Two functional soybean genes encoding p34^{cdc2} protein kinases are regulated by different plant developmental pathways

(cell cycle/functional complementation/*Glycine max*/nodule meristem/*Rhizobium*)

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ABSTRACT   We have isolated two cDNA clones (cdc2-S5 and cdc2-S6) encoding p34^{cdc2} protein kinases, homologs of yeast *cdc2/CDC28* genes, from a soybean nodule cDNA library. The two sequences share 90% sequence homology in the coding regions. The 5' and 3' noncoding regions are distinct from each other, however, indicating that at least two genes encode p34^{cdc2} protein kinases in soybean. Both sequences can rescue the *cdc28* mutation in *Saccharomyces cerevisiae* but rescue it with different efficiency. Genomic Southern analysis showed the existence of two copies for each of these genes, which are not closely linked and are nonallelic. The relative expression level of the two soybean p34^{cdc2} genes varies in different tissues. Expression of cdc2-S5 is higher in roots and root nodules, whereas cdc2-S6 is more actively expressed in aerial tissues, indicating that regulation of these two p34^{cdc2} genes is coupled with plant developmental pathways. Expression of cdc2-S5 is, furthermore, enhanced after *Rhizobium* infection, whereas cdc2-S6 fails to respond, suggesting that cdc2-S5 plays a role in nodule initiation and organogenesis. This latter gene preferentially responds to auxin (α-naph-thaleneacetic acid) treatment, indicating that phytohormones may be involved in the control of cell division mediated by *Rhizobium* infection. Thus, different p34^{cdc2} protein kinases may control cell division in different tissues in a multicellular organism and respond to different signals—e.g., phytohormones.

The development of plants differs fundamentally from that of animals in a number of ways (1). One of these differences is that in plants, a fully differentiated cell is capable of initiating cell division, leading to the formation of new organs (a characteristic known as totipotency). The basic cell-cycle-control mechanism is apparently conserved among eukaryotes (2), including plants (3), but the exact mechanism of cell-cycle control has not yet been determined in plants. Some of the cell-cycle-control genes that can functionally complement the cell-division-control mutations in yeast have recently been isolated from plants (see ref. 3).

A key component of the cell-cycle-control machinery in yeast is the *cdc2/CDC28* gene encoding a serine/threonine protein kinase (2, 4). This protein is involved in both the G$_1$ → S transition, when cells are committed to DNA synthesis, and the G$_2$ → M control, when the cells enter into mitosis (5, 6). In yeast, the activity of cdc2/CDC28 kinase is under the control of both positive and negative regulatory genes and requires interaction with a number of other cellular components, including cyclins (7). Homologs of yeast *cdc2/CDC28* genes encoding p34^{cdc2} protein kinases have been isolated from higher eukaryotes (2, 3). Recently, a subfamily of p34^{cdc2} homologs has been found (8, 9), which encodes cyclin-dependent kinases (CDK2) able to complement *cdc2/*

CDC28 mutations in yeast. These genes appear to be involved in the G$_1$ → S phase control, rather than in the control of G$_2$ → M phase (8). The finding that more than one type of p34^{cdc2} protein kinases is present in higher eukaryotes suggests that the cell-cycle-control mechanism in multicellular organisms is more complex than that in yeast.

The activity of cdc2/CDC28 protein kinases fluctuates in the course of the cell cycle, although the level of the protein seems to remain constant throughout the cell cycle in yeast (10). In multicellular eukaryotes, expression of p34^{cdc2}-encoding genes is higher in actively dividing cells (11–14). Terminally differentiated and senescent cells fail to express the p34^{cdc2} gene in response to mitogen stimulation (15), implying that regulation of the p34^{cdc2} gene is related to the state of cell differentiation. In plants, the level of p34^{cdc2} transcript is higher in meristematic tissues (16).

