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Phosphorylation of Retinoblastoma-Related Protein by the Cyclin D/Cyclin-Dependent Kinase Complex Is Activated at the G1/S-Phase Transition in Tobacco[W]

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In mammals, D-type cyclin-associated kinases mainly regulate the G1/S transition by phosphorylating the retinoblastoma (Rb) protein. We previously demonstrated that in tobacco, cyclin D (Nicta; CycD3;3) is complexed with the PSTAIRE-containing cyclin-dependent kinase (CDKA) from tobacco. Here, we show that Nicta; CycD3;3–associated kinases phosphorylate both the tobacco Rb-related protein (NtRb1) and histone H1. Although NtRb1 kinase activity was detected only during the middle G1- to early S-phase, histone H1 kinase activity was observed as two peaks in G1- to S-phase and G2/M- to M-phase. Importantly, we show that the proportion of cells in the G1-phase was reduced in transgenic Bright Yellow-2 cells overexpressing *Nicta; CycD3;3-GFP*. Mutational analyses revealed that phosphorylation of Thr-191 in Nicta; CycD3;3 possibly is required for both full kinase activity and localization predominantly to the nucleus. These data suggest that Nicta; CycD3;3 acts as a rate-limiting regulator in the G1/S transition by forming active complexes with CDKA or its related kinases to phosphorylate Rb-related protein and potentially plays a novel role during G2/M and mitosis.

INTRODUCTION

Cell division plays a crucial role in the growth and development of multicellular organisms (Meyerowitz, 1997; Polymenis and Schmidt, 1999). Plants have unique properties with respect to cell growth, plasticity of body plan, and organization and development, which suggests that they also may have different controls in place to regulate cell division. Plant cells potentially have the continuous proliferative capacity that is found postembryonically in shoot and root apical meristems. Additionally, plants are able to change their fate in response to environmental signals throughout the life cycle (Deng, 1994; Francis, 1998). However, the external factors that control cell division, and ultimately plant growth, are unknown.

In mammals, changes in the cell cycle machinery play an important role in controlling growth in response to extracellular signals (Sherr, 1994). Progression through the mammalian cell cycle is driven by the periodic activity of cyclin-dependent kinases (CDKs). At the late G1 restriction point, cells must interpret extracellular signals and commit to another round of division or adopt alternative differentiation pathways. The retinoblastoma (Rb) protein binds to members of the E2F transcription factor family, and the resulting Rb-E2F complexes block the transcription of E2F-regulated genes (Dyson, 1998; Kaelin, 1999). In response to growth-promoting signals, cyclin D is synthesized to form active complexes with CDK4 and CDK6, which phosphorylate Rb in mid to late G1-phase, alleviating its repressor function on E2F-controlled gene transcription and stimulating entry into S-phase. Therefore, cyclin D acts as a growth factor sensor that integrates environmental signals with the cell cycle machinery (Sherr, 1994).

Elucidation of the mechanisms that govern the G1/S transition in plants is essential to understanding plant growth and development. The recent identification of plant homologs of Rb-related protein (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997; Nakagami et al., 1999), E2F (Ramirez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000; de Jager et al., 2001), and cyclin D (Dahl et al., 1995; Soni et al., 1995; Nakagami et al., 1999; Sorrell et al., 1999) suggests that the mechanisms that control the G1/S transition in plants are similar to those that act in mammals. mRNA levels of the plant cyclin D genes also are controlled by external growth signals, supporting this idea.

The Arabidopsis cyclin D, *Arath; CycD3;1*, is induced by the plant hormone cytokinin and a carbon source (Suc), whereas *Arath; CycD2;1* is induced by Suc alone (Soni et al., 1995;

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Riou-Khamlichi et al., 1999, 2000). In addition, ectopic expression of *Arath; CycD3;1* alters the requirement for cytokinin in the induction of calli from leaf explants (Riou-Khamlichi et al., 1999). Overexpression of *Arath; CycD2;1* in tobacco reduces the time of G1-phase and increases the overall plant growth rate (Cockcroft et al., 2000). These observations suggest that plant D-type cyclins modulate cell cycle progression in response to extracellular signals during the G1-phase, as is the case in mammals.

Although cyclin D activity is critical for cell cycle control, development, and growth in plants, very little is known about cyclin D activity on Rb-related protein, which is the most likely candidate to play a significant role in the control of the G1/S-phase transition. In mammals, the G1/S transition is controlled by CDK4 and CDK6 associated with cyclin D and CDK2 associated with cyclin E, and the G2/M transition is controlled by cdc2 (CDK1) associated with cyclin A and cyclin B (Pines, 1995). By contrast, CDKA (cdc2a)-associated histone H1 kinase activity was increased at both the G1/S and G2/M boundaries in alfalfa (Magyar et al., 1993, 1997), suggesting that CDKA is involved in both of these transitions in plants.

Plants contain multiple CDKs, including those of the PSTAIRE type, known as cdc2a or CDKA (Doonan and Fobert, 1997; Joubes et al., 2000). We demonstrated previously that the tobacco cyclin D/cdc2a (NtcycD3-1/cdc2Nt1) complex phosphorylates tobacco Rb-related protein (NtRb1) in vitro (Nakagami et al., 1999). In accordance with the nomenclature for plant CDKs (Joubes et al., 2000) and cyclins (Renaudin et al., 1996), we renamed *cdc2Nt1* as *Nicta; CDKA;3* and *NtcycD3-1* as *Nicta; CycD3;3*.

In this study, we demonstrate that Nicta; CycD3;3–associated kinases phosphorylate NtRb1 during the middle G1- to early S-phase and that the phosphorylation of histone H1 occurs at two discrete points within the cell cycle: at the G1/S and G2/M boundaries. Acceleration of the G1-phase progression was observed in tobacco Bright Yellow-2 (BY-2) cells overexpressing *Nicta; CycD3;3-GFP*. Mutational analyses revealed that Thr-191 in Nicta; CycD3;3 is likely to be involved in both full kinase activity in complex with CDKA and its localization to the nucleus.

RESULTS

The Nicta; CycD3;3/Nicta; CDKA;3 Complex Phosphorylates Both Histone H1 and NtRb1

Using a baculovirus expression system, we demonstrated previously that Nicta; CycD3;3 forms an active complex with Nicta; CDKA;3, a member of CDKA (cdc2a) group that exhibits kinase activity against the tobacco Rb-related protein NtRb1 (Nakagami et al., 1999). Because we found that the C-terminal region of NtRb1 (amino acids 823 to 961) was phosphorylated equally by the Nicta; CycD3;3/Nicta; CDKA;3 complex in vitro as the region used previously (amino acids 374 to 961) (Nakagami et al., 1999), we opted to continue our experiments using the C-terminal region as a substrate. In this study, we also evaluated the kinase activity of the Nicta; CycD3;3/Nicta; CDKA;3 complex on histone H1 in vitro.

