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# A study of CDK expression and endocycle induction in *Arabidopsis thaliana*

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**CONTENTS**

## ABBREVIATIONS

| | |
|---|---|
| Arabidopsis; | *Arabidopsis thaliana* |
| BY-2; | Bright Yellow-2 |
| CDK; | cyclin-dependent kinase |
| CDKA; | A-type CDK |
| CDKB; | B-type CDK |
| CHX; | cycloheximide |
| Col; | Columbia ecotype |
| CP; | cisplatin |
| CR; | *cis*-acting region |
| DMSO; | dimethylsulfoxide |
| GUS; | β-glucuronidase |
| HU; | hydroxyurea |
| L*er*; | Lansberg *erecta* ecotype |
| MMS; | methanemethylsulfonate |
| mPS-PI; | modified pseudo-Schiff propidium iodide |
| MS; | Murashige and Skoog |
| PBS; | phosphate buffered saline |
| QC; | quiescent center |
| RAM; | root apical meristem |
| RT-PCR; | reverse transcription-PCR |
| SAM; | shoot apical meristem |
| UTR; | untranslated region |
| UV; | ultraviolet rays |

バイオサイエンス研究科　博士論文要旨

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During the course of plant development, control of cell proliferation and growth is essential to produce organized tissues and organs. The cell cycle machineries are involved in both, since cell division is controlled by the mitotic cell cycle and cell growth is often associated with endoreduplication that is caused by the endocycle. The eukaryotic cell cycle is controlled by the activities of evolutionally conserved protein kinase complexes, which consist of cyclins and cyclin-dependent kinases (CDKs). In plants, A-type and B-type CDKs (CDKA and CDKB) are assumed to mainly control cell cycle progression. While CDKAs are functional orthologs of yeast Cdc2/Cdc28p and expressed throughout the cell cycle, CDKBs are plant-specific CDKs and expressed specifically from the late S to the M phase. Based on amino acid sequences, CDKBs are further classified into two subtypes, CDKB1 and CDKB2. The *Arabidopsis* genome encodes genes for one CDKA and four CDKBs, but regulatory mechanisms governing their expression in tissues and in response to external signals have not been studied well.

To dissect the transcriptional regulation of *CDKA*, I first conducted a promoter analysis of *Arabidopsis CDKA;1*. My analyses revealed that the *CDKA;1* promoter contains several different *cis*-acting regions, such as cell-type specific and general regulatory elements. I found that *Arabidopsis* CDKB2s, but not CDKA or CDKB1s,

contain a possible PEST sequence, which is a hallmark of unstable proteins. Indeed, my results indicated that the protein accumulation of CDKB2 was controlled by ubiquitin-mediated protein degradation in both suspension cultured cells and plant tissues.

Yeast and animal cells possess the DNA damage checkpoint that ensures proper DNA replication and transmission of genomes to daughter cells. Previous studies identified several key factors that are engaged in cell cycle arrest in response to DNA damage, but in plants, genes for corresponding orthologs are missing, implying that plants may have distinct molecular mechanisms to cope with genotoxic stress. My study revealed that DNA double-strand breaks (DSBs) inhibit the mitotic cell cycle and increase the DNA ploidy in *Arabidopsis*. Despite an increase of nuclear DNA content, the number of kinetochore did not change by DSB-inducing treatments, suggesting that endoreduplication occurred by triggering transition to the endocycle. I also found that the expression of *CDKB2*, but not *CDKA* or *CDKB1*, was preferentially downregulated in response to DSBs. A microarray analysis showed a reduced expression of G2/M-related genes, which supports the endocycle induction by skipping the mitosis. In addition, by using *Arabidopsis* mutants that have defects in DNA damage signaling or endoreduplication, I identified an upstream signaling pathway that is essential for the DSB-induced endoreduplication. These results give us insights into the plant-specific regulatory mechanisms underlying survival strategies against genotoxic stress, and the molecular mechanism that controls the transition from the mitotic cell cycle to the endocycle.

## PREFACE

Cell division is a highly regulated process in individual cells: cell growth should be coordinated with cytokinesis, and chromosome duplication must be completed before mitosis in order to keep producing cells in countless rounds of cell divisions. Not only does multicellularity require cell division for growth and development, it imposes extra complexity on regulation of cell division. Cell proliferation in multicellular organisms must be temporally and spatially regulated to accomplish proper development. Most cells that compose an individual stop dividing and differentiate, others actively divide, yet others remain quiescent and are stimulated by endogenous and exogenous signals. Plant meristems continue producing cells throughout a lifetime and other cells also sustain ability to resume and reproduce a whole body, excerting the plant-specific ability of totipotency.

Plant cells are cemented with each other; thus, particularly in plants, the control of cell division plays an essential role in overall growth and organ formation. Molecular genetics using Arabidopsis mutants has revealed that defects in cell cycle progression or inability to maintain cell division in meristems give rise to morphogenesis defects, showing that keeping cell division activity in meristems higher than a particular threshold and orderly progression of the cell cycle are required for proper organogenesis. On the other hand, reverse genetic studies also have reported knockout and overexpression phenotypes of cell cycle regulators in various plant species (Inzé and De Veylder, 2006).

Since cell number and cell size are the determinants for organ shape and size, the relationship between cell proliferation and cell expansion has been an interesting question that has been under debate thus far. In general, dividing cells are relatively small, and they increase their volume by endoreduplication after exiting the mitotic cycle (Inzé and De Veylder, 2006). Endoreduplication is a modified form of the cell cycle wherein the M phase is skipped, indicating that during plant development, the regulation of cell cycle is vital for both

cell proliferation and cell expansion.

Progression of the eukaryotic cell cycle is controlled by activity of protein kinase complexes, which consist of a cyclin-dependent kinase (CDK) as a catalytic subunit and a cyclin as a regulatory subunit, and the activity varies periodically during the cell cycle. CDK activity is required for controlling all aspects of cell cycle progression including commitment, DNA replication, spindle formation, chromosome separation, and cytokinesis (Morgan, 2006). Although a single CDK (Cdc2 in *Schizosaccharomyces pombe*) controls both the G1-to-S and G2-to-M phase transitions in yeast, distinct CDKs are sequentially associated with different cyclins and regulate cell cycle progression in animals (Morgan, 2006). Plants also have different types of CDKs that are classified into six types, namely, CDKA–CDKF (Vandepoele et al., 2002). Among them, the A-type and B-type CDKs are assumed to be crucial for plant cell cycle progression (Inzé and De Veylder, 2006). A-type CDKs (CDKAs) are functional homologs of the fission yeast Cdc2, and they contain a cyclin-binding domain with the canonical PSTAIRE motif. B-type CDKs (CDKBs) are plant-specific CDKs with a divergent cyclin-binding motif (PPTALRE or PS/PTTLRE in CDKB1 or CDKB2, respectively) and, in contrast to CDKAs, they are unable to complement *cdc2* mutations in yeast (Imajuku et al., 1992; Fobert et al., 1996). CDKA is expressed constitutively throughout the cell cycle (Magyar et al., 1997; Sorrell et al., 2001; Menges et al., 2005), whereas the expression of CDKB is restricted to a specific stage, which is from the late S phase to the M phase (Fig. 1A; Segers et al., 1996; Magyar et al., 1997; Umeda et al., 1999; Sorrell et al., 2001; Menges et al., 2005). Based on the expression and kinase activity patterns, CDKAs and CDKBs are assumed to participate in both the G1/S and G2/M transitions, and only the G2/M transition, respectively (Inzé and De Veylder, 2006).

Virtually all tissues and organs except for inflorescence contain endoreduplicated cells in Arabidopsis: they have been found in leaf trichomes (Melaragno et al., 1993), epidermis of

leaf and stem (Galbraith et al., 1991; Melaragno et al., 1993), and hypocotyl (Gendreau et al., 1997). Endoreduplication is observed not only in plants but also other eukaryotes (Edgar and Orr-Weaver, 2001), and a consensus view based on the studies of animals and plants is that the activity of the S-phase CDKs is sustained or upregulated while the activity of the M-phase CDKs is downregulated during the endocycle (Fig. 1B; Sauer et al., 1995; Edgar and Orr-Weaver, 2001; Grafi and Larkins, 2001). In fact, downregulation of CDKA activity by ectopic expression of a CDK inhibitor (De Veylder et al., 2001; Schnittger et al., 2003; Verkest et al., 2005; Weinl et al., 2005), or a dominant-negative CDKB1 (Boudolf et al., 2004) increased the ploidy level in plant cells. Higher mitotic activity achieved by overexpression of *CYCD3;1* (Dewitte et al., 2003), and by mutation in the activator of anaphase-promoting complex/cyclosome (APC/C), *CELL CYCLE SWITCH 52A1* (*CCS52A1*)*/FIZZY RELATED2* (*FZR2)/CDH1* (Larson-Rabin, et al., 2009; Vanstraelen et al., 2009), had inhibitory effects on endoreduplication.

In this thesis, I focus on two essential and contrastive CDKs, *CDKA;1* and *CDKB2;1* of Arabidopsis. In addition to *in planta* expression of these two CDKs, cellular response to DNA damage and associated changes in cell cycle regulators were analyzed to study how plant cell cycle is regulated during development and under stressed conditions.

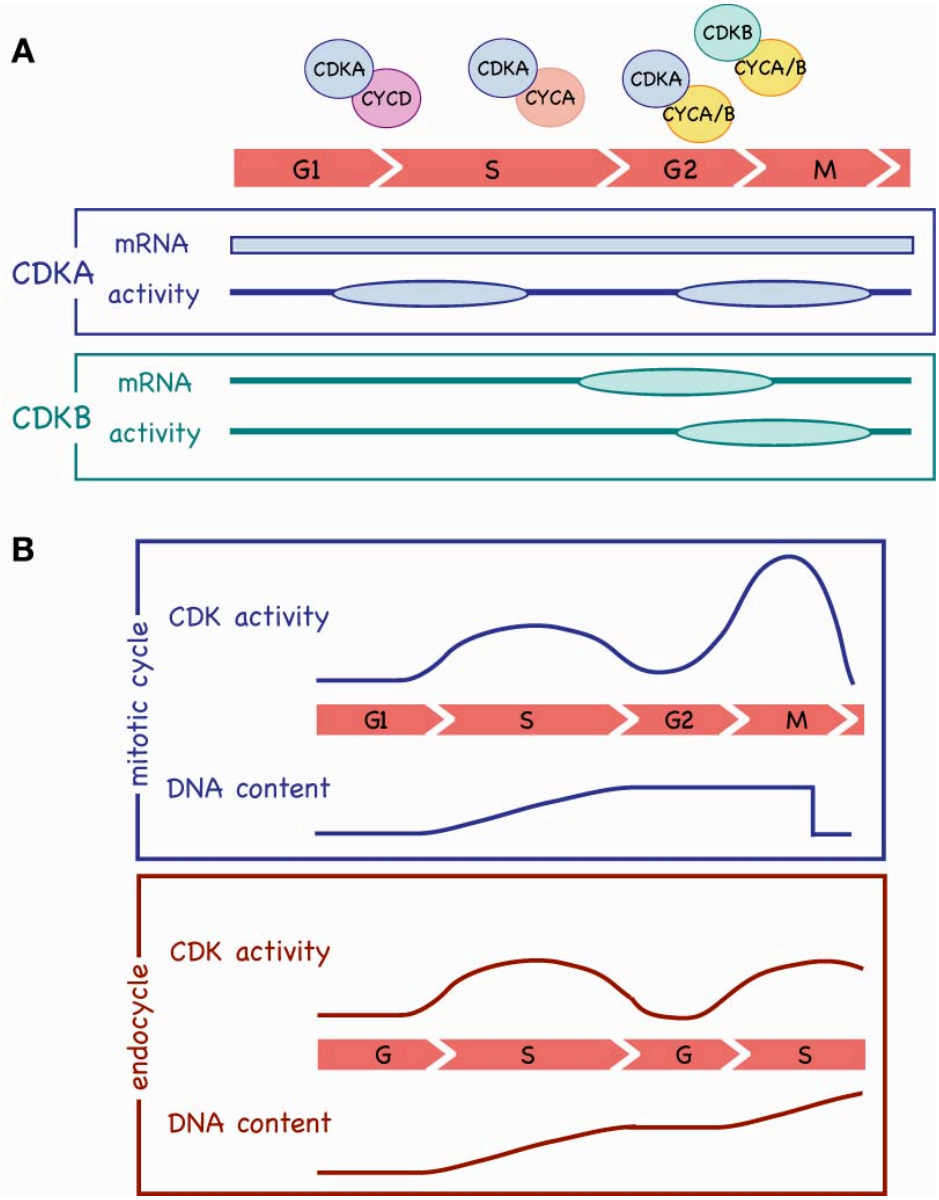



**Figure 1. Transcript accumulation and kinase activities of CDKs in the cell cycle and the endocycle**

(A) Expression and kinase activity of *CDKA* and *CDKB* during the cell cycle.

(B) A schematic representation of CDK activity in the cell cycle and the endocycle.

CHAPTER I

# Quantitative and cell type-specific transcriptional regulation of A-type cyclin-dependent kinase

## 1.1 INTRODUCTION

The Arabidopsis genome encodes a single gene for CDKA, namely, *CDKA;1* (Vandepoele et al., 2002). Several cyclins are known to bind to CDKA;1 and control cell cycle progression; D-type cyclins are assumed to function in mediating internal or external signals to the cell cycle, and A- and B-type cyclins are involved in DNA replication, G2/M transition, and mitotic events (Criqui et al., 2000; Healy et al., 2001; Weingartner et al., 2003; Dewitte et al., 2003; Menges et al., 2006; Kono et al., 2007). The CDK-activating kinase is known to enhance CDKA;1 activity by phosphorylation (Shimotohno et al., 2006), and substitution of the phosphorylated threonine residue with alanine rendered it inactive (Harashima et al., 2007). Hemerly et al. (1995) generated transgenic tobacco plants that overexpressed *CDKA;1* with dominant negative mutations. Compared to wild-type plants, these plants exhibited lower CDKA activities and produced smaller leaves and flowers consisting of larger cells. When an embryo-specific promoter was used to drive the expression of dominant-negative *CDKA;1* in *Arabidopsis*, the phyllotactic pattern and leaf shape were distorted, and some seedlings consisted of one or two cotyledon-like structure(s) (Hemerly et al., 2000). It has been reported that the knock-out mutants of *CDKA;1* exhibited defects in male gametogenesis, which resulted in the production of bicellular pollen grains, and failed in double fertilization and embryogenesis (Nowack et al., 2005; Iwakawa et al., 2006). All these results demonstrate the importance of *CDKA;1* for a broad range of developmental processes during the plant life cycle.

Previous reports have shown that the *CDKA;1* transcripts were accumulated in various tissues of actively dividing cells, such as shoot and root apical meristems, developing leaves, floral organs, and pericycle and vascular tissues (Martinez et al., 1992; Hemerly et al., 1993). Its expression is up-regulated by auxin or cytokinin application and wounding stress in leaves, and is inhibited by abscisic acid treatment of root tissues (Hemerly et al., 1993). Using

tobacco leaf protoplasts for studying the promoter activity of *CDKA;1*, Hemerly et al. (1993) revealed that expression of this gene was induced in the dedifferentiation process, which was triggered by auxin and cytokinin, but it was also enhanced under conditions wherein cells did not actually divide, such as in media containing either auxin or cytokinin. This result indicates that *CDKA;1* expression may not always be linked to actual cell division but precede it. In fact, *CDKA;1* transcripts were detected in the pericycle of roots, in which cells are capable of restarting cell division and producing lateral root primordia (Martinez et al., 1992; Hemerly et al., 1993; Himanen et al., 2002). Therefore, it is likely that the induction of *CDKA;1* expression may be a prerequisite for the activation of post-embryonic cell division during organ formation.

Imajuku et al. (2001) dissected the *CDKA;1* promoter region and identified the regulatory elements required for expression in developing trichomes by using *glabra* mutants. They also demonstrated that the region downstream of the transcriptional start site is required for *CDKA;1* expression in proliferating tissues. Since *CDKA;1* is the sole gene encoding for the yeast Cdc2 ortholog in Arabidopsis, spatial and temporal control of its expression will play a vital role in determining mitotic activity and cell division competency. However, little is known about how its transcription is regulated in different cell types during plant development. Here, to gain more insight into *CDKA;1* expression, I made a dissection series of the *CDKA;1* promoter and analyzed their expression patterns in tissues. My results revealed tissue-specific regulatory regions as well as general quantitative regulatory regions in the promoter. I also identified cell layer-specific transcriptional regulation, which may be involved in proper development of leaves in Arabidopsis.

## 1.2 MATERIALS AND METHODS

### 1.2.1 Plant material

Arabidopsis plants were grown in Murashige and Skoog (MS) medium [0.5× MS salts, 1× MS vitamins, and 2% (w/v) sucrose (pH 6.3)] under continuous light conditions at 23°C. To observe the inflorescences and embryos, I transplanted the seedlings into soil and grew them in a greenhouse under a 15-h light/8-h dark cycle at 22°C. The *phb-1d* mutant (CS3761) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). The *GUS* reporter genes were introduced into *A. thaliana* ecotype Columbia via *Agrobacterium*-mediated transformation (Clough and Bent, 1998). T2 plants were tested for segregation, and at least three independent T3 lines that showed representative *GUS* expression pattern were subjected to *GUS* expression analyses. For analyzing each construct, more than 15 SAMs were sectioned, and more than 30 individuals were used for observations of other tissues. Two independent and representative lines were used for crossing with *phb-1d*, and at least 10 *phb-1d*/+ individuals in F1 generation were analyzed for each line.

### 1.2.2 Plasmid construction for *GUS* expression analysis

The promoter fragments of *CDKA;1* were amplified by PCR using the primers listed in Table 1-1 and cloned into the pENTR/D-TOPO vector (Invitrogen) according to the manufacturer's instruction. Then, each fragment was cloned into pGWB3533 (Nakagawa, unpublished) with LR clonase (Invitrogen) to generate a fusion construct with *GUS*. For construction of 7509F/7R and *proCDKA-CDKA::GUS*, pGWB3 (Nakagawa et al., 2007) was used. Neither of these vectors contains any minimum promoter region. For construction of *proCDKA-CDKA::GUS*, a genomic fragment encompassing the promoter and the coding region was amplified with primers shown in Table 1-1.

### 1.2.3 GUS staining

Plant tissues were incubated in 90% (v/v) acetone at –20°C overnight, and washed in 100 mM

sodium phosphate buffer (pH 7.0). For GUS staining, samples were incubated in a solution [100 mM sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.5 mg/mL 5-bromo-4-chrolo-3-indolyl-β-D-glucuronide (pH 7.0)] at 37 °C for 1 or 24 h, and mounted in a mixture of chloral hydrate, glycerol, and water (8 g:1 mL:2 mL). To make sections of the shoot apices, the GUS-stained samples were dehydrated with an ethanol series, and then the ethanol was substituted with Technovit 7100 (Heraeus Kulzer) solution. After solidification with hardener II (Heraeus Kulzer), they were sectioned at a 6 μm or 10 μm thickness, and mounted in 50% (v/v) glycerol.

**Table 1-1.** Primers used for cloning of *CDKA;1* promoter fragments. Forward primers contain the nucleotides CACC for cloning into the pENTR/D-TOPO vector.

| Construct No. | Primers (5′-3′) forward / reverse |
|---|---|
| 7501 | caccTAATTCATATTATCTGATCTTAAAAAATTTAATTCAAG |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7505 | caccACATATGATTTTCATATTATTGATCATTTATC |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7506 | caccTTTAATTAAAACAACTTTCATATTATTGC |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7507 | caccCTAAAACGTCTTTTTCTAACTTAGATTC |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7508 | caccGTAAAACCAAAACTCTTGATTTATAATTGG |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7509 | caccTATTTTTTTATCATTGAATACCAATCAAAC |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7510 | caccGAGAAAAAAAAAAATAACGAACAAAATTTCC |
| | CAATTCCTGAATAATAAAGCTGAAGTAAAC |
| 7502R | caccACATATGATTTTCATATTATTGATCATTTATC |
| | AATTAAGCTGTATATCAGCACACAACAATC |
| 7503R | caccACATATGATTTTCATATTATTGATCATTTATC |
| | TTTGATTCTTTGGACAGAATTATGAAAATG |
| 7504R | caccACATATGATTTTCATATTATTGATCATTTATC |
| | CTAAAGCGAACAATCATAAACACTGATGAC |
| 7505R | caccACATATGATTTTCATATTATTGATCATTTATC |
| | GAGGAAAACAAGTAGAGATCAGCGACGAAG |
| 7506R | caccACATATGATTTTCATATTATTGATCATTTATC |
| | TTCGCACGGAAAACAAGTTCCTCCTC |
| 7507R | caccACATATGATTTTCATATTATTGATCATTTATC |
| | CACACCGACGGATCCTCTCTCTTCTCTGTG |
| 7509F/7R | caccTATTTTTTTATCATTGAATACCAATCAAAC |
| | CACACCGACGGATCCTCTCTCTTCTCTGTG |
| *proCDKA-CDKA::GUS* | caccTAATTCATATTATCTGATCTTAAAAAATTTAATTCAAG |
| | AGGCATGCCTCCAAGATCCTTG |

## 1.3 RESULTS

### 1.3.1 Expression pattern of *CDKA;1* in various tissues

Imajuku et al. (2001) determined the transcription start site of the *CDKA;1* gene to be at position –679 bp (we considered the A of the initiation codon as +1). The 5′ untranslated region (UTR) contains the first intron, the position of which determined to be from –566 bp to –52 bp (The Arabidopsis Information Resource (TAIR); www.arabidopsis.org, June 2008). While Y patches were present around the transcriptional start site, the typical TATA box was not found in the 150-bp region proximal to the initiation codon by the program PlantPromoterDB (Yamamoto et al., 2006). This suggests that *CDKA;1* may utilize the Y patches instead of a TATA box as core promoter elements (Yamamoto et al., 2006).

To reveal the regulatory mechanisms underlying *CDKA;1* expression, I first cloned the 2690-bp upstream region from the initiation codon and fused it to the β-glucuronidase (*GUS*) gene to create a reporter construct (Fig. 1-1A). Seven-day-old seedlings carrying the reporter gene showed strong *GUS* expression, especially in the roots. The root tips, stele, and vascular tissue of the shoots and stipules were stained by incubation in a GUS-staining buffer for 1 h (Fig. 1-1B, D, E, G). On 24-h incubation, I observed an intense signal in the stomata, which was surrounded by weakly stained mesophyll cells in the leaves (Fig. 1-1C, F). Vertical sections of GUS-stained tissues showed a uniform signal in shoot apical meristems (SAMs) and developing trichomes (Fig. 1-1H, I). GUS expression was also observed to be uniform in developing and mature embryos (Fig. 1-1J–L). In flowers, the reporter gene was highly expressed in the pistils, whereas weaker expressions were detected in the petals, sepals, pollen grains, and filaments (Fig. 1-1M–O). In summary, the strongest promoter activity was observed in root tips, and the second strongest activity was found in root steles and stipules. A weaker but significant level of *GUS* expression was also detected in the SAMs, vascular tissues, developing trichomes, and in the mesophyll and guard cells of leaves.

### 1.3.2 Regulatory elements involved in quantitative control of *CDKA;1* expression

I then dissected the *CDKA;1* promoter and observed *GUS* expression in tissues. Since Imajuku et al. (2001) have reported that the promoter region from –1268 bp to the initiation codon was enough to reproduce the expression pattern driven by longer promoter fragments, I generated a deletion series of the 1290-bp region as shown in Fig. 1-2. The constructs, which were termed 7505, 7506, 7507, 7508, 7509, and 7510, differed with regard to the length of the 5′ region at 100-bp intervals. Seven-day-old seedlings harboring each reporter gene were subjected to GUS staining. I could not find any significant difference in the level and tissue specificity of the expressions of this gene among transgenic lines transformed with the constructs of 7501, 7505, 7506, 7507, 7508, and 7509 (Fig. 1-3). A shorter incubation in a GUS-staining buffer at a lower temperature (20°C, 10 min) also showed no significant difference among these lines (Fig. 1-4). However, *GUS* expression in the 7510 seedlings was lower in both the shoots and roots as compared to that in the above-mentioned lines, although no obvious change was observed in the expression pattern (Fig. 1-3). I could observe similar quantitative differences in the embryos and inflorescences (Fig. 1-5). These results suggest that the region from –890 bp to –791 bp contains *cis*-regulatory element(s) that promote *CDKA;1* expression independently of tissues.