Induction of nodule primodia in roots of leguminous plants provides an example of postembryonic cell division that is both under plant developmental control as well as induced by infection with *Rhizobium*. Application of purified lipooligosaccharide molecules (Nod signals) specified by *Rhizobium* nodulation genes elicits cell division in the root cortex of legume roots and leads to the formation of nodule-like structures (17). Nodule-like structures can also be induced by treatment of legume roots with auxin-transport inhibitors (18). This result suggests that Nod signal molecules may alter the hormone balance in the root, leading, in turn, to the initiation of cortical cell division (19).

We report here on the isolation of two genes encoding homologs of yeast cdc2/CDC28 from soybean.† Both of these sequences complement *cdc28* mutation in yeast but do so with different efficiencies, confirming the functionality of these two genes. The pattern of expression for these two genes differs in root- and shoot-derived tissues and in response to *Rhizobium* infection and to treatment with plant hormones. These two functionally similar p34^{cdc2} genes of soybean appear regulated by two different developmental programs in shoot and root tissues.

MATERIALS AND METHODS

Plant Material and Bacterial and Yeast Strains. Soybean (*Glycine max* L. cv. Prize) seeds were grown in vermiculite in a growth chamber at 28°C. Whole roots were harvested from 3-day-old seedlings. Root tips (2 mm) were used for root meristem RNA preparation. Tissue from the elongation zone and lateral root meristem area was excised from roots of 4-day-old seedlings. Shoot meristem and mature leaf tissue

was harvested from 7-day-old seedlings. Unopened flowers were used for flower RNA preparation. Nodules were collected 21 days after inoculation with *Bradyrhizobium japonicum* (strain 61A76). *Saccharomyces cerevisiae cdc28* mutant strains 4048-21a (20) and cdc28-1N (21) were used for complementation experiments.

Isolation and Analysis of Soybean p34^{cdc2} cDNA Clones. On the basis of the conserved regions of known cdc2/CDC28 homologs (22), two degenerate oligonucleotides, A1, 5′-GGKGARGGHACRTACGG-3′, and A2, 5′-ATCWAT-CAAYAARTTYTG-3′ (see Fig. 1A), were synthesized and used for RNA-PCR (ref. 25) to amplify sequences homologous to cdc2/CDC28 from soybean nodule RNA. One microgram of nodule total RNA was reverse-transcribed into cDNAs that were used as templates for PCR amplification by using a RNA-PCR kit (Perkin–Elmer), according to the manufacturer's instructions. The amplified fragment (378 bp) was cloned into pUC18 for sequencing. This fragment was labeled with [α-³²P]dATP and used for screening a soybean nodule cDNA library made in λZap II (26). Two positive plaques were identified, and their cDNA inserts were excised and cloned into pBluescript SK(−) phagemids. The nucleotide sequences of both clones were determined by dideoxy-nucleotide chain-termination sequencing with Sequenase 2.0 (United States Biochemical).

Complementation of Yeast *cdc28* Mutations. cDNAs of the two soybean cdc2/CDC28 homologs cdc2-S5 and cdc2-S6 were subcloned into an expression vector pEMBLyex4 (27) containing URA3 as a selectable marker. Transformation of *S. cerevisiae cdc28* mutants (strain 4048-21a, ref. 20; strain cdc28-1N, ref. 21) was done by the method of Ito *et al.* (28). Transformants selected on medium without uracil were maintained at the permissive growth conditions (25°C). The ability of soybean cdc2-5 and cdc2-6 cDNAs to complement yeast *cdc28* mutations was tested by shifting the cells to the restrictive growth conditions (37°C). The growth curves of *cdc28* mutant cells transformed with soybean p34^{cdc2} sequences were determined by measuring the cell density of transformants at different time intervals after logarithmic-phase growing cells were shifted to 37°C.

