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Differential Expression of Genes for Cyclin-Dependent Protein Kinases in Rice Plants[1]

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Cyclin-dependent protein kinases (CDKs) play key roles in regulating the eukaryotic cell cycle. We have analyzed the expression of four rice (*Oryza sativa*) CDK genes, *cdc2Os1*, *cdc2Os2*, *cdc2Os3*, and *R2*, by in situ hybridization of sections of root apices. Transcripts of *cdc2Os1*, *cdc2Os2*, and *R2* were detected uniformly in the dividing region of the root apex. *cdc2Os1* and *cdc2Os2* were also expressed in differentiated cells such as those in the sclerenchyma, pericycle, and parenchyma of the central cylinder. By contrast, signals corresponding to transcripts of *cdc2Os3* were distributed only in patches in the dividing region. Counterstaining of sections with 4′,6-diamidino-2-phenylindole and double-target in situ hybridization with a probe for histone H4 transcripts revealed that *cdc2Os3* transcripts were abundant from the G_2 to the M phase, but were less abundant or absent during the S phase. The levels of the Cdc2Os3 protein and its associated histone H1-kinase activity were reduced by treatment of cultured cells with hydroxyurea, which blocks cycling cells at the onset of the S phase. Our results suggest that domains other than the conserved amino acid sequence (the PSTAIRE motif) have important roles in the function of non-PSTAIRE CDKs in distinct cell-cycle phases.

CDKs are Ser/Thr protein kinases involved in the regulation of the eukaryotic cell cycle (for review, see Solomon, 1993; King et al., 1994; Lees, 1995; Morgan, 1995; Pines, 1995). cDNAs encoding CDKs from many organisms have been isolated. A single major CDK has been identified in the fission yeast *Schizosaccharomyces pombe* (CDC2) and in the budding yeast *Saccharomyces cerevisiae* (CDC28) (Hindley and Phear, 1984; Lörincz and Reed, 1984). However, the growing list of CDKs in human cells suggests that during development each CDK in metazoans plays a specific role at a specific time in the cell cycle. CDKs are activated by binding of cyclins and phosphorylation (for review, see Morgan, 1995; Fisher, 1997). Each CDK interacts with a specific subset of cyclins, and the size of this subset varies. For example, CDC28 can associate with many different cyclins, whereas human CDC2 interacts with relatively few (for review, see Nigg, 1995). A short, conserved amino acid sequence in CDKs, PSTAIRE, is responsible for the binding of cyclins that activate CDKs by changing the conformation at the catalytic site (Jeffrey et al., 1995; Morgan, 1996); this sequence also functions in the targeting of CDKs to specific substrates or subcellular locations (Hoffmann et al., 1993; Peeper et al., 1993; Dynlacht et al., 1994).

Plants express different kinds of CDKs; multiple genes for CDKs have been found in Arabidopsis (Ferreira et al., 1991; Hirayama et al., 1991), alfalfa (Hirt et al., 1991, 1993), rice (*Oryza sativa*; Hata, 1991; Hashimoto et al., 1992; Kidou et al., 1994), soybean (Miao et al., 1993), maize (Colasanti et al., 1991), and snapdragon (Fobert et al., 1994). Recently, Fobert et al. (1996) isolated four *cdc2*-related genes from snapdragon, and Magyar et al. (1997) described four homologs of *cdc2* in alfalfa, in addition to *cdc2MsA* and *cdc2MsB*, which had been isolated previously (Hirt et al., 1991, 1993). These findings suggest that different sets of CDK/cyclin pairs might regulate the division of plant cells at each stage of the cell cycle, and that division is not controlled by a single major CDK, as it is in the case of yeast (Doerner, 1994; Ferreira et al., 1994; Murray, 1994; Doonan and Fobert, 1997).

A correlation between the abundance of CDK transcripts and the proliferative state of cells was demonstrated in Arabidopsis, maize, and alfalfa (Colasanti et al., 1991; Hirt et al., 1991; Bergounioux et al., 1992; Martinez et al., 1992; Hemerly et al., 1993). It has also been shown, however, that in Arabidopsis, transcripts of *cdc2aAt* are localized not only in dividing cells but also in differentiated tissues, such as the parenchyma of the vascular cylinder and the pericycle, which contains cells responsible for the formation of lateral roots (Martinez et al., 1992; Hemerly et al., 1993). Moreover, expression of *cdc2aAt* could be induced without cell division in suspension cultures (Hemerly et al., 1993). These results suggest that at least some *cdc2* transcripts might be correlated with the acquisition of the ability to divide rather than with the actual division of cells.

Genes for four different CDKs have been isolated from rice: *cdc2Os1*, *cdc2Os2* (Hashimoto et al., 1992), *rcdc2* (designated *cdc2Os3* in this report) (Kidou et al., 1994), and *R2* (Hata, 1991). *cdc2Os1* and *cdc2Os2* are homologs of *cdc2*, and *R2* is similar to the gene for the CDK-activating kinase,

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Abbreviations: CDK, cyclin-dependent protein kinase; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione *S*-transferase.

which is required for activation of CDK by phosphorylation of a conserved Thr residue in the so-called "T" loop (Morgan, 1995; Umeda et al., 1998; Yamaguchi et al., 1999). The deduced amino acid sequence of Cdc2Os3 showed that it is distinct from proteins in the CDC2/CDC28 family (Kidou et al., 1994), whereas *cdc2Os1* and *cdc2Os2* are closely related to the homologs of *cdc2* that have been isolated from various organisms (Hashimoto et al., 1992). The *cdc2Os1* gene was able to partially complement a temperature-sensitive mutation in the *cdc28* gene in yeast, but *cdc2Os2* and *R2* were unable to complement the same mutation (Hashimoto et al., 1992).

We analyzed transcript levels of genes for CDKs in rice plants by in situ hybridization and found that *cdc2Os3*, which has an altered PSTAIRE sequence, was expressed in a cell-cycle-dependent manner, whereas the other two homologs of *cdc2* with the conserved PSTAIRE motif were expressed throughout the cell cycle. The transcripts and protein products of *cdc2Os3* were abundant from the G_2 to the M phase, an indication that the Cdc2Os3 protein might function in mitosis.