It has been described that the region between the transcriptional start site and the initiation codon contains a regulatory element that functions in proliferating cells (Imajuku et al., 2001). Therefore, I constructed *GUS* reporter genes with 3′-end truncations of the 1290-bp region. The 7505, 7502R, 7503R, 7504R, 7505R, 7506R, and 7507R constructs differed with regard to the length of the 3′ region at 100-bp intervals (Fig. 1-2). In the 7-day-old seedlings, we found a significant decrease in the level of *GUS* expression between the 7502R and 7503R constructs (Fig. 1-6A). A similar reduction was also noted in embryos (Fig. 1-7), indicating the presence of another *cis*-regulatory element between –200 and –101 bp. In the

7503R–7505R lines, the expression in the roots was gradually reduced as the 3′ end became shorter and, in the roots of the 7506R or 7507R plants, I could not detect any *GUS* expression (Fig. 1-6B). Despite the disappearance of *GUS* expression in roots, we could observe GUS signals in young leaves of the 7506R and 7507R lines (Fig. 1-6B), indicating the presence of root-specific regulatory elements that promote *CDKA;1* expression.

### 1.3.3 Presence of a promoter region enhancing *CDKA;1* expression in the leaf epidermis

To examine cell type-specific regulation of *CDKA;1* expression, I made sections of GUS-stained shoot apices. When the 5′-deletion series (7505–7510) were investigated, no difference was found in the expression patterns as compared to that of the full-length construct 7501; *GUS* expression was observed in the SAM, leaf primordia, and developing leaves (Fig. 1-8A). In order to examine whether post-transcriptional regulation is engaged in the *CDKA;1* expression, I generated transgenic lines expressing the CDKA;1::GUS fusion protein under the 2690 bp promoter region (*proCDKA-CDKA::GUS*). The expression pattern in the SAM was almost the same as that of the transcriptional fusion line 7501; namely, the fusion protein was accumulated uniformly in the meristem and leaf primordia, and the leaf epidermis showed slightly stronger expression (Fig. 1-8B). This result indicates that regulatory mechanisms associated with translation or protein stability do not play an important role in the control of spatial expression pattern of *CDKA;1*.

In contrast, I found differential expression patterns in the dissection series with 3′ truncations (Fig. 1-8C). While the 7505 and 7502R lines did not show any difference, 7503R exhibited lower *GUS* expression in the SAM and RAM, and no expression in the inner layers of leaves (Fig. 1-8C, 1-9). The results showed that the epidermis of leaves and trichomes were prominently GUS-stained (Fig. 1-8C). This indicates that the region between –200 bp and –101 bp is associated with *CDKA;1* expression in the SAM and in leaves except for the epidermis. The lack of this region in the 7503R line might cause a significant reduction of

GUS staining in seedlings due to the loss of expression in the inner layers of leaves (Fig. 1-6). The epidermis-specific expression pattern was also observed in 7504R–7507R. However, in the SAM, *GUS* expression was much lower in the 7504R line, and almost no expression was detected in the 7505R–7507R lines (Fig. 1-8C). It should be noted that the GUS signal disappeared not only in the inner layers but also in the L1 layer of the SAM, although the epidermis-specific expression persisted in the leaves of these transgenic lines.

The 7510 construct with a deletion in the region before –790 bp displayed *GUS* expression in any cell type of the leaves (Fig. 1-8A), and 7507R lacking the region after –601 bp still showed epidermal expression of this gene (Fig. 1-8C). Therefore, it is likely that the region between –790 bp and –601 bp contains another regulatory element that promotes *CDKA;1* expression in the epidermis. To examine this idea, I created another reporter construct, 7509F/7R, which carries the region between –890 bp and –601 bp (Fig. 1-2). Although I included the region from –890 bp to –791 bp that promotes *CDKA;1* expression as mentioned above, the GUS signal was very weak as compared to that in 7507R. However, I could again identify epidermis-specific expression (Fig. 1-8D), suggesting that this region contains enough information to promote epidermal expression in leaves.

I then conducted a detailed analysis of the epidermal expression of this gene by using the 7507R reporter gene. Since the longitudinal sections of shoot apices showed biased GUS staining on the abaxial side of the leaf epidermis (Fig. 1-8C), I also created transverse sections of the SAM and leaves. As expected, *GUS* expression was higher on the abaxial side of young leaves, and only a trace level of expression was observed on the adaxial side (Fig. 1-10B). In the SAM, I could not detect *GUS* expression. I then introduced the 7507R reporter gene into the *phbulosa-1d* (*phb-1d*) mutants. The *phb-1d* mutant is known to develop filamentous leaves that lose the abaxial identity (McConnell and Barton, 1998). Although the stipules were stained in a similar manner to the wild-type background, the *GUS* expression in the leaf

epidermis was severely suppressed, and almost no expression was detected in any cell type (Fig. 1-10C, D). This result supports the idea that a regulatory mechanism functions to enhance the *CDKA;1* expression on the abaxial side of the leaf epidermis.

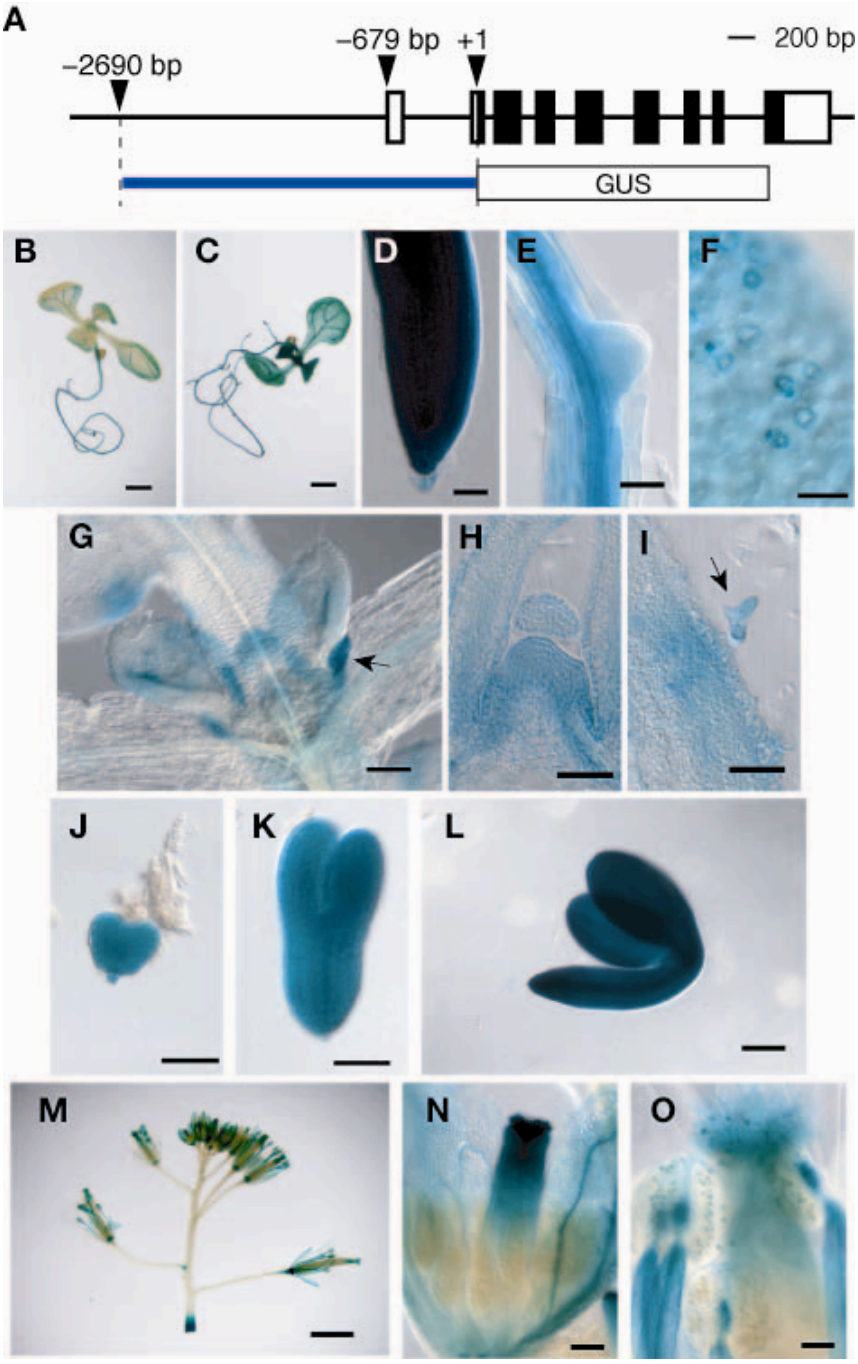



**Figure 1-1. *CDKA;1* expression in various tissues**

(A) The promoter region from –2690 bp to –1 bp (blue bar) was fused to *GUS* and introduced into Arabidopsis plants. The white and black boxes indicate UTRs and protein-coding regions of *CDKA;1*, respectively. (B–G) Seven-day-old seedlings were stained for 1 h (B, D, E, G) or 24 h (C, F) at 37°C: root tip, (D); lateral root primordia, (E); cotyledon, (F); and shoot apex, (G). The arrow indicates a stipule. Shoot apices of 10-day-old seedlings were sectioned and stained for 24 h (H, I). The arrow indicates a developing trichome. Embryos (J–L) and inflorescences (M–O) were stained for 24 h. (N) and (O) show immature and mature flowers, respectively. Bars = 50 μm (A, D–F, H–K), 1 mm (B, C, M), 100 μm (G, L), and 200 μm (N, O).

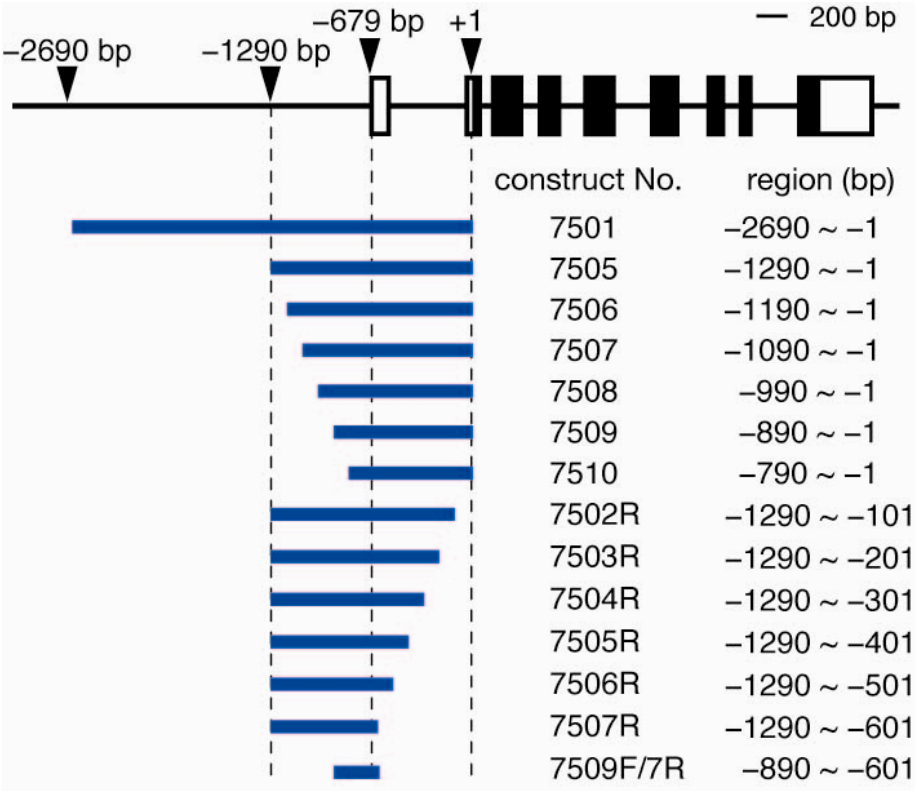



**Figure 1-2. Promoter dissection series of *CDKA;1***

The blue bars indicate promoter regions cloned into the expression vector. In the schematic diagram of the *CDKA;1* genomic region, the white and black boxes indicate UTRs and protein-coding regions, respectively. The construct 7501 is the same as that shown in Fig. 1-1A.

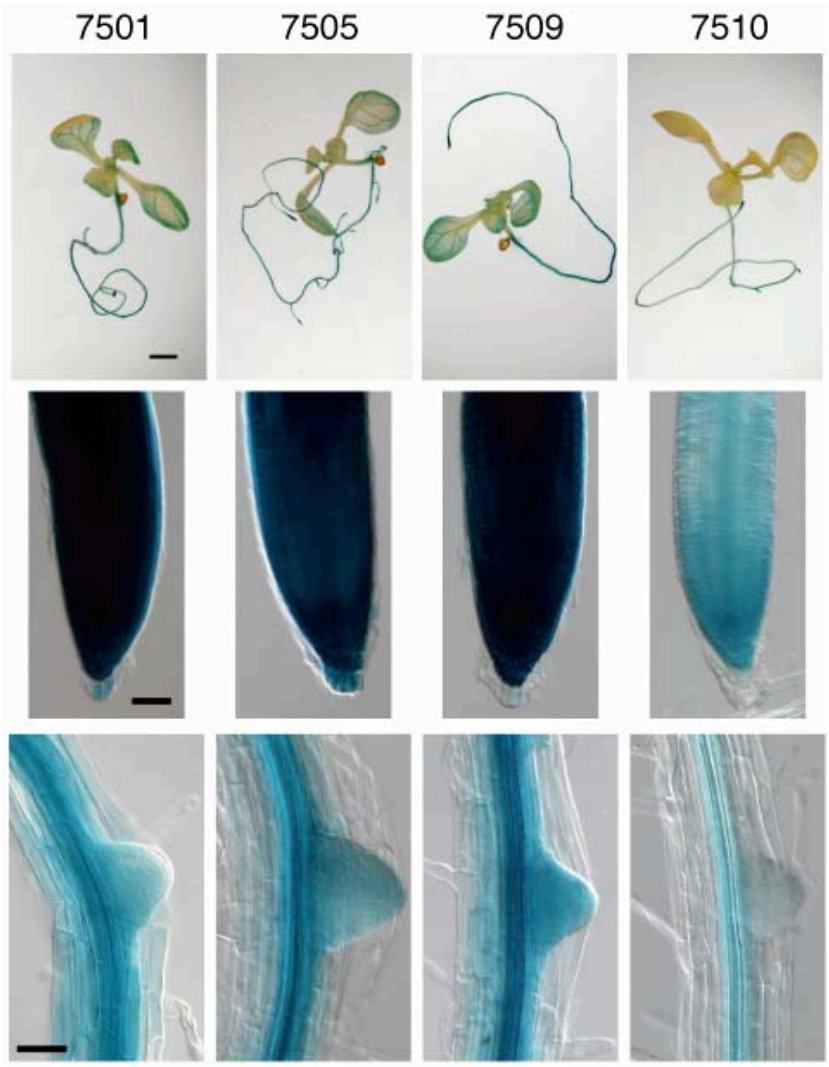



**Figure 1-3. *GUS* expression of the 5′-end dissection series**

Seven-day-old seedlings were stained for 1 h at 37°C. Top row, whole seedlings; middle row, root tips; bottom row, lateral root primordia. Bars for the roots or lateral root primordia equal 50 μm, and that for the whole seedlings equals 1 mm.

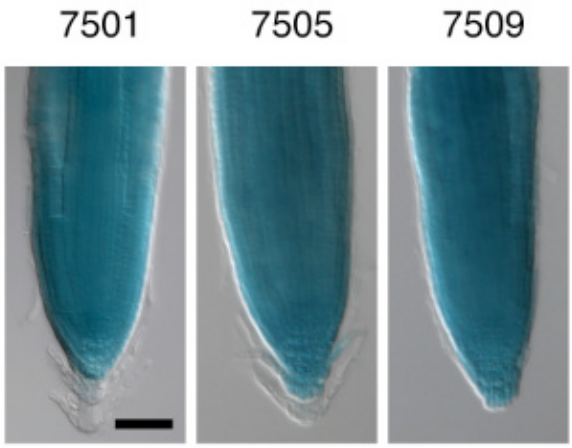



**Figure 1-4. Comparison of *GUS* expression in the 7501, 7505, and 7509 roots**
Root tips of 7-day-old seedlings of the 7501, 7505, and 7509 lines stained at 20°C for 10 min. Bar = 50 μm.

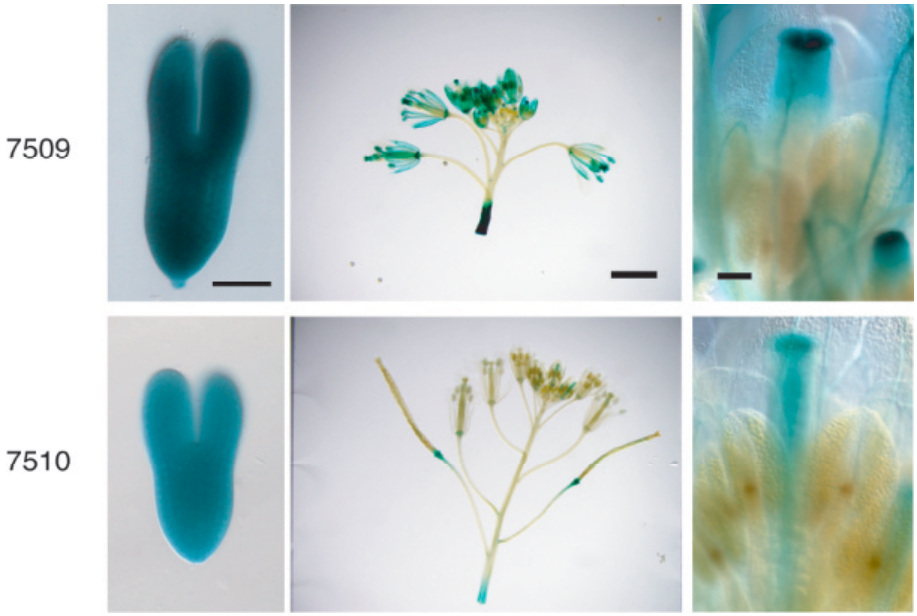



**Figure 1-5. *GUS* expression in embryo and inflorescence of the 7509 and 7510 lines**
Torpedo embryos (left), inflorescences (middle), and flowers (right) of the 7509 and 7510 lines stained at 37°C for 24 h. Bars = 50 μm (left), 2 mm (middle), and 100 μm (right).

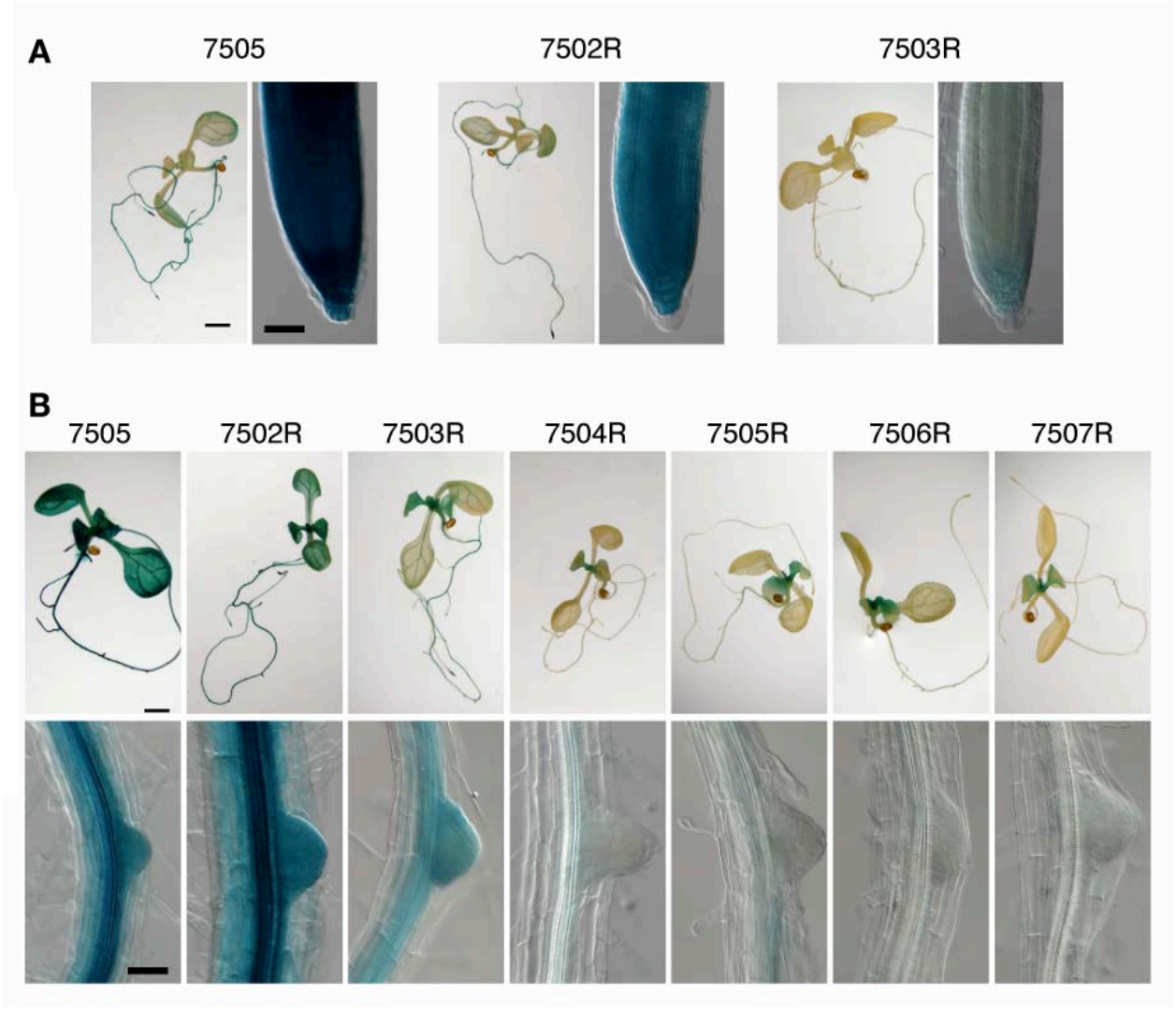



**Figure 1-6. *GUS* expression of the 3′-end dissection series**

Seven-day-old seedlings were stained for 1 h (A) or 24 h (B) at 37°C. (A) left panel, whole seedlings; right panel, root tips. (B) Upper row, whole seedlings; lower row, lateral root primordia. Bars for the roots or lateral root primordia equal 50 μm, and those for the whole seedlings equal 1 mm.

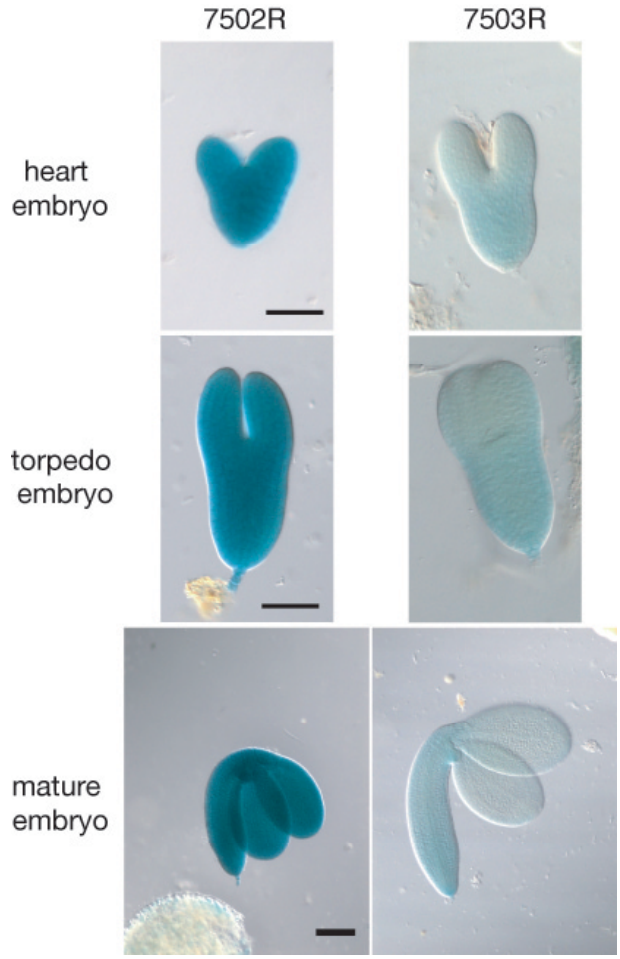



**Figure 1-7. Embryos of the 7502R and 7503R lines**

Samples were stained at 37°C for 24 h. Bars = 50 μm.