His-tagged Nicta;CDKA;3 was coexpressed in insect cells with FLAG-tagged Nicta; CycD3;3 using a baculovirus expression system (Figure 1A). A human cyclin D1/CDK4 complex, used as a control, phosphorylated human Rb but not histone H1 (Figure 1A), consistent with a previous result describing histone H1 as a poor substrate (Kato et al., 1993). By contrast, phosphorylation of both histone H1 and NtRb1 was detected in lysates prepared from insect cells expressing the Nicta; CycD3;3/Nicta; CDKA;3 complex, whereas control lysates containing Nicta; CycD3;3, Nicta; CDKA;3, or vector alone did not exhibit any kinase activity.

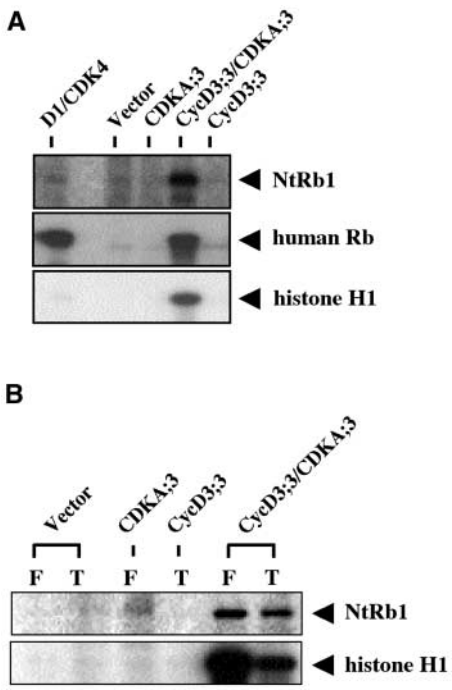

Figure 1. The Nicta; CycD3;3/CDKA Complex Phosphorylates both Histone H1 and Rb-Related Protein in Vitro.

(A) Lysates from insect cells expressing FLAG-tagged Nicta; CycD3;3 and His-tagged Nicta; CDKA;3 were assayed for phosphorylation of histone H1, NtRB1, and human Rb protein. Labeled histone H1 and NtRb1 were resolved on denaturing polyacrylamide gels and visualized by autoradiography. The human cyclin D1/CDK4 complex was used as a control.

(B) Kinase activity of the Nicta; CycD3;3/CDKA complex. Proteins were purified from insect cell lysates with either the anti-FLAG M2 affinity gel (F) or the TALON metal affinity resin (T). Purified proteins were incubated with histone H1 and GST-NtRb1 at 30°C for 10 min in the kinase buffer containing γ-^{32}P-ATP.

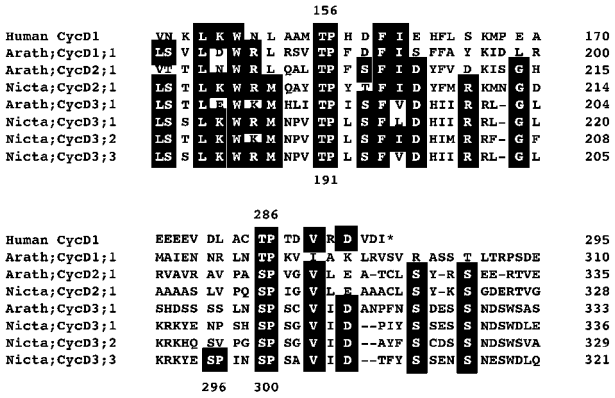

Figure 2. Alignment of the Predicted Amino Acid Sequences of Human Cyclin D1 with Several Plant Cyclin D Proteins within the Putative Phosphorylation Sites.

Amino acids that are identical in at least five of the eight sequences are shown in black boxes. Gaps (dashes) were introduced to optimize the alignment. The asterisk indicates a terminal codon. Numbers above and below the sequences indicate amino acid residues of human cyclin D1 and Nicta; CycD3;3, respectively.

To verify that the Nicta; CycD3;3/Nicta; CDKA;3 complex is responsible for the kinase activity seen in lysates, the complex was purified with an anti-FLAG M2 affinity gel or TALON metal affinity resin. Although proteins purified from cells transfected with Nicta; CycD3;3, Nicta; CDKA;3, or vector alone did not exhibit kinase activity, proteins purified from insect cells expressing both Nicta; CycD3;3 and Nicta; CDKA;3 exhibited kinase activity against histone H1 and NtRb1 (Figure 1B). The presence of a weak signal in Nicta; CDKA;3 alone is most likely the result of complex formation between Nicta; CDKA;3 and endogenous cyclins derived from insect cells, resulting in some weak kinase activity.

Thr-191 of Nicta; CycD3;3 Is Required for Kinase Activity in Vitro

In mammals, cyclin D–dependent kinase activity is subject to multiple regulatory controls. Phosphorylation of Thr-156 in human cyclin D1 is essential for the formation of an active complex with CDK4 and its nuclear localization (Diehl and Sherr, 1997). Phosphorylation of Thr-286 is involved in nuclear export and degradation (Diehl et al., 1997). Alignment of the amino acid sequences of several plant D-type cyclins with that of human cyclin D1 revealed that these putative phosphorylation sites are conserved across the species (Figure 2).

To determine whether these residues also are important for the function of plant D-type cyclins, mutations were introduced into the putative phosphorylation sites in Nicta; CycD3;3. We replaced Thr-191 of Nicta; CycD3;3, which corresponds to Thr-156 of human cyclin D1, with the nonphosphorylatable amino acid Ala (T191A). Thr-191 was further mutated to Ser (T191S) to provide a different phosphorylatable residue. Ser-296 and Ser-300 in Nicta; CycD3;3, which correspond to Thr-286 of human cyclin D1, were replaced with Ala (S296,300A). The triple mutant (T191A,S296,300A), in which all three phosphorylatable sites are mutated, also was constructed.

