Plant Biotechnology 23, 467–473 (2006)

Original Paper

Tobacco RETINOBLASTOMA-RELATED protein is phosphorylated by different types of cyclin-dependent kinases during the cell cycle

Kazue Kawamura[1], Ko Kato[1], Atsuhiko Shinmyo[1], Masami Sekine[2,*]

[1] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan;
[2] Department of Bioproduction Science, Faculty of Bioresources and Environmental Sciences, Ishikawa Prefectural University, Ishikawa 921-8836, Japan
* E-mail: sekine@ishikawa-pu.ac.jp Tel: +81-76-227-7434 Fax: +81-76-227-7410

Received June 29, 2006; accepted July 27, 2006 (Edited by M. Umeda)

Abstract Plants contain a regulatory pathway similar to that involving retinoblastoma (RB) protein in animals. Here, we analyze an *RB* homolog, tobacco *RETINOBLASTOMA-RELATED1* (*NtRBR1*), that encodes a protein possessing 13 potential phosphorylation sites for cyclin-dependent kinases (CDKs). We found that 11 synthetic peptides containing sequences from NtRBR1 were phosphorylated differentially by imunoprecipitates of cyclin/CDK complexes extracted from tobacco BY-2 cells. We raised antibodies that specifically recognize these sites of phosphorylation in NtRBR1. Distinct sites were phosphorylated during the cell cycle, suggesting that NtRBR1 is phosphorylated by different types of cyclin/CDK complexes.

Key words: CDK, cell cycle, RB phosphorylation.

In all eukaryotes, the activities of cyclin-dependent kinases (CDKs) exert major control over transitions from one cell cycle phase to the next (Pines 1999). Two major types of plant CDKs play pivotal roles in cell cycle control (Mironov et al. 1999; Joubes et al. 2000). A-type CDK (CDKA) contains the canonical PSTAIRE motif and can complement mutations in yeast *CDC28/cdc2*, while B-type CDK (CDKB) cannot. CDKBs consist of two subgroups, CDKB1 and CDKB2, and have plant-specific features, including cell cycle-regulated expression and peaks during the G2 phase. Based on sequence similarity and expression patterns, three major classes of cyclins have been identified in plants (Dewitte and Murray 2003). A-type cyclins (CYCAs) are broadly involved during S to M phase, but B-type cyclins (CYCBs) control the G2/M transition. D-type cyclins (CYCDs) are thought to act as mediators linking extracellular and developmental signals to the cell cycle (Meijer and Murray 2000).

E2F transcription factors are key components of cell cycle control in higher eukaryotes (Trimarchi and Lees 2002; Attwooll et al. 2004). In animals, the promoter regions of numerous cell cycle genes that are active during S phase contain multiple E2F-binding sites. E2F regulation involves the retinoblastoma (RB) protein containing the A and B pocket domains, which are conserved in members of the mammalian RB family, p107 and p130. These domains are required for most associated proteins to bind RB (Harbour and Dean, 2000; Cobrinik 2005). RB represses transcription by binding E2F transcription factors and inhibiting their ability to activate transcription. However, CDK-mediated phosphorylation of RB in mid to late G1 phase disrupts the binding of RB to E2F, thereby relieving the repression of E2F-regulated genes that are required for entry into S phase. In addition, the RB-E2F complexes bind to promoter regions and actively repress transcription (Trimarchi and Lees 2002; Attwooll et al. 2004). Therefore, the RB-E2F pathway is involved in the repression or activation of key genes at different stages of development and in different tissues.

A number of plant cDNAs encoding E2F family members and RETINOBLASTOMA-RELATED (RBR) proteins have recently been identified, and evidence is emerging that a similar RBR-E2F pathway plays important roles in controlling the G1/S transition in plants (Gutierrez 2005). For example, immunoprecipitation-coupled kinase assays showed that RBR protein was phosphorylated by CYCD/CDKA complexes during G1 phase (Boniotti and Gutierrez

Abbreviations: BY-2, Bright-Yellow 2; CNBr, cyanogen bromide; CDK, cyclin-dependent kinase; DP, DNA-binding heterodimerization partner protein; E2F, adenovirus E2 promoter-binding factor; RBR, RETINOBLASTOMA-RELATED.
This article can be found at http://www.jspcmb.jp/

2001; Nakagami et al. 2002). In addition, we have demonstrated that transfecting tobacco *E2F* in transient assays activated a reporter gene, and this activation was repressed by co-transfection with the tobacco *RBR* gene (*NtRBR1*) (Uemukai et al. 2005). This repressor activity was cancelled when cells were further co-transfected with *CYCD*, but not with *CYCA* or *CYCB*. Thus, it is likely that CYCD/CDKA phosphorylates RBR, leading to dissociation of RBR from E2F, which results in the transcriptional activation of E2F-controlled S-phase genes in plants.