MATERIALS AND METHODS

Plant Material

Rice (*Oryza sativa* L. var. Yamahoushi) seeds were germinated in water, and seedlings were grown in an incubator at 27°C. In general, seedlings were used for in situ hybridization 1 d after germination. Sections of lateral root primordia were prepared from 7-d-old seedlings. For treatment with compounds that interrupt the cell cycle, roots of 1-d-old seedlings after germination were submerged in water that contained 100 mM hydroxyurea or 0.5% (w/v) colchicine for 26 h. Rice cells in suspension culture were maintained in liquid Murashige-Skoog medium (Glab et al., 1994) on a gyratory shaker (80 rpm) at 27°C, and subcultured at weekly intervals. For treatment with compounds that interrupt the cell cycle, cells in a 5-d-old suspension culture were grown in medium that contained 0.05% (w/v) colchicine or 10 mM hydroxyurea for 36 h.

Preparation of Probes

For preparation of specific RNA probes, individual fragments of cDNAs were subcloned into the pBluescript II SK^- vector (Stratagene) as described below. A pBluescript plasmid carrying *cdc2Os1* cDNA (accession no. X60374) was digested with *Dra*II, and the *Dra*II cDNA fragment was removed to produce a plasmid that contained the 3′-noncoding region (nucleotides 997–1115). A pBluescript plasmid carrying *cdc2Os2* cDNA (accession no. X60375) was digested with *Xho*I, and the *Xho*I fragment (nucleotides 875–1126) containing the 3′-noncoding region was subcloned into the *Xho*I site of pBluescript. A pBluescript plasmid carrying *cdc2Os3* cDNA (accession no. D64036) was digested with *Sac*II and *Eco*RI, and the *Sac*II-*Eco*RI fragment (nucleotides 454–1121) was subcloned into the *Sac*II/*Eco*RI site of pBluescript. A pBluescript plasmid carrying *R2* cDNA (accession no. X58194) was digested with *Bam*HI, and the *Bam*HI cDNA fragment was removed to produce a plasmid that contained the 3′-noncoding region (nucleotides 1421–1764). A cDNA clone encoding histone H4 (accession no. D10397), which had been isolated previously (Uchimiya et al., 1992), was digested with *Eco*RI and *Bam*HI, and the insert was transferred to the *Eco*RI/*Bam*HI site of pBluescript.

We used the plasmids to generate sense and antisense RNA probes by transcription from the T7 and T3 promoters of pBluescript II SK^-. Digoxigenin- and biotin-labeled probes were generated with a digoxigenin RNA-labeling kit in combination with a digoxigenin RNA-labeling mix and a biotin RNA-labeling mix, respectively (Boehringer Mannheim).

In Situ Hybridization

In situ hybridization was performed as described by Hihara et al. (1996) with minor modifications. Tissues were fixed in a solution of 50% ethanol, 5% acetic acid, and 3.7% formaldehyde. Paraffin blocks were cut at 10 μm for cross-sections and at 5 μm for longitudinal sections. The method for double-target in situ hybridization was essentially the same as that described by Kouchi et al. (1995), but we used biotin-labeled RNA probes instead of fluorescein-labeled probes. The biotin-labeled probe for transcripts of histone H4 was detected with a streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim) in combination with Fast Red TR/Naphthol AS-MX (Sigma), and then the digoxigenin-labeled probe for the *cdc2Os3* transcript was detected with digoxigenin-specific antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) in combination with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stock solution (Boehringer Mannheim). Sections were counterstained with DAPI (1 μg mL^{-1} in 0.05 M Tris-HCl, pH 7.0, and 0.5% Triton X-100) after detection of digoxigenin-labeled probes.

Isolation and Purification of GST-Fusion Proteins

The open reading frames of *cdc2Os1*, *cdc2Os2*, and *cdc2Os3* were amplified by PCR with primers that included recognition sequences for specific restriction enzymes, *Bam*HI, *Eco*RI, and *Eco*RI, respectively, at the amino-terminal and carboxy-terminal ends. After digestion with appropriate enzymes, the amplified fragments were ligated to pGEX vectors (Pharmacia). pGEX-2T was digested with *Bam*HI, pGEX-1λT with *Eco*RI, and pGEX-1λT with *Eco*RI, and the fragments were introduced into *Escherichia coli* BL21 cells. The nucleotide sequences of the amplified fragments were confirmed for each construct. The *E. coli* cells were grown in a Luria-Bertani medium to an A_{600} of 0.6 at 27°C, and expression of GST-fusion proteins was induced by the addition of 0.4 mM isopropyl-β-D-galactoside and allowed to continue for 4 h at 27°C. The GST-fusion proteins were purified with glutathione Sepharose 4B (Pharmacia) according to the protocol from the manufacturer.

Immunoblotting with Antibodies against Rice CDKs

Total protein was extracted from rice suspension-cultured cells as described by Magyar et al. (1997). Proteins were fractionated by SDS-PAGE on a 12% polyacrylamide gel and subjected to immunoblotting with an ECL western-blotting detection system (Amersham). Polyclonal antibodies were raised in rabbits against the internal peptides CPEFAKNPTLI and SPDFKNHRIV of Cdc2Os1 and Cdc2Os2, respectively, and against the carboxy-terminal peptide PYFNDVNKELY of Cdc2Os3.

Assay for Histone H1 Kinase

An aliquot of 100 μg of total protein extracted from suspension-cultured cells was incubated with 10 μL of antiserum for 2 h at 4°C, and immune complexes were precipitated with 30 μL of a 50% suspension of protein A-agarose (GIBCO-BRL) for 1 h at 4°C. The immunoprecipitates were washed three times with bead buffer (50 mM Tris-HCl, 5 mM NaF, 250 mM NaCl, 0.1% [w/w] Nonidet P-40, 0.1 mM Na_3VO_4, 5 mM EDTA, and 5 mM EGTA, pH 7.5) containing 10 μg mL^{-1} leupeptin and 0.1 mM benzamidine, and once with kinase buffer (50 mM Tris-HCl, 15 mM $MgCl_2$, 5 mM EGTA, and 1 mM DTT, pH 7.8). A phosphorylation reaction was conducted with each immunoprecipitate in kinase buffer that contained 0.5 mg mL^{-1} histone H1 as a substrate, 0.01 mM ATP, 0.185 MBq of [γ-^{32}P]ATP (167 TBq/mmol, ICN), and 60 μg mL^{-1} cAMP-dependent protein kinase inhibitor (Sigma). After incubation for 15 min at room temperature, the reaction was stopped by the addition of sample buffer for SDS-PAGE, boiled for 5 min, and loaded onto a 12% polyacrylamide gel. Phosphorylated proteins were detected with an imaging plate scanner (BAS1000, Fuji, Tokyo, Japan).