We evaluated whether these mutants form active complexes with Nicta; CDKA;3. His-tagged wild-type and mutant Nicta; CycD3;3 were coexpressed with FLAG-tagged Nicta; CDKA;3 in insect cells (Figure 3A). Equivalent levels of

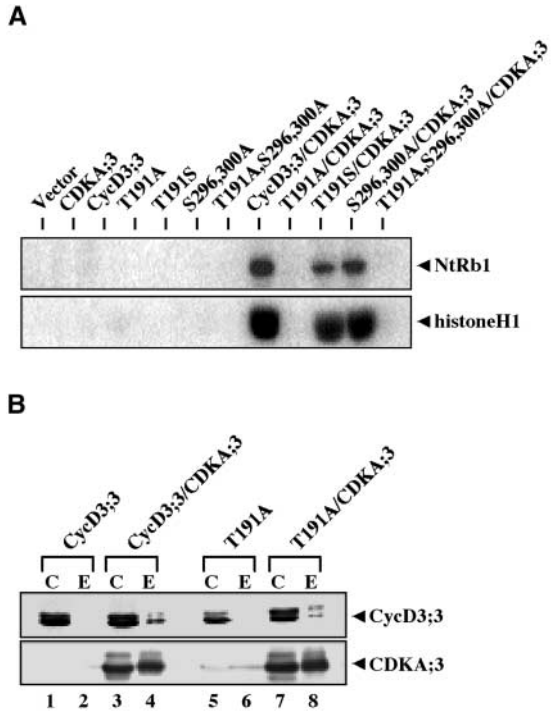

Figure 3. Kinase Activity of Mutant Nicta; CycD3;3 in Vitro.

(A) Lysates from insect cells expressing the indicated proteins were assayed for the phosphorylation of histone H1 and NtRb1. Labeled histone H1 and NtRb1 were resolved on denaturing polyacrylamide gels and visualized by autoradiography.

(B) Mutant Nicta; CycD3;3 forms a complex with Nicta; CDKA;3. Lysates from insect cells expressing FLAG-tagged Nicta; CDKA;3 (lanes 3, 4, 7, and 8) and either His-tagged wild-type Nicta; CycD3;3 (lanes 1 to 4) or His-tagged mutant Nicta; CycD3;3 (T191) (lanes 5 to 8) were purified on an anti-FLAG M2 affinity gel. Crude extracts (C) and elute fractions (E) were separated on 10% SDS–polyacrylamide gels. FLAG-tagged Nicta; CDKA;3 was detected by immunoblotting with an anti-FLAG M2 monoclonal antibody (bottom gel); His-tagged wild-type or mutant Nicta; CycD3;3 were detected by immunoblotting with a His tag–specific antibody (top gel).

wild-type and mutant Nicta; CycD3;3 expression were detected in insect cells by immunoblotting with a His tag–specific antibody (data not shown). Although lysates containing Nicta; CDKA;3 and either wild-type or mutant Nicta; CycD3;3 (T191S or S296,300A) exhibited kinase activity against histone H1 and NtRb1, lysates containing Nicta; CDKA;3 with Nicta; CycD3;3 (T191A) or Nicta; CycD3;3 (T191A,S296, 300A) had no kinase activity. The Nicta; CycD3;3 (T191A) mutant retained the ability to bind Nicta; CDKA;3, as shown by an in vitro binding assay (Figure 3B).

Nicta; CycD3;3 (T191A) Localizes Predominantly to the Cytoplasm

The subcellular localization of wild-type and mutant Nicta; CycD3;3 proteins fused to green fluorescent protein (GFP) was examined in exponentially growing tobacco BY-2 cells and compared with that in BY-2 cells expressing GFP alone. Expression of the GFP-fused proteins was analyzed by immunoblotting with a GFP-specific antibody, and expression of polypeptides corresponding to the expected size was determined for each of the proteins (data not shown).

Fluorescence microscopy of the BY-2 cells expressing GFP alone showed that GFP fluorescence was observed in both the nucleus and the cytoplasm (Figure 4). By contrast, green fluorescence exhibited by Nicta; CycD3;3-GFP and Nicta; CycD3;3 (S296,300A)-GFP was found primarily in the nucleus, whereas that exhibited by Nicta; CycD3;3 (T191A)-GFP and Nicta; CycD3;3 (T191A,S296,300A)-GFP was localized predominantly in the cytoplasm. These results suggest that phosphorylation of Thr-191 is required for the nuclear localization of Nicta; CycD3;3.

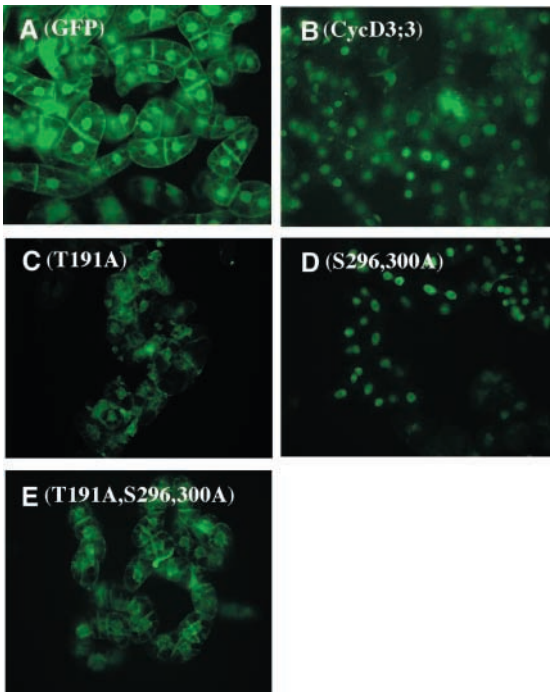

Figure 4. Distribution of GFP Fluorescence in Tobacco BY-2 Cells.

Several transformants of each construct were screened for GFP fluorescence, which was obtained from exponentially growing cells under identical acquisition and image-processing conditions.
(A) GFP.
(B) Nicta; CycD3;3-GFP.
(C) Nicta; CycD3;3 (T191A)-GFP.
(D) Nicta; CycD3;3 (S296,300A)-GFP.
(E) Nicta; CycD3;3 (T191A,S296,300A)-GFP.

Thr-191 of Nicta; CycD3;3 Is Required for Full Kinase Activity in Immunoprecipitates from Cell Extracts

GFP-fused Nicta; CycD3;3 proteins were immunoprecipitated from exponentially growing cells with a GFP-specific antibody and immunoblotted subsequently with an anti-PSTAIRE antibody recognizing the conserved PSTAIRE motif of CDKA. The anti-PSTAIRE antibody cross-reacted with a polypeptide of ~34 kD in immunoprecipitates from the BY-2 cells expressing GFP-fused Nicta; CycD3;3 proteins (Figure 5). However, no band was detected with an anti-PSTAIRE antibody in the case of wild-type BY-2 cells and BY-2 cells expressing GFP alone. This finding suggests that GFP-fused wild-type and mutant Nicta; CycD3;3 proteins bind to CDKA in tobacco cells.