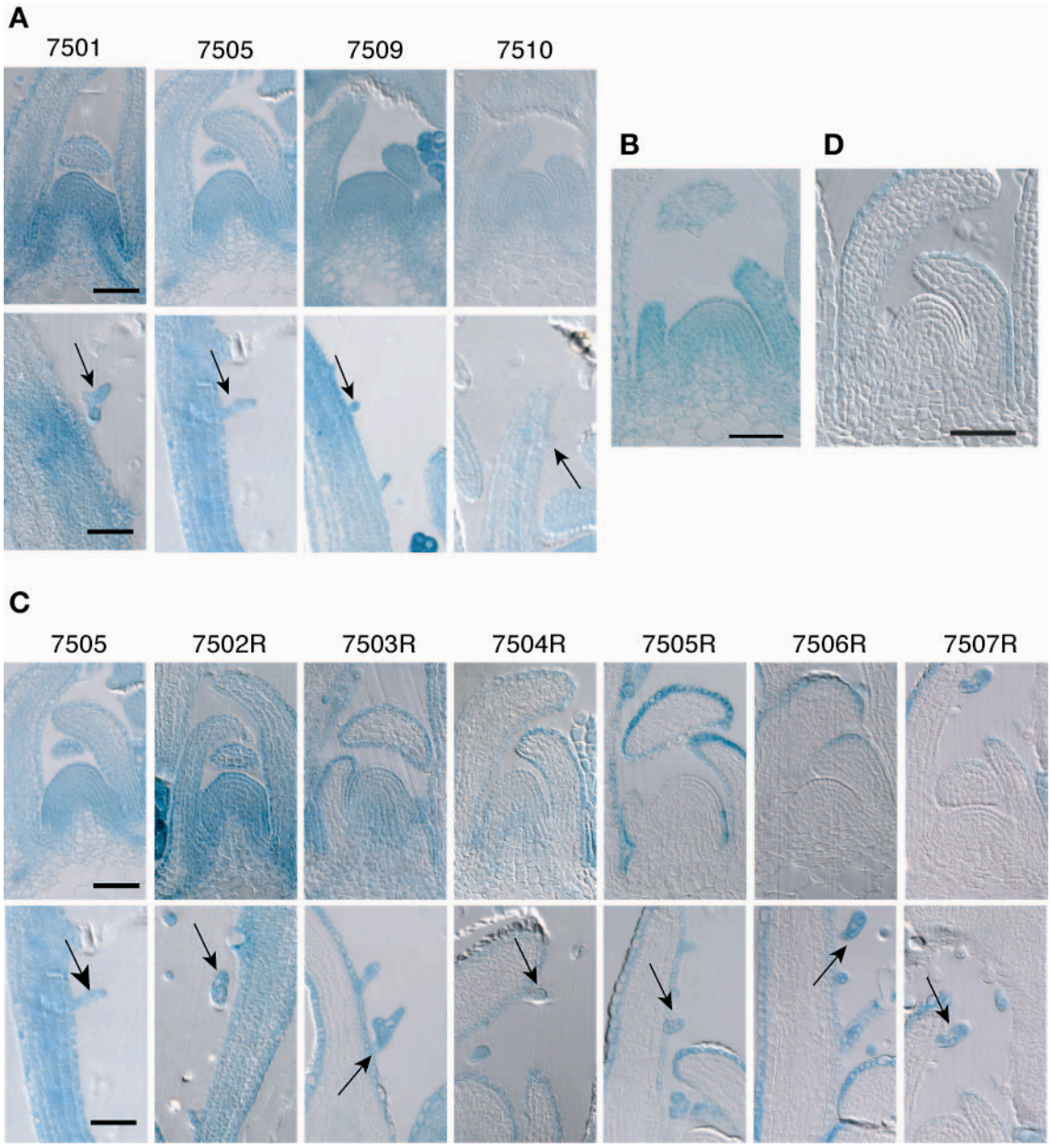



**Figure 1-8. *GUS* expression pattern in shoot apices**

Shoot apices of 10-day-old seedlings were stained for 24 h at 37°C and sectioned at a 6 μm (A–C) or 10 μm (D) thickness. (A, C) Upper row, SAMs and leaf primordia; lower row, developing trichomes (indicated by arrows) on young leaves. (B) The SAM of *proCDKA-CDKA::GUS*. (D) The SAM of 7509F/7R. Bars = 50 μm.

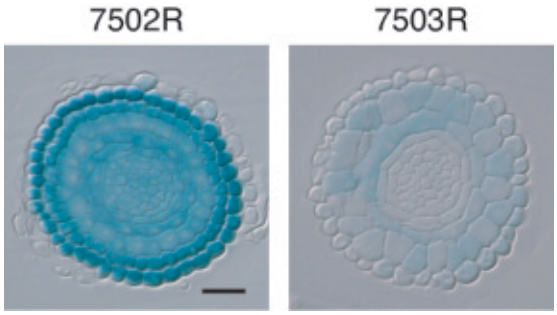



**Figure 1-9. *GUS* expression in the 7502R and 7503R RAMs**

Cross sections of 7-day-old roots of the 7502R and 7503R lines stained at 37°C for 24 h. Root tips were sectioned at a 6 μm thickness. Bar = 20 μm.

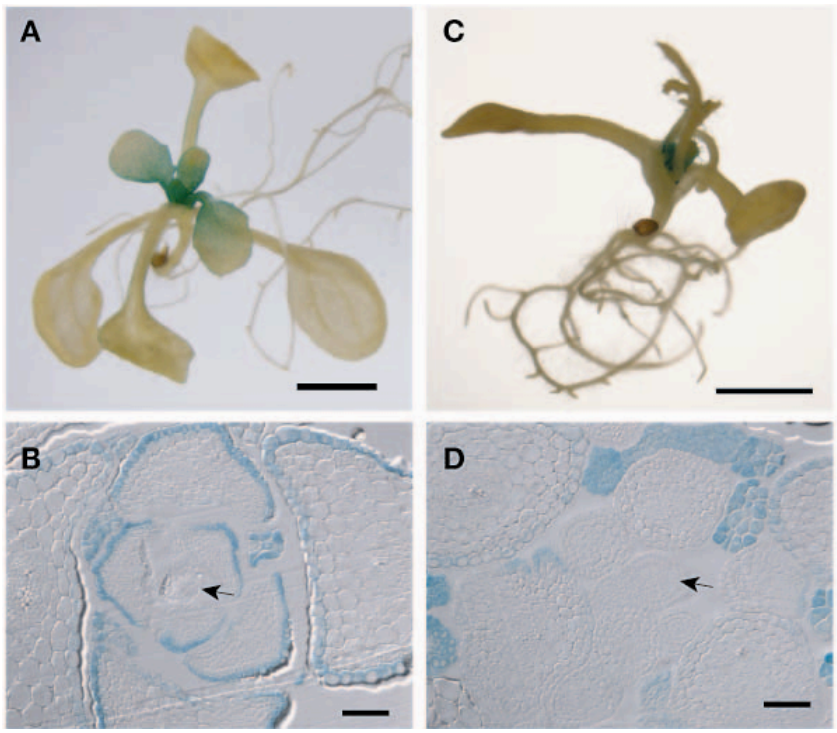



**Figure 1-10. *GUS* expression pattern in the *phb-1d* mutant**

Ten-day-old seedlings of wild-type (A, B) and *phb-1d* mutant (C, D) plants harboring the 7507R construct were stained for 24 h at 37°C. Cross-sections were created from shoot apices (B, D). Arrows indicate SAMs. Bars = 2 mm (A, C) and 50 μm (B, D).

## 1.4 DISCUSSION

### 1.4.1 Quantitative regulation of *CDKA;1* expression

My analyses of the promoter dissection series identified a region from –890 bp to –791 bp, which is involved in quantitative up-regulation of *CDKA;1* expression (Fig. 1-11). The promoter-*GUS* reporter gene lacking the 100-bp region showed much lower expression as compared to the intact promoter construct, and no tissue specificity was found in the reduction of GUS staining in both shoots and roots. This suggests that the 100-bp region may contain a general regulatory element that functions independent of cell type. In the database of PlantPromoterDB (Yamamoto et al., 2006), two *cis*-regulatory elements are annotated in the region between –890 bp and –791 bp—AACCCGGT and CCGGTATA—overlapping sequences of which is known as an abscisic acid (ABA) responsive element (Nelson et al., 1994). However, Hemerly et al. (1993) have reported that *CDKA;1* expression is suppressed by ABA treatment; thus, it is unlikely that these elements function in response to the ABA signal and activate cell division.

A transcription factor termed TEOSINTE-BRANCHED1, CYCLOIDEA, PCF20 (TCP20) is known to bind *in vivo* to a GCCCR motif in the promoter region of *CYCLIN B1;1* and enable a high level of *CYCLIN B1;1* expression at G2/M. (Li et al., 2005). It is also required for high-level expression of ribosomal protein genes by its binding to the GCCCR element in their promoters, suggesting a mechanistic link between the regulation of cell proliferation and cell growth. The *TCP20* gene belongs to class I TCP genes that positively regulate gene expression (Kosugi and Ohashi, 1997); while class II TCP genes, however, negatively control proliferation (Nath et al., 2003). There is no GCCCR element in the promoter region of *CDKA;1*. However, since the amounts of protein such as mitotic cyclins and CDKA;1 may be coordinately regulated in the process of cell division and differentiation, other class I TCP proteins may bind to the region between –890 bp and –791 bp and

quantitatively control *CDKA;1* expression in response to environmental conditions.

### 1.4.2 Cell layer-specific regulation of *CDKA;1* expression

Although *CDKA;1* is expressed in various tissues, this study revealed differential control of its expression in the leaf epidermis. The promoter region from –200 bp to –101 bp seems to enhance *CDKA;1* expression in the SAM and root apical meristems (RAMs), but also in inner layers of the leaf primordia (Fig. 1-11). The 7502R and the 7503R lines lack one of the splicing sites of the first intron (Fig. 1-2), thus I cannot deny the possibility of unsplicing, alternative splicing or the use of alternative translational start sites in the 5′ UTR. However, since it is unlikely that splicing occurs differentially in distinct cell types, I assume that the region from –200 bp to –101 bp has a regulatory function in inner layers of leaves. In contrast, the region from –890 bp to –601 bp was assumed to function in elevating the expression in the leaf epidermis (Fig. 1-11). This region contains no splicing site, suggesting that the possibility of alternative splicing can be ignored. These complementary expression patterns further indicate that *CDKA;1* expression in leaves depends on at least two regulatory elements in the 5′ UTR. L1- and epidermis-specific expressions are known to be regulated by a *cis*-regulatory element, namely, the L1 box, which exists in the promoter of *Arabidopsis thaliana* MERISTEM LAYER 1 (*AtML1*) (Sessions et al., 1999; Abe et al., 2001). In the region from –890 bp to –601 bp, however, I could not find any L1 box-like sequence, suggesting that the mechanisms underlying up-regulation of *CDKA;1* expression in the leaf epidermis are independent of L1 box-mediated signaling.

Recently a few reports indicated that cell division in distinct cell layers is differentially regulated during shoot development. Desvoyes et al. (2006) inactivated RETINOBLASTOMA-RELATED (RBR) protein in an inducible manner in Arabidopsis. The retinoblastoma protein (pRb) is known to bind to the transcription factor E2F and, by suppressing E2F function, it blocks progression from the G1 to the S phase (Weinberg, 1995).

As expected, RBR inactivation caused excess cell divisions and decreased cell size in the leaf epidermis. However, in mesophyll cells, no alteration in cell number or cell size was observed in response to RBR inactivation. This result implies the difference and independence of cell cycle regulation between epidermal and mesophyll cells in leaves. Bemis and Torii (2007) investigated transgenic Arabidopsis plants that ectopically express genes for CDK inhibitors, such as *KIP-RELATED PROTEIN1* and *KIP-RELATED PROTEIN4*. When the expression was driven by the *AtML1* promoter, epidermal cell division was severely inhibited with compensatory cell size enlargement, but normal cell numbers were maintained in the mesophyll and cortex layers. On the other hand, a recent report indicated that the dwarf phenotype of Arabidopsis mutants, which exhibit defects in brassinosteroid biosynthesis or signaling, was rescued by the expression of responsible genes with the *AtML1* promoter (Savaldi-Goldstein et al., 2007). The epidermis-specific expression restored the cell size not only in the epidermis but also in the inner mesophyll layers, suggesting the relay of a non-autonomous signal from the epidermis to the inner tissues. These results indicate that cell division is differentially controlled in the epidermis and underlying tissues, but inter-layer communications in terms of regulation of cell elongation or cell differentiation are present. My present data showed that two distinct *cis*-regulatory elements in the promoter control *CDKA;1* expression in the epidermis and inner layers of leaves. This suggests that CDKA;1 may play a key role not only in cell division but also in coordinating cell differentiation between different cell layers of leaves. This assumption is supported by the previous observation that differentiation of root stem cells was controlled by CDK activities in Arabidopsis (Umeda et al., 2000).

In transgenic plants carrying the 7507R construct, abaxial side-biased *GUS* expression was observed in young leaf primordia. However, when the same reporter construct was introduced into the *phb-1d* mutants, *GUS* expression was suppressed on both sides of the

leaves. In wild-type plants, *PHB* is expressed in the adaxial domain, and the expansion of the expression domain in the gain-of-function mutant *phb-1d* leads to spreading of the adaxial domain and generates radially symmetric leaves lacking abaxial cell types (McConnell and Barton, 1998). Therefore, my result demonstrates that the *CDKA;1* promoter region between –890 bp and –601 bp contains a *cis*-regulatory element that up-regulates the expression on the abaxial side. Since this region is overlapping with the transcription start site, further dissention of the fragment will need careful investigation. Arabidopsis possesses other genetic programs that differentially regulate cell division on each side to guarantee proper leaf expansion. For example, the *ASYMMETRIC LEAVES2* (*AS2*) gene is expressed in the adaxial domain of leaf primordia and represses cell division. The loss-of-function mutants develop downward-curled leaves due to excess cell division on the adaxial side (Iwakawa et al., 2007). Although it remains unknown as to whether the differential regulation of *CDKA;1* expression is linked to the *AS2*-associated network, it is likely that such regulatory mechanisms are involved in adjusting the cell division rate on both sides of epidermal cells and fine-tuning shoot growth and leaf expansion.

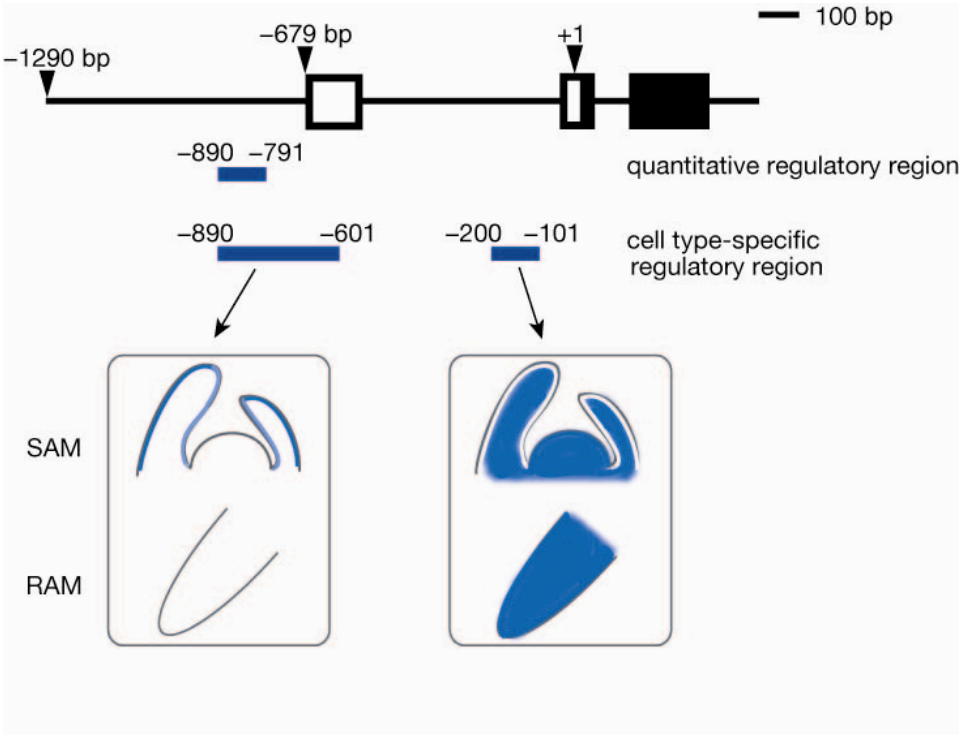



**Figure 1-11. Regulatory regions in the *CDKA;1* promoter**

The blue bars indicate the transcriptional regulatory regions identified in this study. In the diagram of the *CDKA;1* genomic region, the white and black boxes indicate UTRs and protein-coding regions, respectively. Tissues or cell layers in which each regulatory region functions in activating *CDKA;1* expression are schematically shown in blue in the diagrams.

CHAPTER II

# Expression of B2-type cyclin-dependent kinase is controlled by protein degradation

## 2.1 INTRODUCTION

While the A-type CDKs (CDKAs) are functional homologs of yeast Cdc2/Cdc28p, and expressed constitutively throughout the cell cycle, 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).

It is well known that proteolysis plays important roles in cell cycle progression. Of plant cyclins, tobacco CYCA3;1, tobacco CYCB1;1, and Arabidopsis CYCD3;1 have been reported to be degraded through the ubiquitin-proteasome pathway, and tobacco CYCA3;1, tobacco CYCB1;1, and alfalfa CYCB2;2 have been demonstrated to be degraded in a cell cycle-dependent manner (Genshik et al., 1998; Criqui, et al., 2000; Weingertner et al., 2003; Planchais et al., 2004). As for other cell cycle regulators, two components of the prereplicative complex CDC6 and CDT1a, a transcription factor E2Fc, and CDK inhibitor KRPs (KIP-RELATED PROTEINs) are thought to be regulated by the proteasome-mediated pathway (Castellano et al., 2001; Castellano et al., 2004; del Pozo et al., 2002; Verkest et al., 2005; Jakoby et al., 2006).

While the oscillation of CDK transcripts during the cell cycle has been well characterized, only limited information was available for the protein stability of CDKs. This prompted me to analyze the amino acid sequences of CDKAs and CDKBs in order to identify any motif related to posttranslational regulation.

## 2.2 MATERIALS AND METHODS

### 2.2.1 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/L 1-naphthaleneacetic acid, and 0.05 mg/L kinetin (pH 5.8)] at 27°C. Tobacco BY-2 cells were cultured in LSD medium [1× MS salts supplemented with 200 mg/L potassium dihydrogenphosphate, 100 mg/L myo-inositol, 1 mg/L thiamine hydrochloride, 3% (w/v) sucrose, and 0.2 mg/L 2,4-D (pH 5.8)] at 27°C. *Arabidopsis* plants were grown as described in Chapter I.

### 2.2.2 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). The plasmid was then subjected to the LR reaction using the destination vector pGWB3 (Nakagawa et al., 2007) 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 (An, 1985) and Arabidopsis plants (ecotype, Columbia).

### 2.2.3 *in vivo* degradation assay

CHX and MG132 (Calbiochem) 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). 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).

### 2.2.4 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). 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). Semi-quantitative RT-PCR was performed with a TITANIUM One-Step RT-PCR Kit (BD Biosciences). 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.

### 2.2.5 Synchronization of transgenic BY-2 cells

Seven-day-old transgenic BY-2 cells were diluted by adding 10 mL of culture to 95 mL of fresh LSD medium, and treated with 5 mg/L aphidicolin for 24 h. Aphidicolin was removed by washing the cells with 1000 mL of LSD medium, and then resuspended in 95 mL of LSD medium. Mitotic index was calculated from DAPI (4’, 6-diamidino-2-phenylindole)-stained cells fixed with 3.7% (v/v) formaldehyde in PBS.

### 2.2.6 GUS staining of Arabidopsis plants

Ten-day-old plants were transferred to a MS medium with or without 100 μM MG132 and incubated for further 6 h. For zeocin (Invitrogen) treatments, seven-day-old seedlings were transferred onto a 10 μM zeocin-, or 10 μM zeocin and 100 μM MG132-containing MS medium. The seedlings were stained as described in Chapter I, and incubated at 37°C for 3 h for the MG132 treatment, or 24 h for other experiments. Samples were mounted in a mixture of chloral hydrate, glycerol, and water (8 g:1 mL:2 mL).

## 2.3 RESULTS

I 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. 2-1). 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. 2-1).

In order to investigate the stability of CDKB2, I 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. 2-2). In the presence of a specific proteasome inhibitor MG132 together with CHX, no significant reduction was observed (Fig. 2-2), 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.

I 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. 2-3A). 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. 2-3B, 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. 2-3C). 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. 2-3D). 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 (Fig. 2-4). Therefore, I was 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. In the Pro-NT::GUS plants, I observed a patchy pattern of signals in apical meristems, mature embryos, and vasculature, which seems to be well correlated with mitotic activity (Fig. 2-5). By contrast, the *GUS* expression in the Pro-GUS plants was not restricted to mitotic cells, but it showed a relatively uniform expression pattern (Fig. 2-5). When the seedlings were cultured

on an MG132-containing medium, the GUS activity in RAMs became stronger in the Pro-NT::GUS plants, but not in the Pro-GUS plants, which exhibited even lower *GUS* expression than control treatment (Fig. 2-6). These results indicate that proteolysis regulates the accumulation of CDKB2 in Arabidopsis tissues as in the transgenic BY-2 cells.

In order to study responses of these two reporters to genotoxic stress, transgenic seedlings were treated with radiomimetic reagent zeocin. Although both the Pro-GUS and the Pro-NT::GUS roots exhibited reduced *GUS* expression after 8 h-treatment, the GUS expression in the Pro-NT::GUS roots was more dramatically repressed (Fig 2-7A). In the Pro-NT::GUS roots, the GUS expression was restored by treatment with MG132 together with zeocin (Fig 2-7B), suggesting that CDKB2;1 accumulation is regulated by proteasome in response to DNA damage. These results indicate that in addition to the transcriptional regulation, proteolytic regulation plays a major role in downregulation of *CDKB2;1* during the cell cycle and in response to external stress such as DNA damage-induced genotoxic stress.

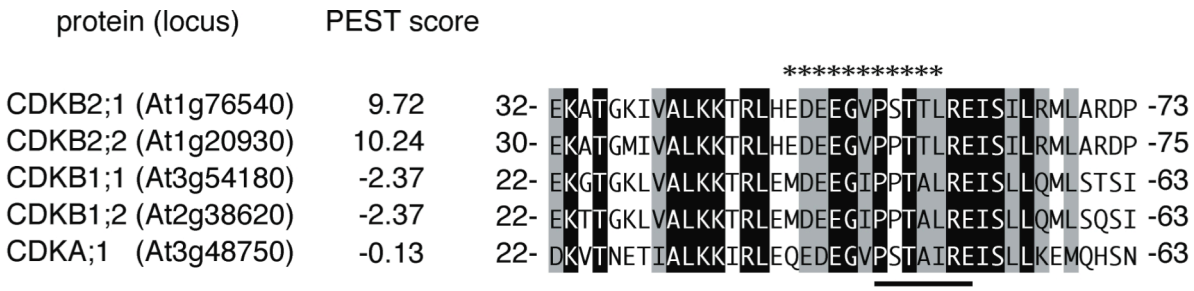



**Figure 2-1. The N-terminal amino acid sequences of Arabidopsis CDKs**

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.

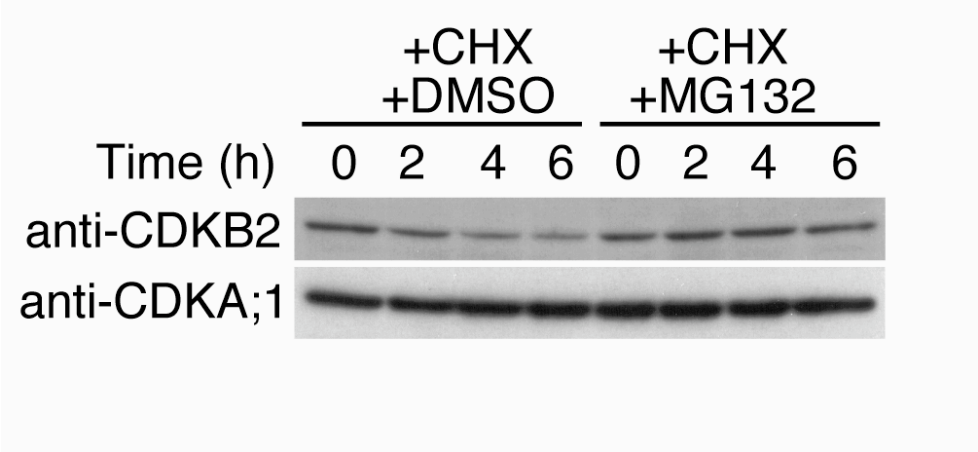



**Figure 2-2. Protein stability of CDKB2 in Arabidopsis cell culture**

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.