Southern Hybridization. Because 3′ noncoding regions of cdc2-S5 and cdc2-S6 are distinct, two pairs of oligonucleotides (S5-1, 5′-GATGGCAAACGTGTTTAT-3′, and S5-2, 5′-ATGAAAAAGTAATATGCA-3′, for cdc2-S5, and S6-1, 5′-CTGAGGTGTCTTATTAGT-3′, and S6-2, 5′-CGGG-TAAATAGGTAACAA-3′, for cdc2-S6) were synthesized to amplify the 3′ noncoding sequences of the two clones (see Fig. 1A) by PCR. The PCR fragments (185 bp specific to cdc2-S5 and 157 bp specific to cdc2-S6) were isolated, labeled with [α-³²P]dATP, and used as probes for Southern hybridization with soybean genomic DNA. Hybridization was done in a phosphate/SDS buffer (29) at 62°C.

Determination of Relative Level of p34^{cdc2} Transcripts by RNA-PCR. The roots of 2-day-old seedlings were spot-inoculated with *B. japonicum* (30) or treated with different concentrations of phytohormones. Rhizobia were grown in liquid medium and pelleted, washed with sterile water, and resuspended in water containing a trace amount of India ink for marking. About 0.5 μl of the cell suspension was delivered to the potential nodule-forming zone (30). *Escherichia coli* DH5αF′ cells were used for mock inoculation as a control. Aliquots of 0.5 μl of phytohormone [α-NAA, 0.01–2 μg/μl, or β-NAA, 0.5 μg/μl, or 6-benzylaminopurine (BAP), 0.5 and 2.0 μg/μl] were applied by spot-inoculation. The root fragments (2 mm) marked by the ink were excised for RNA preparation at different time intervals after inoculation. Total RNA isolated from soybean tissues was used for monitoring expression of the two p34^{cdc2} genes by RNA-PCR (25).

To test the sensitivity and validity of the RNA-PCR method used for determining relative levels of the two p34^{cdc2} tran-

scripts, serial dilutions of total RNA ranging from 1 ng to 1 μg were used for RNA-PCR with the specific primers for each clone, as described above. Amplified fragments were separated on 3% agarose gel, transferred onto GeneScreen (NEN) membrane, and probed with ³²P-labeled fragments specific to soybean p34^{cdc2} genes. The conditions for PCR amplification were 94°C for 1 min, 48°C for 45 sec, 72°C for 1 min for 18 cycles. Under these conditions, the amount of amplified products was proportional to the RNA input (see Fig. 4A). The intensity of specific signals on the blots was quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Cloning and Characterization of Two Soybean cDNAs Encoding p34^{cdc2} Protein Kinases. Several highly conserved sequence domains have been identified in all p34^{cdc2} protein kinases (22). Two degenerate oligonucleotide primers (see *Materials and Methods*) corresponding to the conserved sequence motifs (GEGTYG and QNLLID) were synthesized and used for PCR with cDNAs reverse-transcribed from soybean nodule total RNA as template. The deduced amino acid sequence of the 378-bp PCR fragment obtained revealed extensive homology with other known p34^{cdc2} protein kinases (22). By using the 378-bp fragment as a probe, two positive clones (cdc2-S5 and cdc2-S6) were isolated by screening a total of 1.3 × 10⁶ recombinant plaques from a soybean nodule
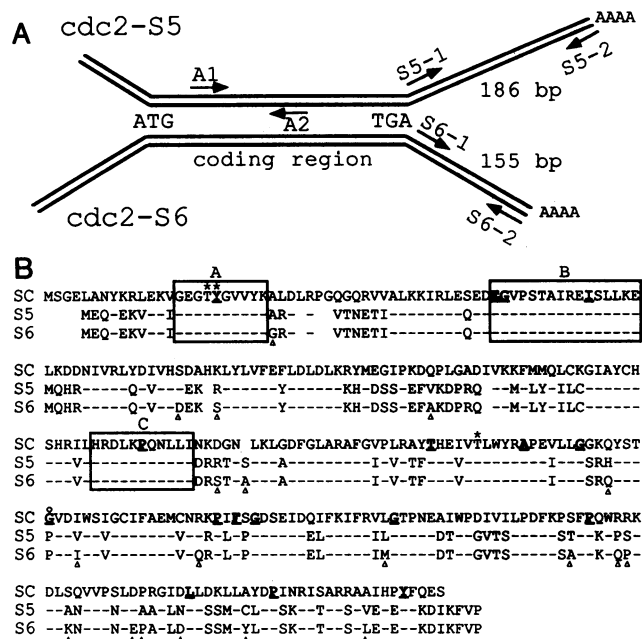