Phosphorylation of histone H1 was detected in immunoprecipitates prepared from BY-2 cells expressing Nicta; CycD3;3-GFP and Nicta; CycD3;3 (S296,300A)-GFP, whereas immunoprecipitates from BY-2 cells expressing either Nicta; CycD3;3 (T191A)-GFP or Nicta; CycD3;3 (T191A, S296,300A)-GFP demonstrated only weak kinase activity (Figure 5). Phosphorylation of NtRb1 also was reduced significantly in immunoprecipitates from BY-2 cells expressing Nicta; CycD3;3 (T191A)-GFP compared with cells expressing Nicta; CycD3;3-GFP. These results suggest that phosphorylation of Thr-191 in Nicta; CycD3;3 is required for full kinase activity in immunoprecipitates from cell extracts.

Nicta; CycD3;3-Associated Kinases Phosphorylate Rb-Related Protein during the Middle G1- to Early S-Phase

To examine whether NtRb1 is phosphorylated by Nicta; CycD3;3-associated kinases from cells in G1- and S-phases, tobacco BY-2 cells were synchronized using the DNA polymerase α inhibitor aphidicolin and the anti-tubulin drug propyzamide (Planchaisa et al., 2000). After removal of the inhibitors, BY-2 cells were harvested at 2-h intervals, and cell cycle progression was evaluated by measuring cellular DNA content using a laser scanning cytometer (LSC) (Figure 6A).

Nicta; CycD3;3–associated kinases were obtained by immunoprecipitation with an antibody raised against the 13 N-terminal amino acids of Nicta; CycD3;3 (Nakagami et al., 1999). Immunoprecipitates obtained with the Nicta; CycD3;3–specific antibody exhibited kinase activity against both histone H1 and NtRb1. Phosphorylation of histone H1 occurred in two peaks at G1- to S-phase and G2/M- to M-phase, whereas NtRb1 kinase activity was present only during G1- to S-phase (Figure 6C). The immunoprecipitated kinase activity resulted from Nicta; CycD3;3–associated kinases, because preimmune serum immunoprecipitated only background kinase activity and no phosphorylated bands were detected in the assay without the addition of the substrates (data not shown).

Because the yeast p13^{SUC1} protein binds various plant CDKA-related kinases (De Veylder et al., 1997), we examined the ability of p13^{SUC1}-bound kinases to exhibit kinase activity on NtRb1. The kinases that associated with p13^{SUC1} demonstrated two peaks of histone H1 kinase activity, as did the immunoprecipitates obtained with the Nicta; CycD3;3–specific antibody, consistent with the results reported for alfalfa (Magyar et al., 1993, 1997). Importantly, phosphorylation of NtRb1 by p13^{SUC1}-associated kinases occurred with remarkable specificity at G1- to S-phase, and very weak activity was found at G2/M-phase compared with the activity against histone H1. It should be noted that p13^{SUC1}-bound kinases did not phosphorylate NtRb1until 8 h, although Nicta; CycD3;3–associated kinases exhibited kinase activity from 6 h and peaked at 8 to 12 h (Figure 6C).

There is growing evidence that Rb also has effects during S-phase and mitosis in mammals (Lukas et al., 1999; Zhang et al., 2000). Therefore, tobacco BY-2 cells were synchronized using aphidicolin only to assay Nicta; CycD3;3–associated kinases and p13^{SUC1}-bound kinases during S- to G1-phase more precisely. After removal of aphidicolin, BY-2 cells were harvested at 2-h intervals followed by measurement of cellular DNA content using a LSC (Figure 6B). The removal of aphidicolin resulted in synchronous progression through the cell cycle, with a clear peak of the mitotic index (~35%) after 8 to 10 h.

Although Nicta; CycD3;3–associated kinases phosphorylated histone H1 during S-phase to the next (G1) phase, their kinase activities were reduced twice at 2 to 4 h and 12 h. However, NtRb1 was phosphorylated only at 14 h, corresponding to the middle to late G1-phase (Figure 6D), which is consistent with the result obtained by synchronization of BY-2 cells treated with aphidicolin and propyzamide (Figure 6C). p13^{SUC1}-bound kinases also phosphorylated histone H1 during all phases tested, and their kinase activities were reduced at 2 to 4 h and 12 to 14 h, whereas NtRb1 was phosphorylated only at 0 to 2 h, corresponding to middle to late S-phase (Figure 6D). Thus, Nicta; CycD3;3–associated kinases phosphorylate NtRb1 during a very short period in middle G1-phase to early S-phase, and p13^{SUC1}-bound kinases exhibit kinase activity on NtRb1 during late G1-phase to late S-phase.

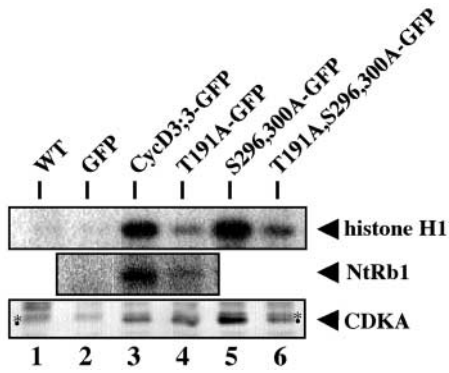

Figure 5. Thr-191 in Nicta; CycD3;3 Is Essential for Full Kinase Activity in Immunoprecipitates from Cell Extracts.

Immunoprecipitates from transformed tobacco BY-2 cells prepared with the GFP-specific antibody were assayed for histone H1 and NtRb1 phosphorylation. Labeled histone H1 and NtRb1 were resolved on denaturing polyacrylamide gels and visualized by autoradiography. CDKA was detected by immunoblotting with an anti-PSTAIRE antibody followed by immunoprecipitation with a GFP-specific antibody. Asterisks indicate nonspecific bands, and dots represent CDKA. Lane 1, wild-type (WT) BY-2 cells; lane 2, GFP; lane 3, Nicta; CycD3;3-GFP; lane 4, Nicta; CycD3;3 (T191A)-GFP; lane 5, Nicta; CycD3;3 (S296,300A)-GFP; lane 6, Nicta; CycD3;3 (T191A,S296,300A)-GFP.

Overexpression of Nicta; CycD3;3 Reduces the Proportion of Cells in G1-Phase

Transient overexpression of human cyclin D1 can shorten the G1-phase (Diehl and Sherr, 1997), whereas mutant cyclin D1 (T156A) inhibits the ability of human cells to enter S-phase in a dominant fashion. Therefore, we examined the effects of the overexpression of tobacco *Nicta; CycD3;3* by generating transgenic BY-2 cells that stably express GFP-fused wild-type or mutant Nicta; CycD3;3 driven by the 35S promoter of *Cauliflower mosaic virus*, and the cell cycle progressions of these transformants were determined with a LSC (Figure 7).