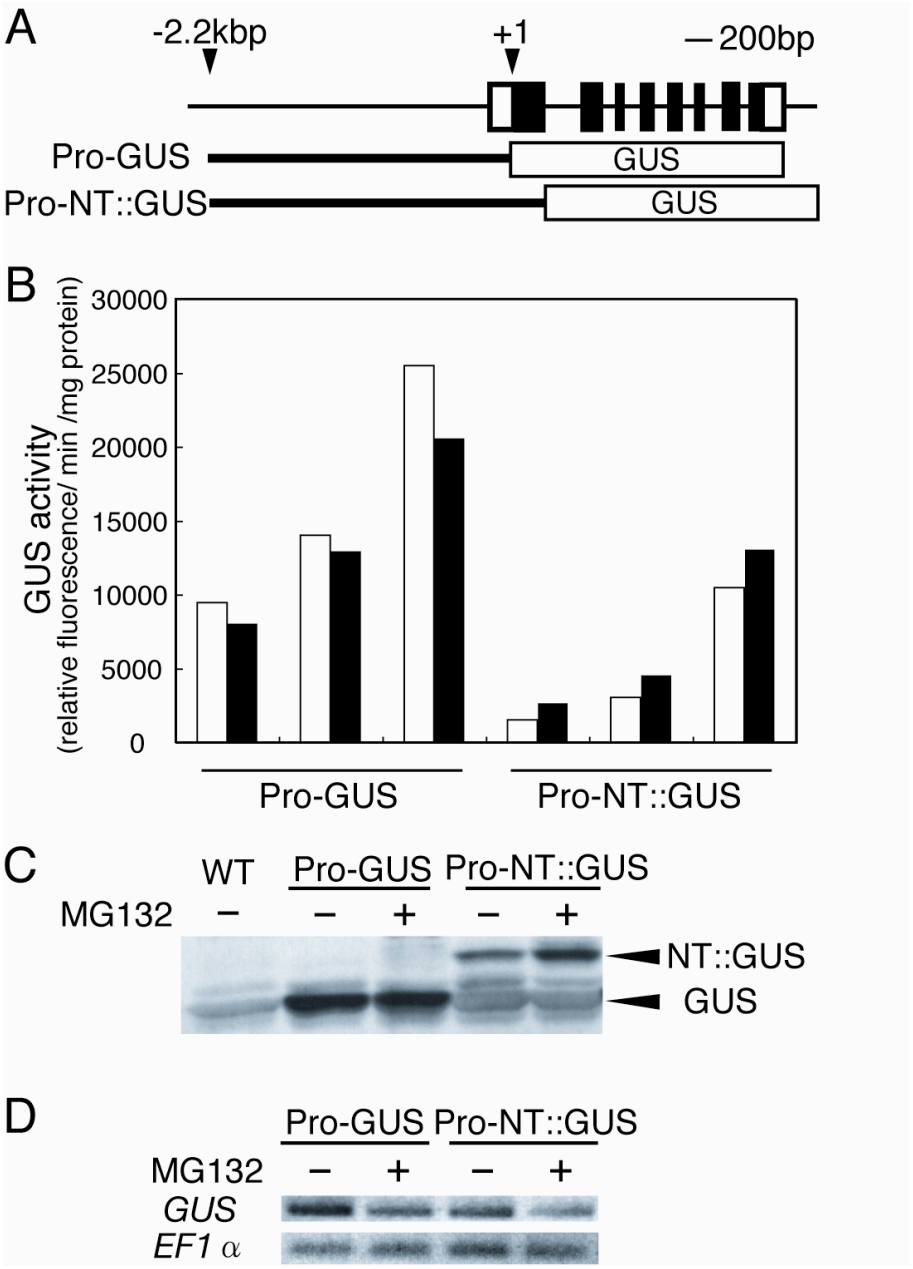



**Figure 2-3. Protein stability of GUS constructs in tobacco BY-2 cells**

(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.

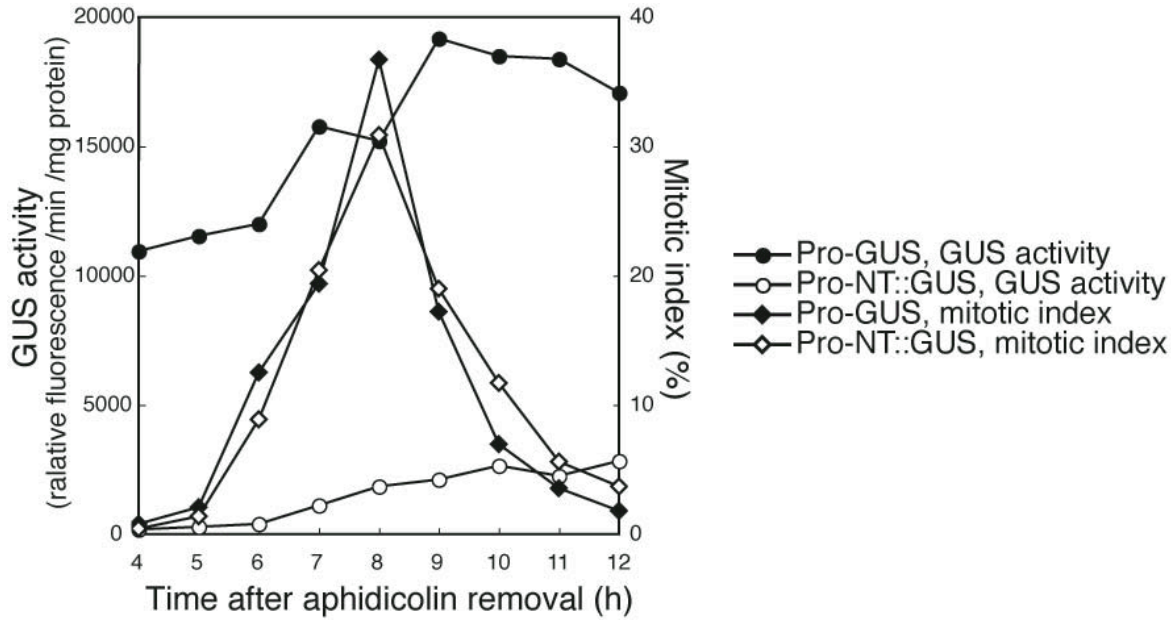



**Figure 2-4. GUS activities in synchronized tobacco BY-2 cells**

Aphidicolin-synchronized Pro-GUS and Pro-NT::GUS cells were applied to GUS activity measurements. Mitotic index was calculated at each time point for monitoring progression of the cell cycle.

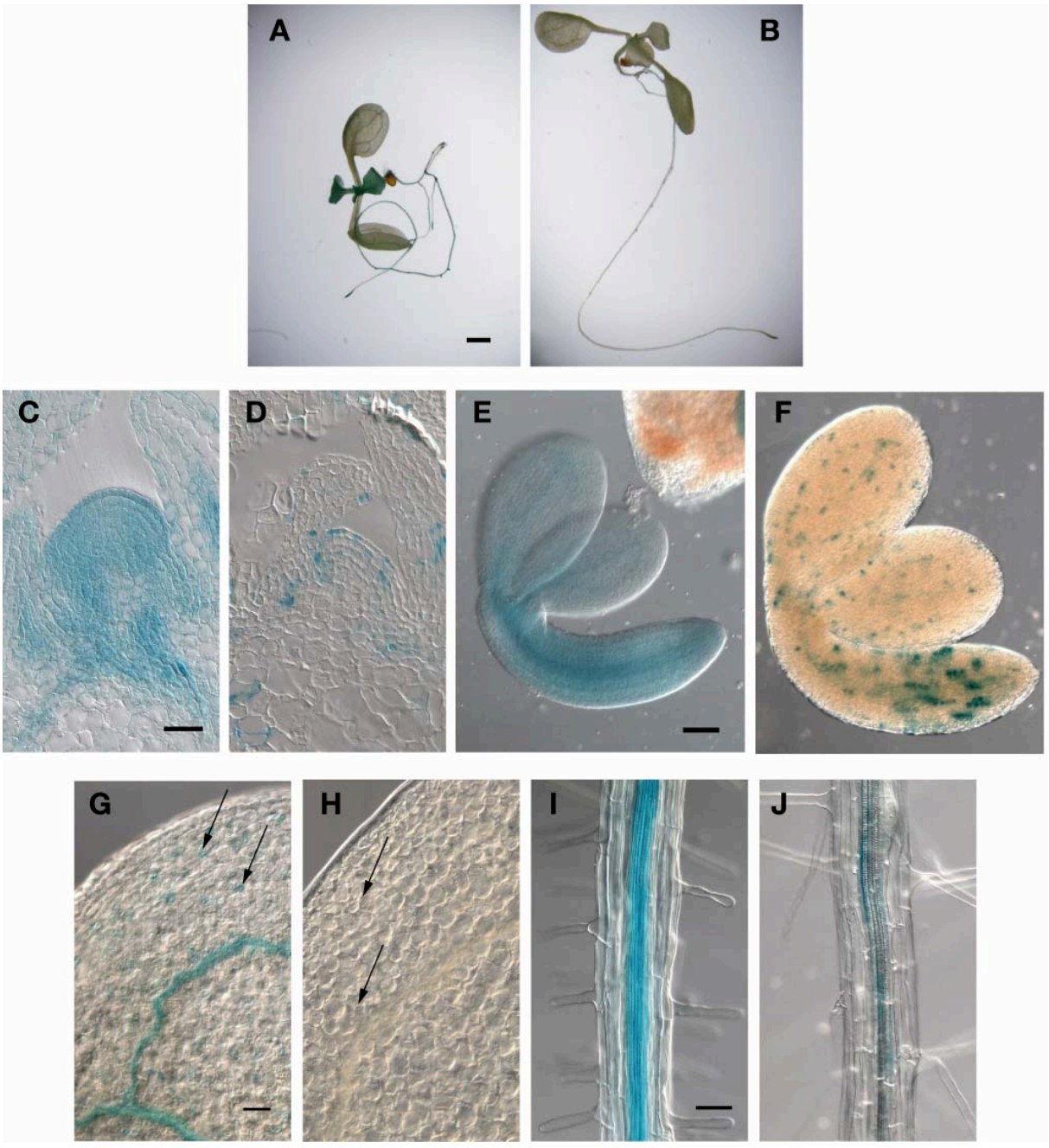



**Figure 2-5. GUS staining of transgenic Arabidopsis tissues**

The Pro-GUS (A, C, E, G, I) and the Pro-NT::GUS (B, D, F, H, J) plants are shown. Seven-day-old seedlings (A, B, G-J), SAMs of ten-day-old seedlings (C, D), mature embryos (E, F) were observed. Arrows in (G) and (H) show stomata. Bar in (A) is 1 mm, others are 50 μm.

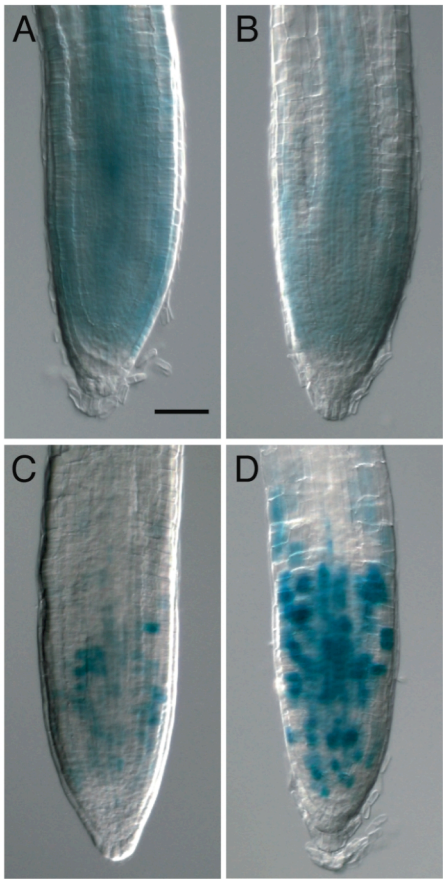



**Figure 2-6. The NT::GUS protein is degraded by proteasome in RAMs**

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.

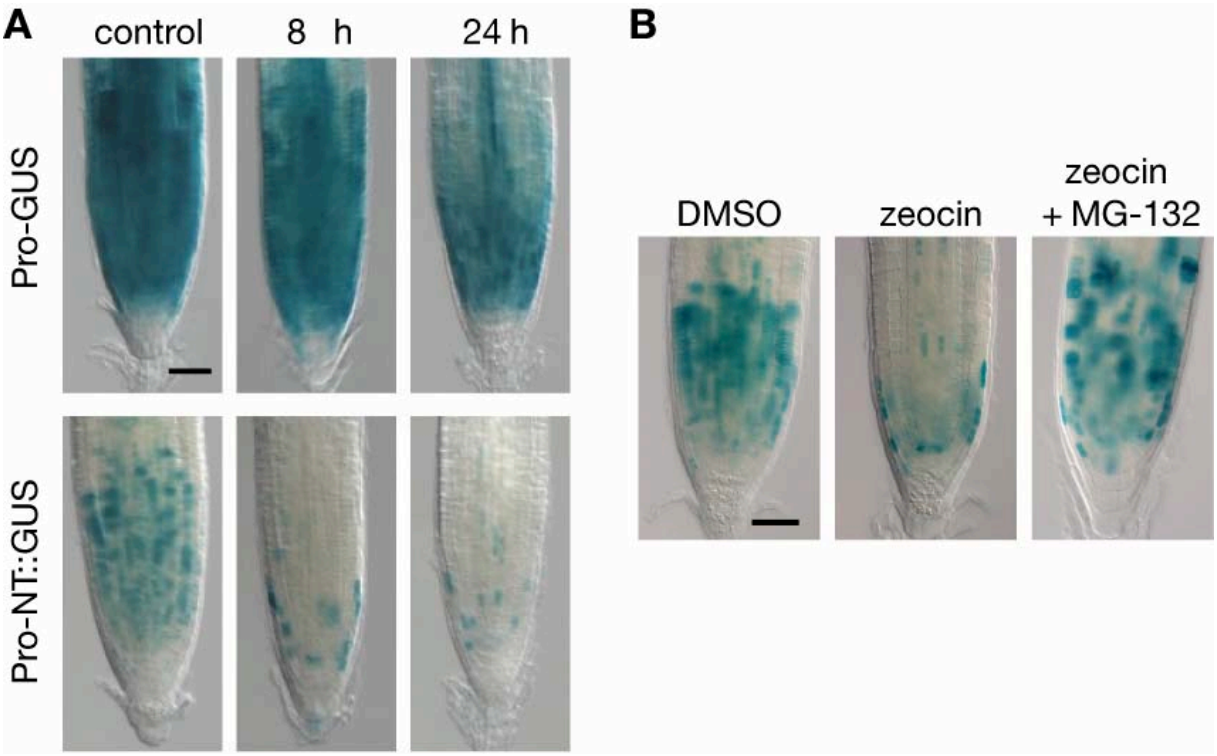



**Figure 2-7. Accumulation of CDKB2 is suppressed by zeocin**

Seven-day-old seedlings of the Pro-GUS and the Pro-NT::GUS lines were transferred to zeocin-containing plates. GUS staining of plants treated with 10 μM zeocin for 8 or 24 h (A). MG132 was added with zeocin to a medium and incubated for 8 h to observe GUS expressions (B). Bars are 50 μm.

## 2.4 DISCUSSION

I demonstrated that the first exon of CDKB2;1 can act as a transferrable degron in transgenic BY-2 cells and Arabidopsis root tips, using two reporter constructs. Both of these constructs include the 0.9-kb promoter region of *CDKB2;1* that previously shown to be sufficient to produce the G2-to-M phase-specific expression by 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, I propose that the periodic accumulation of CDKB2;1 is determined not only by a transcriptional control, but also through proteasome-mediated protein degradation.

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 so 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.

I also demonstrated that the NT::GUS protein is degraded by the proteasome in response to DNA damage. Considering the inhibitory effect of DNA damage to cell cycle progression (Weinert and Hartwell, 1988), this result may give us a hint not only to reveal physiological significanse of CDKB2 degradation but also to elucidate the molecular mechanisms of DNA damage checkpoint. Further studies on upstream signaling factors and machinery associated with CDKB2 degradation will reveal how DNA damage checkpoint functions in response to genotoxic stress in plants.

## CHAPTER III

# DNA damage controls the mitotic cell cycle and the endocycle

## 3.1 INTRODUCTION

Genetic information in genomes must be faithfully copied and distributed to two daughter cells in each of countless cell divisions. Eukaryotic cells duplicate their chromosomes with high fidelity and have molecular machinery that ensures that chromosomal segregation occurs after completion of replication. However, genome integrity is continuously threatened by external stresses such as genotoxic stress, and by endogenous causes such as errors in DNA replication, and attack by reactive oxygen species (Sancar et al., 2004; De Bont and van Larebeke, 2004). There are several kinds of DNA damage: DNA double-strand breaks (DSBs), single-strand breaks (SSBs), base excision, and nucleotide damages such as oxidation, alkylation, depurination, deamination and cross-linking (Sancar et al., 2004). In order to convey correct genomic information to daughter cells, these damages must be sensored and repaired before mitosis or replication.

To coordinate DNA repair activities with cell cycle progression, cells need checkpoint mechanisms that inhibit cell division while DNA damages are being repaired. Studies of animal cells and yeasts revealed several key factors that are important for arresting the cell cycle in response to DNA damage: two sensor kinases ATM (ataxia-telangiectasia-mutated) and ATR (ataxia-telangiectasia- and Rad3-related) trigger the checkpoint responses, whereas inhibitors of cyclin-dependent kinases (CDKs) and DNA damage repair genes are activated at the downstream of these kinases (Abraham, 2001; Kurz and Lees-Miller, 2004). ATM is thought to be specialized for the response to DSBs, whereas ATR is required for the responses to many other different forms of DNA damage. The ATM response to DSBs depends on a complex of three proteins named MRN complex (Mre11, Rad50 and Nbs1) in vertebrates (Lee et al., 2005). When DSB is bound by the MRN complex, ATM is recruited to the site of the DSB and activated by interaction with the MRN complex. The response to DSBs is partly dependent on ATR activity because nuclease activity of the MRN complex causes resection,

which exposes single-stranded DNAs. ATR binds SSBs via single-stranded DNA binding RPA (replication protein A) and ATRIP (ATR interacting protein) (Zou et al., 2003). The binding of ATM or ATR to sites of DNA damage is accompanied by the recruitment of other proteins, which include repair enzymes and proteins that helps those enzymes. In addition to them, two adaptor kinases named Chk1 and Chk2 in vertebrates are also recruited to the complex and activated through phosphorylation by ATM or ATR (Kurz and Lees-Miller, 2004).

These four kinases, ATM, ATR, Chk1 and Chk2, then phosphorylate several proteins that are involved in cell cycle arrest or apoptosis (Fig 3-1). In vertebrate cells, p53 is stabilized by phosphorylation by ATM, ATR, Chk1 or Chk2 (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999; Hirao et al., 2000; Chehab et al., 2000; Shieh et al., 2000). p53 is a gene regulatory protein that alters the rate of transcription of target genes. Depending on the cell type and other factors, p53 causes either cell-cycle arrest or apoptosis (Vousden and Lu, 2002). The p53 action arrests the cell cycle mainly at the G1 phase by promoting the expression of the CDK inhibitor p21, but also the G2/M phase by promoting the expression of 14-3-3σ in response to DNA damage (El-Deiry et al., 1993; Hermeking et al., 1997). The 14-3-3σ protein associates with the CDK-activating phosphatase CDC25, and excludes CDC25 from nuclear CDK/cyclin complexes (Lopez-Girona et al., 1999). The G2/M arrest in yeasts and vertebrate cells is due to the degradation of CDC25 triggered by phosphorylation by Chk1/Chk2 (Karlsson-Rosenthal et al., 2006). In addition, the CDK-inhibitory kinase WEE1 is stabilized at the downstream of ATM/ATR by inhibition of upstream kinase Plk1 (Watanabe et al., 2004).

Although *Arabidopsis* possesses ATM, ATR and WEE1 homologs (Garcia et al., 2003; Culligan et al., 2004; Sorrell et al., 2002), lack of orthologs of some other key regulators such as p53, Chk, and CDC25 (Table 3-1) implies that plants probably have different mechanisms

to cope with DNA damage. Divergent functions of ATM, ATR and WEE1 between animal/fungi and plants are also suggested by functional analysis of these proteins: in contrast to embryonic lethality caused by ATR knockout in animals (Brown and Baltimore, 2000; de Klein et al., 2000), an Arabidopsis null mutant of *ATM* or *ATR* is viable (Garcia et al., 2003; Culligan et al, 2004), and loss-of-function mutations in the Arabidopsis *WEE1* resulted in no obvious defect under normal growth conditions (De Schutter et al., 2007). In addition, a recent study revealed that a NAC domain family protein, which is unique to plants, is involved in DNA damage signaling (Yoshiyama et al., 2009), implying again, the presence of plant-specific pathways and mechanisms in DNA damage response.

Cell division control in plants has a couple of unique features. Position and timing of cell division is important for plant cells, because they are cemented with each other by the cell wall. In addition, endoreduplication occurs during the course of differentiation in various plants (Nagl, 1976; De Rocher et al., 1990). These characteristics indicate that plants can grow either by cell division or cell growth associated with endoreduplication. In Arabidopsis shoot apices or root tips, meristems are composed of small cells, and cell size gradually increases as cells get further from meristems. This organized array of cells indicates that there is a switch mechanism from the mitotic cell cycle to the endocycle as cells are pushed away from meristems.

Although reverse genetics have identified molecules required for DNA damage signaling and repair, cellular responses including regulation of the cell cycle in response to DNA damage in plants largely remain to be studied. Considering the difference between yeasts or animal cells and plant cells cell cycle regulation, I hypothesized that the plant cell cycle has unique mechanisms to cope with DNA damage.

**Table 3-1: DNA damage signaling factors in vertebrates, *S. cerevisiae*, and Arabidopsis**

| Vertebrate | *S. cerevisiae* | *A. thaliana* | Reference for Arabidopsis |
|---|---|---|---|
| Sensor kinases | | | |
| ATM | Tel1 | ATM | Garcia et al. (2003) |
| ATR | Mec1 | ATR | Culligan et al. (2004) |
| ATR regulatory subunit | | | |
| ATRIP | Ddc2/Lcd1 | HUS2/ATRIP | Sakamoto et al. (2009); Sweeney et al. (2009) |
| MRN complex | | | |
| Mre11 | Mre11 | AtMre11 | Bundock and Hooykaas (2002) |
| Rad50 | Rad50 | AtRAD50 | Gallego et al. (2001) |
| Nbs1 | Xrs2 | AtNBS1 | Waterworth et al. (2007) |
| Mediator | | | |
| Chk1 | Chk1 | – | – |
| Chk2 | Rad53 | – | – |
| CDK activators and inhibitors | | | |
| CDC25 | Mih1 | – | – |
| p21 | – | – | – |
| WEE1 | Swe1 | WEE1 | Sorrell et al. (2002) |
| Transcription factor | | | |
| p53 | – | – | – |
| – | – | SOG1 | Yoshiyama et al. (2009) |

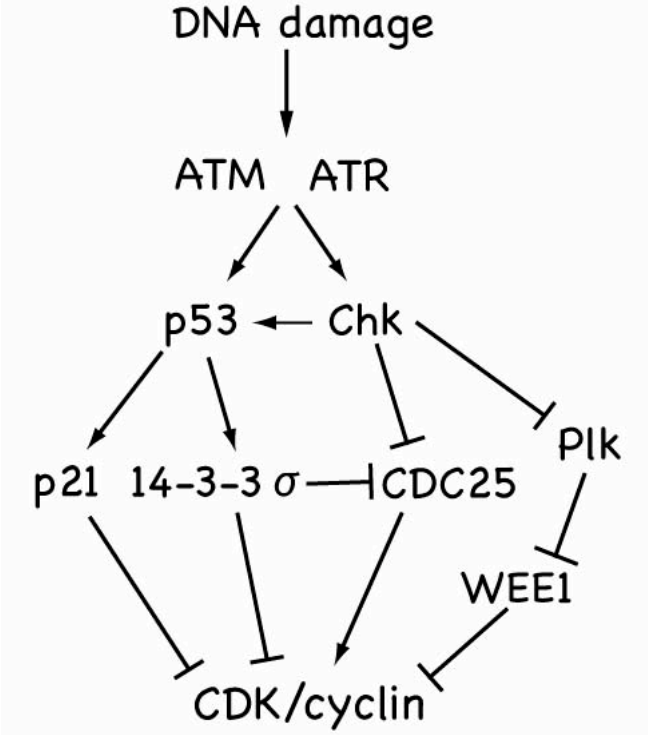



**Figure 3-1. DNA damage signaling pathways in vertebrates**
A schematic model of downstream pathway of vertebrate ATM/ATR is shown.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Plant materials and growth conditions

Arabidopsis plants were grown on a vertically oriented MS medium [0.5× MS salts, 1× MS vitamins, 2% (w/v) sucrose, and 0.8% (w/v) gellan gum or agar (pH 6.3)] under continuous light conditions at 23°C. The *xpf-2* or the *xpf-2 sog1-1* seedlings were grown under lamps with UV-absorbing filters (NK system), Arabidopsis MM2d cells were cultured in a LSD medium [1× MS salts supplemented with 200 mg/L potassium dihydrogenphosphate, 100 mg/L myo-inositol, 1 mg/L thiamine hydrochloride, 3% (w/v) sucrose, and 0.2 mg/L 2,4-D (pH 5.8)] at 27°C.