RESULTS

Expression of *cdc2Os1*, *cdc2Os2*, and *R2* throughout the Dividing Region of Rice Roots

cDNAs encoding four different CDKs have been isolated from rice, and the corresponding genes have been designated *cdc2Os1*, *cdc2Os2*, *rcdc2*, and *R2*, respectively (Hata, 1991; Hashimoto et al., 1992; Kidou et al., 1994). In this paper *rcdc2* is referred to as *cdc2Os3*, since it was the third homolog of *cdc2* to be identified. To investigate the level of transcripts of these genes, we prepared root sections from rice seedlings and subjected them to in situ hybridization. RNA probes were prepared from cDNAs such that the respective probes were specific for each CDK, as determined by northern analysis (M. Umeda, unpublished data), and labeled with digoxigenin.

As shown in Figure 1, signals corresponding to transcripts of *cdc2Os1*, *cdc2Os2*, and *R2* were detected in the root apex. A relatively uniform distribution of signals was observed for the transcripts of these three genes. In the upper parts of the root, no signals were detected in the cortex. For a more detailed investigation, root cross-sections were prepared and allowed to hybridize with each probe. Figure 2a shows the results for *cdc2Os1*. On sections that included the root cap and the quiescent center, signals were detected in the dividing cells of the root cap but not in the quiescent center or in the differentiated cells of the root cap (section 5). Uniform signals were observed in the dividing region of the root apex (sections 3 and 4). However, in the upper region, *cdc2Os1* transcripts were restricted to the sclerenchyma and to inner files of cells that included the pericycle and central cylinder (section 2). No signals were detected in the xylem poles. The same pattern of distribution of transcripts was observed with the probe

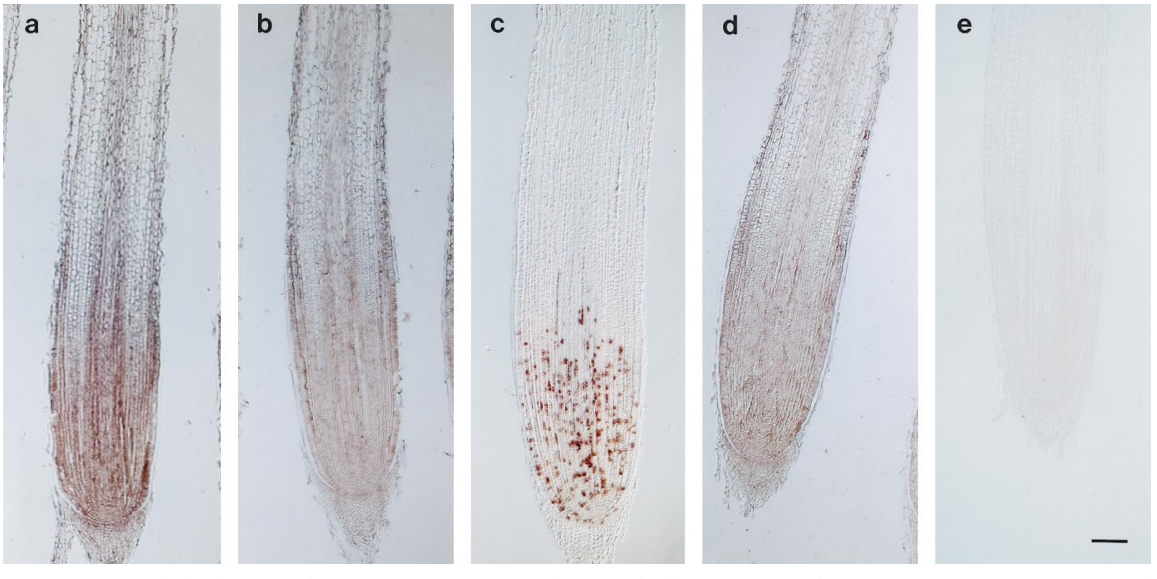

Figure 1. In situ hybridization of rice root apices with probes specific for transcripts of *cdc2Os1*, *cdc2Os2*, *cdc2Os3*, and *R2*. Longitudinal sections of roots were allowed to hybridize with digoxigenin-labeled RNA probes. Hybridization signals are visible as brownish-purple staining. a, *cdc2Os1* antisense probe; b, *cdc2Os2* antisense probe; c, *cdc2Os3* antisense probe; d, *R2* antisense probe; and e, *cdc2Os1* sense probe. Bar = 100 μm.

