Studies on functional roles of CDK-activating kinases in *Arabidopsis* development

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Contents

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

CAK: CDK-activating kinase

CAKAK: CAK-activating kinase

Cdc: Cell division cycle

CDK: cyclin-dependent kinase

CDKA: A-type CDK

CDKB: B-type CDK

CDKC: C-type CDK

CDKD: D-type CDK

CDKE: E-type CDK

CDKF: F-type CDK

CHX: cyclohexamide

CTD: carboxy-terminal domain

CYCA: A-type cyclin

CYCB: B-type cyclin

CYCC: C-type cyclin

CYCD: D-type cyclin

CYCE: E-type cyclin

CYCH: H-type cyclin

CYCT: T-type cyclin

DAG: days after germination

DAP: days after pollination

DMSO: dimethyl sulfoxide

FG: female gametophyte stage

h; hour

KRP: Kip-Related Protein

MAT1: ménage á trois 1

ORF: open reading frame

PMI: pollen mitosis I

PMII: pollen mitosis II

RACE: rapid amplification of cDNA ends

Rb: Retinoblastoma protein

RBR: Retinoblastoma-related Protein

RAM: root apical meristem

RNAPII: RNA polymerase II

SAM: shoot apical meristem

TFIIH: Transcription factor II H

X-gluc: 5-bromo-4-chloro-3-indoryl-β-D-glucuronide

General introduction

Cell cycle regulation by cyclin-dependent kinases (CDKs) in yeasts and animals

Cell proliferation is the most fundamental event in all living organisms. In unicellular organisms, the control of proliferation plays an important role in the perception of the nutrient conditions and sporulation. In multicellular organisms, the regulation of cell division is one of the important mechanisms that underlie several essential processes, including cell fate determination, differentiation, organ development, growth, cell death, carcinogenesis, etc.

'Replication' and 'division' are the key for cells to proliferate. These two steps are regulated by cyclin-dependent kinases, which referred to as CDKs in eukaryotes. CDKs are serine/threonine protein kinases, forming active complexes with cyclin (CYC), and phosphorylate the substrates controlling cell proliferation (for a review, see Dorée and Hunt, 2002). CDKs are involved in many events associated with cell cycle progression, such as DNA replication, chromosome segregation, cytokinesis, centrosome amplification, and so on .

Cell cycle consists of four stages, namely G1 phase, S phase, G2 phase and M phase. G0 is a physiological state occupied by resting or terminally differentiated cells that have exited from the cell cycle, especially at G1 phase. Cells increase in size in G1 phase. The G1 checkpoint control mechanism ensures that everything is ready for DNA synthesis. DNA replication occurs during S (DNA synthesis) phase. During G2 phase, cells continue to grow. The G2 checkpoint control mechanism ensures that everything is ready to enter the M (mitosis) phase and divide. Cell growth stops at M phase and cellular energy is focused on the orderly division into two daughter cells. A checkpoint in the middle of mitosis ensures that the cell is ready to complete cell division. After cell division, each of the daughter cells enters the interphase of a new cycle.

In budding and fission yeasts, Cdc28 and Cdc2 were identified as the sole CDK directly regulating cell cycle regulation (Simanis and Nurse, 1986; Wittenberg and Reed, 1988). Cdc28 controls both G1/S and G2/M by changing binding partners, cyclins, in a cell cycle-dependent manner. Whereas the protein level of Cdc28 is constant, nine cyclins are known to activate Cdc28 in a cell cycle-specific manner. Cln1, Cln2, and Cln3 are the G1/S cyclins, and six Clbs regulate G2/M progression (for a review, see Andrews and Measday, 1998) (Fig 0-1). Fission yeast Cdc2 is known to associate with four different cyclins; Cig1, Cig2, Puc1 and Cdc13. The amount of Cdc2 remains constant throughout the cell cycle, while cyclin levels oscillate. Therefore, the cell cycle-specific activity of Cdc2 is determined in part by its partner cyclins. Among them, Cig2 is the major S phase cyclin. It accumulates late in G1 phase and disappears upon exit from S phase. Cdc13 is required for Cdc2 activity at the onset of M phase. The Cdc13 protein level is low in G1 phase but increases in G2 phase, and is maintained until the end of M phase (for a review, see Moser and Russell, 2000) (Figure 0-1)

In animals, the situation is more complicated. Five CDKs regulate the cell cycle directily, namely CDK1, CDK2, CDK3, CDK4 and CDK6. A-, B-, C- and D-type cyclins are known to bind to these CDKs with a high specificity (for a review, see Satyanarayana and Kaldis, 2009). CDK2, CDK4 and CDK6 were identified as G1/S CDKs (Figure 0-1). CDK4 and CDK6 control the G1/S progression by associating with cyclin D, which is expressed in response to mitogenic signals. Activation of S phase gene expressions requires the relief of another inhibitory pathways, consisting of the retinoblastoma (Rb) protein and the transcription factors E2F and DP in animals. Rb family members bind to and suppress the activity of E2F-DP transcription factors, blocking the synthesis of S phase genes. Hyper-phosphorylation of Rb by

CDK4/6-CYCD releases E2F from Rb and facilitates S phase progression (Kato *et al*., 1994; Meyerson and Harlow, 1994).