The RBR-E2F pathway also plays important roles in plant development. Overexpression of *E2Fa-DPa* (De Veylder et al. 2002; Kosugi and Ohashi 2003) or *CYCD3* (Dewitte et al. 2003) in *Arabidopsis* resulted in uncontrolled cell proliferation and delayed differentiation, and had severe effects on plant development. Additionally, particle bombardment of tobacco BY-2 cells with the maize *ZmRBR1* gene suppressed cell division, while overexpression of *RepA*, encoding a virus-derived protein that separates RBR from its physiological partners, stimulated cell division (Gordon-Kamm et al. 2002). Inhibition of RBR function by virus-induced gene silencing prolonged cell proliferation and also delayed cell differentiation in the leaves and stems, indicating that RBR positively regulates cell cycle exit (Park et al. 2005). Using the *RepA*-inducible system to inactivate RBR function, it has been reported that cells respond differently to RBR inactivation in terms of regulating their cell division and endoreplication potential during *Arabidopsis* leaf development (Desvoyes et al. 2006). Recently, experiments with a T-DNA tagging mutant of *Arabidopsis* containing a single *RBR* gene (*AtRBR1*) showed that RBR is required for female gametogenesis prior to fertilization (Ebel et al. 2004). Using conditional loss-of-function and gain-of-function approaches, as well as overexpression of *CYCD3* and *E2Fa* along with genetic interactions, Wildwater et al. (2005) showed that these factors function in the RBR-E2F pathway to regulate the size and state of the root stem cell.

Nevertheless, little is currently known about the protein levels of RBR in plants. *In vitro* binding assays revealed that maize RBR protein interacts with *Arabidopsis* CYCD3 and other cellular or virus-derived factors (Ach et al. 1997; Dewitte and Murray 2003). Transient assays with a plasmid encoding myc-tag-fused maize RBR showed that RBR protein is localized to the nucleus (Ach et al. 1997). *Arabidopsis* plants overexpressing *CYCD3* contain much higher levels of AtRBR1 mRNA and protein than are found in wild type, which may reflect a feedback mechanism that normally regulates CYCD3 activity, because the *CYCD3* promoter contains an E2F-binding site (Dewitte et al. 2003). Like the regulation of RB activity by phosphorylation in animals, maize RBR protein undergoes changes in level and phosphorylation state during development of endosperm cells (Grafi et al. 1996).

To examine the protein characteristics of tobacco NtRBR1, we have first analyzed protein levels during the growth phase. We found that hypo-phosphorylated NtRBR1 is present at very low levels during proliferation, whereas it accumulated abundantly during stationary phase. We then synthesized 11 peptides containing putative phosphorylation sites in NtRBR1, and *in vitro* kinase assays revealed that immunoprecipitates prepared with antibodies against CYCA and CDKA phosphorylate almost all the peptides, whereas CDKB-associated kinases phosphorylate strongly only those peptides whose native sequences are located in the spacer region between the A and B domains. Using phospho-specific antibodies, we further found that distinct sites in NtRBR1 were phosphorylated during the cell cycle. These results suggest that RBR function is regulated by phosphorylation by different types of cyclin/CDK complexes during the cell cycle.

Materials and methods

Plant materials and growth conditions

Tobacco BY-2 cells were cultured at 27°C in a modified Linsmaier and Skoog medium as previously described (Nagata et al. 1992).