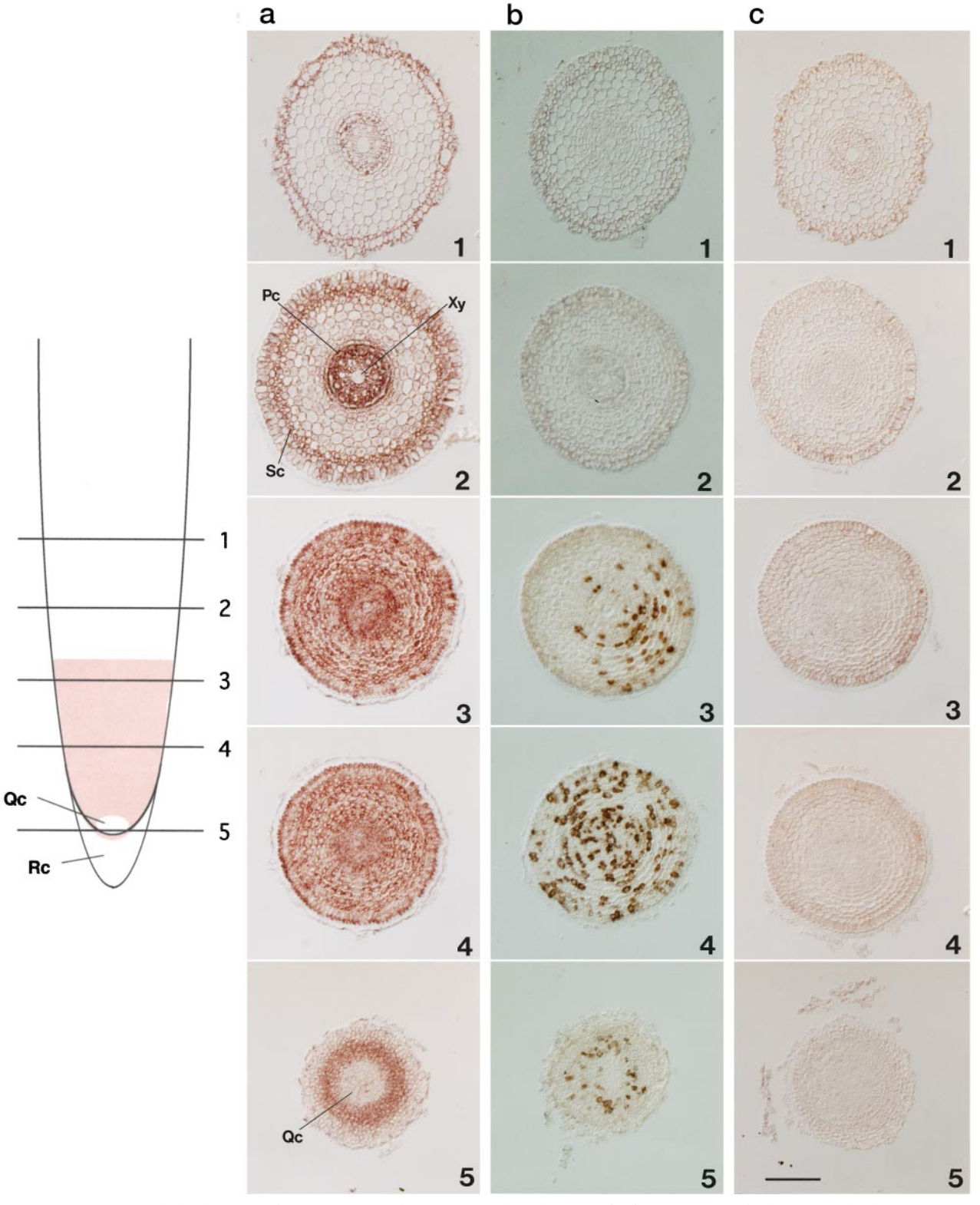

Figure 2. In situ hybridization of cross-sections of rice roots with probes specific for transcripts of *cdc2Os1* and *cdc2Os3*. Cross-sections were prepared from each part of the root, as shown schematically by red shading in the region of dividing cells in the drawing on the left. Numbers indicate positions from which sections were prepared. Pc, Pericycle; Qc, quiescent center; Rc, root cap; Sc, sclerenchyma; Xy, xylem. a, *cdc2Os1* antisense probe; b, *cdc2Os3* antisense probe; and c, *cdc2Os1* sense probe. Bar = 100 μm.

for transcripts of *cdc2Os2*, but signals were weaker than those for the *cdc2Os1* probe (data not shown). These results suggest that *cdc2Os1* and *cdc2Os2* were expressed in the sclerenchyma, pericycle, and parenchyma of the central cylinder in the differentiated zone of roots, as well as in the dividing region. The uniform signals due to each transcript indicated that *cdc2Os1*, *cdc2Os2*, and *R2* were probably expressed throughout the cell cycle. The control sense probe gave no signals on either longitudinal sections or cross-sections (Figs. 1e and 2c).

Dependence of Expression of *cdc2Os3* Transcripts on the Phase of the Cell Cycle

When the probe was specific for transcripts of *cdc2Os3*, we observed a patchy pattern of signals in the root apex where the other transcripts had given uniform signals (Fig. 1c). The level of *cdc2Os3* transcripts was higher than those of *cdc2Os1* and *cdc2Os2*. A patchy pattern was also apparent in the region near the shoot meristem at the base of the stem (Fig. 3a). Moreover, the primordium for lateral root formation gave a similar patchy pattern in a small number of dividing cells (Fig. 3b). In the actively dividing region, the division of cells was poorly synchronized, so neighboring cells were unlikely to be at the same stage of the cell cycle. Accordingly, these results suggest that *cdc2Os3* is expressed at particular phases of the cell cycle.

Cross-sections of roots also gave patchy patterns of *cdc2Os3* signals throughout the dividing region of the root apex (Fig. 2b, sections 3–5); however, when we investigated the differentiated cells in the upper region, we found almost no signals on cross-sections (Fig. 2b, sections 1 and 2). Therefore, it is likely that transcripts of *cdc2Os3* were restricted to dividing cells of roots and were not expressed in the differentiated cells. By contrast, *cdc2Os1* and *cdc2Os2* were expressed in several differentiated tissues as well as in dividing regions (Fig. 2a).

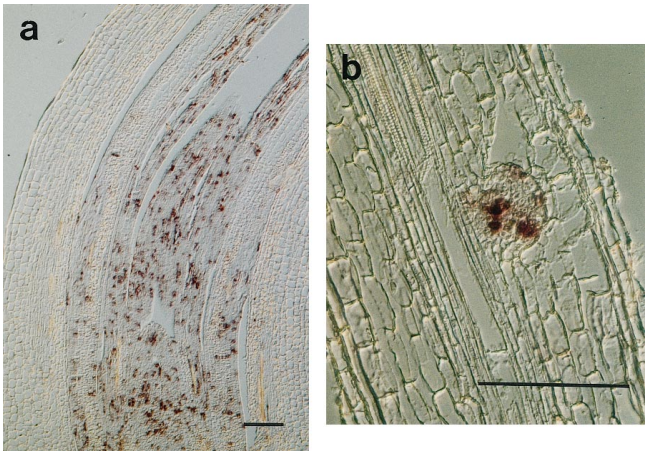

Figure 3. In situ hybridization of a region near the shoot meristem and the primordium for formation of lateral roots with a probe specific for transcripts of *cdc2Os3*. Longitudinal sections were allowed to hybridize with the digoxigenin-labeled *cdc2Os3* antisense probe. a, Region near the shoot meristem. b, Primordium arising from the pericycle in the initial stages of formation of a lateral root. Bars = 100 μm.

