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Expression of B2-type cyclin-dependent kinase is controlled by protein degradation in *Arabidopsis thaliana*

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Abbreviations: BY-2, Bright Yellow-2; CDK, cyclin-dependent kinase; CDKA, A-type CDK; CDKB, B-type CDK; CHX, cycloheximide; DMSO, dimethylsulfoxide; GUS, β-glucuronidase; RT-PCR, reverse transcription-PCR.

Running title: Protein degradation of B2-type CDK in *Arabidopsis*

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Subject areas: gene structure and expression

Abstract: The eukaryotic cell cycle is controlled by cyclin-dependent kinases (CDKs). Plants possess six types of CDK, among which the B-type CDK (*CDKB*) is expressed specifically from the late S- to the M-phase. We demonstrate that the expression of *Arabidopsis CDKB2* is under the control of protein degradation machinery. The β-glucuronidase fused to a putative PEST motif of CDKB2 was unstable in tobacco Bright Yellow-2 cells and *Arabidopsis* plants, and its degradation was arrested by the proteasome inhibitor MG132. We propose that the abundance of CDKB2 protein is regulated not only at the transcriptional level, but also through proteasome-mediated protein degradation.

Keywords: *Arabidopsis thaliana* — cyclin-dependent kinase — PEST motif — protein degradation

Cell cycle progression in eukaryotes is controlled by the activities of evolutionarily conserved protein kinase complexes, which consist of cyclins and cyclin-dependent kinases (CDKs). *Arabidopsis* CDKs have been classified into six types, namely CDKA–CDKF, among which the A- and B-type CDKs are assumed to be crucial for cell cycle progression (for a review, see De Veylder et al. 2003). The A-type CDKs (CDKAs) are functional homologs of yeast Cdc2/Cdc28p, and contain a conserved PSTAIRE motif that is important for cyclin binding. *CDKA* is expressed constitutively throughout the cell cycle and is assumed to play a key role in both the G1-to-S and G2-to-M transitions (Hemerly et al. 1995). The B-type CDKs (CDKBs) are plant-specific CDKs with a divergent PSTAIRE sequence and are further classified into two subtypes, CDKB1 and CDKB2. CDKA, but not CDKB, can complement yeast *cdc2/cdc28* mutations (Imajuku et al. 1992, Fobert et al. 1996), suggesting that CDKB plays a distinct role in cell cycle regulation. The expression of *CDKB*s is under strict cell cycle control: *CDKB1* is expressed from the late S- to the M-phase, while *CDKB2* is expressed from the G2- to the M-phase (Magyar et al. 1997, Umeda et al. 1999, Porceddu et al. 2001, Menges et al. 2005).

While the oscillation of CDK transcripts during the cell cycle has been well characterized, we have only limited information about the protein stability. This prompted us to analyze the amino acid sequences of CDKAs and CDKBs in order to identify any motif related to posttranslational regulation. We discovered that *Arabidopsis* CDKB2;1 and CDKB2;2 contain possible PEST sequences, which are known to target themselves for proteolytic degradation and therefore reduce their half-lives (for a review, see Rechsteiner and Rogers 1996). These motifs can be identified by the PESTfind program; a score above five is considered to be of real biological interest (Rogers et al. 1986, Rechsteiner and Rogers 1996). In the region near the altered PSTAIRE motif, CDKB2;1 and CDKB2;2 display PEST scores of 9.72 and 10.24, respectively, which is comparable to that of the D-type cyclin CYCD3;1 (score = 10.84) (Fig. 1). Since CYCD3;1 is known to be degraded through the

ubiquitin-proteasome pathway (Planchais et al. 2004), our results suggested that CDKB2 may also be unstable *in vivo*. In contrast, CDKA;1, CDKB1;1, and CDKB1;2 do not have typical PEST sequences (Fig. 1).

In order to investigate the stability of CDKB2, we performed an *in vivo* degradation assay using an *Arabidopsis* cell culture. The total protein from cell suspensions was immunoblotted with an anti-CDKB2 antibody. Owing to the 90% identity of the antigen peptides, this antibody may recognize both CDKB2;1 and CDKB2;2. When *de novo* protein synthesis was blocked with cycloheximide (CHX), the level of CDKB2 protein gradually decreased from 2 h after application (Fig. 2). In the presence of a specific proteasome inhibitor MG132 together with CHX, no significant reduction was observed (Fig. 2), suggesting that CDKB2 may be degraded via the ubiquitin-proteasome pathway in plant cells. In contrast, the CDKA;1 level remained unchanged until 6 h after application, indicating the stable nature of this protein.