The cell lines that overexpress *Nicta; CycD3;3* (*T191A*)*-GFP*, named T191A, had no discernible cell cycle effects on growing cells compared with the control cells that express GFP alone, named GFP (Figure 7, Table 1). By contrast, overexpression of *Nicta; CycD3;3-GFP*, named CycD3;3, and *Nicta; CycD3;3* (*S296,300A*)*-GFP*, named S296,300A, both of which form the active complexes with CDKA, reduced the proportion of cells in G1-phase and increased the proportion of S-phase cells (Figure 7, Table 1). Furthermore, the doubling times of CycD3;3 and S296,300A were reduced relative to that of GFP. For the S296,300A mutant, this was particularly marked, corresponding to a 25% reduction in doubling time. These results suggest that Nicta;

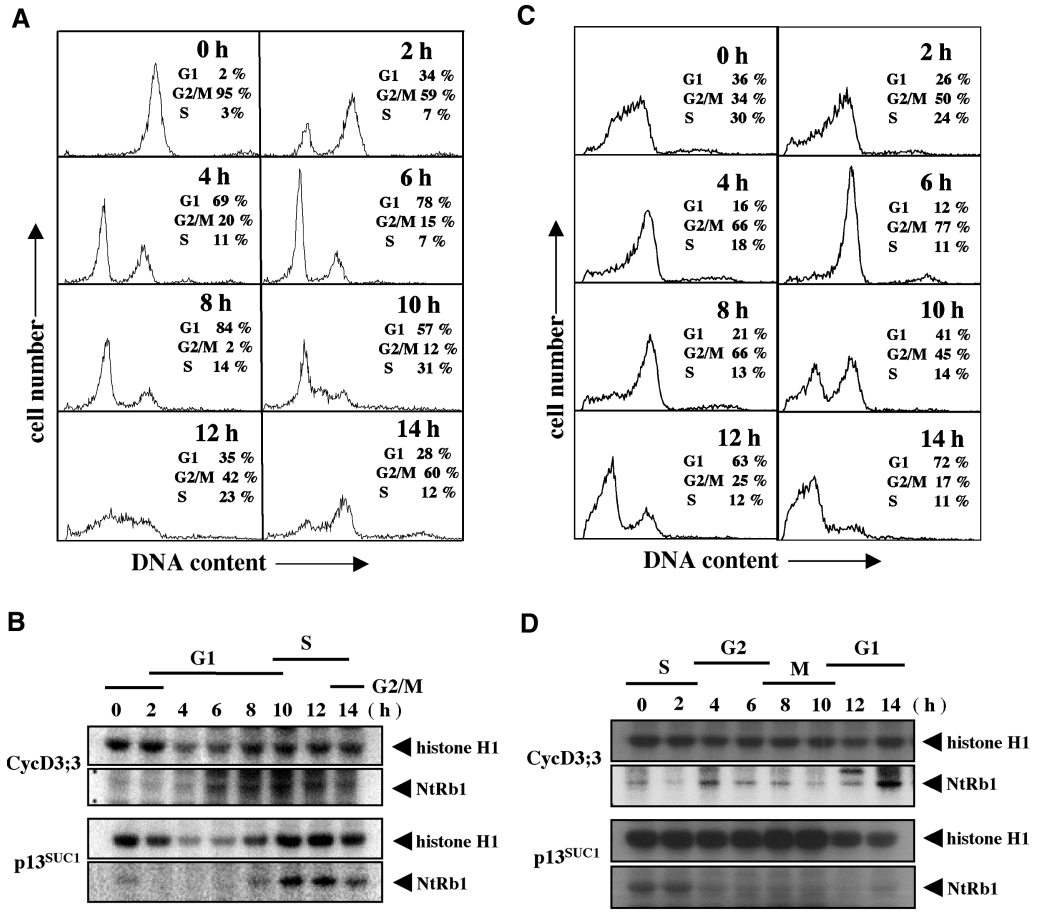

Figure 6. Rb-Related Protein Phosphorylation Is Increased at the G1/S-Phase Transition.

(A) and **(B)** DNA histograms determined by LSC analysis after releasing the G2/M block imposed by treatment with both aphidicolin and propyzamide **(A)** and after releasing the S-phase block by treatment with aphidicolin **(B)**. Cell cycle profiles were determined without counting the polyploid cells. The percentages of cells in G1-, S-, and G2/M-phase are shown in each panel.

(C) and **(D)** Anti-Nicta; CycD3;3 immunoprecipitates and the p13^{SUC1}-bound proteins prepared from synchronized tobacco BY-2 cells were assayed for kinase activity against histone H1 and NtRb1. Labeled histone H1 and NtRb1 were resolved on denaturing polyacrylamide gels and visualized by autoradiography.

CycD3;3–associated kinases promote the cell cycle progression from G1- to S-phase and that Nicta; CycD3;3 regulates the G1/S transition in tobacco cells.

We also measured the cell number for each cell line for 8 days after subculturing under identical conditions in culture medium supplemented with the antibiotic kanamycin (Figure 8). S296,300A showed faster growth and reached higher cell density than CycD3;3. T191A grew more slowly than both S296,300A and CycD3;3 during the first 4 days, but ultimately it reached the highest cell density. We did not observe a clear difference in cell size between these transgenic cell lines and wild-type BY-2 cells, so overexpression of *Nicta; CycD3;3* and its mutants appears to have no effect on cell size.

DISCUSSION

In plants, D-type cyclins respond to signals such as cytokinin and Suc at the G1/S boundary (Riou-Khamlichi et al., 1999, 2000). Therefore, cellular mechanisms that sense extracellular signals must be integrated with the cell cycle control machinery during the G1/S transition in plants. Although the existence of cyclin D– and Rb-related genes suggests that the G1/S control mechanisms in plants may be similar to those in mammals, this is by no means confirmed (Gutierrez, 1998; de Jager and Murray, 1999; Mironov et al., 1999). In particular, the phosphorylation of Rb by plant cyclin D complexes prepared from cell extracts has not been

demonstrated. In this study, we have demonstrated that, in tobacco, Nicta; CycD3;3–associated kinases phosphorylate Rb-related protein during middle G1-phase to early S-phase.

Mammalian cyclin D associates with CDK4 and CDK6, which contain the sequence P(I/L)ST(V/I)RE, to form active complexes that selectively phosphorylate Rb protein (Sherr, 1994). By contrast, in tobacco, Nicta; CycD3;3 forms an active complex with Nicta; CDKA;3 that has kinase activity against NtRb1 in vitro (Nakagami et al., 1999). Here, we have shown that CycD3;3 complexes phosphorylate both histone H1 and NtRb1 in immunoprecipitates from cell extracts (Figures 6C and 6D).