Detection of Abundant Transcripts of *cdc2Os3* from the G2 to the M Phase of the Cell Cycle

To identify the stage of the cell cycle at which *cdc2Os3* was expressed, we counterstained sections with the DNA-specific dye, DAPI (Nacalai, Kyoto, Japan), which reveals the extent of condensation of nuclei. As shown in Figure 4, mitotic cells with condensed nuclei were usually positive for *cdc2Os3* transcripts, although the levels of the transcript seemed to depend on the stage of mitosis. When 200 metaphase cells with condensed chromosomes at the equatorial plane were counted for signals corresponding to *cdc2Os3* transcripts, almost all of the cells (99%) contained significant amounts. Similarly, almost all of the anaphase cells (99%) with two daughter chromosomes also contained the transcripts, but signals were weaker than those in metaphase cells. Moreover, signals from cells that were forming cell plates were much less intense than signals from cells at the early stage of mitosis (Fig. 4). These results suggest that expression of *cdc2Os3* might extend to the M phase and end with the completion of mitosis. By contrast, about 90% of the cells that expressed *cdc2Os3* transcripts did not contain condensed nuclei, an indication that *cdc2Os3* was also expressed during the preceding G_2 phase and the prophase of mitosis.

To determine whether *cdc2Os3* was expressed during the S phase, we performed double-labeling experiments in which each section was hybridized with probes specific for transcripts of a gene for histone H4 and *cdc2Os3*. The probe for histone H4 transcripts was labeled with biotin and positive signals were recognized as having a red coloration; the *cdc2Os3* probe was labeled with digoxigenin, and positive signals were recognized as having a brownish-purple coloration. Figure 5 shows examples of sections after color development. We allowed many sections to hybridize with both probes. Almost all cells (99%) with signals corresponding to *cdc2Os3* transcripts were negative for histone H4 transcripts, suggesting that the timing of the expression of the two genes did not overlap or overlapped for only a very short period of the cell cycle. Our results indicated that transcripts of *cdc2Os3* were abundant from the G_2 to the M phase but not during the S phase.

Differential Expression of *cdc2Os3* in Response to Compounds That Interrupt the Cell Cycle

We analyzed the transcription regulation of *cdc2Os3* in further detail with compounds that interrupt the cell cycle. One-day-old seedlings were transferred to water containing hydroxyurea, which blocks cycling cells at the onset of the S phase, or containing colchicine, which inhibits the formation of spindle fibers and blocks cells in mitosis. After treatment for 26 h, root sections were prepared and subjected to in situ hybridization. Roots treated with hydroxyurea showed no signals in the region of dividing cells (Fig. 6b) where a patchy pattern had been observed on sections of untreated seedlings (Fig. 6a). By contrast, after

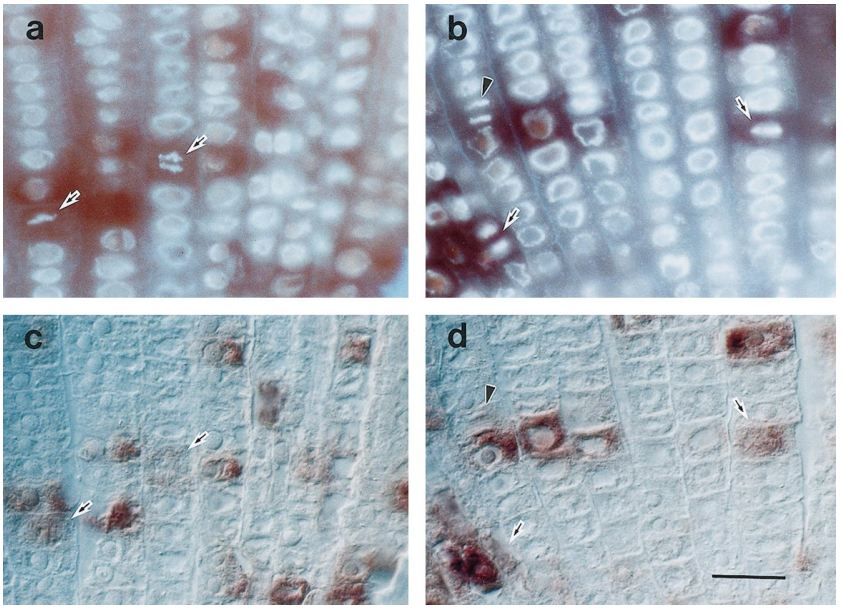

Figure 4. Correlation between expressions of *cdc2Os3* and mitosis. Sections of root apex were probed with the digoxigenin-labeled *cdc2Os3* antisense probe and then counterstained with DAPI. Images were viewed by epifluorescence (a and b) and bright-field (c and d) microscopy. Arrows indicate mitotic cells with condensed nuclei, and arrowheads indicate cells forming cell plates. Bar = 20 μm.

treatment with colchicine, *cdc2Os3* transcripts were still detected at the root tips (Fig. 6c). Since colchicine caused radial expansion close to the root tip and promoted the vacuolation of cells, the signals corresponding to *cdc2Os3* transcripts were restricted to a small region in the root apex. These results support the hypothesis that *cdc2Os3* is expressed from the G_2 to the M phase but is not expressed during the transition from the G_1 to the S phase.

To analyze expression at the protein level, we raised polyclonal antibodies in rabbits against peptides specific to each *cdc2* homolog. Since the Cdc2Os1-specific antibodies produced a high background on immunoblots, we chose antibodies against Cdc2Os2 and Cdc2Os3 for further analysis. When we used recombinant Cdc2 proteins fused to GST for immunoblotting, the preparations of antibodies specifically recognized GST-Cdc2Os2 and GST-Cdc2Os3, respectively (Fig. 7a). Neither preparation of antibodies cross-reacted with GST (data not shown). Therefore, we used these antibodies to investigate the differential expression of Cdc2Os2 and Cdc2Os3 proteins.