Protein methods

Procedures for protein extraction, SDS-PAGE, and Western blot analysis are described in Nakagami et al. (1999; 2002). Protein concentrations were determined by the Bradford (1976) method using BSA as a standard. Antibody was used at 1/500 to 1/1,000 dilution and incubated with Western blots overnight at room temperature. Polyclonal antibodies against CYCA1;1, CYCD3;3, CDKA and CDKB were prepared as described (Nakagami et al. 1999; Sorrell et al. 2001). Anti-NtRBR1 polyclonal antiserum (αRBRC) was generated by immunizing rabbits with purified GST-tagged C-terminal NtRBR1 (amino acids 823 to 961). Antibodies were purified on a protein A-Sepharose (GE Healthcare, Piscataway, NJ) column followed by an affinity column of purified C-terminal NtRBR1 conjugated with CNBr-activated Sepharose 4B (GE Healthcare). Anti-phospho-specific polyclonal antibodies (αRBR-pS666, -pS713, -pS900) were raised at Sigma Genosys Japan (Osaka, Japan) against peptides containing the corresponding phosphorylated serine residues. Antisera obtained from the immunized rabbits were purified using column chromatography of CNBr-activated Sepharose 4B conjugated with the corresponding phosphorylated peptides. The affinity-purified antibodies were then passed through a column of CNBr-activated Sepharose 4B conjugated with the corresponding peptides, to remove contaminating antibodies which could bind with unphosphorylated antigen.

In vitro kinase assays

In vitro kinase assays using purified proteins and

imunoprecipitated CDKs from BY-2 cells were performed as previously reported (Nakagami et al. 1999; 2002). Those using synthetic peptides were conducted with the following modification. Substrate peptides were synthesized and HPLC-purified by Kurashiki Boseki (Osaka, Japan). Synthetic peptides (5 μg) were incubated with immunoprecipitated CDKs at 30°C for 30 min in R buffer [20 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$, 4.5 mM β-mercaptoethanol, 1 mM EGTA, 1 mM DTT, 1 mM PMSF] that contained 92.5 KBq of [γ-^{32}P-]ATP in a final volume of 10 μl. After samples were denatured in SDS sample buffer containing 9 M urea, they were electrophoresed at 25°C on a 20% PAGE gel as described (Yim et al. 2002), and phosphorylated peptides were detected by autoradiography.

Insect cell culture and baculovirus infection

The phosphorylation site mutants (N-psm, S-psm, C-psm) were constructed by PCR-based site-directed mutagenesis. Vectors encoding N-terminal His-tagged NtRBR1 and its phosphorylation site mutants were generated by ligating appropriate PCR products into pFastBac HTb (Invitrogen, Carlsbad, CA). Full construction details are available from the authors. Insect cells (Sf9) were cultured and infected with baculovirus as described (Nakagami et al. 1999; 2002).

Results and discussion

Accumulation of hypo-phosphorylated NtRBR1 during stationary phase

We produced the antibody αRBRC against the C-terminal region of NtRBR1. To verify that the antibody specifically detects the intended protein, it was used to probe Western blots of purified His-tagged full-length NtRBR1; the antibody probes were used both directly and in competition with purified GST or GST-fused C-terminal region of NtRBR1 (NtRBRC) (Figure 1A). αRBRC detected the ~110-kDa NtRBR1 protein, and a very faint band, perhaps attributable to incomplete elimination of NtRBR1, was detected when the antibody was preincubated with purified GST-NtRBRC.

We next performed Western blots of suspension-cultured tobacco BY-2 cells with αRBRC (Figure 1B). As a control, the CDKA antibody was used to probe the blot and CDKA was detected equally in both the active growth and stationary phases, indicating that almost equal amounts of proteins were loaded in each lane. In contrast, CDKB accumulated after 3 days, but declined after 7 days. Using αRBRC, a single band of about 110 kDa was detected, and no shifted band was observed as is the case for mammalian RB protein. In animals, RB activity is regulated by phosphorylation, and cyclin D/CDK4,6 and cyclin E/CDK2 phosphorylate non- and hypo-phosphorylated (active) RB to form hyper-phosphorylated (inactive) RB. Hyper-phosphorylated RB is usually detected as a slower-migrating band. We also raised a specific antibody against phospho-serine