We then examined the contribution of the putative PEST motif to proteolysis. The 2.2-kb promoter region of *CDKB2;1* was fused to the β-glucuronidase (GUS) gene (Pro-GUS). The same promoter containing the first exon was also fused in-frame to the GUS gene (Pro-NT::GUS) (Fig. 3A). It should be noted that the PEST sequence identified above resides in the first exon of *CDKB2;1*. These constructs were introduced into tobacco Bright Yellow-2 (BY-2) cells, and stably transformed single calli were cultured in liquid medium. Since the GUS activities varied with each transformant, three independent lines exhibiting different levels of GUS activity were subjected to analysis. As shown in Fig. 3B, the GUS activities were relatively higher in the Pro-GUS cells compared to the Pro-NT::GUS cells. MG132 treatment of the Pro-NT::GUS cells increased the GUS activity to 125%–150% of that of the dimethylsulfoxide (DMSO)-treated cells, while the activity was decreased to 81%–93% in the Pro-GUS cells. This is in agreement with immunoblotting using anti-GUS antibody: the level of NT::GUS protein was significantly elevated by MG132 application, while in the Pro-GUS

cells a high level of GUS protein was detected irrespective of MG132 treatment (Fig. 3C). A semi-quantitative reverse transcription-PCR (RT-PCR) demonstrated that the transcripts of both *GUS* and *NT::GUS* were slightly decreased in the presence of MG132 (Fig. 3D). These results suggest that the PEST sequence in the first exon is associated with protein degradation by proteasomes. When the Pro-NT::GUS cells were synchronized with aphidicolin, a periodic change in GUS activity was observed, with the maximum activity occurring from the late G2- to the M-phase. However, in the Pro-GUS cells, the GUS activities were maintained high during the cell cycle, possibly because of the carry-over of GUS protein from the previous round of the cell cycle (data not shown). Therefore, we were unable to identify a phase-specific requirement for the machinery that is engaged in CDKB2 degradation.

The same reporter constructs were introduced into *Arabidopsis* plants and *GUS* expression was detected in the root tips. In the Pro-NT::GUS plants, we observed a patchy pattern of signals, whereas the *GUS* expression in the Pro-GUS plants was relatively uniform (Fig. 4A, C). When the seedlings were cultured on an MG132-containing medium, the GUS activity became stronger in the Pro-NT::GUS plants, but not in the Pro-GUS plants, which exhibited even less *GUS* expression (Fig. 4B, D). This result is consistent with the expression in BY-2 cells described above. Previously, we reported that the 0.9-kb promoter region of *CDKB2;1*, which is included in the above constructs, was sufficient to produce the G2-to M-phase-specific expression (Kono et al. 2003). Nonetheless, the Pro-GUS plants did not exhibit a patchy pattern of signals in the root meristem. Based on these results, we propose that the periodic accumulation of CDKB2;1 is determined not only by a transcriptional control, but also through proteasome-mediated protein degradation.

Recently, Zhiponova et al. (2006) cloned a mitosis-specific promoter of alfalfa *Medsa;CDKB2;1* and fused it to *GUS* in order to observe the expression pattern *in planta*. The *GUS* expression was relatively uniform at the root tips, which is similar to that observed in our Pro-GUS plants. However, a computational search of alfalfa CDKs for potential PEST

sequences revealed that Medsa;CDKB2;1 also has a high PEST score of 9.76, whereas Medsa;CDKA;1, Medsa;CDKA;2, and Medsa;CDKB1;1 have no sequence with a score above zero. Similarly, in *Antirrhinum*, only CDKB2 possesses a potential PEST motif. Therefore, regulatory mechanisms mediated through protein degradation may be a common feature of CDKB2 that enable this protein to exhibit G2-to M-phase-specific functions during the cell cycle. It is well known that proteasome-mediated pathways also control other cell cycle regulators such as cyclins, CDK inhibitors, and E2F transcription factors (Genschik et al. 1998, Criqui et al. 2000, del Pozo et al. 2002, Weingartiner et al. 2003, Verkest et al. 2005, Magyar et al. 2005, Jakoby et al. 2006). Some of these are highly unstable proteins; for example, CYCD3;1 exhibited a half-life of 7 min when protein synthesis was blocked by CHX (Planchais et al. 2004). Although CDKB2 was not as unstable as CYCD3;1, our results indicated that protein degradation mechanisms are a prerequisite for the strict control of CDKB2 protein abundance. Further studies will reveal whether the PEST motif in CDKB2 is crucial for cell cycle progression and cell proliferation in plant tissues.