FIG. 1. (A) Schematic presentation of two soybean p34^{cdc2} protein kinase cDNAs showing high sequence homology in the coding region and unique 5′ and 3′ noncoding regions. A1 and A2 are degenerate oligonucleotide primers used for PCR amplification of a 378-bp fragment that was then used for screening a soybean nodule cDNA library. (i) S5-1 and S5-2 and (ii) S6-1 and S6-2 are two pairs of gene-specific primers used for PCR amplification of a particular sequence. (B) Structural homology between the two soybean p34^{cdc2} protein kinases (S5 and S6) and their comparison with the *S. cerevisiae* CDC28 protein kinase (SC; ref. 23). Three highly conserved domains including the ATP-binding region (box A), PSTAIR motif (box B), and catalytic domain (box C) are indicated. The conserved phosphorylation sites are marked by stars. Open arrowheads indicate the positions of amino acids that differ between the two soybean clones. Positions of amino acids that are essential for the activity of yeast cdc2/CDC28 protein kinases (24) are underlined. The open circle indicates substitution of a glycine essential in yeast protein by proline in soybean homologs.
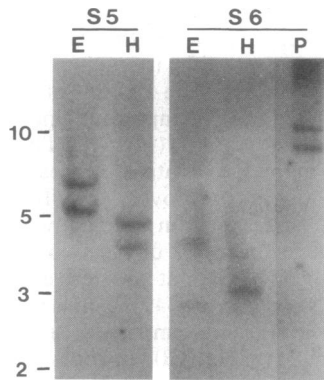
FIG. 2. Southern blot of soybean genomic DNA probed with cdc2-S5-(S5) and cdc2-S6-(S6) specific sequences. E, *Eco*RI; H, *Hin*dIII; P, *Pst* I. Molecular size is indicated in kbp.

cDNA library constructed in λZap II (26). Sequence analysis revealed that both sequences encode p34^{cdc2} protein kinases, as they share 61–65% and 80–88% sequence identity to yeast *cdc2/CDC28* and different plant p34^{cdc2} cDNA sequences, respectively (data not shown). The two sequences display 90% sequence identity with each other in the coding region. However, both the 5′ and 3′ noncoding regions of the two clones are unique (Fig. 1A), suggesting that they represent two distinct genes encoding p34^{cdc2} protein kinases. Southern blot analysis of soybean genomic DNA probed with the cdc2-S5- and cdc2-S6-specific sequences further confirmed the presence of at least two p34^{cdc2} genes (Fig. 2). There are two copies of each of these nonallelic genes in the soybean genome, and they are not closely linked. The homology in the coding region between cdc2-S5 and cdc2-S6 is higher than that between other plant p34^{cdc2} sequences (3). Thus, cdc2-S5

and cdc2-S6 appear to be closely related p34^{cdc2} genes rather than being members of two different subfamilies encoding cdc2-related protein kinases as recently identified in other systems (8, 9, 12, 31).

Soybean cdc2-S5 and cdc2-S6 are composed of 294 amino acids, 5 amino acids shorter than yeast CDC28 (Fig. 1B). Both sequences contain the highly conserved regions, including an ATP-binding region, a catalytic domain for protein kinases, and the hallmark sequence motif PSTAIR of the *cdc2/CDC28* gene family (22). In *Schizosaccharomyces pombe*, 17-amino acid residues of cdc2 protein kinase have been shown essential for function of this protein (24). All these essential amino acid residues are conserved in both soybean p34^{cdc2} clones, except one at position 184, where a glycine in the yeast sequence is substituted by proline in soybean (Fig. 1B). This proline residue is conserved among plant and animal p34^{cdc2} kinases, however (13, 14, 32, 33).