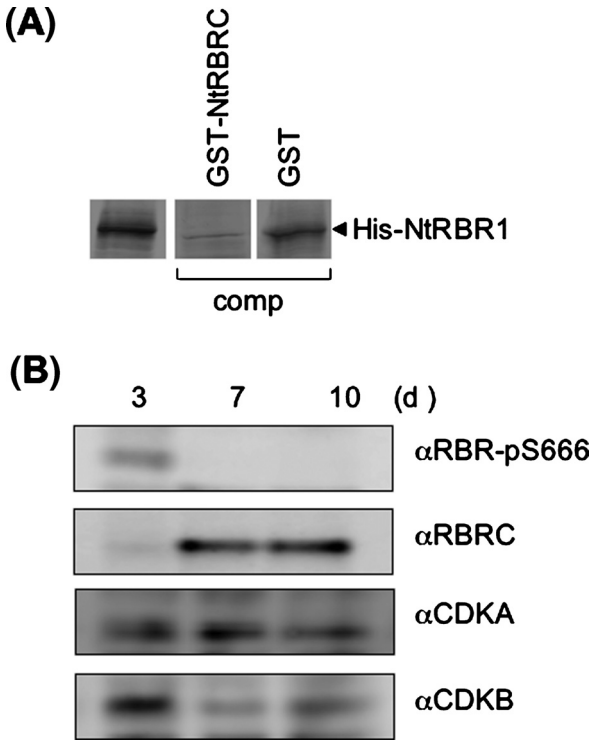

Figure 1. Levels of NtRBR1 protein during the growth cycle of BY-2 cells. (A) Western blots of NtRBR1 demonstrating specificity of the antibody (αRBRC) raised against the C-terminal region of NtRBR1. His-tagged full-length NtRBR1 was expressed in insect cells and purified by affinity chromatography. αRBRC was competed with purified GST-fused C terminus of NtRBR1 (GST-RBRC) or, as a negative control, GST. (B) Western blots showing levels of NtRBR1, CDKA and CDKB during exponential growth (day 3) and in the stationary phase (days 7 and 10). Hypo-phosphorylated NtRBR1 was detected using αRBRC, and αRBR-pS666 detected phosphorylated NtRBR1.

residues in NtRBR1 (described below). The phospho-specific antibody (αRBR-pS666) detected a single band migrating at the same position as that detected with αRBRC (Figure 1B).

Next, the level of NtRBR1 was examined during the growth cycle of BY-2 cells, which reach maximum cell density after 6 days. Although the level of NtRBR1 recognized with αRBRC remained very low during the growth phase, it accumulated dramatically after 7 and 10 days, corresponding to the stationary phase (Figure 1B). By contrast, the phospho-specific antibody detected strong signals after 3 days, but the signals were hardly detectable during the stationary phase. These results suggested that αRBRC could recognize hypo-phosphorylated NtRBR1 accumulated during the stationary phase, but not preferentially hyper-phosphorylated NtRBR1 accumulated in actively dividing cells. In animals, accumulation of hypo-phosphorylated RB protein is thought to assist in achieving and/or maintaining the post-mitotic state associated with terminal differentiation of many tissue types, as well as in protection from apoptosis (Halaban

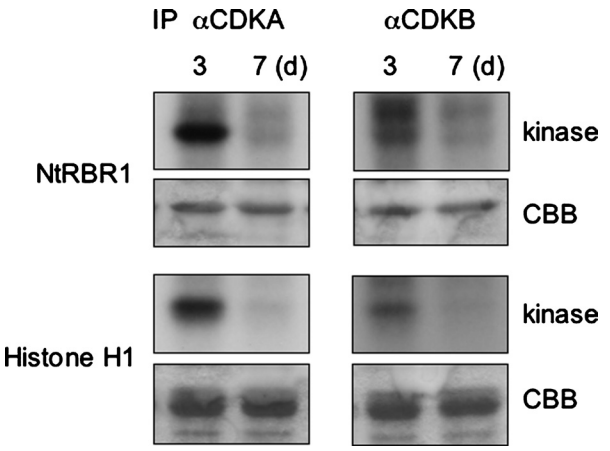

Figure 2. Hypo-phosphoyrlated NtRBR1 is phosphorylated by CDKA- and CDKB-associated kinase from actively growing cells. Protein kinase assays showing phosphorylation of NtRBR1 and histone H1 with CDKA and CDKB immunoprecipitates prepared from actively growing (day 3) and stationary phase (day 7) BY-2 cells. GST-RBRC and histone H1 were detected by CBB staining to ensure that equivalent amounts of substrates were present in each reaction.

2005).

NtRBR1 is phosphorylated by cyclin/CDK complexes in actively growing cells

Immunoprecipitation-coupled kinase assays have shown that CDKA associated with CYCDs phosphorylates RBR protein in plants (Boniotti and Gutierrez 2001; Nakagami et al. 2002). To examine whether the other main plant group of CDKs, CDKBs, is also able to phosphorylate RBR, we carried out immunoprecipitation-coupled kinase assays with antibodies against both CDKA and CDKB. Since CDKB-associated kinases show histone H1 kinase activity (Boudorf et al. 2004), we also included histone H1 as a substrate. As shown in Figure 2, NtRBR1 and histone H1 were phosphorylated by both CDKA and CDKB immunoprecipitates prepared from actively growing cells, but very weak phosphorylation was observed with the immunoprecipitates from stationary phase. These results are consistent with the finding that phosphorylated NtRBR1 was present predominantly in actively dividing cells (Figure 1A).