Materials and Methods

Plant growth conditions

Arabidopsis MM2d cells were cultured in MSS medium [1× Murashige and Skoog's (MS) medium, 3% (w/v) sucrose, 0.5 mg liter^{-1} 1-naphthaleneacetic acid, and 0.05 mg liter^{-1} kinetin (pH 5.8)] at 27°C. Tobacco BY-2 cells were cultured in LSD medium [1× MS salts supplemented with 200 mg liter^{-1} potassium dihydrogenphosphate, 100 mg liter^{-1} myo-inositol, 1 mg liter^{-1} thiamine hydrochloride, 3% (w/v) sucrose, and 0.2 mg liter^{-1} 2,4-D (pH 5.8)] at 27°C. *Arabidopsis* plants were grown on MS medium [0.5× MS salts, 1× MS vitamins, 2% (w/v) sucrose, and 0.8% (w/v) agar (pH 6.3)] under continuous light conditions at 23°C.

Plasmid constructions

The *CDKB2;1* promoter region and the first exon, which extend from 2170 bp upstream to 225 bp downstream of the start codon, were PCR-amplified and cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA). The plasmid was then subjected to the LR reaction using the destination vector pGWB3 (a gift from Dr. T. Nakagawa) to generate a translational fusion with *GUS* at the C-terminus (Pro-NT::GUS). In the case of Pro-GUS, the binary construct was prepared with the promoter fragment, which extends from 2170 bp to 1 bp upstream of the start codon. The resultant plasmids were used for the *Agrobacterium*-mediated transformation of BY-2 cells and *Arabidopsis* plants (ecotype, Columbia-0).

In vivo *degradation assay*

CHX and MG132 (Calbiochem, Darmstadt, Germany) were dissolved in ethanol and DMSO, respectively, and each used at a final concentration of 100 μM. After the addition of these inhibitors, four-day-old MM2d cells were further cultured for several hours. Cell protein extracts were subjected to immunoblotting by using an ECL western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA). The anti-CDKA;1 antibody was raised against the C-terminal FKDLGGMP peptide of *Arabidopsis* CDKA;1. The anti-CDKB2 antibody was described by Kono et al. (2003).

GUS assay and expression analyses with tobacco BY-2 cells

Four-day-old transgenic BY-2 cells were cultured in the presence or absence of MG132 for 6 h and total protein was extracted in GUS buffer [50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) Sarkosyl NL-97, and 10 mM β-mercaptoethanol]. One hundred microliters of extract was mixed with an equal volume of 1 mM 4-methylumbelliferyl-β-D-glucuronide in GUS buffer, and incubated for 20 min at 37°C. Five

microliters of each reaction was mixed with 1 ml of 0.2 M sodium carbonate, and the fluorescence was quantified by a fluorescence spectrometer (model F-4500; HITACHI, Tokyo, Japan). The GUS activity was estimated from the fluorescence readings, which were divided by the protein concentrations and incubation times. The same protein extract was also used for immunoblotting with an anti-GUS antibody (Molecular Probes, Eugene, OR, USA). Semi-quantitative RT-PCR was performed with a TITANIUM One-Step RT-PCR Kit (BD Biosciences, Mountain View, CA, USA). Two hundred nanograms of RNA and the following primers were used: 5′-GCGTTTCGATGCGGTCACTCATTAC-3′ and 5′-CGCTAGTGCCTTGTCCAGTTGCAAC-3′ for *GUS*, and 5′-GGTAGGATACAACCCTGACAAGATC-3′ and 5′-GGCTCATTAATCTGGTCAAGAGCATC-3′ for *EF1α*. The PCR conditions were 1 cycle at 50°C for 60 min and at 94°C for 5 min; 23 cycles (for *GUS*) or 19 cycles (for *EF1α*) at 94°C for 30 s, at 65°C for 30 s, and at 68°C for 60 s; and 1 cycle at 68°C for 2 min.

GUS staining of Arabidopsis *plants*

Ten-day-old plants were transferred to MS medium with or without MG132 and incubated for further 6 h. The seedlings were then immersed in 90% (v/v) acetone overnight at −20°C, washed with 100 mM sodium phosphate buffer (pH 7.0), and stained in the same buffer containing 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide at 37°C for 3 h. Samples were mounted in a mixture of chloral hydrate, glycerol, and water (8 g:1 ml:2 ml).

Acknowledgements

We thank Dr. Tsuyoshi Nakagawa (Shimane University) for the pGWB3 vector. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (grant no. 17027007 and no. 18056006) and a Grant-in-Aid for Scientific Research (B) (grant no.

16370019) from the Ministry of Education, Sports and Culture, Science and Technology of Japan, and by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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Legends to figures

Fig. 1 The N-terminal amino acid sequences of *Arabidopsis* CDKs. Asterisks represent regions with a high PEST score in CDKB2. The PSTAIRE motif is underlined. Amino acids in black and gray boxes indicate identical and similar residues, respectively. Amino acid positions are indicated at the N- and C-terminal ends of each sequence.