Rice cells in suspension culture were treated with hydroxyurea or colchicine, and total protein was extracted from cells and subjected to immunoblotting. As shown in Figure 7b, antibodies against Cdc2Os2 and Cdc2Os3 immunoreacted with proteins of 40 and 36 kD, respectively. The level of Cdc2Os2 (p40) protein was unchanged after treatment with the two compounds (Fig. 7b). By contrast, hydroxyurea, which arrests cells at the onset of the S phase, reduced the level of Cdc2Os3 (p36). Colchicine had no effect on the level of Cdc2Os3 protein (Fig. 7b). Next we

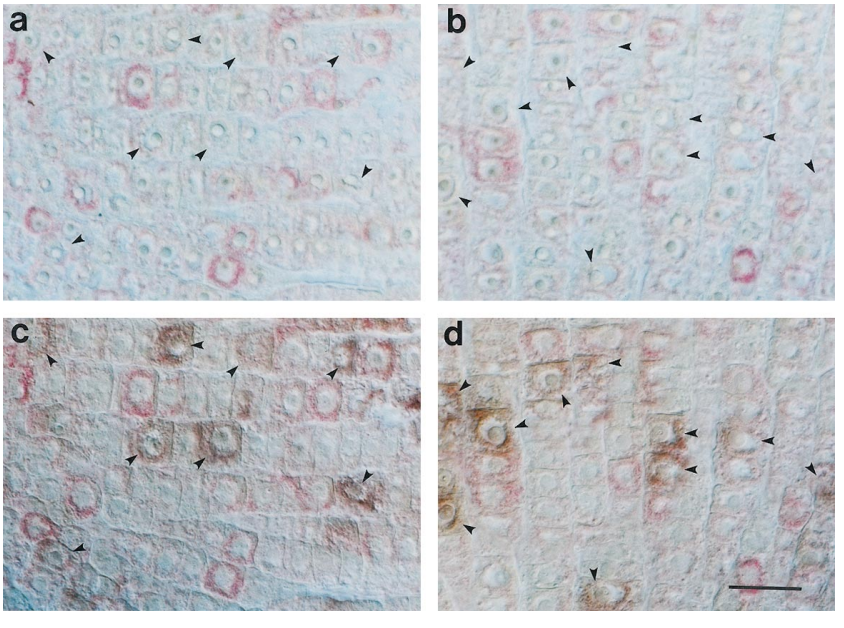

Figure 5. Double-labeling of the root apex for transcripts of *cdc2Os3* and of a gene for histone H4. a and b, Detection of hybridization signals with the biotin-labeled antisense probe for histone H4 transcripts (red). c and d, Same section after detection of the digoxigenin-labeled *cdc2Os3* probe (brownish-purple). Arrowheads indicate cells labeled with the *cdc2Os3* antisense probes. Bar = 20 μm.

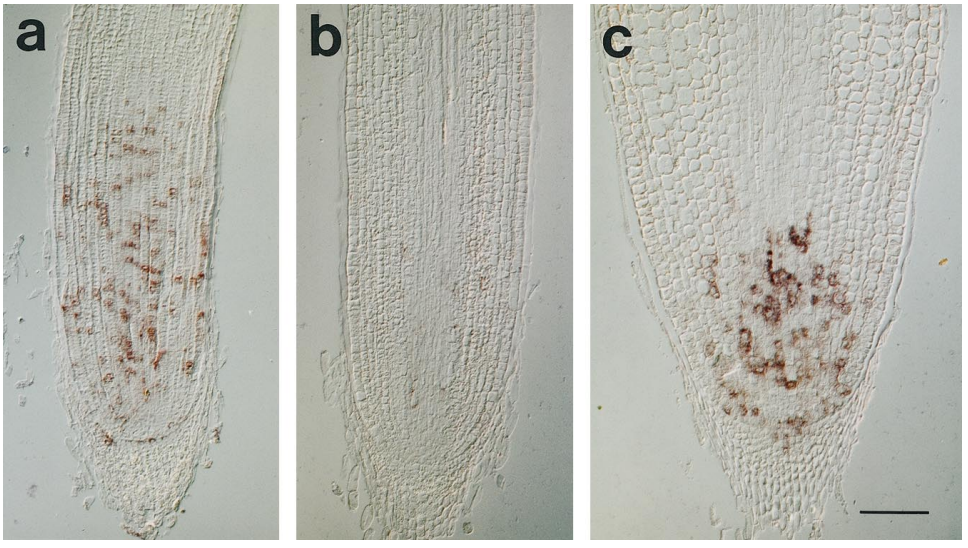

Figure 6. In situ hybridization of root apices after treatment with cell-cycle blockers. Longitudinal sections of root apices were prepared from seedlings that had been treated with hydroxyurea or colchicine, and probed with the digoxigenin-labeled *cdc2Os3* antisense probe. a, Control root (not treated with blockers of the cell cycle); b, root treated with hydroxyurea; and c, root treated with colchicine. Details of treatments are given in the text. Bar = 100 μm.

immunoprecipitated endogenous Cdc2 proteins with the antibodies, and subjected the immunoprecipitates to kinase assays with histone H1 as the substrate. Although the kinase activity associated with Cdc2Os2 was detected in both samples, Cdc2Os3-specific antibodies allowed recovery of histone H1-kinase activity from colchicine-treated cells but not from hydroxyurea-treated cells (Fig. 7c). These results suggest that the levels of Cdc2Os3 protein and its associated histone H1-kinase activity were high in G_2/M-arrested cells but low at the entry to the S phase.

DISCUSSION

Although synchronization of rice cells in suspension culture has been reported (Ohtsubo et al., 1993; Sauter, 1997), it is difficult to investigate phase-specific expression of genes in such cells because of low efficiency of synchronization. Sauter (1997) partially synchronized rice cells in suspension culture and performed northern hybridizations with some rice *cdc2* genes as probes. Levels of *cdc2Os2* and *R2* transcripts were slightly elevated after the release from a hydroxyurea block, but the changes did not prove unequivocally that *cdc2Os2* and *R2* were expressed in a G_1/S-phase-specific manner (Sauter, 1997). Moreover, the mitotic index of the cell culture was below 5% (Sauter, 1997). We analyzed the expression of rice genes for CDKs by in situ hybridization of root sections, and found that transcripts of *cdc2Os1*, *cdc2Os2*, and *R2* were uniformly detectable in dividing cells of roots. Thus, accumulation of *cdc2Os1*, *cdc2Os2*, and *R2* transcripts was not strictly related to particular stages of the cell cycle, although the levels might change slightly during the cell cycle. Our results indicate that in situ hybridization is a powerful tool for studies of the cell cycle in rice plants, as it is in snapdragon (Fobert et al., 1994, 1996) and soybean (Kouchi et al., 1995).