NtRBR1 is phosphorylated differentially by distinct types of cyclin/CDK complexes

The phosphorylation sites of mammalian RB have been defined using synthetic peptides containing part of the sequence of RB, and these experiments showed that the consensus motif for phosphorylation by cyclin D/CDK4 is different from that for phosphorylation by cyclin E/CDK2 (Kitagawa et al. 1996). To investigate the sites of phosphorylation in NtRBR1, we prepared 11 peptides, which collectively contain 13 potential phosphorylation

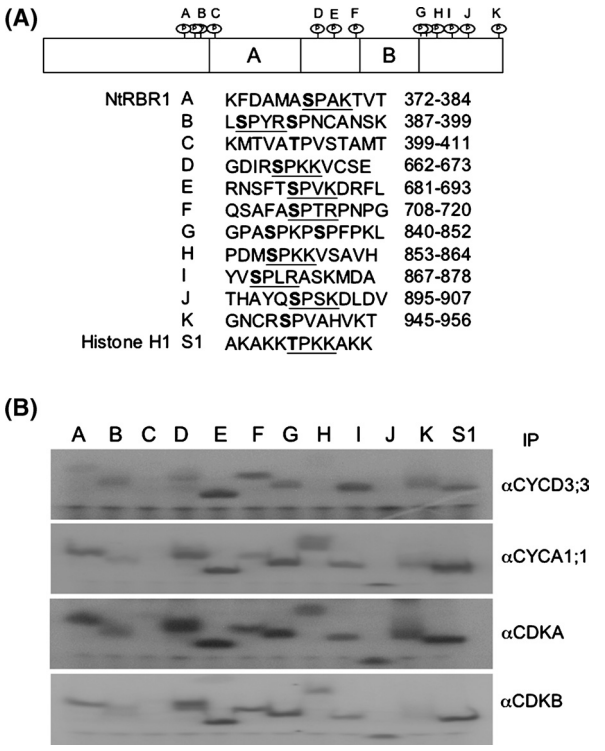

Figure 3. Differential phosphorylation of synthetic peptides by immunoprecipitated CDKs from BY-2 cells. (A) A schematic representation of NtRBR1 including the conserved A and B domains is shown, and the positions of potential sites of phosphorylation by CDKs are indicated by vertical lines above the boxes. Synthetic peptides that contained sequences from NtRBR1 (A-K) or histone HI (S1) are also shown. Ser and Thr residues potentially phosphorylated by CDKs are in bold type. The consensus motifs for phosphorylation by CDKs, S or T-P-X-K/R, in these peptides are underlined. Amino acid residues corresponding to the sequence in NtRBR1 are given on the right. (B) Protein kinase assays showing phosphorylation of synthetic peptides that contained sequences from NtRBR1 (A-K) or histone HI (S1). These peptides were individually incubated with immunoprecipitates of αCYCD3;3, αCYCA1;1, αCDKA and αCDKB. The phosphorylated peptides were subjected to SDS-PAGE and detected by autoradiography.

sites for CDKs in NtRBR1 (Figure 3A), and tested them for phosphorylation by incubating them with immunoprecipitates prepared from actively growing BY-2 cells using antibodies against CYCA1;1, CYCD3;3, CDKA and CDKB (Figure 3B). Most of these synthetic peptides contained S/T-P-X-K/R (X is any amino acid), which is the consensus sequence for phosphorylation by CDKs (Holmes and Solomon 1996). Since the molar concentrations of these peptides varied slightly (5 μg of each 12- to 13-amino acid peptide was added to the reactions), and since we did not evaluate the quantities of precipitated kinases, these experiments allow only rough comparisons to be made. Except for the C peptide, all the peptides, including the S1 peptide containing a part of the sequence of histone H1, were efficiently phosphorylated to varying extents by immunoprecipitates with the antibodies against CYCA1;1 and CDKA (Figure 3A). In contrast, immunoprecipitates with the CDKB

antibody strongly phosphorylated only the D, E, F, and G peptides, while virtually no phosphorylation was observed for the B, C, and K peptides. Moreover, immunoprecipitates with the CYCD3;3 antibody preferentially phosphorylated the E, F, and I peptides. These results suggested that native NtRBR1 might be phosphorylated differentially by distinct types of cyclin/CDK complexes.

