle (see above), accumulation of *cdc2a* and *cdc2b* transcripts cannot be strictly related to specific stages of the cell cycle. Levels of these transcripts appear to be relatively low and only slightly higher in regions of high cell division, a phenomenon that may be related to the small size and high cytoplasmic density of meristematic cells rather than to real differences in expression. Taken together, the data indicate that expression of *cdc2a* and *cdc2b* transcripts is not as tightly coupled to cell division as that of *cdc2c*, *cdc2d*, or mitotic cyclins. Comparison of PSTAIRE-containing *cdc2* and mitotic cyclin gene expression in other plant species has yielded similar conclusions (Ferreira et al., 1994b; Renaudin et al., 1994) and led to the proposal that expression of PSTAIRE *cdc2* tran-

scripts may be correlated with the competence of cells to divide rather than to division itself (Hemerly et al., 1993; Doerner, 1994). Additional factors, such as those involved in posttranslational regulation of Cdks, are presumably required for activating the plant proteins and triggering cell division. The relative contribution of transcriptional control in regulating the activity of plant *cdc2*-encoded proteins remains to be determined.

In general, the expression pattern of Antirrhinum *cdc2a* and *cdc2b* transcripts is similar to that reported for an Arabidopsis *cdc2a* promoter–β-glucuronidase gene fusion (Hemerly et al., 1993) and that of the Arabidopsis *cdc2a* mRNA, as revealed by in situ hybridization (Martinez et al., 1992). There are two important exceptions. Unlike Martinez et al. (1992), we did not find any evidence of stratification of transcript within floral meristems or of a reciprocal relationship between the expression of PSTAIRE-containing *cdc2* and floral homeotic gene transcripts. In fact, labeling consecutive serial sections for *cdc2a* or *cdc2b* and the B class floral homeotic gene *def* (Schwarz-Sommer et al., 1992) clearly demonstrated that the *cdc2* transcripts are expressed throughout the *def* domain of floral meristems (data not shown).

Expression of Antirrhinum *cdc2* genes in yeast revealed that *cdc2a* and *cdc2b* encode functional homologs of p34^{cdc2}. They have the ability to partially complement the temperature-sensitive *cdc2-33^{ts}* mutation in fission yeast, and *cdc2b* induced a semi-*wee* phenotype in a *cdc2$^+$* background. Complementation was apparent when the cells were observed under the microscope and found to have reduced cell lengths. However, these cells were unable to form colonies when grown at the restrictive temperature. This finding may indicate that the Antirrhinum proteins are limited in their ability to substitute for the yeast p34^{cdc2} or, as suggested by their expression at the permissive temperature, that they have deleterious effects on yeast cell division. Although *cdc2c* and *cdc2d* did not complement the *cdc2-33^{ts}* mutation, their expression at the permissive temperature did result in the production of elongated cells. This phenotype is similar to that of the mutant grown at the restrictive temperature, suggesting that cells have lost their ability to divide in the presence of a functional p34^{cdc2}, presumably due to the interference from the Antirrhinum proteins. To our knowledge, similar phenotypes have not been reported when other non-PSTAIRE p34^{cdc2} homologs were overexpressed in yeast cells. Elucidating the mechanism by which these genes interfere with yeast cell division may be helpful in determining their functions.

Although the results from gene expression in yeast suggest a possible role for the Antirrhinum proteins in the control of cell division or proliferation, their precise functions remain to be determined by testing in plants. Overexpression of a dominant negative Arabidopsis *cdc2a* mutant has revealed a role for this gene in controlling plant cell division (Hemerly et al., 1995). Its expression in Arabidopsis was lethal, whereas tobacco plants containing low levels of the protein had reduced frequencies of cell divisions and much larger cells. Nevertheless, these plants underwent normal morphogenesis and

histogenesis, suggesting that cell division and plant development can be uncoupled (Hemerly et al., 1995). No significant phenotypic changes were noted in plants overexpressing wild-type cdc2a or a mutated protein in which Thr-14 and Tyr-15 were substituted for Ala-14 and Phe-15. Therefore, the potential involvement of Thr-14 phosphorylation in regulating plant p34^{cdc2} activity and the significance of Ala-14 in Antirrhinum p34^{cdc2b} remain unknown.