Fig. 2 Protein stability of CDKB2 in *Arabidopsis* cell culture. Four-day-old MM2d cells were treated with CHX and MG132 and cultured for the indicated times. As a control for MG132, DMSO was used. Twenty micrograms of total protein was subjected to immunoblotting with anti-CDKA;1 or anti-CDKB2 antibodies.

Fig. 3 Protein stability of GUS constructs in tobacco BY-2 cells. (A) Pro-GUS and Pro-NT::GUS constructs. Open and filled boxes represent untranslated regions and exons of *CDKB2*, respectively. (B) GUS activities of four-day-old Pro-GUS and Pro-NT::GUS cells. Three independent lines were tested. Filled and blank bars indicate GUS activities of cells treated with MG132 and DMSO for 6 h, respectively. (C) Immunoblotting of protein extracts from cells described in (B). Anti-GUS antibody was used. Note that, in some Pro-GUS cell lines, a slight reduction in the protein level was detected by MG132 application (data not shown). (D) Amounts of *GUS* transcripts in the same cultures as (B) and (C) were examined by semi-quantitative RT-PCR. *EF1α* transcripts were detected as a quantitative control. In (C) and (D), representative results among three independent lines are shown.

Fig. 4 GUS staining of transgenic *Arabidopsis* root tips. Pro-GUS plants (A, B) and Pro-NT::GUS plants (C, D) were treated with DMSO (A, C) or MG132 (B, D) for 6 h. Bar = 50 μm.

Fig. 1

protein (locus)	PEST score	
		************
CDKB2;1 (At1g76540)	9.72	32- EKATGKIVALKKTRLHEDEEGVPSTTLREISILRMLARDP -73
CDKB2;2 (At1g20930)	10.24	30- EKATGMIVALKKTRLHEDEEGVPPTTLREISILRMLARDP -75
CDKB1;1 (At3g54180)	-2.37	22- EKGTGKLVALKKTRLEMDEEGIPPTALREISLLQMLSTSI -63
CDKB1;2 (At2g38620)	-2.37	22- EKTTGKLVALKKTRLEMDEEGIPPTALREISLLQMLSQSI -63
CDKA;1 (At3g48750)	-0.13	22- DKVTNETIALKKIRLEQEDEGVPSTAIREISLLKEMQHSN -63

Fig. 2

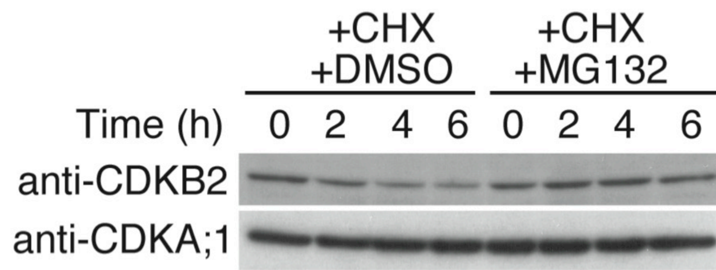

Fig. 3

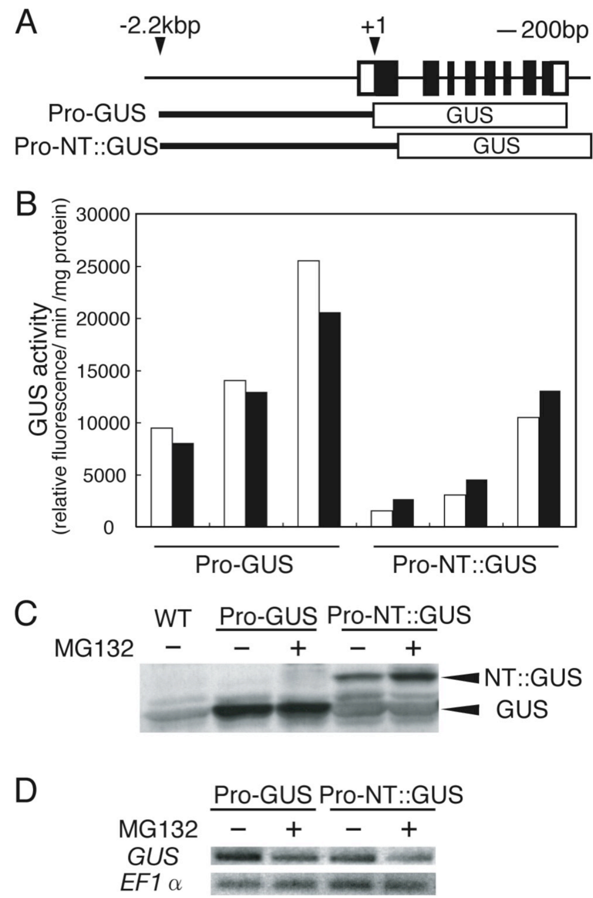

Fig. 4