Preparation of antibodies specifically recognizing phosphorylated sites of NtRBR1

To analyze the phosphorylation state of specific sites in NtRBR1, we raised antibodies (αRBR-pS666, -pS713, -pS900) against phospho-Ser-666, -Ser713, -Ser900 in NtRBR1 as described in Materials and methods. Selectivity of the antibodies was verified using mutant His-tagged NtRBR1 proteins (N-psm, S-psm, C-psm) in which the putative phosphorylation sites were replaced with a non-phosphorylatable amino acid, alanine; N-psm contains mutations in sites 1-4, S-psm mutations in sites 5-7, and C-psm mutations in sites 8-13 (Figure 4A).

Confirming a previous report (Kato et al. 1994), His-tagged NtRBR1 proteins were phosphorylated in the insect cells (Figure 4A). Anti His-tagged antibody recognized all the His-tagged NtRBR1 proteins, indicating that these proteins were equivalently produced in the insect cells. Both αRBR-pS666 and αRBR-pS713 recognized wild type NtRBR1, N-psm and C-psm, but neither recognized S-psm. In contrast, αRBR-pS900 recognized wild type NtRBR1, N-psm and S-psm, but did not recognize C-psm. These data strongly suggested that these antibodies specifically detected the corresponding phosphorylated serine residues in NtRBR1.

Distinct sites in NtRBR1 are phosphorylated differentially during the cell cycle

Finally, we determined the phosphorylation state of the sites in NtRBR1 during the cell cycle. BY-2 cells were treated with aphidicolin and the antitubulin drug propyzamide to synchronize cells from M phase (Plachais et al. 1997). After removal of the inhibitors, samples were taken every 2 h and cell cycle progression was monitored by measuring changes in DNA content (Figure 4B). As shown in Figure 4B, the actin control was roughly constant, confirming that almost equal amounts of proteins were loaded in each lane. CDKA was constitutively expressed during the cell cycle, but the level of CDKB started to increase at 12 h, corresponding to G2/M phase, and declined after 18 h. Additionally, CDKA and CDKB appear to be present as two bands, suggesting that different phosphorylated forms accumulated differentially during the cell cycle. To examine the expression of hypo-phosphorylated NtRBR1, αRBRC was used to probe Western blots of

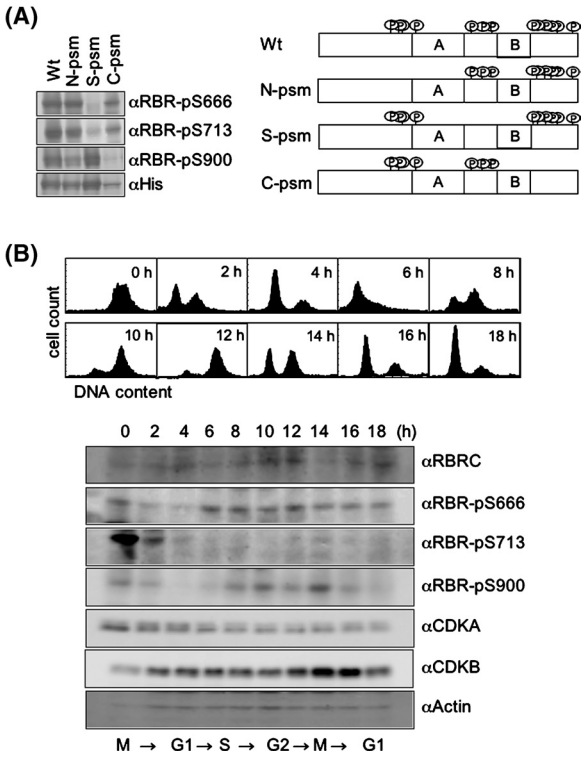

Figure 4. Cell cycle-dependent phosphorylation of Ser residues in NtRBR1. (A) Western blots of mutant NtRBR1 proteins demonstrating specificity of the antibodies raised against the phosphorylated sites of NtRBR1. His-tagged wild type (Wt) NtRBR1 and the mutant proteins (N-psm, S-psm, C-psm) were expressed in insect cells and purified by affinity chromatography. Western blots were conducted with the His-tagged antibody and αRBR-pS666, -pS713, -pS900 antibodies. A schematic representation of NtRBR1 and its phosphorylation mutants is shown on the right. The A and B pocket domains are shown, and the sites of putative phosphorylation by CDKs are indicated by vertical lines above the boxes. (B) DNA histograms showing changes in DNA content after BY-2 cells were synchronized with aphidicolin and propyzamide (upper panel). Samples from the same time points shown in the upper panel were used for Western blots with the CDKA and CDKB antibodies. Western blots were also conducted with αRBRC and αRBR-pS666, -pS713, -pS900 antibodies. Actin antibody was used as a control. S, S phase; G2, G2 phase; M, mitosis; G1, G1 phase.