All Arabidopsis lines except for *xpf-2*, *xpf-2 sog1-1*, and *35S-CYCD3;1* were derived from the Columbia ecotype (Col). The *xpf-2* mutant (Jian et al., 1997) and transgenic plants carrying the *35S-CYCD3;1* (Dewitte et al., 2003) are in Landsberg *erecta* (L*er*) background, and *xpf-2 sog1-1* (Preuss et al., 2003) is Col/L*er* hybrid. Other T-DNA lines used in this study are as follows: *atm-2* (Garcia et al., 2003), *atr-2* (SALK_032841; Culligan et al., 2004), *ccs52a1-1* (SALK_083656; Lammens et al., 2008), *ccs52a2-1* (SALK_001978; Lammens et al., 2008), *mid-1* (Kirik et al., 2007), and *wee1-3* (SALK_039890; De Schutter et al., 2007).

The genomic fragment of *CDKB2;1* (4.0-kb, including the 2.2-kb promoter region) were PCR amplified with primers 5′-AAAAAGCAGGCTGTCTTTTGCTTGTTCCCTAAC-3′ and 5′-AGAAAGCTGGGTAGAGAGAGGACTTTTCTGGCAG-3′. It was reamplified and cloned into pDONR221 with the PCR-mediated Gateway directional BP cloning system (Invitrogen), and then transferred into the pGWB3 destination vectors (Nakagawa et al., 2007) with LR clonase II (Invitrogen). Resultant plasmids were introduced into Col plants via *Agrobacterium*-mediated transformation (Clough and Bent, 1998). The *proCDKA-CDKA::GUS* and the *proCDKB1-CDKB1::GUS* are described in Chapter I and

Minamisawa (2008), respectively. The *CYCB1;2::GUS* line was described in Culligan et al. (2006).

### 3.2.2 DNA damage induction

Zeocin (Invitrogen) and hydroxyurea (nacalai tesque) were dissolved in water, and stored at −20°C. Cisplatin (Wako) was freshly dissolved in DMSO. These inhibitors were added to media just before use. UV-C irradiation was performed with Stratalinker 1800 (Stratagene), and then seedlings were incubated in the dark in order to prevent photolyase activity.

### 3.2.3 mPS-PI stain

Cells in root tips were stained according to the method described Truernit et al. (2008) with some modifications. Whole seedlings were fixed with 50% (v/v) methanol/ 10% (v/v) acetic acid at 4°C for at least 12 h. After washed with water, samples were treated with 1% (w/v) periodic acid solution for 40 min. Washed samples were then stained for 1 to 3 h with propidium iodide with Schff reagent (100 mM sodium metabisulphite/ 0.15 N HCl/ 10 μg/mL propidium iodide), which was prepared just before use. Stained roots were mounted with chloral hydrate solution [a mixture of chloral hydrate, glycerol, and water (8 g: 1 mL: 2 mL)], and observed with confocal microscope (FV1000, Olympus) with 488 nm multi Argon laser. Cell area and distance from QC were measured with the MBF ImageJ software (www.macbiophotonics.ca/imagej/) by tracing contour of cells.

### 3.2.4 Ploidy measurement

Ploidy levels were measured by a ploidy analyzer PA (Partec) and CyStain UV precise P (Partec) according to the manufacturer's instruction. At least 10,000 nuclei were used for each measurement.

### 3.2.5 Immunostaining of Arabidopsis cultured cells

Arabidopsis MM2d cells were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min.

Cells were collected by gravity-dependent sedimentation. Fixed cells were washed in PBS and incubated in an enzyme mixture containing 2% (w/v) Cellulase Onozuka RS and 0.5% (w/v) Pectolyase Y-23 for 30 min at 37°C to digest the cell wall. The cells were resuspended in Galbraith buffer [45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(*N*-morpholino) propane sulfonic acid (MOPS), 0.1% (v/v) Triton-X 100, pH 7.3; Galbraith et al., 1983] and filtered through a 30-mm nylon mesh. Isolated nuclei were centrifuged onto microscope slides coated with amino-silane (s8111; Matsunami Glass) using Cytospin (Cytospin 3; Shandon Scientific) at 452 x *g* for 5 min. Cells were permeabilized by 15 min incubation with PBS containing 0.5% (v/v) Triton X-100 at room temperature and washed further two times in PBS. After blocking in PBS containing 1% (w/v) BSA for 20 min, cells were incubated with primary antibodies at the following dilutions in PBS overnight at 4°C; 1:500 dilutions of rabbit polyclonal antibodies against HTR12 (Talbert et al., 2002). After being washed twice in PBS, cells were incubated with secondary antibodies at the following dilutions in PBS for 3 h at room temperature; 1:200 dilutions of Alexa 488-conjugated goat anti-rabbit IgG antibodies (Invitrogen). Cells were washed twice in PBS, and then mounted with 2 μg/mL DAPI solution.

The slides were placed on the inverted platform of a fluorescence microscope (IX-81; Olympus) equipped with a cooled charged-coupled device (CCD) camera (CoolSNAP HQ2; Roper Scientific, USA). Images were acquired with a 40 × objective lens (UApo/340, NA 1.35, oil immersion). Image processing was performed with the following software; MetaMorph (Universal Imaging Corporation) and MBF ImageJ software. The fluorescent signals were counted using the Cell Counter plug-in of the MBF ImageJ.

### 3.2.6 RT-PCR and immunoblotting

Total RNA from 0.5-cm root tips of 5-day-old seedlings was extracted using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. Semi-quantitative RT-PCR

was performed with cDNA synthesized with Super Script II reverse transcriptase (Invitrogen). Twenty nanograms of total RNA and the primers listed in Table 3-2 were used for each PCR. The PCR conditions were 94°C for 2 min; 20 cycles (for *TUB4*), 22 cycles (for *CDKA;1*, *PCNA*s), 23 cycles (for *HISTONE H4*) or 25 cycles (for *BRCA1*, *RAD51*, *CDKB1;1*, *CDKB2;1*, *CYCB1;1*, *CYCA3;1*, *AtNACK1*, *KNOLLE*) at 94°C for 30 s, at 60°C for 45 s, and at 72°C for 60 s; and 1 cycle at 72°C for 2 min.

Immunoblotting was performed as described in Chapter II. The anti-CDKB1 antibody was described by Takatsuka et al. (2009).

### 3.2.7 Microarray analysis

The analysis was performed with the Agilent *Arabidopsis* 3 Oligo Microarray for 44K Microarray analysis (Agilent Technologies). Zeocin treatments of MM2d cells were biologically duplicated, and total RNA was extracted using the NucleoSpin RNA Plant (MACHEREY-NAGEL) as described in the manufacturer's instructions. Each RNA sample was labeled for Cy3-cRNA probes, according to the instructions for one color experiment. The hybridized and washed material on each glass slide was scanned by an Agilent DNA microarray scanner (model G2505B; Agilent Technologies). The location and delineation of every spot in the array were established by Feature Extraction Software version 9.5.3.1 (Agilent Technologies). Fluorescence intensity was integrated through filtration and normalization, and the p-value of each spot was calculated using default parameters. After quality check by the GeneSpring software (Agilent Technologies), the number of probes in the first and the second experiments were 24,526 and 27,028, respectively. The intersectional 23,338 probes, which corresponded to 62% of the initial set (37,538 probes), were applied to the Quantile Normalization (Bolstad, 2001), and used for succeeding analysis. In statistical analysis for detecting genes whose expression levels changed significantly, data at 0, 12, 18, 24, 30, and 48 h were used. For the nonparametric two-way analysis of variance, the

Friedman test was applied to detect significant genes in two factors of time and treatments. In addition, false discovery rates (FDR) of multiple hypothesis testing was estimated by using the procedure described in Benjamini and Hochberg (1995). At the threshold of FDR < 0.01, the number of genes with significant difference between the zeocin and the control treatments was 3,678.

### 3.2.8 GUS staining

Seven- or five-day-old seedlings were stained and observed as described in Chapter I.

**Table 3-2. Primers used for RT-PCR analysis**

| Gene name | Primers (5′-3′) | |
|---|---|---|
| | Forward | Reverse |
| *TUB4* | AGAGGTTGACGAGCAGATGA | GGCTGTAGCATCTTGGTACT |
| *BRCA1* | CGAGGAAGGATCTCTTGCAG | GCACTAGTGAACCCCAGAGG |
| *RAD51* | GAAGGAGCAGACAAAGTGAG | GGTGAGATGGAAGTGATAGG |
| *CDKA;1* | TGCATATTTGCTGAGATGATCAGCCAAAAG | AGGCATGCCTCCAAGATCCTTG |
| *CDKB1;1* | CTACCAAATCCAATCTCTATCTC | GTAAACATGCCAGTCACGCAG |
| *CDKB2;1* | ATGGACGAGGGAGTTATAGCAGTTTC | GTGCAATATCCCGTGACCATGGCAGAATG |
| *CYCB1;1* | TGGCCACCACAGGTGGAGG | TGTTGCTTCCATTGCTGATACG |
| *PCNA*s* | GTAGCATTGGTGACACAGTTGTGATC | GTGTCTTCTTCTTCTTCAATCTTAGG |
| *HISTONE H4* | TCTAAGAGACAACATTCAAGGAATC | ATCATTAATCCATTACAATGCTG |
| *CYCA3;1* | GTCGTACTCGGAGAGCTTCCAAACT | CAGGCATTGTCGCTACACACTTGAA |
| *AtNACK1* | CGTGCCCTTAAACTCCTTGA | GCTCTCCGATTTCCATTTCCA |
| *KNOLLE* | CACACTAATCAGAAGAGTGAAAAAGATG | GGGGATGACAACAATGAGAATG |

*Primers for *PCNA*s amplify both *PCNA1* and *PCNA2*.

## 3.3 RESULTS

### 3.3.1 DNA double-strand breaks cause mitotic inhibition and cell expansion through endoreduplication in Arabidopsis roots

In order to study cellular response to DSBs in *Arabidopsis* seedlings, I treated roots by transferring seedlings onto media that contain a radiomimetic reagent, zeocin. Growth of roots was inhibited in a dose-dependent manner, and roots ceased to grow around 48 h after transfer onto a medium containing 10 μM zeocin (Fig 3-2A). Production of DSBs was confirmed by examining DNA damage-responsive genes (Fig 3-2B). Coinciding with growth inhibition, the expression of the mitotic cyclin *CYCB1;2* was suppressed in response to zeocin (Fig 3-2C). At the 10 μM condition, cells in root apical meristems started to enlarge around 20 h after transfer, and the cells became even larger at 30 h (Fig 3-3A). To investigate whether this change is reversal or not, 8 or 24 h-treated seedlings were re-transferred onto zeocin-free media. Although lateral roots emerged and grew, root tips that were treated for 24 h did not recover growth (Fig 3-4). Not all the roots treated for 8 h were arrested, but root growth rate was smaller than that of non-treated seedlings, indicating partial effects on cell division by 8 h treatment. This result shows that irreversible changes in cell division activity happened in root meristematic cells between 8 and 24 h after zeocin application. Quantification of cell area by mPS-PI staining (Truernit et al., 2008) confirmed the microscopic observations (Fig 3-3B). Cells with more distant from QC were more enlarged, which is in contrast to the almost constant size in the control without zeocin. In addition, this enlargement was not restricted to epidermis, and a similar but milder effect was observed in cortex cell files (Supplemental fig 1).

Since cell size is usually correlated with nuclear ploidy (Melaragno et al., 1993; Gendreau et al., 1997; Sugimoto-Shirasu and Roberts, 2003), nuclear DNA contents of zeocin-treated root tips was analyzed. Nuclear DNA content of epidermal cells, which were

measured by quantification of tdTomato::HISTONE H2B fluorescence intensity, showed an increase after 24 h treatment (Supplemental fig 2A, B). Increase of nuclear DNA content may be caused by endoreduplication or endomitosis.

Endoreduplication is achieved by increasing nuclear DNA content through the modified cell cycle called endocycle, which is assumed as a cell cycle without mitosis (Edgar and Orr-Weaver, 2001). Chromosomes number was counted by visualizing kinetochore proteins HTR12 (Talbert et al., 2002) with the tdTomato fluorescent protein. Although nuclear volume increased by zeocin treatment as shown in Supplemental fig 2B, number of kinetochores did not respond to zeocin treatment (Supplemental fig 2C), suggesting that DSBs trigger the endoreduplication in root meristematic cells. In fact, nuclear ploidy in root tips increased after zeocin treatment (Supplemental fig 2D).

### 3.3.2 DNA double-strand breaks induce endoreduplication independently of cell differentiation

As differentiating Arabidopsis roots also contain endoreduplicated cells, I then used Arabidopsis cultured cells to uncouple zeocin-induced endoreduplication from cell differentiation and to focus only on mitotic cells. As in root tips, the cultured cells became larger and had an enlarged nucleus when zeocin was added to the culture medium (Fig 3-5A). To distinguish polyploid cells from endoreduplicated cells, chromosomal numbers in each cell were investigated by immunostaining nuclei with anti-HTR12 antibody. Chromosome numbers of MM2d cells have two peaks around 30 and 60, corresponding to the previous report that showed that they are hexaploids (Menges and Murray, 2002). I found that both nuclear ploidy and nuclear size increased after zeocin treatment (Fig 3-5B, C). However, zeocin treatment did not increase the kinetochore number (Fig 3-5C). These results suggest that DSBs cause cell enlargement and halt of cell division by inducing endoreduplication in root tips. Analysis with cultured cells also showed that endoreduplication induced by DSB

occurs independently of differentiation state of cells.

### 3.3.3 DNA damage-induced endoreduplication may be specific to DSBs

DNA molecules in cells suffer from various kinds of DNA damage. To compare responses to DSB and other kinds of DNA damage, root growth and epidermal cell area were measured after 24 h treatment with gamma rays, ultraviolet rays (UV), hydroxyurea (HU), cisplatin (CP), or methanemethylsulfonate (MMS), which causes DSBs, pyrimidine dimmers, replication stall, DNA crosslinking, or alkylation, respectively. Although root growth after gamma irradiation was not so affected as the other reagents (Fig 3-6A), increase of cell area was most prominent and similar to zeocin-treated roots (Fig 3-6B; see Fig 3-3B for zeocin-treated roots). I found little enlargement of cells in UV-, HU-, or CP-treated roots (Fig 3-6B). MMS-treated roots exhibited slight increase in cell size. DSBs might be produced by repair activities provoked by non-DSB damage (Strumberg et al., 2000). Considering cell area in root meristems and root growth, growth of gamma- or zeocin-treated roots may result not only from cell division but also from endoreduplication, whereas growth of UV-, HU-, or CP-treated roots may be attributed to cell division (Fig 3-6A). Considering the possibility that inhibition of DNA replication by HU hindered cells from endoreduplicating, I tested whether HU could suppress the cell enlargement caused by zeocin. Combined treatment with zeocin and HU showed that the HU concentration used in this experiment did not block cell enlargement caused by zeocin (Fig 3-6C), indicating that HU might partially but not completely inhibit DNA replication. These comparison revealed that DSBs readily induce endoreduplication, and that the induction of endoreduplication may be specific to the response to DSBs.

### 3.3.4 Gene expression and cell cycle progression during DSB-induced endoreduplication

To examine effects of DSBs on cell cycle progression in more detail, I developed partial synchronization system by using MM2d cells. When seven-day-old MM2d culture, in which

around 80% of cells are arrested at G1 phase, was subcultured into fresh media, the cells re-start the cell cycle synchronously (Fig 3-7). The progression of the cell cycle was analyzed by monitoring ploidy distribution: the highest proportion of 12C cells appears around 12 h, which implies that cells need 12 h for progression from the G1 entry to the G2/M transition. Application of zeocin at the point of subculturing caused delay in cell cycle progression, and zeocin-treated cells reached G2/M transition at around 18 h after subculture (Fig 3-7). Accumulation of 12C cells was also detected at 24 and 48 h, and 24C cells appeared after 48 h, whereas the peak of 6C was barely recognizable at 48 h and 72 h. This change in ploidy distribution implies inhibition of M phase entry, which is associated with the transition from the mitotic cell cycle to the endocycle.

This partial synchronization system was used for a microarray analysis in order to study responses in gene expression. MM2d cells were collected at 0, 12, 18, 24, 30, and 48 h for control treatment, and at 12, 18, 24, 30, 48, and 72 h after zeocin addition. Among 23,338 genes whose expressions were detected in the biological duplicates, the Friedman test identified 3,678 genes that responded significantly to zeocin. A comparison with the results from aphidicolin-synchronized MM2d cells (Menges et al., 2005) revealed that expressions of 82.9% of the mitotic genes were affected by zeocin treatment, whereas only two (11.1%) of the S phase genes showed significant changes (Fig 3-8A). Of 77 genes that encode regulators of the cell cycle, 30 genes exhibited significant changes, and expressions of almost all the mitotic genes were downregulated (Table 3-3). This biased effect on mitotic genes agreed with the ploidy data (Fig 3-7) and the idea that the endocycle is a type of the cell cycle that lacks M phase. Expressions of *WEE1* and *CYCB1;1* were induced by zeocin treatment (Fig 3-8B), as previously reported (Culligan et al., 2006; De Schutter et al., 2007). The CDK inhibitor *SMR5*, which has reported to be induced by genotoxic stress (Peres et al., 2007), was also highly upregulated (Fig 3-8B). When focused on the genes that have phase-specific

expression patterns, G2/M genes such as *CYCA1;1, AtNACK1/HINKEL,* and *KNOLLE* exhibited elevating pattern in zeocin-free medium, but were suppressed in zeocin-containing medium (Fig 3-8B). G1/S genes (*CYCA3;1*, *PCNA*s, *E2F*a) did not exhibit significant changes (Fig 3-8B). RT-PCR experiments confirmed similar G2/M biased effects of zeocin in root tips, expressions of *AtNACK1/HINKEL* and *KNOLLE* were severely inhibited whereas those of *CYCA3;1* and *PCNA* were not (Fig 3-9).

Among A- and B-type CDKs, only *CDKB2;1* showed a significant change in gene expression by zeocin treatment (Table 3-3). To examine the amount of *CDKB2;1* in more detail, I used actively dividing three-day-old culture instead of partially synchronized cells. When treated with zeocin, 12C cells accumulated at 24 h, and 24C cells appeared at 48 h after zeocin addition (Fig 3-10A). In the absence of zeocin, 24C cells never appeared (data not shown). I employed specific antibodies for CDKB2, CDKB1 or CDKA to examine accumulation of these proteins. The amount of CDKB2 protein decreased at 24 and 48 h after addition of zeocin, although that of CDKB1 or CDKA protein did not so drastically change (Fig 3-10B). Results of RT-PCR showed that transcripts of *CDKB2;1* decreased in response to zeocin, but transcripts of *CDKB1;1* and *CDKA;1* did not. These results demonstrate that *CDKB2* expression is specifically suppressed when DSBs occur.

These results are consistent with those from root tips (Fig 3-11). In order to visualize CDK proteins in root apical meristems, I used marker constructs for these CDK genes. CDKB2;1 accumulation was suppressed as early as 8 h after transfer onto zeocin-containing plates whereas GUS activity of CDKA;1::GUS and CDKB1;1::GUS did not show a change (Fig 3-11A). The reduction in CDKB2;1 accumulation should not a result of meristem dysfunction, because the 8 h-treatment was enough to affect root growth, but it did not stop cell division (Fig 3-4). Gamma irradiation caused similar reduction in expression (Fig. 3-11B), suggesting that the observed downregulation is not specific to zeocin treatment but to DSB in

general.

### 3.3.5 Signaling pathways required for DNA-damage induced endoreduplication

In order to reveal regulators and pathways involved in DSB-induced endoreduplication, I then investigated various Arabidopsis mutants defective in DNA damage signaling and endoreduplication. Of the signaling factors shown in Fig 3-1, Arabidopsis has homologs for *ATM*, *ATR*, and *WEE1* (Table 3-1). Two sensor kinases *ATM* and *ATR* are assumed to have distinct and additive functions: ATM has a major role in coping with DSBs whereas ATR is associated with the response to single-stranded DNA (Sancar et al., 2004). WEE1 kinase and the transcription factor SOG1 (SUPPRESSOR OF GAMMA RESPONSE 1) have been proposed to function at the downstream of ATM and ATR (De Schutter et al., 2007; Yoshiyama et al., 2009). Root growth of *atm-2* and *atr-2* was more sensitive to 2 μM zeocin than wildtype (Fig 3-12A), and growth of the *atm-2 atr-2* roots was also inhibited by 2 μM zeocin (Fig 3-12B). Despite the hypersensitivity of *wee1-3* to HU (De Schutter et al., 2007; Fig 3-13), its response to zeocin was indistinguishable from that of wildtype (Fig 3-12A). Root growth of *sog1-1* was only slightly affected on 2 μM zeocin plates (Fig 3-12C). Unlike the hypersensitivity in root growth, root epidermal cells of *atm-2* and *atr-2* were enlarged similarly as in wildtype seedlings (Fig 3-14). Considering the suppression of zeocin-induced cell growth in the *atm-2 atr-2* double mutant, ATM and ATR are thought to have redundant function in DSB-induced endoreduplication (Fig 3-15). Cell area of *wee1-3* also increased as in wildtype (Fig 3-14), suggesting that *WEE1* may not function in DSB-induced endoreduplication. On the contrary, cell area in *sog1-1* did not increase after 24 h treatment with 10 μM zeocin (Fig 3-14). In addition, the CDKB2 marker expression was not suppressed in the *sog1-1* roots (Fig 3-16). These results suggest that *SOG1* is required for the DSB-induced endoreduplication and that cells in *sog1-1* continue to divide irrespective of DSBs.

As for endoreduplication mutants, I found that the *midget-1* (*mid-1*) mutant sustains growth on 2 μM zeocin plates and that cell area in these mutants did not respond to zeocin (Fig 3-17). MID/BIN4 was reported to physically interact with a component of the type VI topoisomerase that is known to be required for endoreduplication (Hartung et al., 2002; Sugimoto-Shirasu et al., 2002; Yin et al., 2002; Sugimoto-Shirasu et al., 2005; Kirik et al., 2007; Breuer et al., 2007). Among components that consist the type VI topoisomerase complex, A and B subunit, which are designated as *AtSPO11-3/BIN5/RHL2* and *AtTOP6B/BIN3/HYP6*, respectively, in Arabidopsis, are conserved from archae, while *RHL1* and *MID/BIN4* are unique components to plants (Sugimoto-Shirasu et al., 2005; Kirik et al., 2007). The result *MID/BIN4* indicates that molecular mechanisms involved in the DSB-induced endoreduplication are at least partially shared with those required for endoreduplication during developmental processes.

Some of the cell cycle regulators have been shown to have a role in balancing the mitotic cell cycle and the endocycle (Inzé and De Veylder, 2006). The activator of anaphase-promoting complex/cyclosome (APC/C), *CCS52A* has been reported to promote endocycle onset in Arabidopsis. Although two *CCS52A*s of Arabidopsis, namely *CCS52A1* and *CCS52A2*, have been shown to be expressed in different tissues and have divergent functions (Vanstraelen et al., 2009), a mutation in either *CCS52A* reduced the ploidy level in leaves and ectopic expression elevated the DNA ploidy in various tissues (Lammens et al., 2008; Larson-Rabin, et al., 2009). Cellular enlargement was observed in *ccs52a1-1* as in wildtype, although *ccs52a2-1* showed hypersensitivity in both root growth and cell enlargement (Fig 3-18). Although overexpression of *CYCD3;1* has been reported to inhibit endoreduplication (Dewitte et al., 2003), I could not detect a difference in zeocin response in terms of root growth or cell enlargement (Fig 3-19). These observations show that ectopic higher mitotic activity or the mutation in *CCS52A* does not suppress the DSB-induced

endoreduplication.