Functionally distinct groups of PSTAIRE-containing proteins have been isolated from humans and Drosophila (Stern et al., 1993; van den Heuvel and Harlow, 1993), whereas pairs of genes encoding PSTAIRE-containing proteins from plants are known to differ in their ability to complement specific mutant alleles of yeast cdc2 (Hashimoto et al., 1992; Hirt et al., 1993; Miao et al., 1993). Based on sequence similarity alone, it is not possible to establish which if any of the Antirrhinum proteins correspond to known PSTAIRE-containing proteins, such as p34^{cdc2} (Cdk1), Cdk2, or Cdk3.

With respect to the cdc2c and cdc2d genes, the most compelling evidence for their involvement in regulating plant cell division or proliferation is the cell cycle–specific accumulation of their transcripts. Levels of most gene transcripts remain relatively constant during the cell cycle (McKinney and Heintz, 1991), and to our knowledge, genes showing transcriptional regulation during the cell cycle have all been implicated in cell cycle progression (for examples, see McKinney and Heintz, 1991; Müller, 1995). The fact that the expression of cdc2d transcripts is specific to the G2 and M phases is also intriguing, because none of the Cdks characterized to date, other than p34^{cdc2}, appears to be expressed specifically or to function at these stages of the cell division cycle.

In summary, we isolated two cdc2-related genes encoding PSTAIRE-containing proteins and two genes capable of encoding novel proteins containing unique sequences within this motif. We showed that transcripts from individual members of a plant cdc2 family can be differentially expressed within apices: those from the novel genes accumulate at distinct phases of the cell cycle, whereas expression of the PSTAIRE-encoding genes does not appear to be linked to particular phases of the cell cycle or to be as closely correlated with cell division in general. The differences in sequence conservation, expression patterns, and ability to complement a yeast cdc2 mutation all suggest that plants contain at least two classes of cdc2-related genes. The availability of cDNA clones for these and other putative cell division genes should provide useful tools for elucidating their functions and the possible interactions between plant Cdks, cyclins, and other proteins.

METHODS

Gene Isolation and Sequence Analysis

Methods used to isolate, clone, and sequence cdc2-related cDNAs were according to Fobert et al. (1994). Isolation of the missing 5' end of cdc2d was accomplished by polymerase chain reaction (PCR) amplification of the cDNA library, with an oligonucleotide specific to the 5' end of the gene and an oligonucleotide specific to the λ vector arms.

Both DNA strands were sequenced using the dideoxy nucleotide method, and sequences were analyzed using the LASERGENE for WINDOWS software (DNASTAR Inc., Madison, WI) version 3.05.

In Situ Hybridization

Methods used for single- and double-labeling in situ hybridization were as outlined in Fobert et al. (1994). Restriction fragments containing the entire cDNA (for cdc2a and cdc2b) or 5' segments (cdc2c and cdc2d) were subcloned into pGEM4Z (Promega Inc., Madison, WI) to provide templates for T7 or SP6 polymerases to generate sense and antisense RNA. For cdc2c, the 5' segment consisted of a 0.8-kb EcoRI-HindIII fragment, and for cdc2d, a 0.5-kb EcoRI-SstI fragment.

The proportion of cells labeled with the cdc2c and cdc2d probes was estimated by photographing histological sections that were counterstained with 4',6-diamidino-2-phenylindole and counting the number of labeled and unlabeled nuclei on at least 6 to 10 individual sections. The frequencies of mitotic cells labeled with the cdc2c and cdc2d probes were determined by counting the numbers of labeled and unlabeled mitotic cells (~100 for each probe) directly under the microscope with use of oil immersion. Data were obtained from different apices hybridized in independent labeling experiments. To estimate the relative frequencies of cells labeled with histone H4 and cdc2c, alone or in combination, a total of 382 labeled cells was tallied.