synchronized BY-2 cells. We found that low levels of hypo-phosphorylated NtRBR1 could be detected in actively dividing cells (Figure 4B). The levels oscillated during the cell cycle, and gradually increased from 10 h after removal of the inhibitors. Although phosphorylation of Ser666 was detected throughout the cell cycle and gradually increased from 6 h, significant phosphorylation of Ser713 was observed at 0 h and after 4 h but was hardly detectable during the cell cycle. The phosphorylation of Ser900 oscillated dramatically during the cell cycle and began to increase at 8 h. Thus, Ser666 and Ser900 were phosphorylated during G1 to M phase, but Ser713 was virtually unphosphorylated during that period, suggesting that these sites are phosphorylated variably *in vivo* by different types of cyclin/CDK complexes in a cell cycle-dependent manner.

These results further support the suggestion that the

function of NtRBR1 is regulated by distinct types of cyclin/CDK complexes during the cell cycle. In animals, human RB contains 16 potential CDK phosphorylation sites and at least seven of these sites (Ser-249, -807, -811, and Thr-252, -373, -821, and -826) have been shown to be phosphorylated *in vivo* (Harbour and Dean, 2000). Sequential phosphorylation of RB by cyclin D/CDK4 and cyclin E/CDK2 during G1 phase is a major control element, regulating passage of cells into S phase by preventing RB from binding and inactivating E2F (Harbour and Dean, 2000; Cobrinik 2005). It will be interesting to clarify whether such sequential phosphorylation of RBR also plays an important role during the plant cell cycle. Elucidation of the mechanisms regulating the levels of phosphorylation in RBR protein in response to developmental signals may therefore provide considerable insight into the functions of RBR during plant development.

Acknowledgements

The authors wish to thank Dr. Kazuya Yoshida for helpful discussions and suggestions throughout this work. The authors also wish to thank Dr. Ian Smith for critical reading of the manuscript. This research was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology. K.K. was supported by a Grant-in-Aid for 21st Century COE Research.

References

Ach RA, Durfee T, Miler AB, Taranto P, Hanley-Bowdoin L, Zambryski PC, Gruissem W (1997) *RRB1* and *RRB2* encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. *Mol Cell Biol* 17: 5077–5086

Attwooll C, Lazzerini Denchi E, Helin K (2004) The E2F family: specific functions and overlapping interests. *EMBO J* 23: 4709–4716

Boniotti MB, Gutierrez C (2001) A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in *Arabidopsis*, a CDKA/cyclin D complex. *Plant J* 28: 341–350

Boudolf V, Barroco R, Engler Jde A, Verkest A, Beeckman T, Naudts M, Inze D, De Veylder L (2004) B1-type cyclin-dependent kinases are essential for the formation of stomatal complexes in *Arabidopsis thaliana*. *Plant Cell* 16: 945–955

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254

Cobrinik D (2005) Pocket proteins and cell cycle control. *Oncogene* 24: 2796–2809

Desvoyes B, Ramierez-Parra E, Xie Q, Chua NH, Gutierrez C (2006) Cell type-specific role of the retinoblastoma/E2F pathway during *Arabidopsis* leaf development. *Plant Physiol* 140: 67–80

De Veylder L, Beeckman T, Beemster GT, de Almeida Engler J, Ormenese S, Maes S, Naudts M, Van Der Schueren E, Jacqmard A, Engler G, Inze D (2002) Control of proliferation, endoreduplication and differentiation by the *Arabidopsis* E2Fa-DPa transcription factor. *EMBO J* 21: 1360–1368

Dewitte W, Murray JAH (2003) The plant cell cycle. *Annu Rev Plant Biol* 54: 235–264

Dewitte W, Riou-Khamlichi C, Scofield S, Healy JM, Jacqmard A, Kilby NJ, Murray JAH (2003) Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. *Plant Cell* 15: 79–92

Ebel C, Mariconti L, Gruissem W (2004) Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. *Nature* 429: 776–780