Functional Characterization of Soybean p34^{cdc2} Genes. To determine the functionality of the soybean cdc2-S5 and cdc2-S6, the ability of these genes to rescue a *cdc28* mutation in *S. cerevisiae* was tested. Both cdc2-S5 and cdc2-S6 inserts were cloned into the *Pst* I site of a yeast shuttle vector pEMBLyex4 (27) under control of the *GAL-CYC1* promoter. The resulting plasmids, pYCDC2-S5 and pYCDC2-S6, were introduced into the two temperature-sensitive *cdc28* mutants of yeast, 4078-21a (20) and cdc28-1N (21). Transformants containing either pYCDC2-S5 or pYCDC2-S6 grew at the nonpermissive temperature on yeast synthetic medium without uracil addition (Fig. 3 A and B). The *Arabidopsis* p34^{cdc2} sequence (14) used as a control also restored the *cdc28* mutation of strain 4078-21a (Fig. 3 A and B). Constructs containing cdc2-S5 and cdc2-S6 in antisense orientation or vector pEMBLyex4 alone did not complement the *cdc28* mutation (Fig. 3 A and B). Similar results were obtained when strain cdc28-1N was used for the complementation assay
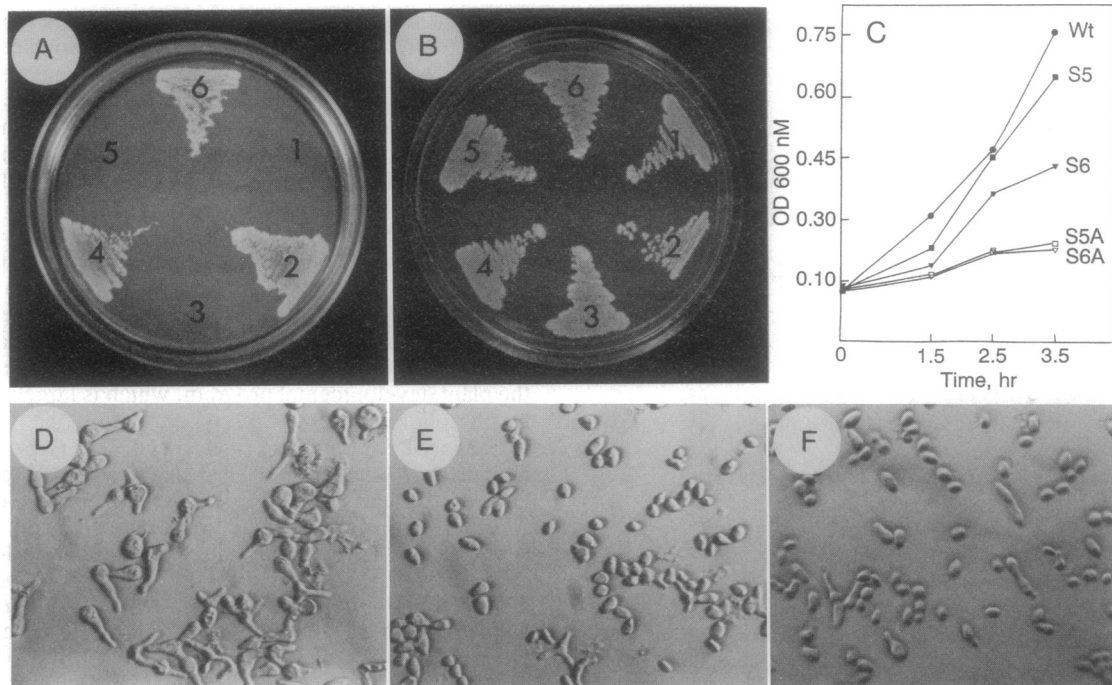