Expression of Plant cdc2 Genes in Yeast

Entire coding regions were amplified by PCR, using oligonucleotide primers containing recognition sites for SalI (5' oligonucleotide) or NotI (3' oligonucleotide) at the 5' end. For cdc2d, the 5' end obtained by PCR rapid amplification of cDNA ends was joined to the truncated cDNA by PCR-based gene stitching (Newton and Graham, 1994). This technique was also used to remove a SalI site within the coding region of cdc2d. Typically, PCR was performed in a 50-µL volume with ~5 ng of plasmid DNA as starting material, 200 µM deoxynucleotide triphosphates, 200 ng each of oligonucleotide primer, and 2.5 units of recombinant Pfu DNA polymerase (Stratagene Inc., La Jolla, CA) in the manufacturer-supplied reaction buffer. Cycling conditions consisted of 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C for 10 to 15 cycles, with an additional 5-min extension at 72°C after the final cycle. Amplified bands were gel-purified by using the Qiaex DNA gel extraction kit (Qiagen Inc., Chatsworth, CA) and ligated without further treatment into pBC-SK+ (Stratagene) digested with EcoRV or HincII. Recombinant plasmids containing desired inserts were digested with SalI and NotI, and inserts were ligated into derivatives of pREP1, pREP41, and pREP81 (Basi et al., 1993) containing a NotI site within the multiple cloning site polylinker. Strains of fission yeast (Schizosaccharomyces pombe) cdc2-33 leu1-32 (Carr et al., 1989) and leu1-32 were transformed with 100 to 500 ng of Qiagen-purified DNA by electroporation (Prentice, 1990), and transformants were selected on synthetic glucose medium lacking leucine.

For functional testing of Antirrhinum majus cdc2 genes, individual colonies from the different recombinant constructs were grown overnight in EMM medium (Bio-101 Inc., Vista, CA) lacking leucine and supplemented with 2.0 µM thiamine, rinsed in thiamine-free medium, diluted into media containing or lacking thiamine, and grown for 9 hr

at the permissive temperature (27°C) with moderate agitation. At this point, aliquots were removed and plated onto agar-solidified media with or without thiamine, and these specimens were incubated at 27 or 35°C until colonies appeared on control plates. One-half of the liquid cultures were returned to 27°C, and the other half were transferred to 35°C and grown overnight with moderate agitation. Cells were fixed and stained as described by Moreno et al. (1991) and photographed under dark field using a 40× objective.

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REFERENCES

Basi, G., Schmid, E., and Maundrell, K. (1993). TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene **123**, 131–136.

Bradley, D., Carpenter, R., Sommer, H., Hartley, N., and Coen, E. (1993). Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of Antirrhinum. Cell **72**, 85–95.

Buck, V., Russell, P., and Millar, J.B.A. (1995). Identification of a cdk-activating kinase in fission yeast. EMBO J. **14**, 6173–6183.

Carpenter, R., Copsey, L., Vincent, C., Doyle, S., Magrath, R., and Coen, E.S. (1995). Control of flower development and phyllotaxy by meristem identity genes in Antirrhinum. Plant Cell **7**, 2001–2011.

Carr, A.M., MacNeill, S.A., Hayles, J., and Nurse, P. (1989). Molecular cloning and sequence analysis of mutant alleles of the fission yeast *cdc2* protein kinase gene: Implications for *cdc2*+ protein structure and function. Mol. Gen. Genet. **218**, 41–49.

Damagnez, V., Mäkelä, T.P., and Cottarel, G. (1995). *Schizosaccharomyces pombe* Mop1-Mcs2 is related to mammalian CAK. EMBO J. **14**, 6164–6172.

Doerner, P.W. (1994). Cell cycle regulation in plants. Plant Physiol. **106**, 823–827.

Espinosa, F.H., Ogas, J., Herskowitz, I., and Morgan, D.O. (1994). Cell cycle control by a complex of the cyclin HCS26 (PCL1) and the kinase PHO85. Science **266**, 1388–1391.

Ferreira, P.C.G., Hemerly, A.S., de Almeida Engler, J., Bergounioux, C., Burssens, S., Van Montagu, M., Engler, G., and Inzé, D. (1994a). Three discrete classes of *Arabidopsis* cyclins are expressed during

different intervals of the cell cycle. Proc. Natl. Acad. Sci. USA **91**, 11313–11317.