We found that *cdc2Os1* and *cdc2Os2* were expressed also in the sclerenchyma, pericycle, and parenchyma of the central cylinder in the differentiated zone of roots. In parts of Arabidopsis roots beyond the apical meristem, expression of *cdc2aAt* is restricted to the parenchyma of the vascular cylinder and to the pericycle cells (Martinez et al., 1992; Hemerly et al., 1993). Rice *cdc2Os1* and *cdc2Os2* are closely related to *cdc2aAt* of Arabidopsis at the amino acid level (Fig. 8), and may also be correlated with the ability of these cells to divide (Hemerly et al., 1993). The pericycle is a differentiated tissue, but retains the potential to divide and is responsible for lateral root formation. The expression of *cdc2Os1* and *cdc2Os2* in the parenchyma of the central cylinder suggests that this cell layer might engage in some mitotic activity that contributes to the thickening of primary roots in rice plants. The rice-specific expression of these two *cdc2* genes in the sclerenchyma remains to be investigated.

In contrast to transcripts of *cdc2Os1*, *cdc2Os2*, and *R2*, transcripts of *cdc2Os3* were distributed with a patchy pattern in the dividing region of the root apex. Such a pattern was also observed in the region near the shoot meristem and in the primordia for lateral root formation. Counterstaining of sections with DAPI indicated that almost all of the cells with mitotic nuclei contained *cdc2Os3* transcripts, while cells forming cell plates had trace levels of transcripts. In double-labeling experiments with probes specific for transcripts of a gene for histone H4 and *cdc2Os3*, signals did not overlap, an indication that expression of *cdc2Os3* does not extend to the S phase. Furthermore, treatment of seedlings with hydroxyurea, which blocks cells in the early S phase, inhibited the patchy expression of *cdc2Os3* at the root apex, whereas transcripts were still detectable in roots treated with colchicine, which blocks cells in mitosis. The patchy pattern on colchicine-treated

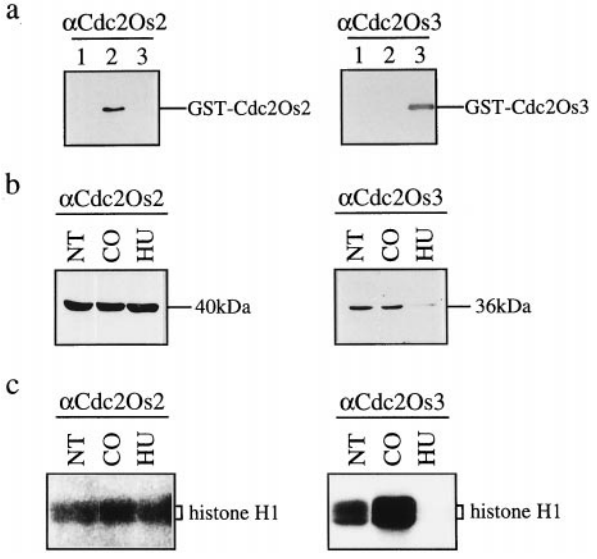

Figure 7. Changes in the level of the Cdc2Os3 protein in response to cell-cycle blockers. a, Specific cross-reactions of the GST-Cdc2Os2 and GST-Cdc2Os3 fusion proteins with antibodies. One microgram of each GST-fusion protein was subjected to immunoblotting with antibodies against Cdc2Os2 or Cdc2Os3. Lanes 1, GST-Cdc2Os1; lanes 2, GST-Cdc2Os2; and lanes 3, GST-Cdc2Os3. b, Immunological detection of Cdc2Os2 (p40) and Cdc2Os3 (p36) in suspension-cultured rice cells that had been treated with cell-cycle blockers. Total protein was extracted from cultured cells after no treatment (NT) and after treatment with colchicine (CO) or hydroxyurea (HU). Twenty micrograms of each sample was subjected to immunoblotting with Cdc2Os2- or Cdc2Os3-specific antibodies. c, Histone H1-kinase activities in the immunoprecipitates obtained with the antibodies. Total protein was extracted from cultured cells as described above and used for immunoprecipitation with Cdc2Os2- or Cdc2Os3-specific antibodies. Immunoprecipitates were subjected to the histone H1-kinase assay, and phosphorylated histone H1 was detected.

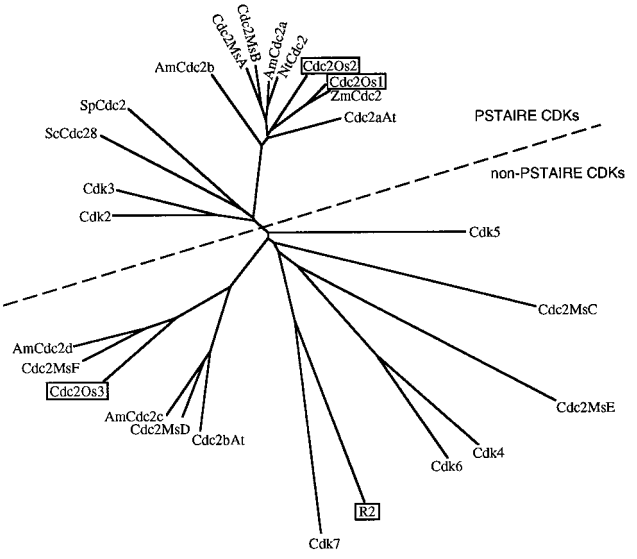

Figure 8. Phylogenetic tree for members of the CDK protein family. The tree was constructed using the CLUSTAL software program (Higgins et al., 1992), with sequences selected from the databases. Rice CDKs are boxed. Amcdc2a-d, CDKs of snapdragon; Cdc2aAt and Cdc2bAt, CDKs of Arabidopsis; Cdc2MsA-F, CDKs of alfalfa; NtCdc2, CDK of tobacco; ZmCdc2, CDK of maize; ScCdc28, Cdc28 of *S. cerevisiae*; SpCdc2, Cdc2 of *S. pombe*; Cdk2–7, human CDKs.

sections may have reflected the partial synchrony of cell division in this case.