FIG. 3. Functional complementation of *S. cerevisiae cdc28* mutation by two soybean cdc2/CDC28 homologs and efficiency of complementation. Growth of yeast *cdc28* mutant (strain 4048-21a) at the restrictive temperature (37°C; A) and at the permissive temperature (25°C; B) containing pEMBLyex4 vector alone (*1*); pYCDC2-S5 (*2*); pYCDC2-S5 in antisense orientation (*3*); pYCDC2-S6 (*4*); pYCDC2-S6 in antisense orientation (*5*); *Arabidopsis* p34^{cdc2} homolog pCDC2Ara (*6*; ref. 14). (C) Growth kinetics of *cdc28* mutant cells harboring various plasmid constructs grown at the restrictive temperature. Wt, wildtype yeast cells containing pEMBLyex4 vector; S5, pYCDC2-S5; S5A, pYCDC2-S5 in antisense orientation; S6, pYCDC2-S6; S6, pYCDC2-S6 in antisense orientation. (*D–F*) Morphological phenotypes of cdc28-1N harboring pEMBLyex4 alone (*D*), showing elongated cells unable to divide; pCDC2-S5 (*E*) and pCDC2-S6 (*F*), showing round-cell phenotype indicative of dividing cells.

(Fig. 3 *D–F*). These studies demonstrated that the proteins encoded by soybean cdc2-S5 and cdc2-S6 genes possess the same function as the *CDC28* gene product in budding yeast, and thus both sequences are *bona fide* soybean functional homologs of the *cdc2/CDC28* genes. Growth characteristics of yeast strain 4078-21a harboring either pYCDC2-S5 or pYCDC-S6 showed different efficiencies in rescuing the *cdc28* mutation (Fig. 3*C*). Soybean cdc2-S5, containing yeast *cdc28* mutant cells, grew much faster than those harboring cdc2-S6 at the restrictive temperature (Fig. 3*C*). Because the activity of cdc2/CDC28 protein kinase depends on the interaction with specific cyclins and other components of maturation-promoting factor (ref. 7), the apparent differences in abilities of the two soybean p34^{cdc2} sequences to rescue the *cdc28* mutation imply different roles or regulation of these two sequences in cell-cycle control.

The Two Soybean p34^{cdc2} Genes are Differentially Expressed in Aerial Tissues and Roots and Root Nodules in Soybean. To address the relevance of the two cdc2/CDC28 homologs of soybean in cell-cycle control during plant development, we determined the relative expression level of the two soybean p34^{cdc2} transcripts in different tissues by using RNA-PCR. Taking advantage of the distinct 3' noncoding regions in the cdc2-S5 and cdc2-S6 genes (Fig. 1*B*), two pairs of specific oligonucleotide primers were synthesized (see *Materials and Methods*) to amplify the respective transcripts. As expected, a fragment of 185 bp was specifically amplified by using a pair of primers based on cdc2-S5 sequence, whereas a 157-bp fragment was detected with primers for cdc2-S6 (Fig. 4*B*, lanes S5 and S6, respectively). Fragments corresponding to

FIG. 4. (*A*) Determination of linearity between PCR products and RNA input by RNA-PCR. Total RNA from root ranging from 0.001 μg (lane 1); 0.005 μg (lane 2); 0.01 μg (lane 3); 0.05 μg (lane 4); 0.1 μg (lane 5); and 1 μg (lane 6) was used for RNA-PCR with specific primers for cdc2-S5 (S5) and cdc2-S6 (S6) as described. (*B*) Differential expression of two soybean p34^{cdc2} genes in different tissues. S5 and S6 sequences were amplified from total RNA (0.05 μg) from root by using specific primers for S5 and S6 separately (see Fig. 1*A*). RNA (0.05 μg each) from shoot meristem (Sm), root meristem (Rm), root elongation (Re), lateral root-forming zone (R1), mature leaf (L), total root (R), nodule (N), and flower (F) was used for RNA-PCR with mixed primers for both S5 and S6 sequences. Consistent results were obtained in four independent experiments.