CDK2-CYCE functions mainly at the late G1/S phase, and its action is almost complementary to CDK4/6-CYCD (Figure 0-1). The complex phosphorylates Rb on different sites from CDK4/6-CYCD, indicating a distinct function in the downstream signalling (Kitagawa *et al*., 1996). These findings suggested that CDK4/6-CYCD and CDK2-CYCE act co-operatively to inactivate the negative regulators of S phase progression, and allow the formation of the CDK2-CYCA complex at the onset of S phase (Sherr and Roberts, 2004). The CDK2-CYCA complex drives the cell cycle through S phase by phosphorylating substrates that initiate DNA replication (Coverley *et al*., 2002). Also, CDK2-CYCA is required for the activation of the mitotic CDK1 and the activating phosphatase for CDK1 (Mitra and Enders, 2004).

Entry into mitosis is triggered by the activation of Cdc2-like protein kinases that require mitotic cyclins such as CYCB in animals. The mitotic CDK-CYCB complex in animal cells is also known as a maturation promoting factor (MPF) and minimally consists of CDK1 bound to CYCB (Draetta *et al*., 1989) (Figure 0-1). The activated CDK1-CYCB complex subsequently phosphorylates numerous substrates that facilitate the mitotic events like nuclear envelope breakdown and spindle assembly (Nigg, 2001). Additionally, activated CDK1-CYCB regulates the anaphase-promoting complex/cyclosome (APC/C), a major component of the ubiquitin-dependent proteolytic machinery (Golan *et al*., 2000). Recently, about 200 proteins were identified as potential substrates for human CDK1, and some of them were shown to be phosphorylated in a CDK1-dependent manner (Ubersax *et al*., 2003).

CYCC binds to CDK3 to stimulate Rb phosphorylation during the G0/G1

transition, and this phosphorylation is required for cells to exit from G0 efficiently, indicating that CDK3 functions in the entry into cell cycle from the static G0 phase (Ren and Rollins, 2004)

Regulation of CDK activity by CDK-activating kinases (CAKs)

A CDK-activating kinase (CAK) phosphorylates the threonine residue on the T-loop region, which is widely conserved among CDKs of eukaryotes. The T-loop blocks the entry of the substrate into the catalytic cleft when the threonine residue is unphosphorylated (for review, see Nigg, 1996; Draetta, 1997) (Figure 0-2). The phosphate on the threonine is inserted into a cationic pocket beneath the T-loop, and it acts as the central node for a network of hydrogen bonds spreading outwards to stabilize neighbouring interactions in both CDK and cyclin. Thus, the principal function of T-loop phosphorylation is probably stabilizing the protein-substrate interaction. This phosphoregulatory system is particularly important in the activation of a subset of CDK-cyclin complexes such as CDK1-CYCB, where cyclin binding alone barely affects the activity.

The catalytic subunit of CAK belongs to the CDK family and has a preference for the phospho-acceptor site of TXXVVTL (in which the first threonine is phosphorylated and where X indicates lack of conservation); it is termed CDK7/p40^{MO15} in vertebrates, and its regulatory subunit is named cyclin H (CYCH) (Fisher and Morgan, 1994; Labbé *et al*., 1994; Mäkelä *et al*., 1994). When alone, CDK7 has a low CAK activity; however, in the presence of CYCH, CDK7 activity is significantly stimulated. In addition to CYCH, MAT1 (ménage á trois 1)—a RING finger protein—also interacts with CDK7 to assemble the CDK7-CYCH complex (Devault *et*

al., 1995; Tassan *et al*., 1995). Unlike other CDKs, CAK exhibits another function in controlling basal transcription as well as CDK activation (Figure 0-3). That is, it phosphorylates the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. In mammalian cells, the CTD contains up to 52 repeats of the consensus heptapeptide YSPTSPS. Null mutation in CDK7, either *in vivo* or *in vitro*, led to a drastic inhibition of CTD kinase activity (Mäkelä *et al*., 1995; Tirode *et al*., 1999). It is known that the amino (N)-terminal RING finger domain of MAT1 is a requisite for CTD phosphorylation (Busso *et al*., 2000), but not for the CAK activity *in vitro* (Tassan *et al*., 1995). Rather, its carboxy (C)-terminal region, which interacts with the CDK7-CYCH complex, is responsible for the stimulation of the CAK activity (Tassan *et al*., 1995).