In order to have a clue to elucidating physiological importance of DSB-induced endoreduplication, I employed tetraploid plants and examined whether increased DNA amount per nucleus has any effect on sensitivity to DSB. While roots growth of diploid seedlings was inhibited on 2 μM zeocin plates, tetraploid roots grew comparably to the control without zeocin (Fig 3-20). This result demonstrates that increased nuclear DNA amount or higher copy number of genes alleviates the inhibitory effect of DSB on cell division.

**Table 3-3: Transcriptional response of cell cycle regulators to zeocin in MM2d cells**

| Synonym | Significant response | Expression pattern during the cell cycle* |
|---|---|---|
| *CDKA;1* | no | |
| *CDKB1;1* | no | |
| *CDKB1;2* | no | |
| *CDKB2;1* | decrease | mitotic |
| *CDKB2;2* | no | mitotic |
| *CDKC;1* | no | |
| *CDKC;2* | no | |
| *CDKD;1* | no | |
| *CDKD;2* | no | |
| *CDKD;3* | no | |
| *CDKE;1* | no | |
| *CDKF;1* | no | |
| *CDKG;1* | no | |
| *CDKG;2* | no | |
| *CKS1* | decrease | |
| *CKS2* | no | |
| *CYCA1;1* | decrease | mitotic |
| *CYCA1;2* | no | |
| *CYCA2;1* | decrease | |
| *CYCA2;2* | no | mitotic |
| *CYCA2;3* | decrease | |
| *CYCA2;4* | no | |
| *CYCA3;1* | no | S phase |
| *CYCA3;2* | no | S phase |
| *CYCA3;3* | no | |
| *CYCA3;4* | no | |
| *CYCB1;1* | increase | |
| *CYCB1;2* | decrease | mitotic |
| *CYCB1;3* | decrease | mitotic |
| *CYCB1;4* | decrease | mitotic |
| *CYCB1;5* | decrease | mitotic |
| *CYCB2;1* | decrease | mitotic |
| *CYCB2;2* | decrease | mitotic |
| *CYCB2;3* | decrease | |
| *CYCB2;4* | decrease | mitotic |
| *CYCB2;5* | no | mitotic |
| *CYCB3;1* | decrease | mitotic |
| *CYCD1;1* | no | |
| *CYCD2:1* | no | |
| (Continued) | | |
| *CYCD3;1* | decrease | |
| *CYCD3;2* | decrease | |
| *CYCD3;3* | no | |
| *CYCD4;1* | no | |
| *CYCD4;2* | increase | |
| *CYCD5;1* | no | |
| *CYCD6;1* | no | |
| *CYCD7;1* | no | |
| *CYCH;1* | no | |
| *E2Fa* | no | |
| *E2Fb* | no | |
| *E2Fc* | no | |
| *KRP1* | no | |
| *KRP2* | decrease | |
| *KRP3* | no | |
| *KRP4* | no | |
| *KRP5* | decrease | |
| *KRP6* | increase | |
| *KRP7* | no | |
| *SIM* | increase | |
| *SMR1* | increase | |
| *SMR2* | no | |
| *SMR3* | increase | |
| *SMR4* | increase | |
| *SMR5* | increase | |
| *SMR6* | no | |
| *SMR7* | increase | |
| *SMR8* | no | |
| *SMR9* | no | |
| *SMR10* | increase | |
| *SMR11* | no | |
| *SMR12* | no | |
| *SMR13* | no | |
| *RBR* | no | |
| *WEE1* | increase | |
| *CDC6* | no | |
| *CDT1a* | decrease | |
| *CDT1b* | no | |

* Expression pattern during the cell cycle was defined according to the data of Menges et al. (2005).

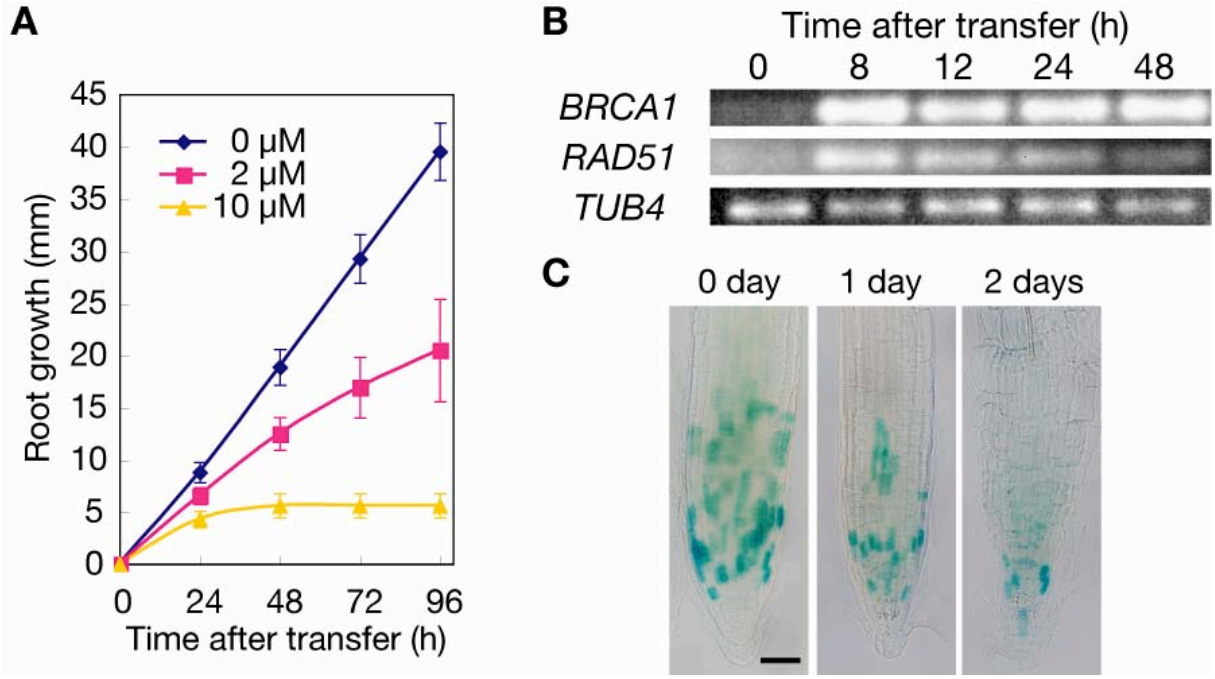



**Figure 3-2. DSBs cause growth arrest and inhibit mitosis**

(A) Five-day-old seedlings were transferred onto zeocin-containing plates (n ≧ 28). Root growth was affected dose-dependently. Error bars represent standard deviations in (A) and subsequent graphs. (B) Induction of DNA damage-responsible genes *BRCA1* and *RAD51* in root tips treated with 10 μM zeocin. Five-day-old seedlings were subjected to the treatment and RNAs from 0.5 cm root tips were used for RT-PCR. Forty root tips were used at each time point. (C) Expression of the CYCB1;2-GUS fusion protein under the *CYCB1;2* promoter was suppressed on 10 μM zeocin plates. The bar in (C) shows 50 μm.

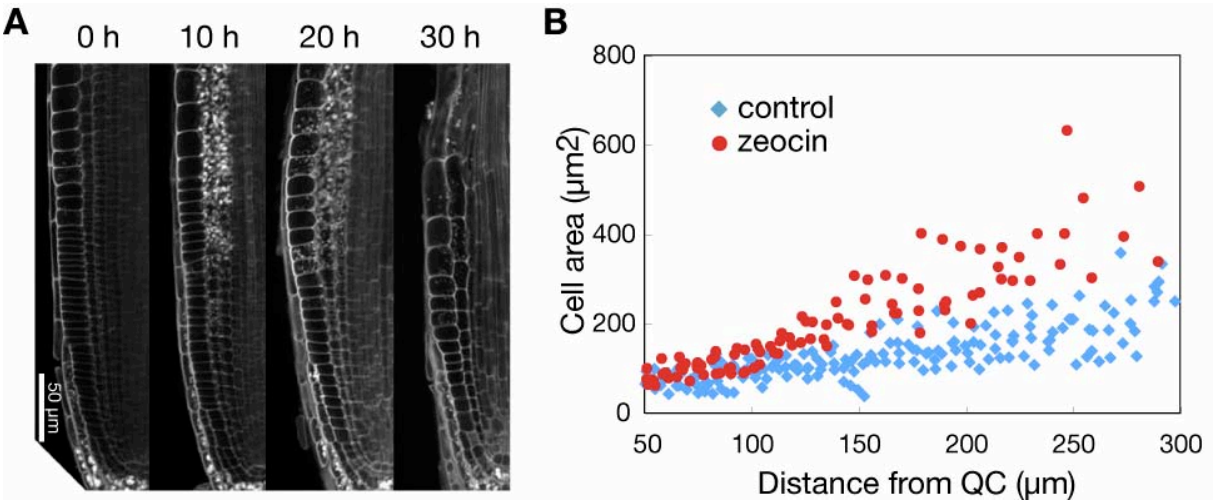



**Figure 3-3. DSBs induce cell enlargement in Arabidopsis RAMs**

(A) Cell enlargement occurred in 10 μM zeocin-treated RAMs. Confocal images of mPS-PI stained roots. Time after transfer is indicated on the top of each picture. The bar shows 50 μm. (B) Cell area in epidermal cell file was quantified after 24 h treatment with or without 10 μM zeocin. Five individuals were used for quantification in each condition.

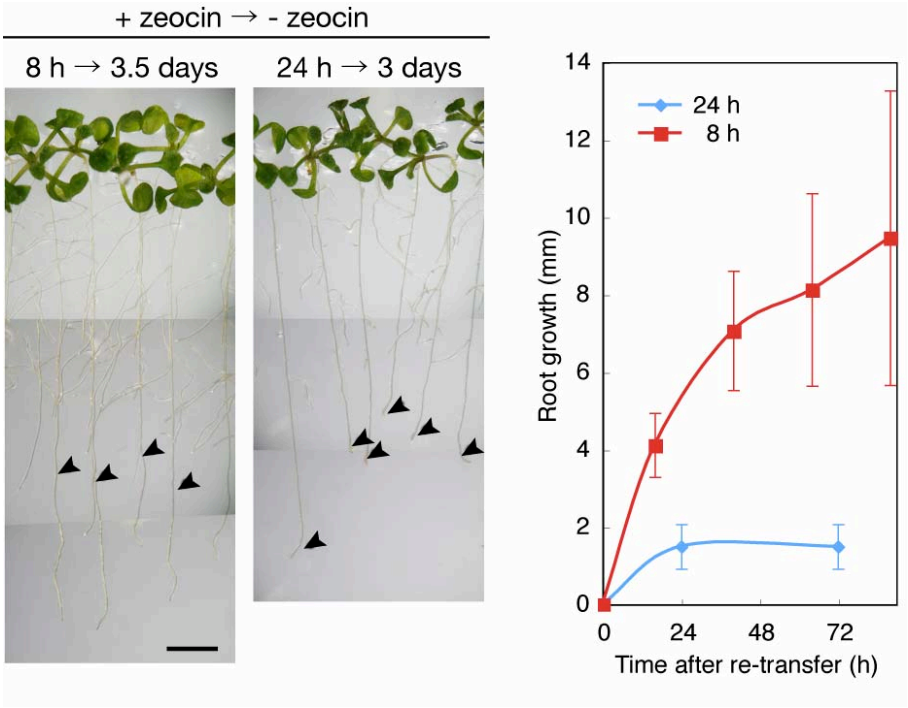



**Figure 3-4. Recovery of root growth after transfer to zeocin-free conditions**

Five-day-old seedlings were transferred onto 10 μM zeocin plates and incubated for 8 or 24 h and then re-transferred onto zeocin-free media (n = 34 for each treatment). Arrowheads on pictures indicate the positions of root tips at the re-transfer. Bar = 0.5 cm.

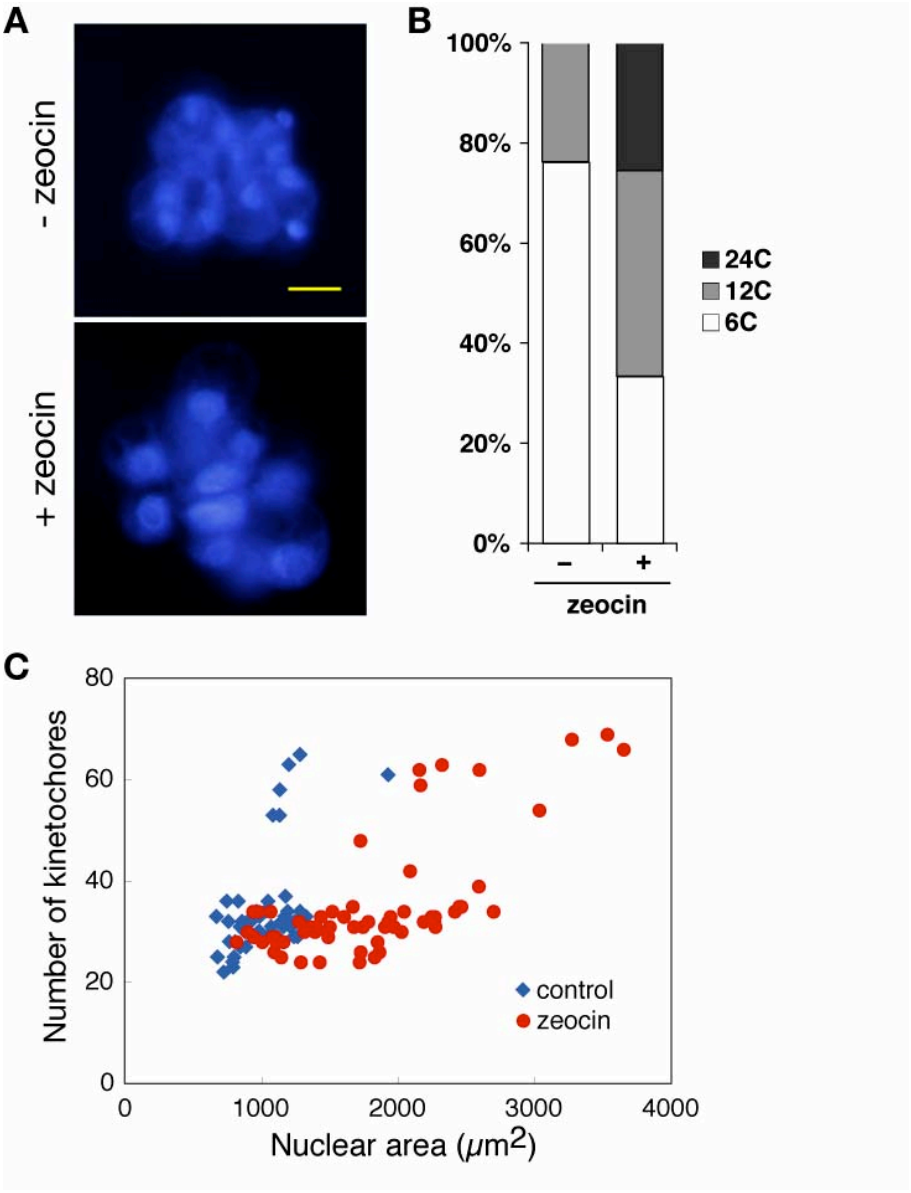



**Figure 3-5. Zeocin induces endoreduplication in Arabidopsis cultured cells**

(A) Three-day-old MM2d cells were treated with 50 μM zeocin for 48 h, and stained with DAPI. The bar indicates 20 μm. (B, C) After 72 h treatment of freshly subcultured cells with zeocin, nuclear ploidy (B) and kinetochore numbers (C) were analyzed.

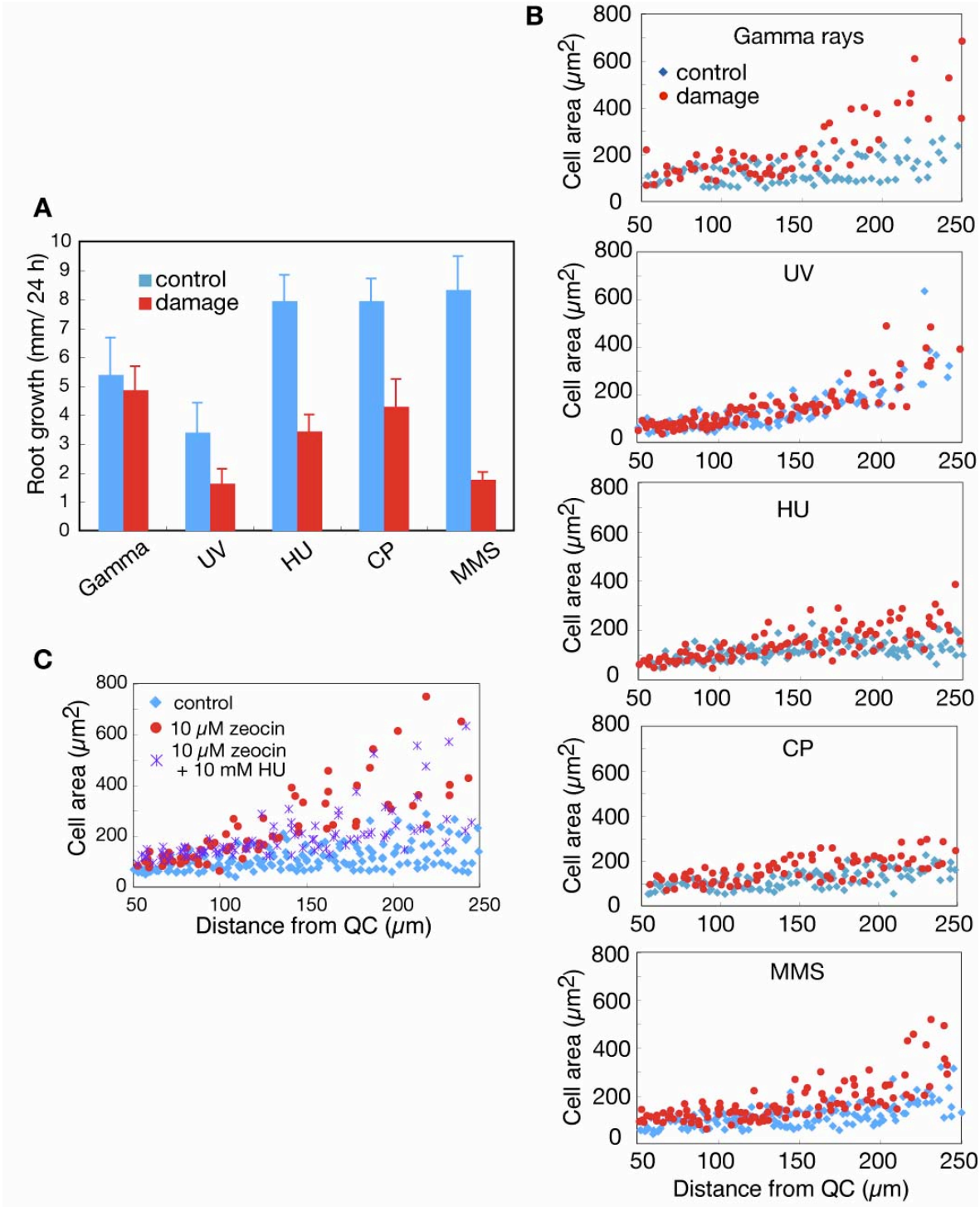



**Figure 3-6. DNA-damage induced endoreduplication is specific to DSBs**

(A, B) Five-day-old seedlings were irradiated with 150 Gy gamma or 1 kJ/m$^2$ ultraviolet rays (UV), or transferred onto 10 mM hydroxyurea (HU)-, 50 μM cisplatin (CP)-, or 100 ppm methanemethylsulfonate (MMS)-containing medium, and incubated for 24 h. Root growth (A, n ≧ 20) and cell area in the epidermal cell file of four (gamma rays and CP) or five individuals (UV, HU, and MMS) (B) were quantified. (C) Epidermal cell area after 10 mM HU and 10 μM zeocin treatment was also measured (n ≧ 5).

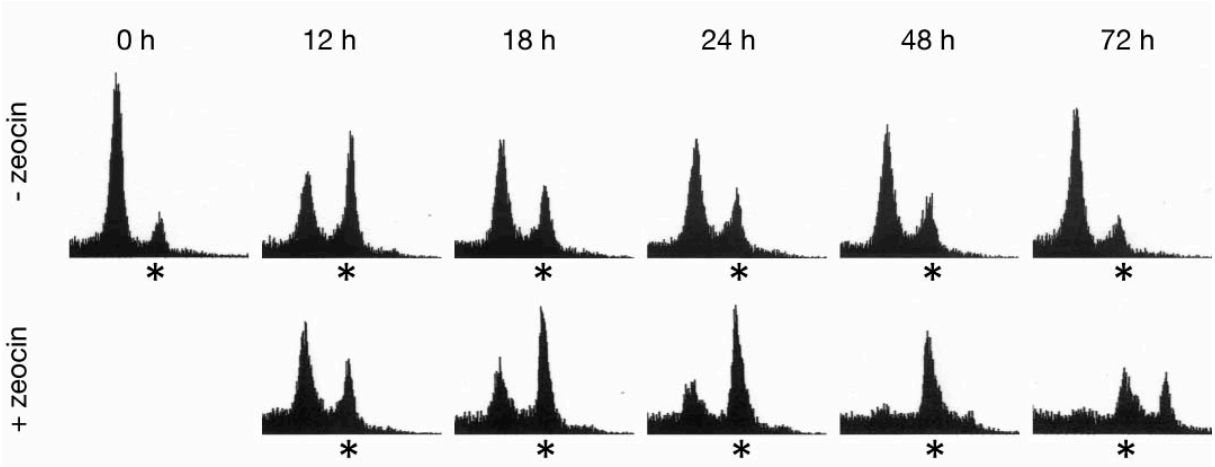



**Figure 3-7. Effect of zeocin on cell cycle progression**

Seven-day-old culture of MM2d cells was subcultured to fresh media with or without 50 μM zeocin. DNA ploidy analysis was performed. Positions of 12C peaks are indicated with asterisks.

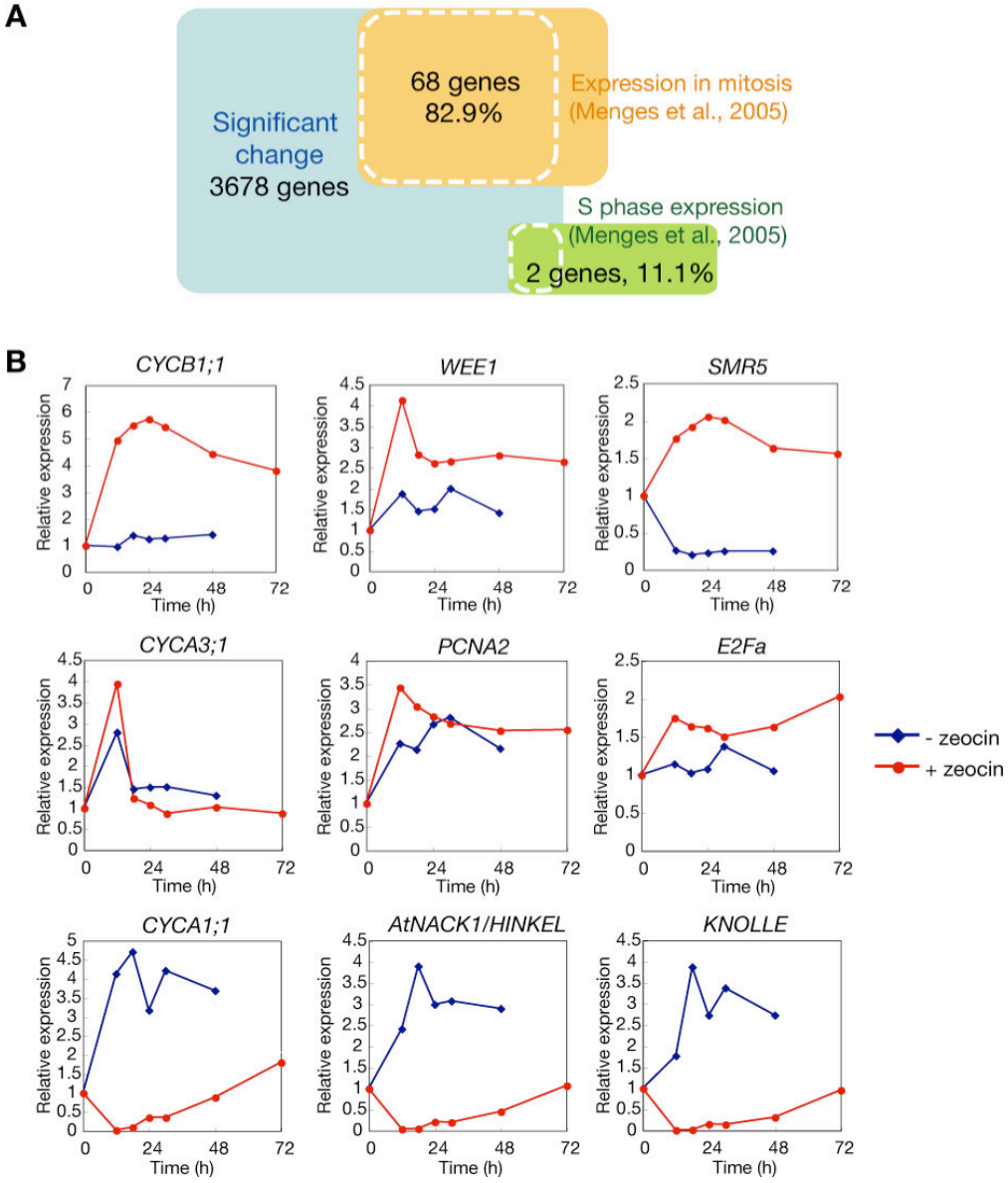



**Figure 3-8. G2/M-biased effect of zeocin on the cell cycle**

(A) Genes with significant change in expression were compared with results of the microarray analysis with synchronized MM2d cells. (B) Expression patterns of cell cycle regulators. Names of genes are indicated at the top of each graph. *TUB4* was used as a control.