Arabidopsis CycD2 and CycD3 proteins have been shown to interact with the PSTAIRE-containing CDKA in vivo and to phosphorylate histone H1 (Cockcroft et al., 2000; Riou-Khamlichi et al., 2000; Sandra Healy et al., 2001), but we report a plant D-type cyclin-associated kinase that is capable of phosphorylating both histone H1 and Rb-related protein. During the course of this work, Boniotti and Gutierrez (2001) reported that a plant Rb-related protein is phosphorylated by kinase complexes that contain Arabidopsis CycD2 and CDKA, but they were unable to conclude that the CycD2/CDKA complexes were the responsible components.

Rb contains several functional domains. Domains A and B are highly conserved from human to plants, and they form a "pocket" domain that binds many cellular factors, which is critical to the function of Rb (Weinberg, 1995). In mammals, sequential phosphorylation of Rb, by cyclin D/CDK4,6 complexes and cyclin E/CDK2 complex, are required to prevent Rb from binding and inactivating E2F (Harbour et al., 1999). It should be noted that the kinase complexes prepared from Arabidopsis phosphorylate the *A/B* pocket and the C-terminal region as well as the C-terminal region alone of maize Rb-related protein, but the complexes were not capable of phosphorylating Rb substrate containing only the *A/B* pocket region (Boniotti and Gutierrez, 2001).

Table 1. Characteristics of Tobacco BY-2 Cell Lines

Cell	Doubling Time (h)	Percentage of Cells in Cell Cycle Phases		
		G1	S	G2/M
GFP	40.6 ± 4.3	50 (20.3)	14 (5.7)	36 (14.6)
CycD3;3-GFP	36.6 ± 4.4	37 (13.5)	27 (9.9)	36 (13.2)
T191A-GFP	41.4 ± 7.6	48 (19.9)	14 (5.8)	38 (15.7)
S296,300A-GFP	29.9 ± 3.7	31 (9.3)	32 (9.6)	37 (11.1)

The doubling time of tobacco BY-2 cells transformed with each construct were determined by measuring cell numbers. Data shown are means ± SD obtained from at least four sampling times with three independent samples for each time point. The percentage of cells in G1-, S-, and G2/M-phases was determined by LSC analysis. The data presented are a representative sample of many experiments. Mean times for each cell cycle phase were calculated assuming that all cells in the culture were cycling and are shown in hours.

These findings suggest that phosphorylation within the plant *A/B* pocket region may be allowed by its conformational change caused primarily by phosphorylation in the C-terminal region. We used the C-terminal region alone as a substrate, and it remains to be determined whether the timing and pattern of phosphorylation of intact Rb-related protein are different from those of the C-terminal region alone. In addition, it will be interesting to determine the relative contributions of each of 13 putative phosphorylation sites in tobacco Rb-related protein to the release of important regulators from the pocket, which may stimulate the commitment to progress through G1- into S-phase.

In agreement with the findings that *Nicta; CycD3;1* and *Nicta; CycD2;1* transcripts accumulate during G2/M- to M-phase (Sorrell et al., 1999), the Nicta; CycD3;3–associated kinases phosphorylated histone H1 at both the G1/S and G2/M boundaries, suggesting a possible role for D-type cyclins in G2/M- to M-phase regulation in plants.

Because the fission yeast protein $p13^{SUC1}$ binds largely to CDKA-related proteins containing a conserved PSTAIRE motif (De Veylder et al., 1997), we tested whether $p13^{SUC1}$-bound kinases could phosphorylate NtRb1. Surprisingly, although $p13^{SUC1}$-bound proteins in plants exhibited kinase activity against Rb-related protein, this activity was observed mainly with $p13^{SUC1}$-bound kinases purified from late G1- to S-phase cells. Because the $p13^{SUC1}$-associated kinases presumably contain Nicta; CycD3;3, NtRb1 kinase activity of the $p13^{SUC1}$-associated kinases was present at later times than Nicta; CycD3;3–associated NtRb1 kinase activity (Figures 6C and 6D).

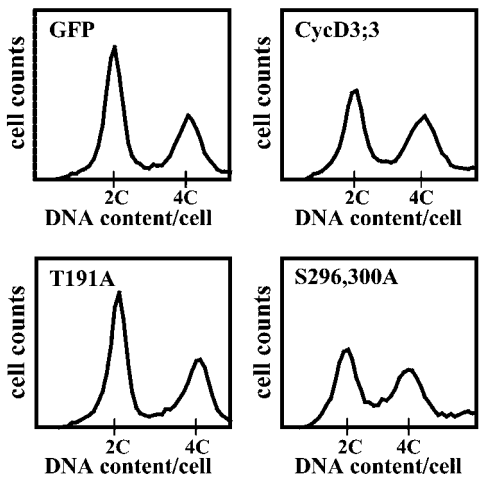

Figure 7. Overexpression of Nicta; CycD3;3 Reduces the Proportion of G1-Phase Cells.

DNA histograms for each transformant were determined by LSC analysis from exponentially growing cells. Representative samples from many replicate experiments are shown.

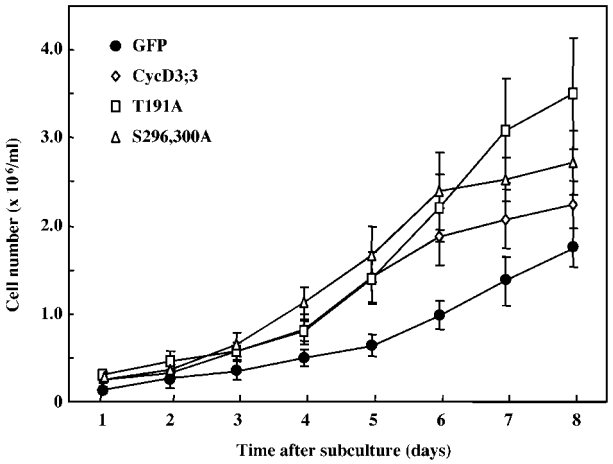

Figure 8. Growth Curve of Tobacco BY-2 Cell Lines Overexpressing Nicta; CycD3;3 and Its Mutants.

Early stationary-phase (7 days) cells were subcultured, and cell growth was monitored for cell lines overexpressing Nicta; CycD3;3-GFP (CycD3;3; open diamonds), Nicta; CycD3;3 (T191A)-GFP (T191A; open squares), Nicta; CycD3;3 (S296,300A)-GFP (S296, 300A; open triangles), and GFP alone (GFP; closed circles) by determination of the number of cells each day after subculturing. Data shown are from representative cell lines derived from each clone selected from several cell lines. Error bars indicate SE ($n = 15$).