The heterotrimeric CAK complex also exists as an intrinsic component of the general transcription factor TFIIH, which is involved in the initiation and elongation of transcription (Roy *et al*., 1994; Serizawa *et al*., 1995; Shiekhattar *et al*., 1995), and DNA repair (Frit *et al*., 1999). Mammalian TFIIH is a multiprotein complex of nine subunits consisting of two major subcomplexes: the core TFIIH (XPB, p34, p44, p52 and p62) and the CAK trimeric complex (Roy *et al*., 1994; Adamczewski *et al*., 1996). The remaining subunit, namely, XPD helicase, is associated with either the core or the kinase complex and is assumed to anchor the CAK to the core TFIIH (Drapkin *et al*., 1996; Reardon *et al*., 1996; Coin *et al*., 1999). The trimeric CAK complex is mainly involved in cell cycle regulation. In contrast, when associated with the core TFIIH, it is involved in transcription (Rossignol *et al*., 1997; Yankulov and Bentley, 1997).

In the fission yeast *Schizosaccharomyces pombe*, CAK consists of Mcs6/Crk1/Mop1, Mcs2 and Pmh1, which are closely related to CDK7, cyclin H and

MAT1, respectively, and it phosphorylates Cdc2 (Damagnez *et al*., 1995). The Mcs6-Mcs2-Phm1 trimeric complex is a part of TFIIH and phosphorylates the CTD of RNA polymerase II (Buck *et al*., 1995; Damagnez *et al*., 1995; Lee *et al*., 1999) (Figure 0-3). Similarly, in the budding yeast *Saccharomyces cerevisiae*, Kin28 is the closest relative of CDK7; it is associated with the cyclin H homologue Ccl1 and the MAT1 homologue Rig2/Tfb3 for the phosphorylation of the CTD as components of TFIIH (Feaver *et al*., 1994; Cismowski *et al*., 1995; Svejstrup *et al*., 1996; Faye *et al*., 1997; Feaver *et al*., 1997). However, this trimeric complex does not exhibit Cdc28 kinase activity. Another 44-kDa monomeric kinase, designated Cak1/Civ1, is involved in the activation of Cdc28 through T-loop phosphorylation *in vivo* (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996). It shows a very low sequence similarity to other CDKs and does not possess the CTD kinase activity, indicating that CDK and CTD phosphorylations are controlled by distinct kinases in the budding yeast (Cismowski *et al*., 1995). There are indications that Cak1 is also required for the activation of Kin28, suggesting that Cak1 influences transcription as well as cell division (Kimmelman *et al*., 1999) (Figure 0-3).

Vertebrate CDK7 and fission yeast Mcs6 are also phosphorylated at serine and threonine residues within the T-loops, similar to other CDKs. In fission yeasts, Csk1 has been identified as the second kinase with CAK activity, and it was observed to phosphorylate Mcs6 on the T-loop activation site (S165) and activate the Mcs6-Mcs2 complex *in vivo* (Hermand *et al*., 1998; 2001; Lee *et al*., 1999). Thus, Csk1 was defined as a CAK-activating kinase (CAKAK), i.e. an upstream activating kinase of Mcs6 (Figure 0-3). Kimmelman *et al*. (1999) demonstrated that in budding yeasts, Cak1 phosphorylates the T-loop of Kin28 and thereby stimulates its CTD kinase activity. This

suggests that despite their low sequence similarity, budding yeast Cak1 and fission yeast Csk1 perform similar functions by the phosphorylation of Kin28 and Mcs6, respectively. In fact, detailed phylogenetic analyses revealed that Cak1 and Csk1 were included in the same family with significant bootstrap support (Liu and Kipreos, 2002) (Figure 0-4).