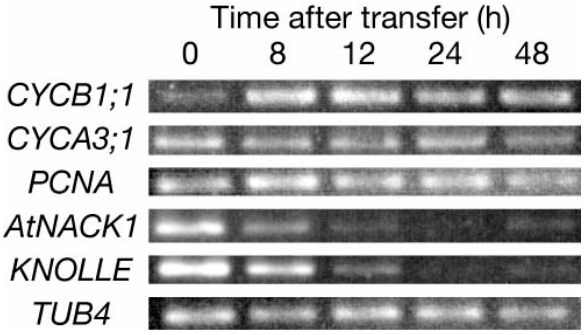



**Figure 3-9. Expression of cell cycle regulators in root tips in response to zeocin**

Five-day-old seedlings were transferred onto 10 μM zeocin plates and 0.5 cm root tips were collected for RNA extraction at the time indicated. Forty root tips were used at each time point.

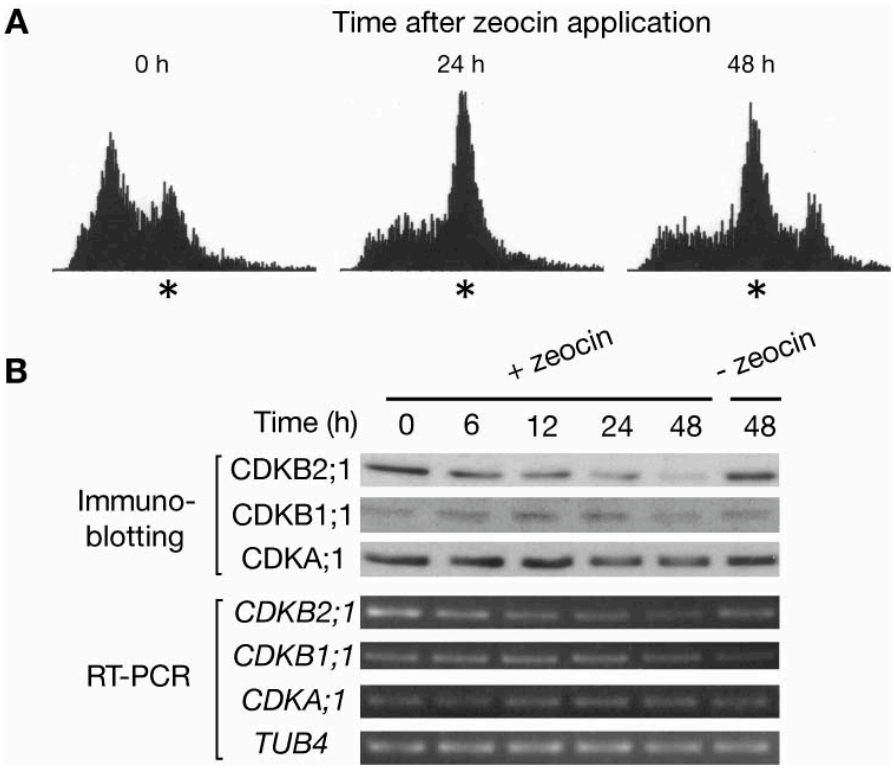



**Figure 3-10. Responses of CDK expression to zeocin in MM2d cells**

(A) Zeocin was added to three-day-old MM2d cells, and nuclear ploidy was analyzed at the time indicated. Positions of 12C peaks are indicated with asterisks. (B) Protein or RNA was extracted from the cells treated in (A). Immunoblotting was performed with specific antibodies against each CDK. Twenty micrograms of total proteins were applied for immunoblotting.

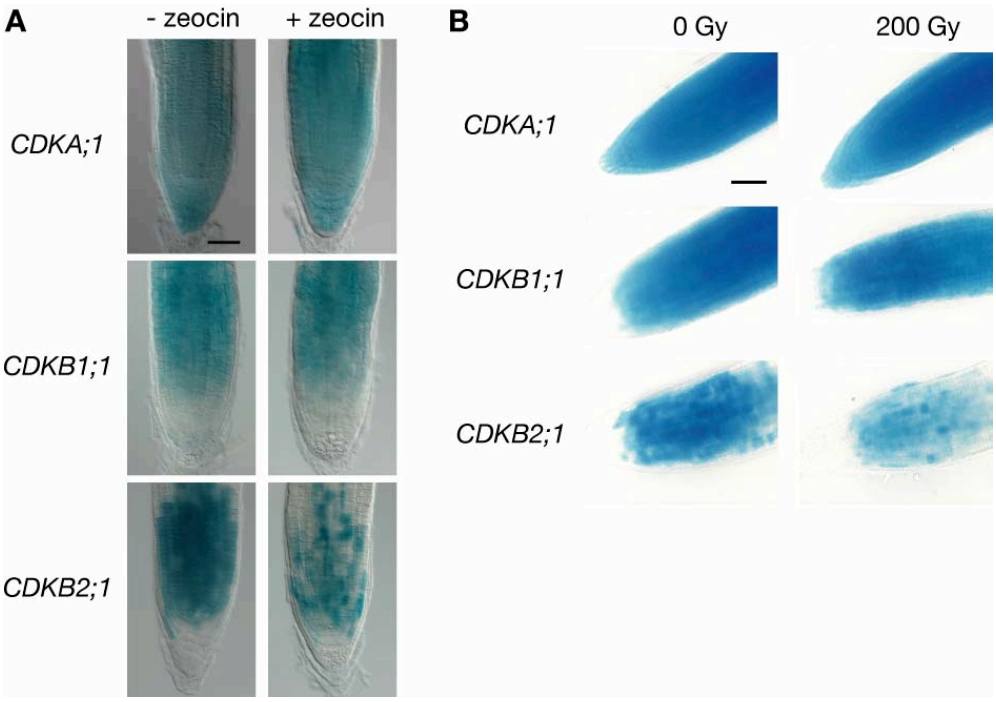



**Figure 3-11. Responses of CDK expression to DSBs in root tips**

(A) Seven-day-old seedlings of proCDK-CDK::GUS were transferred to 10 μM zeocin-containing plates and treated for 8 h. (B) Seven-day-old seedlings were subjected to gamma radiation. Samples were fixed at 8 h after irradiation. Bars show 50 μm.

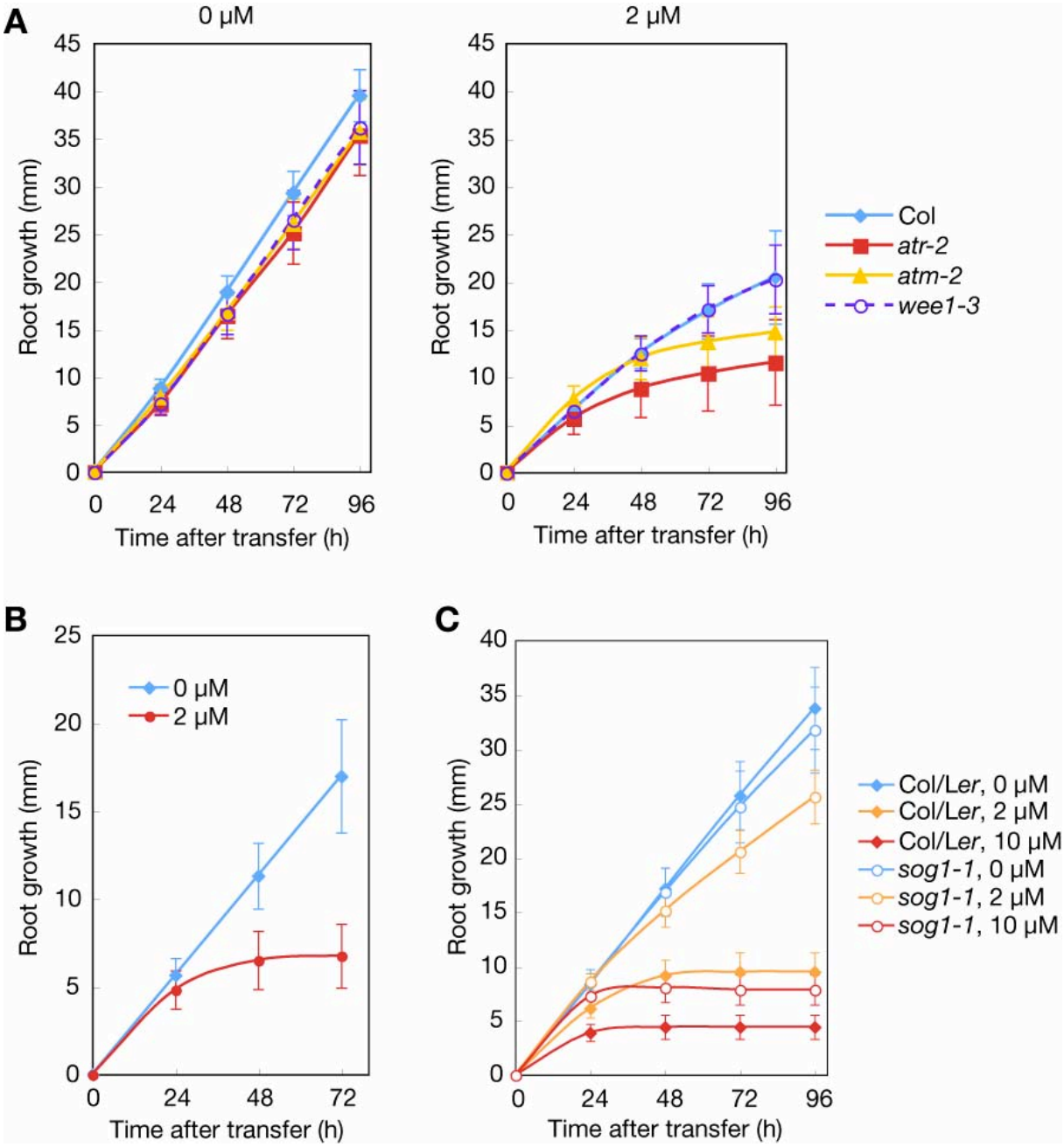



**Figure 3-12. Root growth of *sog1-1* is less sensitive to zeocin**

Five-day-old seedlings were transferred onto zeocin plates, and root growth was measured. (A) Root growth of the *atm-2*, *atr-2* and *wee1-3* mutants with or without 2 μM zeocin (n ≧ 19) (B) Root growth of the *atm-2 atr-2* double mutant with or without 2 μM zeocin (n = 23 and 14, at 0 and 2 μM treatment, respectively). (C) Response of *sog1-1* root growth to zeocin (n ≧ 26). The hybrid of Col and L*er* (Col/L*er*) was used as a control of *sog1-1*.

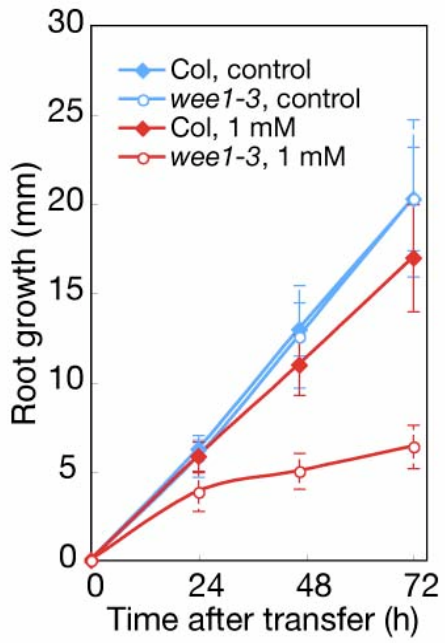



**Figure 3-13. The *wee1-3* mutants showed hypersensitivity to HU**

Five-day-old seedlings were transferred onto 1 mM HU plates, and root growth was measured (n = 11 for Col, and 12 for *wee1-3*, for each condition).

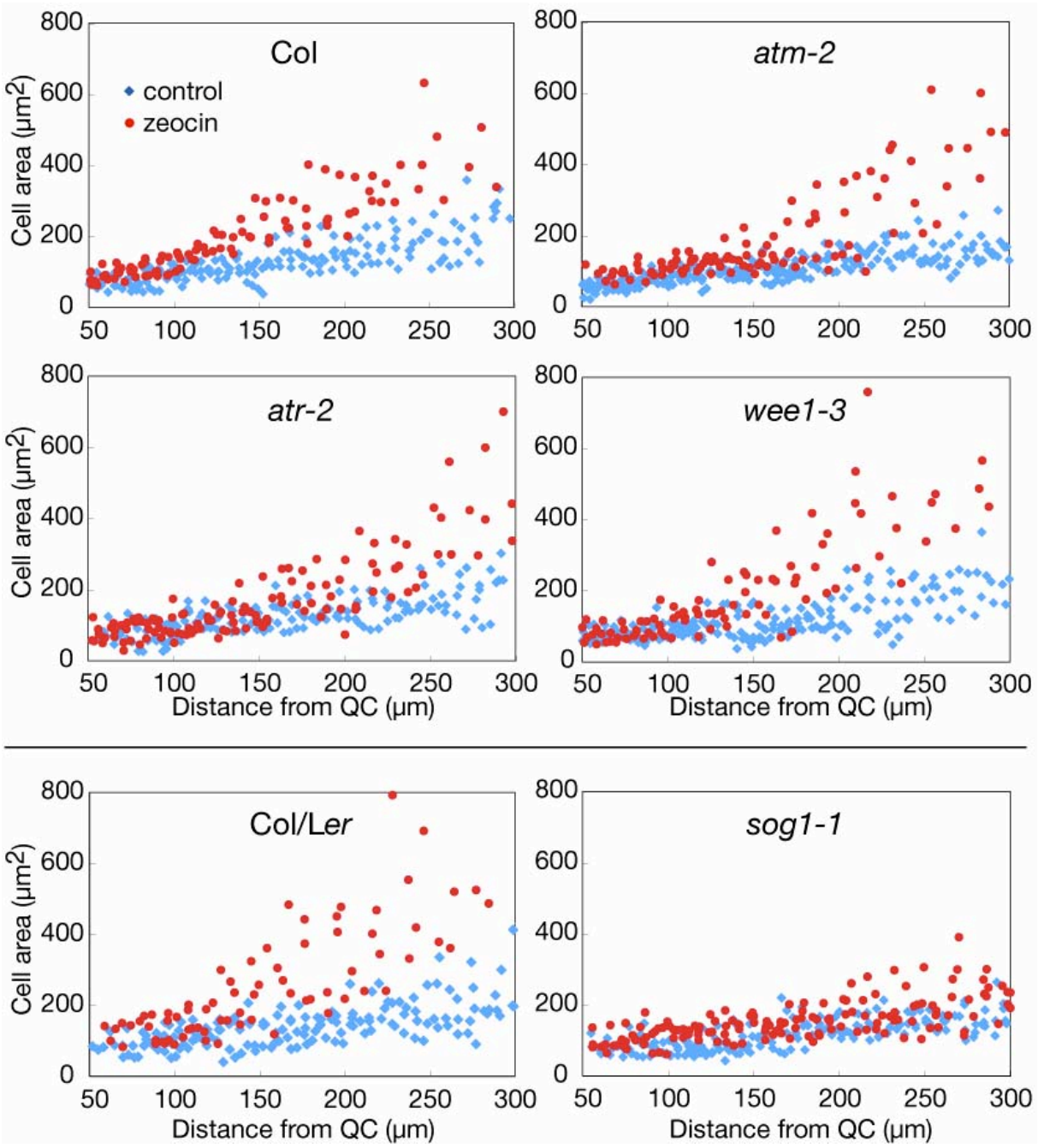



**Figure 3-14. SOG1 is required for DSB-induced endoreduplication**

Five-day-old seedlings were treated with 10 μM zeocin. Cell area in epidermal cell file was quantified with mPS-PI stain after 24 h treatment. The hybrid of Col and L*er* (Col/L*er*) was used as a control of *sog1-1*. Five individuals were used for quantification in each condition.

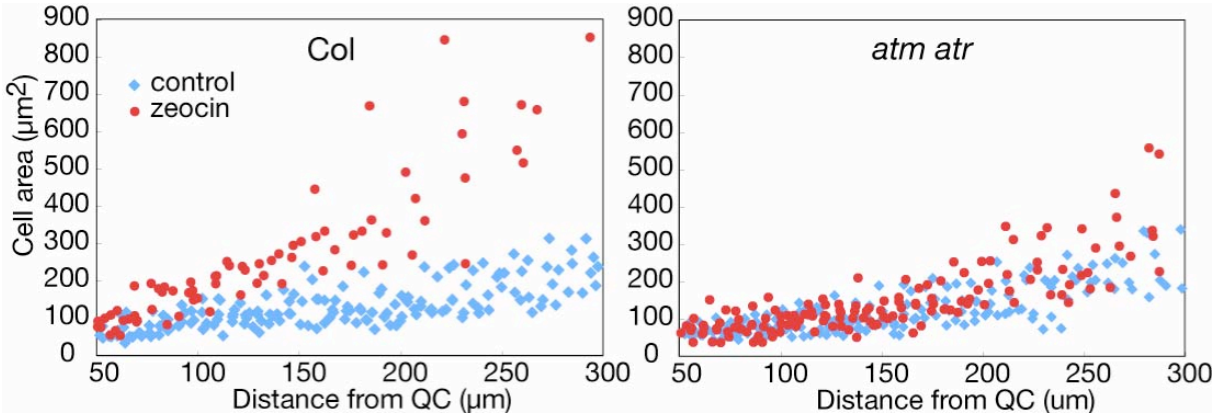



**Figure 3-15. Cell growth in response to zeocin in the *atm-2 atr-2* double mutant**

Five-day-old seedlings were treated with 10 μM zeocin. Cell area in epidermal cell file was quantified with mPS-PI stain after 24 h treatment. Five individuals were used for quantification in each condition.

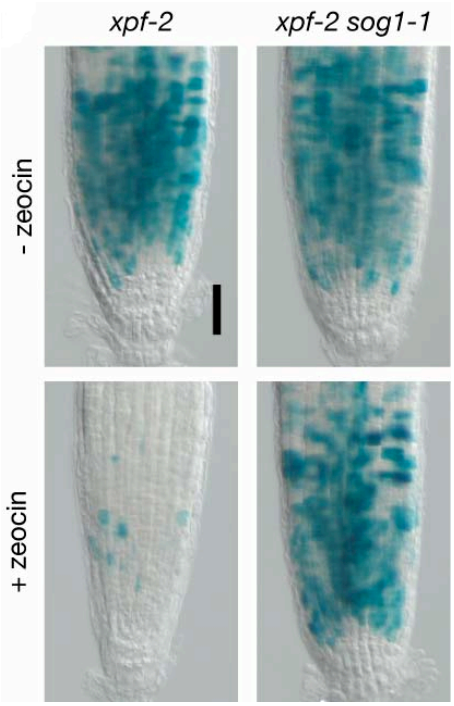



**Figure 3-16. Expression of the *CDKB2* marker in the *sog1-1* mutants**

Seven-day-old seedlings harboring the Pro-NT::GUS construct (see Chapter II for detail) were transferred onto media with or without 10 μM zeocin plates, and incubated for 24 h. The bar shows 50 μm.

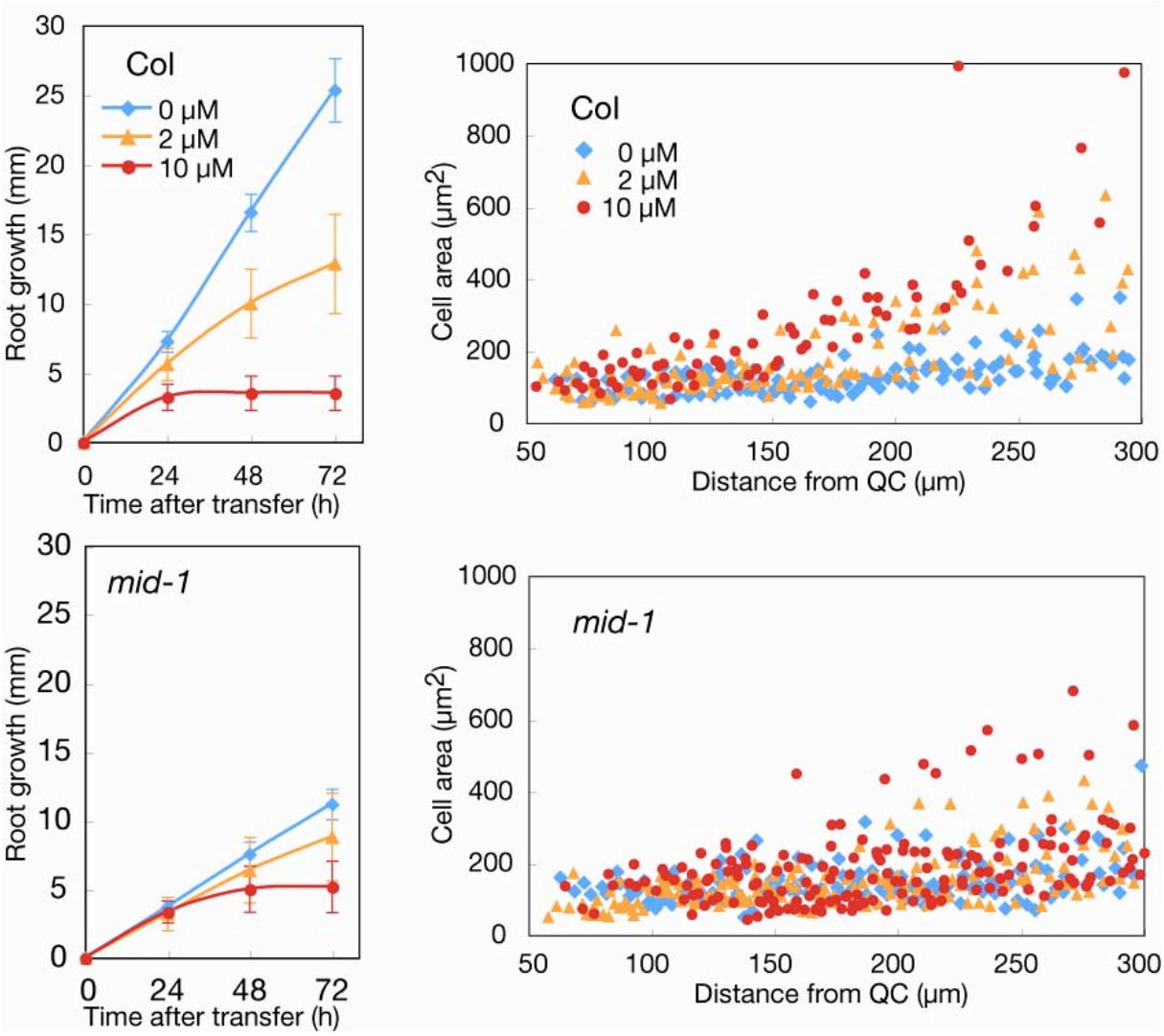



**Figure 3-17. Cell growth in response to zeocin in a TOPO VI mutant**

Five-day-old seedlings were transferred onto a 10 μM zeocin-containing medium, and incubated for 24 h. Root growth (n ≧15), and cell area in epidermal cell file (n ≧ 4) were quantified in each condition.