Transcripts of *cdc2Os3* appeared to be abundant from the G_2 to the M phase but almost disappeared when cells had completed mitosis at telophase. However, we cannot exclude the possibility that *cdc2Os3* might be expressed more than once during the cell cycle but discontinuously. Transcripts of *cdc2Os3* were detected in the dividing region of the root, whereas the expression was observed in a wide region near the shoot meristem. More detailed experiments are required to investigate whether *cdc2Os3* is specifically expressed in dividing cells.

Both *cdc2Os1* and *cdc2Os2* include the characteristic PSTAIRE domain (Hashimoto et al., 1992) and are classified as PSTAIRE CDKs on the phylogenetic tree (Fig. 8). PSTAIRE CDKs, such as the products of *cdc2aAt* in Arabidopsis, *Amcdc2a* and *Amcdc2b* in snapdragon, and *cdc2MsA* and *cdc2MsB* in alfalfa are expressed throughout the cell cycle (Martinez et al., 1992; Fobert et al., 1996; Magyar et al., 1997). Therefore, the products of *cdc2Os1* and *cdc2Os2* can also be classified as PSTAIRE CDKs in terms of their pattern of expression. Several plant CDKs with altered PSTAIRE motifs have been reported: the products of *Amcdc2c* and *Amcdc2d* in snapdragon (Fobert et al., 1996); *cdc2MsC*, *cdc2MsD*, *cdc2MsE*, and *cdc2MsF* in alfalfa (Magyar et al., 1997); and *cdc2bAt* in Arabidopsis (Imajuku et al., 1992). Rice *cdc2Os3* encodes a PPTALRE sequence that is the same as those of AmCdc2c, Cdc2MsD, and Cdc2bAt (Table I). However, when Cdc2Os3 was compared with the other non-PSTAIRE CDKs in the whole region, it was close to *Amcdc2d* and *cdc2MsF*, rather than to AmCdc2c, Cdc2MsD, or Cdc2bAt (Fig. 8).

Transcripts of *cdc2*s belonging to the group including c*dc2Os3* are abundant from the G_2 to the M phase (this study; Fobert et al., 1996; Magyar et al., 1997). On the other hand, *Amcdc2c* is expressed from the mid-S phase to the early-M phase (Fobert et al., 1996), and *cdc2bAt* is prefer-

Table I. *Amino acid sequences in the PSTAIRE region of planta non-PSTAIRE CDKs*

Amino acids that are conserved in the PSTAIRE sequence are shown in bold type. Amcdc2c-d, CDKs of snapdragon; Cdc2bAt, CDK of Arabidopsis; Cdc2MsC-F, CDKs of alfalfa.

CDKs expressing during the G_2-to-M phase	
Cdc2Os3	EGV**PPTALRE**VSLLRM
AmCdc2d	EGV**PPTTLRE**VSLLRM
Cdc2MsF	EGV**PPTTLRE**VSILRM
CDKs expressing during the S-to-M phase	
AmCdc2c	EGV**PPTALRE**VSLLQM
Cdc2MsD	EGV**PPTALRE**VSLLQM
Cdc2bAt	EGI**PPTALRE**ISLLQM
CDKs expressing throughout the cell cycle	
R2	EGVNF**TALRE**IKLLKE
Cdc2MsC	EGF**PITAIRE**ILILKK
Cdc2MsE	DGVSP**TAIRE**IMLLRE

entially expressed during the S and G_2 phases (Segers et al., 1996). Transcripts of *cdc2MsD* are abundant at the G_2/M phase and are also detected just after alfalfa cells are released from arrest by aphidicolin (Magyar et al., 1997). Accordingly, we propose that the products of *cdc2Os3*, *Amcdc2d*, and *cdc2MsF* form a distinct subclass of non-PSTAIRE CDKs that are preferentially expressed from the G_2 to the M phase. Our results also indicate that domains other than the PSTAIRE region are important for the function of non-PSTAIRE CDKs in distinct cell-cycle phases. The alfalfa genes *cdc2MsC* and *cdc2MsE* and the rice gene *R2*, which encode divergent PSTAIRE sequences, are expressed throughout the cell cycle, and the encoded amino acid sequences are distinct from those of other CDKs (Table I; Fig. 8; Magyar et al., 1997).

The level of the Cdc2Os3 protein (p36) was reduced by treatment of cultured cells with hydroxyurea but not with colchicine. The histone H1-kinase activity associated with Cdc2Os3 was correlated with the level of the protein. However, small amounts of Cdc2Os3 protein (p36) were still detectable in hydroxyurea-treated cells. We do not know whether Cdc2Os3 protein is actually present at the onset of the S phase or if the partial synchronization allowed detection of a small amount of the protein. Nevertheless, it seems that the level of Cdc2Os3 fluctuates during the cell cycle according to the level of the transcript. It is likely that expression of *cdc2Os3* is controlled at the transcriptional level and that the Cdc2Os3 protein accumulates from the G_2 to the M phase.

Yeast *cdc2/cdc28* mutants have been rescued by the overexpression of plant genes for PSTAIRE CDKs, such as *cdc2aAt* of Arabidopsis (Ferreira et al., 1991; Hirayama et al., 1991), *cdc2Os1* of rice (Hashimoto et al., 1992), *cdc2MsA* and *cdc2MsB* of alfalfa (Hirt et al., 1991, 1993), *Amcdc2a* and *Amcdc2b* of snapdragon (Fobert et al., 1996), *cdc2ZmA* of maize (Colasanti et al., 1991), and *cdc2-S5* and *cdc2-S6* of soybean (Miao et al., 1993). No plant CDK with an altered PSTAIRE sequence was able to rescue such yeast mutants. We overexpressed the rice *cdc2Os3* gene in a mutant of *Schizosaccharomyces pombe*, *cdc2–33* (Carr et al., 1989), and several mutants of *Saccharomyces cerevisiae*, such as *cdc28–1N* (Surana et al., 1991), *cdc28–4*, and *cdc28–13* (Reed, 1980). However, the temperature sensitivity of each strain was not rescued by the overexpression of *cdc2Os3*, which was expressed from either a single-copy or a multicopy vector (data not shown). Therefore, non-PSTAIRE CDKs might have distinct functions in the cell cycle and form active complexes with particular cyclins specific to plants. Indeed, plants have several specific subclasses of cyclins (for review, see Renaudin et al., 1996), and it is likely that a mitotic cyclin that is expressed preferentially from the G2 to the M phase controls the activity of Cdc2Os3.

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