One possible explanation for this finding is that the weak activity of the Nicta; CycD3;3–associated kinases bound to $p13^{SUC1}$ would not be detected during middle to late G1-phase against this background. Alternatively, other complexes than CycD3;3/CDKA also might contribute to phosphorylate NtRb1 at the G1/S transition. There are at least two CycD3 genes and one CycD2 gene in tobacco (Sorrell et al., 1999), so other CycD3- and CycD2-associated kinases could be candidates.

By contrast, two peaks of histone H1 kinase activity were observed at the G1/S and G2/M boundaries. This result is consistent with reports that alfalfa histone H1 kinase activity was increased at both G1- to S-phase and G2/M- to M-phase, suggesting that CDKA forms different complexes in the G1/S- and G2/M-phases (Magyar et al., 1993, 1997).

$p13^{SUC1}$-bound proteins recovered from synchronized cells in various cell cycle phases contained equivalent levels of CDKA, as determined by immunoblotting with an anti-PSTAIRE antibody (data not shown). This finding suggests that the activities of CDKA-associated kinases may be regulated by several different cyclins and, more importantly, by additional regulatory factors, such as CDK-activating kinase and CDK inhibitors (Umeda et al., 1998; Wang et al., 1998; Yamaguchi et al., 1998; 2000; Lui et al., 2000; De Veylder et al., 2001).

Presuming that NtRb1 is a preferred target of the Nicta; CycD3;3–associated kinases, it is likely that the kinase complexes undergo transport into the nucleus. Fluorescence microscopy of transgenic cells revealed the localization of Nicta; CycD3;3-GFP to the nucleus, whereas Nicta; CycD3;3 (T191A)-GFP remained predominantly in the cytoplasm (Figure 4). Human cyclin D1 accumulates in the nucleus during G1-phase, disappearing subsequently from nuclei during DNA synthesis (Diehl and Sherr, 1997). It will be interesting to determine the identity of the kinase responsible for the phosphorylation of Nicta; CycD3;3.

After overexpression of *Nicta; CycD3;3-GFP*, the proportion of G1-phase cells decreased in growing cells (Figure 7). In addition, overexpression of *Nicta; CycD3;3* (*S296,300A*)-*GFP* resulted in a reduction in the lengths of G1- and G2/M-phases and significantly reduced the doubling time (Table 1), suggesting that Nicta; CycD3;3–associated kinase activity not only promotes the G1/S transition but also plays a potential role during G2/M- and M-phase. In contrast, overexpression of *Nicta; CycD3;3* (*T191A*)-*GFP* had no discernible cell cycle effects; rather, it prolonged the doubling time. It should be noted that Nicta; CycD3;3 (T191A)–associated kinase activity is very weak, compared with that of both wild-type and mutant Nicta; CycD3;3 (S296,300A) in immunoprecipitates from cell extracts (Figure 5).

In plants, Nicta; CycD3;3/CDKA complexes exhibit kinase activity against Rb-related protein during middle G1-phase to early S-phase, and phosphorylation of histone H1 is increased at both G1- to S-phase and G2/M- to M-phase. This is in sharp contrast to findings in mammals, in which cyclin D–associated kinases mainly regulate the G1/S transition through the phosphorylation of Rb proteins. Although it remains to be determined whether the phosphorylation of Rb-related protein stimulates the G1/S transition in plants, our data provide further evidence that Rb-related protein plays a potentially significant role in plant cell cycle control.

METHODS

Plant Material and Cell Cycle Synchronization

Tobacco cells (*Nicotiana tabacum* cv Bright Yellow-2 [BY-2]) were cultured in a modified Linsmaier and Skoog medium as described previously (Nakagami et al., 1999). Cells were synchronized using 5 μM aphidicolin (Wako Pure Chemical, Osaka, Japan) and 3 μM propyzamide (Wako Pure Chemical) essentially as described by Nagata et al. (1992).

Cell Cycle Analysis of Tobacco BY-2 Cells

Tobacco BY-2 cells were fixed in 70% ethanol and stored at −20°C before analysis. After rehydration in PBS (7.4 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, and 150 mM NaCl), cells were treated with 0.1 mg/mL RNase A (Sigma) for 30 min at room temperature, and DNA was stained subsequently with 50 μg/mL propidium iodide (Sigma) for 30 min at room temperature. Cytometric analysis on 5×10^3 cells was

performed using a laser scanning cytometer (LSC101; Olympus, Tokyo, Japan) according to the manufacturer's instructions. Cell numbers were measured as described by Sorrell et al. (1999) in triplicate for each of the three samples removed each day.

Construction of Nicta; CycD3;3 Mutants

Nicta; CycD3;3 was mutagenized using PCR-based site-directed mutagenesis. Forward primers containing the mutated codon and reverse primers were generated tail to tail for ligation of the PCR products as follows: forward, 5′-TGAATCCTGTGGCGCCACTTTCAT-3′ (T191A; codon 191 underlined); reverse, 5′-TGAATCCTGTGGAGCCACTTTCAT-3′ (T191E); forward, 5′-TGAATCCTGTGTCGCCACTTTCAT-3′ (T191S); reverse, 5′-TCCTCCACTTTAAAGAGGACAATA-3′ (T191); forward, 5′-CAAGTATGAGGCGCCAATAAATGCGCCAAGTGCTG-3′ (S296,300A; codons 296 and 300 underlined); reverse, 5′-CGCTTGTGTGAAATAGAGTTGTAA-3′. All PCR-generated sequences were verified by sequencing.

Construction of Binary Plasmids and Transformation of Tobacco BY-2 Cells

Sequences encoding wild-type and mutant *Nicta; CycD3;3* were amplified with the following primers: forward, 5′-GTCGACATGGGAATACAACACAATGAG-3′; reverse, 5′-GGTACCGCGAGGGCTGCCAACAGCATA-3′. The DNA fragments generated by PCR were inserted into the SalI and KpnI sites of a modified pTH-2 vector (pTH-2SK) to generate mutant constructs fused to the N terminus of a modified green fluorescent protein (GFP; sGFP[S65T]) gene (Niwa et al., 1999). These chimeric genes were cloned into the binary vector pBI121 under the control of the 35S promoter of *Cauliflower mosaic virus*. Vector pTH-2SK was constructed by the insertion of an oligonucleotide (5′-GTCGACAGGCCTGGTACCGGGGCCATGG-3′) into the SalI and NcoI sites of the pTH-2 vector to generate additional StuI and KpnI sites. Transformation of tobacco BY-2 cells was performed as described by An (1995).