both cdc2-S5 and cdc2-S6 were coamplified when the two pairs of primers were included in the same reaction, showing that the primers are specific to each p34^{cdc2} transcript of soybean (Fig. 4 *A* and *B*). The amount of amplified product was proportional to the RNA input, ranging from 1 ng to 1 μg used in the RNA-PCR reaction (Fig. 4*A*). This result provided a way to determine the relative expression level of the soybean p34^{cdc2} genes. Moreover, amplification of the two fragments in the same reaction tube allowed us to compare the relative expression levels of the two genes in different tissues and during development with less experimental error.

The transcripts of the two p34^{cdc2} genes were higher in root (Rm) and shoot (Sm) meristem tissues and lower in differentiated leaf tissue (L; Fig. 4*B*). In roots, both cdc2-S5 and cdc2-S6 transcripts were higher in the root meristem (Rm) and lateral root-forming zone (R1) and lower in the elongation zone (Re), where cells are more differentiated (Fig. 4*B*). Thus, expression of the two cdc2/CDC28 homologs of soybean appears to correlate with cell proliferation. However, in contrast to animal p34^{cdc2} genes, soybean cdc2-S5 and cdc2-S6 were expressed at a low level in fully differentiated cells. The p34^{cdc2} gene is not expressed in terminally differentiated tissues of chicken (11) or senescent human cells (15). Differences in the expression pattern of p34^{cdc2} gene in plants compared with that in animals may reflect differences in the status of cell differentiation. Most plant cells retain developmental plasticity, even when fully differentiated, whereas development of animal cells is usually irreversibly determined.

Quantification of the relative level of transcripts of cdc2-S5 and cdc2-S6 showed a correlation between expression of each gene with different plant developmental states and tissue types. The relative expression level of cdc2-S6 was higher in shoot-derived organs, whereas the transcript of cdc2-S5 is consistently higher in the root-derived organs (Fig. 4*B*). Thus, the two soybean p34^{cdc2} genes are differentially regulated in shoot and root tissues during plant development.

Evolution of genes with redundant function seems a common mechanism for plants to adapt to different developmental programs or to cope with environmental stress. Redundant genes have been identified for many fundamental processes of plant life, including those involved in biosynthesis of amino acids (34, 35) and the plant hormone ethylene (36, 37), as well as housekeeping genes (38). These genes are functionally redundant but differentially expressed during development. Therefore, different isoforms of these gene products may fulfill nonoverlapping functions during development (34–37). The differential regulation of the two functionally redundant genes for p34^{cdc2} suggests that the cell-division-control mechanism in plants has been integrated into the plant developmental program, which is largely controlled by phytohormones.