Ferreira, P.C.G., Hemerly, A.S., de Almeida Engler, J., Van Montagu, M., Engler, G., and Inzé, D. (1994b). Developmental expression of the Arabidopsis cyclin gene *cyc1At*. Plant Cell **6**, 1763–1774.

Fobert, P.R., Coen, E.S., Murphy, G.J.P., and Doonan, J.H. (1994). Patterns of cell division revealed by transcriptional regulation of genes during the cell cycle in plants. EMBO J. **13**, 616–624.

Forsburg, S., and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Annu. Rev. Cell Biol. **7**, 227–256.

Hanks, S.K. (1987). Homology probing: Identification of cDNA clones encoding members of the protein-serine kinase family. Proc. Natl. Acad. Sci. USA **84**, 388–392.

Hashimoto, J., Hirabayashi, T., Hayano, Y., Hata, S., Ohashi, Y., Suzuka, I., Utsugi, T., Toh-E, A., and Kikuchi, Y. (1992). Isolation and characterization of cDNA clones encoding *cdc2* homologues from *Oryza sativa*: A functional homologue and cognate variants. Mol. Gen. Genet. **223**, 10–16.

Hata, S. (1991). cDNA cloning of a novel cdc2+/CDC28-related protein kinase from rice. FEBS Lett. **279**, 149–152.

Heichman, K.A., and Roberts, J.M. (1994). Rules to replicate by. Cell **79**, 557–562.

Hemerly, A.S., Ferreira, P., de Almeida Engler, J., Van Montagu, M., Engler, G., and Inzé, D. (1993). *cdc2a* expression in Arabidopsis is linked with competence for cell division. Plant Cell **5**, 1711–1723.

Hemerly, A.S., de Almeida Engler, J., Bergounioux, C., Van Montagu, M., Engler, G., Inzé, D., and Ferreira, P. (1995). Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J. **14**, 3925–3936.

Hirt, H., Páy, A., Bögre, L., Meskiene, I., and Heberle-Bors, E. (1993). *cdc2MsB*, a cognate *cdc2* gene from alfalfa, complements the G1/S but not the G2/M transition of budding yeast *cdc28* mutants. Plant J. **4**, 61–69.

Imajuku, Y., Hirayama, T., Endoh, H., and Oka, A. (1992). Exon-intron organization of the *Arabidopsis thaliana* protein kinase genes *CDC2a* and *cdc2b*. FEBS Lett. **304**, 73–77.

Irie, K., Nomoto, S., Miyajima, I., and Matsumoto, K. (1991). *SGV1* encodes a CDC28/cdc2-related kinase required for a Gα subunit-mediated adaptive response to pheromone in *S. cerevisiae*. Cell **65**, 785–795.

Jacobs, T.W. (1995). Cell cycle control. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**, 317–339.

Kaffman, A., Herskowitz, I., Tjian, R., and O'Shea, E.K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. Science **263**, 1153–1156.

King, R.W., Jackson, P.K., and Kirschner, M.W. (1994). Mitosis in transition. Cell **79**, 563–571.

Lahti, J.M., Xiang, J., Heath, L.S., Campana, D., and Kidd, V.J. (1995). PITSLRE protein kinase activity is associated with apoptosis. Mol. Cell. Biol. **15**, 1–11.

Lapidot-Lifson, Y., Patinkin, D., Prody, C.A., Ehrlich, G., Seidman, S., Ben-Aziz, R., Benseler, F., Eckstein, F., Zakut, H., and Soreq, H. (1992). Cloning and antisense oligodeoxynucleotide inhibition of a human homolog of *cdc2* required in hematopoiesis. Proc. Natl. Acad. Sci. USA **89**, 579–583.

Lew, J., and Wang, J.H. (1995). Neuronal cdc2-like kinase. Trends Biochem. Sci. **20**, 33–37.

Martinez, M.C., Jørgensen, J.-E., Lawton, M.A., Lamb, C.J., and Doerner, P.W. (1992). Spatial pattern of *cdc2* expression in relation to meristem activity and cell proliferation during plant development. Proc. Natl. Acad. Sci. USA **89**, 7360–7364.