Preparation of the GST-NtRb1 Fusion Protein

A fusion protein composed of NtRb1 (amino acids 823 to 961) and glutathione *S*-transferase (GST) was expressed by pGEX-4T-1. *NtRb1* was amplified with the following primers: forward, 5′-GGATCCGAGCTTGCACCTGCTGGA-3′; reverse, 5′-GTCGACTAAGACTCAGGCTGCTCAGT-3′. The DNA fragments generated by PCR were inserted into the BamHI and SalI sites of pGEX-4T-1. GST-NtRb1 and GST–human Rb (kindly provided by Jun-ya Kato, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, Japan) were expressed in *Escherichia coli*, purified with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech), and concentrated by lyophilization.

Insect Cell Culture and Baculovirus Infection

Spodoptera frugiperda (Sf9) cells were cultured and infected with baculovirus as described previously (Nakagami et al., 1999). A FLAG tag was fused to the N terminus of Nicta; CDKA;3 (Setiady et al., 1996). *Nicta; CDKA;3* was amplified with the following primers: forward, 5′-TCTAGAATGGACCAGTATGAAAAAG-3′; reverse, 5′-CTCGAGTCACGGAACATACCCAATA-3′. The DNA fragments generated by PCR were inserted into the XbaI and XhoI sites of pFLAG-1 (Eastman Kodak), yielding pFLAG-1–CDKA;3. To clone the FLAG-tagged fragment of *Nicta; CDKA;3* into pFastBac1 (Gibco BRL), *FLAG-CDKA;3* was amplified with the following primers: forward, 5′-GCGGCCGCATGGACTACAAGGACGACGAT-3′; reverse, 5′-CTCGAGTCACGGAACATACCCAATA-3′. The DNA fragments generated by PCR were inserted into the NotI and XhoI sites of pFastBac1.

A His tag was fused to the N terminus of wild-type and mutant Nicta; CycD3;3. Sequences encoding wild-type and mutant *Nicta; CycD3;3* were amplified with the following primers: forward, 5′-GGATCCATGGGAATACAACACAATGAG-3′; reverse, 5′-GTCGACTTAGCGAGGGCTGCCAACA-3′. The DNA fragments generated by PCR were inserted into the BamHI and SalI sites of pFastBac HTb (Gibco BRL). Recombinant bacmid, intermediate plasmid, was isolated and transfected into Sf9 cells using a liposome-mediated transfection kit (Gibco BRL).

In Vitro Kinase Assay

His–Nicta; CycD3;3 and FLAG–Nicta; CDKA;3 were coexpressed in insect cells, and the corresponding proteins were purified with the TALON metal affinity resin (Clontech, Palo Alto, CA) and the anti-FLAG M2 affinity gel (Sigma) according to the manufacturers' instructions. At 72 h after infection, 3×10^6 infected insect cells were lysed by sonication in 500 μL of the kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 25 mM β-glycerophosphate, and 2 mM sodium orthovanadate) and then cleared by centrifugation. Kinase reactions were initiated by adding the lysates to the kinase buffer containing 2.5 μg of histone H1 (Gibco BRL) or GST-NtRb1 and 370 kBq of γ-^{32}P-ATP (~167 TBq/mmol; ICN, Costa Mesa, CA). After incubation at 30°C for 10 min, the samples were resolved on denaturing polyacrylamide gels. Phosphorylated histone H1 and GST-NtRb1 were detected by autoradiography.

Detection of Complex Formation

His–Nicta; CycD3;3 (wild type or T191A) and FLAG–Nicta; CDKA;3 were coexpressed in insect cells. Recombinant proteins were purified from lysates using an anti-FLAG M2 affinity gel (Sigma). Eluted fractions were denatured in gel sample buffer, separated on 10% SDS–polyacrylamide gels, and immunoblotted with a His tag–specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to detect His–Nicta; CycD3;3 (wild type or T191A) proteins.

Protein Extraction and Immunoprecipitation

Tobacco BY-2 cells were lysed by sonication in extraction buffer (25 mM Tris-HCl, pH 7.6, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 50 μg/mL *N*-tosyl-L-phenylalanine chloromethyl ketone, 5 μg/mL pepstatin A, 10 μg/mL aprotinin, 5 μg/mL antipain, 10 μg/mL soybean trypsin inhibitor, 0.1 mM benzamidine, 10 mM NaF, 25 mM β-glycerophosphate, and 2 mM sodium orthovanadate) and cleared subsequently by centrifugation. Protein concentrations were determined with Protein Assay CBB solution (Nacalai Tesque, Kyoto, Japan) using BSA as a standard.

Proteins were immunoprecipitated from 300-μg protein extracts

using 4 μg of Nicta; CycD3;3–specific antibody (Nakagami et al., 1999) and a GFP-specific antibody (Boehringer Mannheim). After incubation for 2 h on ice, 20 μL of 50% (v/v) protein A–Sepharose 4FF beads (Amersham Pharmacia Biotech) was added, and the mixture was incubated for an additional 1 h on ice. The beads then were washed three times in extraction buffer followed by three washes in kinase buffer (Nakagami et al., 1999). Binding of $p13^{SUC1}$ was achieved by adding 20 μL of 50% (v/v) $p13^{SUC1}$-agarose beads (Calbiochem) to the protein extracts for 2 h on ice.

Kinase assays were performed on proteins immobilized on protein A–Sepharose beads or $p13^{SUC1}$-agarose beads. The reaction was initiated by adding 10 μL of kinase buffer containing 2.5 μg of histone H1 (Gibco BRL) or GST-NtRb1 and 370 kBq of γ-^{32}P-ATP (~167 TBq/mmol; ICN). After incubation for 30 min at 30°C, samples were resolved on denaturing polyacrylamide gels. Phosphorylated histone H1 and GST-NtRb1 proteins were detected by autoradiography.

After denaturation of beads in gel sample buffer, proteins were separated on 10% SDS–polyacrylamide gels, and cyclin-dependent kinase was detected by immunoblotting with an anti-PSTAIRE antibody (Santa Cruz Biotechnology).

Fluorescence Microscopy

Expression of the GFP-fused proteins in cells mounted on slides was observed using a fluorescence microscope (BX50; Olympus) equipped with a standard fluorescence filter set. Images were acquired with a cooled charge-coupled device color digital camera (SPOT; Seki Technotron, Tokyo, Japan) and exported to Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA).

Accession Numbers

The accession numbers for the sequences shown in Figure 2 are as follows: human CycD1 (M64349); Arath; CycD1;1 (X83369); Arath; CycD2;1 (X83370); Nicta; CycD2;1 (AJ011892); Arath; CycD3;1 (X83371); Nicta; CycD3;1 (AJ011893); Nicta; CycD3;2 (AJ011894); and Nicta; CycD3;3 (BAA76478).

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