Enhanced Expression of Soybean cdc2-S5 Gene After Inoculation by *Rhizobium* and Treatment with Phytohormones. One of the early events in nodule formation elicited by *Rhizobium* infection is induction of cortical cell division of the host cells. The possible roles of cdc2-S5 and cdc2-S6 were studied by monitoring expression of each transcript after spot-inoculation on soybean roots with *Rhizobium* or spot-application of phytohormones. Transcripts of cdc2-S5 increased 3- to 4-fold after inoculation with *B. japonicum*, as compared with mock inoculation (Fig. 5). In contrast, the expression of cdc2-S6 was not much affected (Fig. 5). This result is consistent with the higher level expression of cdc2-S5 in roots and nodules (Fig. 4*B*). The enhanced cdc2-S5 expression is *Rhizobium*-specific, because mock inoculation with *E. coli* did not increase the level of cdc2-S5 transcript (Fig. 5), further supporting the observation that expression of two soybean p34^{cdc2} genes is differently regulated. The induction of the cdc2-S5 gene is temporally and
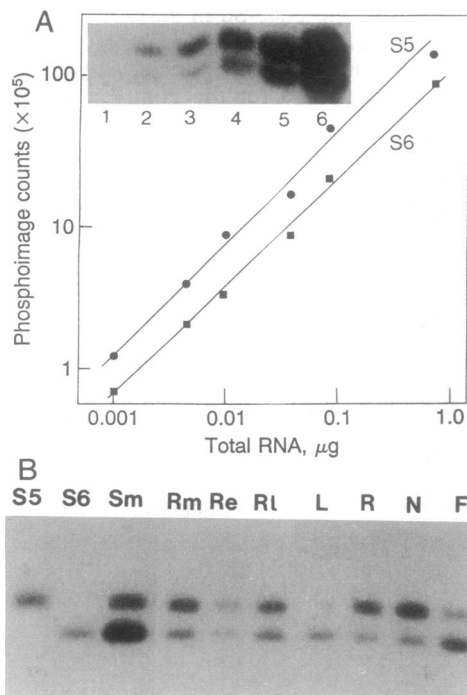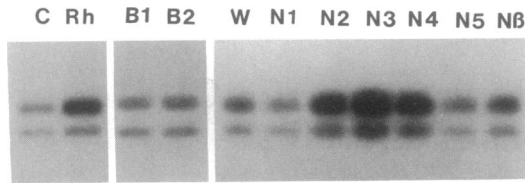
FIG. 5. Expression of soybean p34^{cdc2} genes after *Rhizobium* inoculation or phytohormone treatment. RNA (0.05 μg each) from 3-day-old roots spot-inoculated with *E. coli* (*C*); *B. japonicum* (Rh); or after treatment with BAP, 0.5 (B1) and 2.0 μg/μl (B2); water only (W); or different concentrations of α-NAA, 0.01 (N1); 0.1 (N2); 0.5 (N3), 1.0 (N4), and 2.0 (N5) μg/μl; or β-NAA 0.5 μg/μl (Nβ) was used for RNA-PCR as in Fig. 5. All lanes had mixtures of both S5 and S6 primers. The hormone treatment and *Rhizobium* inoculation were each for 24 hr. Three additional experiments with *Rhizobium* and six additional experiments with hormones gave similar results.

spatially correlated with the initiation of cortical cell divisions after inoculation with *Rhizobium* (30). Expression of cyclin B is also enhanced by *Rhizobium* infection (ref. 39; unpublished data). Thus, the host cell division is induced by *Rhizobium* via activating genes controlling cell-cycle progression.

Application of NAA on the root elongation zone, which is the site for *Rhizobium* infection, also revealed a differential regulation of expression of cdc2-S5 and cdc2-S6 genes. The preferential enhancement of cdc2-S5 gene expression in roots treated with 0.1–0.5 μg/μl concentrations of α-NAA and not by β-NAA (Fig. 5) indicates that up-regulation of plant p34^{cdc2} genes in root cells is mediated by auxins. This result has also been shown for the p34^{cdc2} gene in *Arabidopsis* roots (16) and cell-suspension culture of alfalfa treated with 2,4-dichlorophenoxyacetic acid (32). Mitogen-stimulated p34^{cdc2} gene expression has been observed in mammalian cells (15). On the other hand, application of BAP to the soybean roots did not affect the expression of any of these genes (Fig. 5). This result is consistent with the fact that soybean roots do not respond to BAP application for shoot differentiation as do roots of lotus (D.P.S.V., unpublished data). The up-regulation of cdc2-S6 in shoot-derived tissues remains to be determined by using tissue that responds to cytokinin. With the availability of a homolog of the *cdc2* gene expressed specifically in root meristems, it may now be possible to determine whether *Rhizobium* Nod factors directly affect the cell-cycle-control mechanism or whether this response is mediated by auxins (16, 19).

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