Maundrell, K. (1990). *nmt1* of fission yeast. J. Biol. Chem. **265**, 10857–10864.

McKinney, J.D., and Heintz, N. (1991). Transcriptional regulation in the eukaryotic cell cycle. Trends Biochem. Sci. **16**, 430–435.

Measday, V., Moore, L., Ogas, J., Tyers, M., and Andrews, B. (1994). The PCL2 (ORFD)-PHO85 cyclin-dependent kinase complex: A cell cycle regulator in yeast. Science **266**, 1391–1395.

Meskiene, I., Bögre, L., Dahl, M., Pirck, M., Ha, D.T.C., Swoboda, I., Heberle-Bors, E., Ammerer, G., and Hirt, H. (1995). *cycMs3*, a novel B-type alfalfa cyclin gene, is induced in the G_0-to-G_1 transition of the cell cycle. Plant Cell **7**, 759–771.

Meyerson, M., and Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14**, 2077–2086.

Meyerson, M., Enders, G.H., Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L.-H. (1992). A family of human cdc2-related protein kinases. EMBO J. **11**, 2909–2917.

Miao, G.-H., Hong, Z., and Verma, D.P.S. (1993). Two functional soybean genes encoding p34^{cdc2} protein kinases are regulated by different plant developmental pathways. Proc. Natl. Acad. Sci. USA **90**, 943–947.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194**, 795–823.

Müller, R. (1995). Transcriptional regulation during the mammalian cell cycle. Trends Genet. **11**, 173–178.

Newton, C.R., and Graham, A., eds (1994). PCR: Introduction to Biotechniques. (Oxford, UK: BIOS Scientific Publishers).

Pines, J. (1993). Cyclins and cyclin-dependent kinases: Take your partners. Trends Biochem. Sci. **18**, 195–197.

Pines, J. (1994). Protein kinases and cell cycle control. Semin. Cell Biol. **5**, 399–408.

Prentice, H.L. (1991). High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res. **20**, 621.

Reed, S.I. (1992). The role of p34 kinases in the G1 to S-phase transition. Annu. Rev. Cell Biol. **8**, 529–561.

Renaudin, J.-P., Colasanti, J., Rime, H., Yuan, Z., and Sundaresan, V. (1994). Cloning of four cyclins from maize indicates that higher plants have three structurally distinct groups of mitotic cyclins. Proc. Natl. Acad. Sci. USA **91**, 7375–7379.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lönnig, W.-E., Saedler, H., and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene *deficiens*: Evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J. **11**, 251–263.

Sherr, C.J. (1993). Mammalian G1 cyclins. Cell **73**, 1059–1065.

Sherr, C.J. (1994). G1 phase progression: Cyclin on cue. Cell **79**, 551–555.

Shuttleworth, J., Godfrey, R., and Colman, A. (1990). p40^{MO15}, a *cdc2*-related protein kinase involved in negative regulation of meiotic maturation of *Xenopus* oocytes. EMBO J. **9**, 3233–3240.

Simon, M., Seraphin, B., and Faye, G. (1986). *Kin28*, a yeast split gene coding for a putative protein kinase homologous to *CDC28*. EMBO J. **5**, 2697–2701.

Simon, R., Carpenter, R., Doyle, S., and Coen, E. (1994). Fimbriata controls flower development by mediating between meristem and organ identity genes. Cell **78**, 99–107.

Soni, R., Carmichael, J.P., Shah, Z.H., and Murray, J.A.H. (1995). A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell **7**, 85–103.

Staiger, C., and Doonan, J. (1993). Cell division in plants. Curr. Opin. Cell Biol. **5**, 226–231.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development, 2nd ed. (Cambridge, UK: Cambridge University Press).

Stern, B., Ried, G., Clegg, N.J., Grigliatti, T.A., and Lehner, C.F. (1993). Genetic analysis of the Drosophila *cdc2* homolog. Development **117**, 219–232.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science **262**, 2050–2054.

Xiang, J., Lahti, J.M., Grenet, J., Easton, J., and Kidd, V.J. (1994). Molecular cloning and expression of alternatively spliced PITSLRE protein kinase isoforms. J. Biol. Chem. **269**, 15786–15794.