Gordon-Kamm W, Dilkes BP, Lowe K, Hoerster G, Sun X, Ross M, Church L, Bunde C, Farrell J, Hill P, Maddock S, Snyder J, Sykes L, Li Z, Woo YM, Bidney D, Larkins BA (2002) Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. *Proc Natl Acad Sci USA* 99: 11975–11980

Grafi G, Burnett RJ, Helentjaris T, Larkins BA, DeCaprio JA, Sellers WR, Kaelin WG (1996) A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. *Proc Natl Acad Sci USA* 93: 8962–8967

Guterrez C (2005) Coupling cell proliferation and development in plants. *Nat Cell Biol* 7: 535–541

Halaban R (2005) Rb/E2F: A two-edged sword in the melanocytic system. *Cancer Metast* 24: 339–356

Harbour JW, Dean DC (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev* 14: 2393–2409

Holmes JK, Solomon MJ (1996) A predictive scale for evaluating cyclin-dependent kinase substrates. A comparison of p34cdc2 and p33cdk2. *J Biol Chem* 271: 25240–25246

Joubes J, Chevalier C, Dudits D, Heberle-Bors E, Inze D, Umeda M, Renaudi JP (2000) CDK-related protein kinases in plants. *Plant Mol Biol* 43: 607–620

Kato J, Matsuoka M, Strom DK, Sherr CJ (1994) Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol Cell Biol* 14: 2713–2721

Kitagawa M, Higashi H, Jung H-K, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Segawa K, Yoshida E, Nishimura S, Taya Y (1996) The consensus motif for phosphorylation by cyclin Dl-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *EMBO J* 15: 7060–7069

Kosugi S, Ohashi Y (2003) Constitutive E2F expression in tobacco plants exhibits altered cell cycle control and morphological change in a cell type-specific manner. *Plant Physiol* 132: 2012–2022

Meijer M, Murray JAH (2000) The role and regulation of D-type cyclins in the plant cell cycle. *Plant Mol Biol* 43: 621–633

Mironov V, De Veylder L, Van Montagu M, Inze D (1999) Cyclin-dependent kinases and cell division in plants–The nexus. *Plant Cell* 3: 29–41

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. *Int Rev Cytol* 132: 1–30

Nakagami H, Sekine M, Murakami H, Shinmyo A (1999) Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D *in vitro*. *Plant J* 18: 243–252

Nakagami H, Kawamura K, Sugisaka K, Sekine M, Shinmyo A (2002) Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. *Plant Cell* 14: 1847–1857

Park J-A, Ahn J-W, Kim Y-K, Kim SJ, Kim J-K, Kim WT, Pai, H-S

(2005) Retinoblastoma protein regulates cell proliferation, differentiation, and endoreduplication in plants. *Plant J* 42: 153–163

Pines J (1999) Four-dimensional control of the cell cycle. *Nat Cell Biol* 1: 73–79

Planchais S, Glab N, Trehin C, Perennes C, Bureau JM, Meijer L, Bergounioux C (1997) Roscovitine, a novel cyclin-dependent kinase inhibitor, characterizes restriction point and G2/M transition in tobacco BY-2 cell suspension. *Plant J* 12: 191–202

Sorrell DA, Menges M, Healy JM, Deveaux Y, Amano C, Su Y, Nakagami H, Shinmyo A, Doonan JH, Sekine M, Murray JAH (2001) Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar Bright Yellow-2 cells. *Plant Physiol* 126: 1214–1223

Trimarchi JM , Lees JA (2002) Transcription: sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 3: 11–20

Uemukai K, Iwakawa H, Kosugi S, de Jager S, Kato K, Kondorosi E, Murray JAH, Ito M, Shinmyo A, Sekine M (2005) Transcriptional activation of tobacco E2F is repressed by co-transfection with the retinoblastoma-related protein: cyclin D expression overcomes this repressor activity. *Plant Mol Biol* 57: 83–100

Wildwater M, Campilho A, Perez-Perez JM, Heidstra R, Blilou I, Korthout H, Chatterjee J, Mariconti L, Gruissem W, Scheres B (2005) The *RETINOBLASTOMA-RELATED* gene regulates stem cell maintenance in *Arabidopsis* roots. *Cell* 123: 1337–1349

Yim SK, Ahn T, Kim JS, Yun CH (2002) Polyacrylamide gel electrophoresis without a stacking gel: application for separation of peptides. *Anal Biochem* 305: 277–279