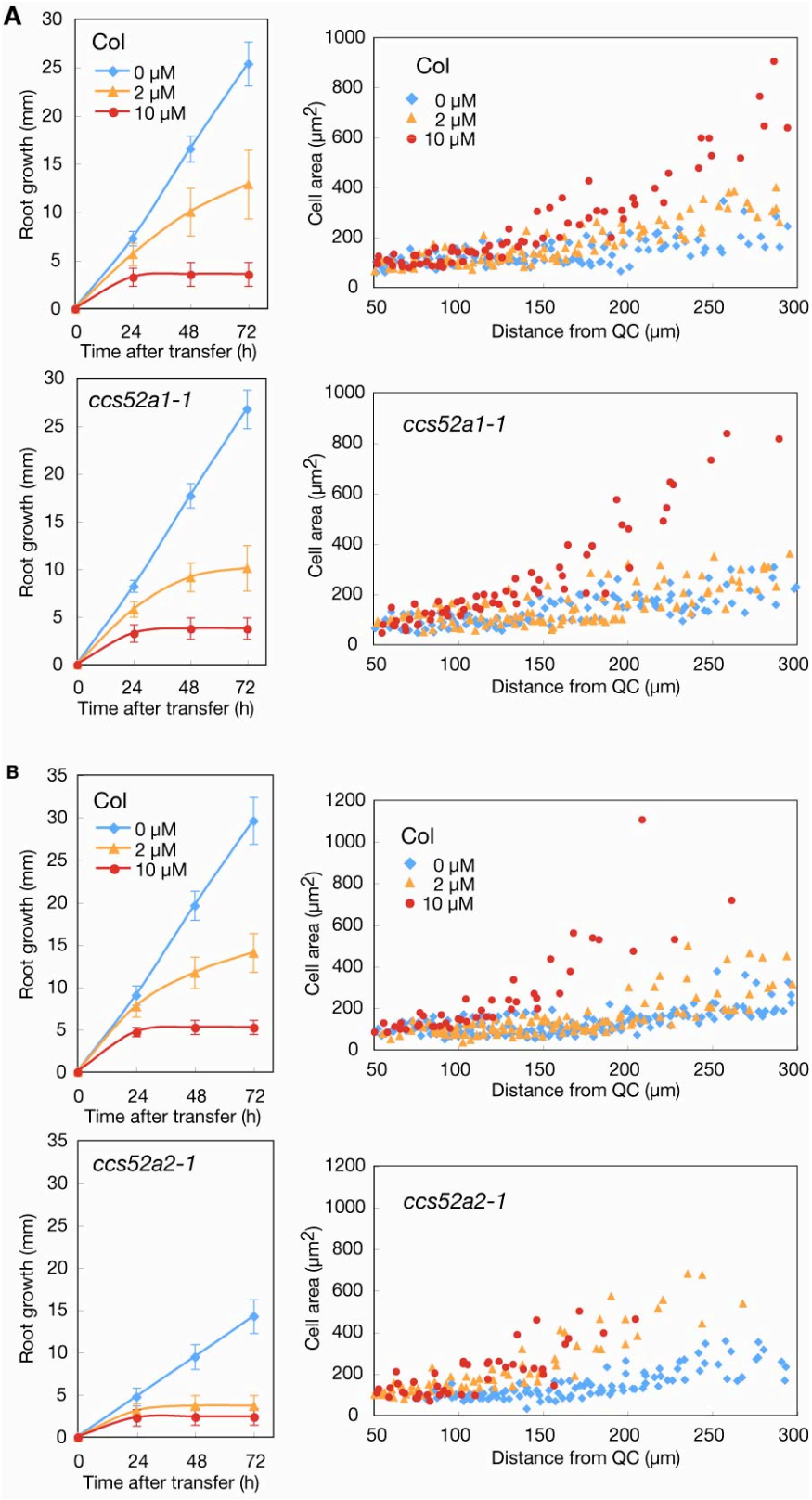



**Figure 3-18. Cell growth in response to zeocin in *ccs52a* mutants**

Five-day-old seedlings were transferred onto a 10 μM zeocin-containing medium, and incubated for 24 h. Root growth (n ≧ 13 (A) or 9 (B)), and cell area in epidermal cell file (n ≧ 4) were quantified in *ccs52a1-1* (A), and *ccs52a2-1* (B).

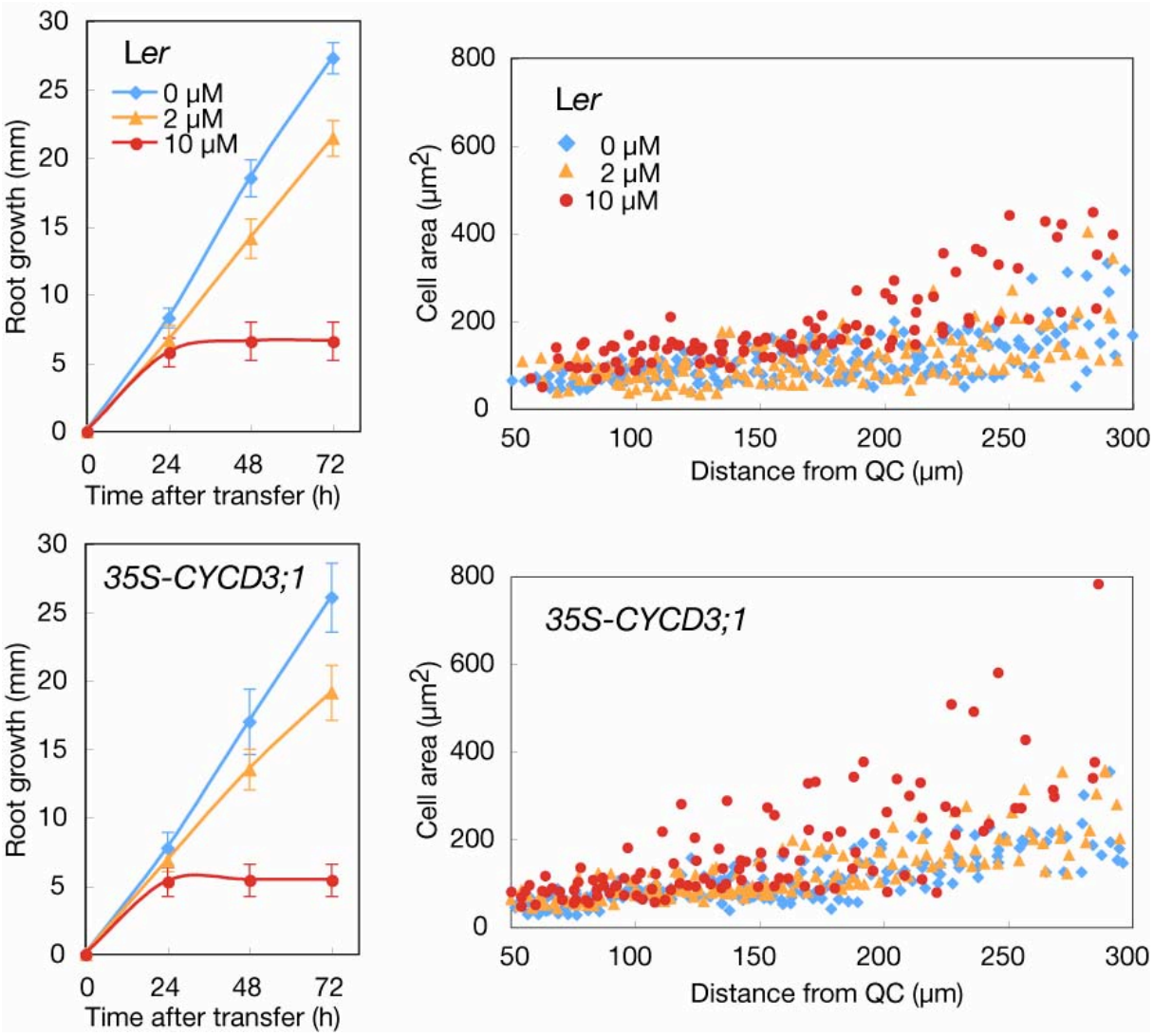



**Figure 3-19. Cell growth in response to zeocin the *CYCD3;1* overexpressing plant**

Five-day-old seedlings were transferred onto a 10 μM zeocin-containing medium, and incubated for 24 h. Root growth (n ≧ 11), and cell area in epidermal cell file (n = 5) were quantified.

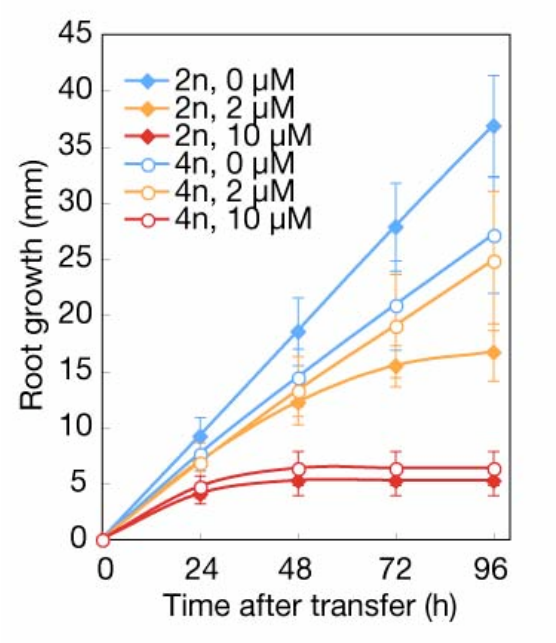



**Figure 3-20. Tetraploidy conferred tolerance to zeocin.**

Five-day-old seedlings were transferred onto 0, 2 or 10 µM zeocin plates (n ≧ 15).

## 3.4 DISCUSSION

### 3.4.1 DNA damage-induced endoreduplication in Arabidopsis

My results demonstrated that DNA damage inhibits mitosis (Fig 3-2A, C) and induces endoreduplication (Fig 3-5 and Supplemental fig 2) in both meristematic cells in root tips and undifferentiated cultured cells. Although a flowcytometric analysis has previously shown gamma irradiation-induced endoreduplication previously (Hefner et al., 2006), my detailed cellular analysis at earlier time points after zeocin treatment demonstrated a clear relationship between DNA damage and induction of endoreduplication. In addition, the results in MM2d cells demonstrated that the DSB-induced endoreduplication can occur independently of differentiation state of cells (Fig 3-5). Hefner et al. (2006) showed that the mutation in DNA Ligase IV (*Lig4*), which is involved in DSB end joining (Calsou et al., 2003; Friesner et al., 2003), suppresses gamma rays-induced endoreduplication. Considering that majority of DSBs are repaired by end joining (Gisler et al., 2002), DSBs may be repaired by Lig4 before initiating endoreduplication, and induction of endoreduplication may not be a consequence of errors or inability of DNA repair.

Induction of endoreduplication implies that DNA damage affects mainly the G2 and M phase progression whereas it does minor effects on G1 and S phase progression. Indeed, although genes that have a peak of expression at the S phase or are required for the S phase progression did not respond to zeocin, mitotic genes were repressed when DSB occurs by zeocin treatment (Fig 3-8 and 3-9). In vertebrate system, DNA damage causes either cell-cycle arrest or apoptosis (Vousden and Lu, 2002). The vertebrate cell cycle can be arrested at both G1 and G2 phase, which is mainly controlled by p53, and WEE1 and CDC25, respectively (Sancar et al., 2004). Interestingly, in cancer cells that lack p53/p21 pathway, endoreduplication occurs in response to DNA damage (Waldman et al., 1996; Bunz et al., 1998). This implies that the lack of the G1 checkpoint results in an induction of

endoreduplication in response to DNA damage in both vertebrates and Arabidopsis cells.

It should be noted that we have only limited information concerning when and how many DSBs occur in untreated plant cells. Besides such estimation of DSB frequency, the mechanisms suggested here must be validated in future by using less artificial conditions.

### 3.4.2 Molecules and signaling pathways involved in DNA damage-induced endoreduplication

Inhibition of mitosis by inducing endoreduplication indicates that Arabidopsis cells have a checkpoint mechanism at the G2/M phase. This checkpoint mechanism may be plant-specific since functional orthologs of CDC25 have not yet identified in plants, and a recent report demonstrated that WEE1 function is dispensable for plant growth under normal conditions (De Schutter et al., 2007; Dissmeyer, et al., 2009). Considering the hypersensitivity of *wee1-3* to HU (Fig 3-13; De Schutter et al., 2007), *WEE1* may have a role in response to replication stress, but it seems to be unrelated to the response to DSB and DSB-induced endoreduplication (Fig 3-12 and 3-14). Another important plant-specific player at the G2/M transition is CDKB (Inzé and De Veylder, 2006). It is interesting to hypothesize that, instead of phospho-regulatory mechanisms, CDKB may be quantitatively regulated at mRNA and protein levels. The lack of response of *CDKB1* to DNA damage may be related to its expression pattern from the late S-to-the M phase, which is earlier and longer than that of *CDKB2* (Magyar et al., 1997; Mészáros et al., 2000; Menges et al., 2002; Menges et al., 2005).

I demonstrated that *SOG1* is required for induction of endoreduplication when DSBs were generated (Fig 3-14). *SOG1* has reported to be required for expression of gamma ray-inducible genes (Yoshiyama et al., 2009). Given the fact that most of the genes that required ATM for their induction also required SOG1 for upregulation in response to gamma rays, and that ATM was properly activated in *sog1-1*, these two proteins are assumed to

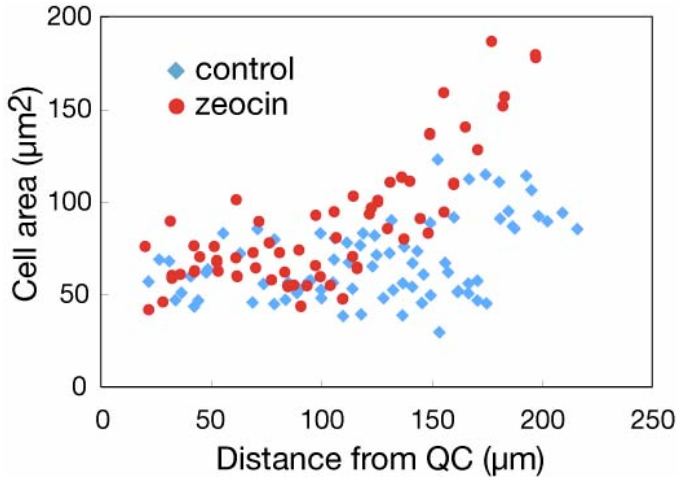
function sequentially or simultaneously (Yoshiyama et al., 2009). *SOG1* and *ATR* are also thought to work in concert since the *xpf atr* phenocopied gamma-resistant growth of the *xpf sog1* mutant (Yoshiyama et al., 2009). In fact, DSB-induced cell enlargement was suppressed in the *atm-2 atr-2* double mutant as in the *sog1-1* mutant (Fig 3-14 and 3-15). These results indicate that ATM and ATR cooperatively activate SOG1 or these three factors concurrently function in DSB-induced endoreduplication. As for sensitivity of root growth to zeocin, the *atm-2 atr-2* roots exhibited sensitivity to 2 μM zeocin, by which the *sog1-1* roots were only slightly affected (Fig 3-12B, C), implying different effects of those mutations on long-termed response of the RAMs. Such a difference may be attributed to activation of some of the ATM/ATR substrates that does not require transcriptional activation mediated by SOG1.

The DSB-induced endoreduplication seems to employ the TOPO IV complex that is involved in endoreduplication during development (Figure 3-17). Although *CCS52A*s are also known to promote endoreduplication (Lammens et al., 2008; Larson-Rabin, et al., 2009), DSB-induced cell growth was not suppressed in *ccs52a* mutants (Fig 3-18). As for *CCS52A1*, this is probably because of the spatial expression pattern around the elongation zone excluding the RAM (Vanstraelen et al., 2009). On the other hand, *CCS52A2* is expressed in the RAMs and shown to be required for maintenance of the RAMs (Vanstraelen et al., 2009), probably by facilitating proper transition from the M phase to the G1 phase. The reason of *ccs52a2*'s hypersensitivity to zeocin in terms of cell growth is not clear, but it is likely that reduced supply of auxin from meristem enhances onset of endoreduplication, as I have demonstrated that auxin deficiency promotes endoreduplication (Ishida et al., 2010). Previous reports suggested that higher mitotic activities achieved by overexpression of *CYCD3;1* reduced endoreduplication levels in transgenic plants (Dewitte et al., 2003). However, the overexpression of *CYCD3;1* did not prevent the DSB-induced endoreduplication (Fig 3-19), even though overexpression of *CYCD3;1* induces ectopic mitosis in trichomes (Schnittger et

al., 2002). This may result from unidentified difference in molecular mechanisms between DSB-induced endoreduplication and endoreduplication in trichomes. Combined overexpression of cyclins or a cyclin and a CDK may elucidate which factors to be downregulated for induction of endoreduplication.

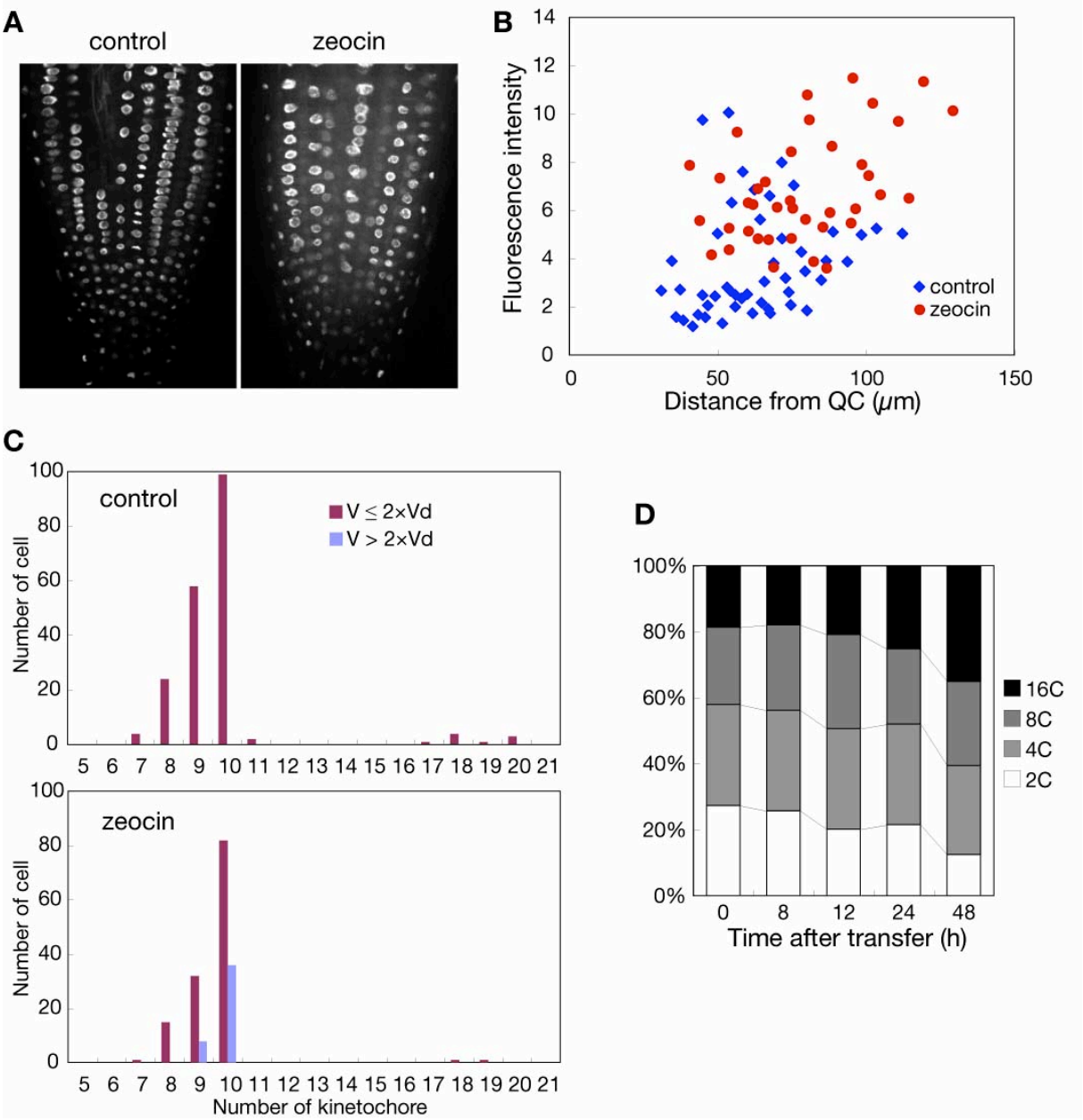
Physiological significance of endoreduplication in non-stressed condition has not studied well, which makes it more difficult to address the importance of the DSB-induced endoreduplication. However, it is tempting to speculate that molecular mechanisms engaged in endoreduplication during cell differentiation process might be applied to prevent proliferation of cells with impaired genomes. Another possibility is that multiplication of genomes may facilitate maintaining cellular metabolism when DNA damage occurs. Indeed, I showed that tetraploid roots were more tolerant to zeocin (Fig 3-20), and a UV-resistant mutant, *uvi4* (*UV-B insensitive 4*) shows high ploidy level (Hase et al., 2006), indicating that increased ploidy confers DNA damage tolerance. Further functional studies on molecules involved in the DSB-induced endoreduplication will reveal physiological importance of the endoreduplication not only in response to genotoxic stress but also during continuous development in plants, and will give an insight into regulatory mechanisms underlying the transition from the mitotic cycle to the endocycle.

## 3.5 SUPPLEMTENTAL DATA




**Supplemental figure 1. Cortex cell growth in response to zeocin**

Cell area in cortex cell files was quantified after 24 h treatment with 10 µM zeocin (n = 3). This experiment was performed by Dr. Kurihara and Dr. Matsunaga (Osaka University).




**Supplemental figure 2. Zeocin triggers endoreduplication in Arabidopsis roots**

Five-day-old seedlings harboring *RPS5A-tdTomato::HISTONE H2B* (A) were transferred onto 10 μM zeocin plates, and incubated for 24 h. The transgenic line was generated by Dr. Kurihara (Osaka University). (B) Fluorescence of tdTomato was quantified after 24 h treatment with or without zeocin (n = 5). Intensity of the fluorescent signal was quantified with the ImageJ software by selecting images in which each nucleus appeared in the maximum sizes. (C) Histograms showing kinetochore numbers that were counted by visualizing the HTR12 protein (n = 3). The *tdTomato::HTR12* binary vector was constructed by Kurihara (2008). The fluorescence of tdTomato was observed with a fluorescence microscope (IX-81; Olympus) using a 60 × objective lens (UPlanSApo, NA 1.20, water immersion). The fluorescent signals were counted using the MetaMorph. Kinetochore numbers are shown in two series according to volume of the virtual nucleus (V; volume

surrounded by HTR12 signals). The ‘Vd’ stands for volume of a diploid nucleus estimated from volume of virtual nuclei in cells adjacent to QC. (D) Seven-day-old seedlings were transferred onto 10 μM zeocin plates, and nuclear ploidy was measured by using root tips of 0.5 cm length at indicated time points. Experiments of (A), (B), and (C) were performed by Dr. Matsunaga (Osaka University), and (D) is adopted from Minamisawa (2008).

## CONCLUSIONS

This study first focused on regulation of *CDKA* and *CDKB2* expressions. In Arabidopsis tissues, the CDKA expression is mainly regulated at the transcriptional level whereas the CDKB2 expression is controlled by proteolysis as well as transcriptional regulation. My analyses revealed that the *CDKA;1* promoter contains several different *cis*-acting regions, which contains cell-type specific and general regulatory elements. My results indicated that ubiquitin-mediated protein degradation of CDKB2 plays a important role during cell cycle progression and in response to DNA damage. Analysis of DNA damage response in mitotic cells revealed that DNA double-strand breaks inhibit the mitotic cell cycle and induce the endoreduplication, which is accompanied with preferential effects on expression of G2/M-related genes. The expression of *CDKB2*, but not *CDKA* or *CDKB1*, was downregulated in response to DSBs. In addition, by using Arabidopsis mutants that have defects in DNA damage signaling or endoreduplication, I identified *SOG1* as upstream factors essential for the DSB-induced endoreduplication. Further analysis of genes involved in expression of CDKs will give us insights into molecular mechanism that controls cell division in plant tissues and the transition from the mitotic cell cycle to the endocycle.

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