The functions of CAKs in the development of yeasts and animals

It is reported that CAK has essential roles in the development of yeasts and animals. However, contribution of CAK to the regulation of cell cycle and transcription is still unclear. The *cdk7* null mutants of *Drosophila* were lethal before or soon after the initiation of pupation. It was argued that this lethality was probably due to loss of activity of CDK1-CYCA/B complexes, since the activity of CDK2-CYCE complex was normally maintained. This suggests the possibility that CDK7 is required for CDK1 activation but not for CDK2 activation. It was unclear that CDK7 was required for regulation of transcription *in vivo*, since it was not examined whether transcriptional machinery is impaired or not in this report. (Larochelle *et al*., 1998). In contrast to this report, Leclerc *et al*. (2000) reported that the major function of CDK7 is to regulate transcription but not to activate CDKs in *Drosophila*. In *Caenorhabditis elegans*, the RNA-mediated interference of CDK7 resulted in an embryonic lethal phenotype. Partial loss of CDK7 activity led to a general decrease in CTD phosphorylation, and severe loss of CDK7 activity blocked all cell divisions, although CDK activity was not investigated. This result suggests that CDK7 affects the viability via controlling both cell cycle and transcription (Wallenfang and Seydoux, 2002). Rossi *et al*. (2001) showed that the disruption of MAT1 led to peri-implantation lethality in mice. Although MAT1-deficient cells showed a decrease of phosphorylation in CTD, *de novo* transcription was not

defective, suggesting that MAT1-modulating transcription was not essential for the viability. These cells also showed a defect in the progression into S phase. Although this indicates that MAT1 was required for cell cycle progression, it was not clear which CDK activity was decreased in the cells because of experimental difficulty. In spite of this controversial situation about the requirement of CDK7 for transcription and cell cycle regulation, it is clear and common that CDK7 activity is indispensable during early developmental stages in animals among these reports.

In fission yeast, 2 kinases are involved in Cdc2 phosphorylation and activation (Buck *et al*., 1995; Damagnez *et al*., 1995; Hermand *et al*., 1998; 2001; Saiz and Fisher, 2002). Mcs6 is an orthologue of CDK7 and has both CDK and CTD kinase activities. The lethality of the *mcs6* null mutants indicates that it plays an essential role in cell division and/or basal transcription (Buck *et al*., 1995). Csk1, which functions as CAKAK, is not essential for the viability. However, *Csk1*-deficient cells showed the lethality, when a weak allele of *mcs6* mutation were added (Saiz and Fisher, 2002). This suggests that the two kinases play an essential role in a redundant manner. In budding yeast, both Kin28 and Cak1 have been reported as essential genes (Feaver *et al*., 1994; Espinoza *et al*., 1996), suggesting that CAK activity is also a requisite in budding yeast.

CDKs in plants

Similar to vertebrates, plants express several types of CDKs and cyclins (Mironov *et al*., 1999); thus, different sets of CDK/cyclin pairs might regulate plant cell division at each stage of the cell cycle. Vandepoele *et al*. (2002) identified 61 core cell cycle genes in the *Arabidopsis* genome, and additional cyclin-like genes were updated later (Wang *et al*., 2004). Based on the primary structure, plant CDKs have been classified into six types:

CDKA to CDKF (Joubés *et al*., 2000; Vandepoele *et al*., 2002). Among these, CDKA and CDKB are directly involved in cell cycle progression (Figure 0-1and Table 0-1). CDKA contains a conserved PSTAIRE motif, which is an important domain for cyclin binding. In addition, it apparently plays a role in both G1/S and G2/M phase progression. The expression of CDKB, which contains altered PSTAIRE sequences, is restricted from the late S to M phases. In contrast, CDKC and CDKE are assumed to play critical roles in transcriptional control (Table 0-1). CDKC is closely related to vertebrate CDK9 and forms a complex with cyclin T (CYCT) to function as a positive regulator of transcription (Barrôco *et al*., 2003; Fülöp *et al*., 2005). In humans, CYCT-CDK9 was identified as the catalytic subunit of positive transcription elongation factor b (P-TEFb) (Marshall and Price, 1995; Zhou *et al*., 1998). CDKE is a homologue of mammalian CDK8, which interacts with cyclin C (CYCC) and exerts a negative effect on transcription as a component of the RNA polymerase II holoenzyme (Leclerc *et al*., 1996; Maldonado *et al*., 1996; Rickert *et al*., 1996). CDKD and CDKF are CDK-activating kinases as described below in detail.

The functions of CDKs in plant development

In *Arabidopsis*, CDKA;1 is the sole gene that encodes CDKA and is expressed throughout the cell cycle (Menges *et al*., 2005; Vandepoele *et al*., 2002). The *cdka;1* null mutants were defective in pollen mitosis II, showing a gametophyte lethal phenotype (Iwakawa *et al*., 2006; Nowack *et al*., 2006). Hemerly *et al*. (2000) showed that *Arabidopsis* plants overexpressing a dominant negative form of CDKA;1 had severe defects during embryogenesis. These reports indicate that CDKA;1 has an essential function in both gametogenesis and embryogenesis. It has also been reported

that CDKA;1 plays an important role in post-embryonic development. Tobacco seedlings overexpressing a dominant negative form of *Arabidopsis* CDKA;1 consisted of larger and fewer cells (Hemerly *et al*., 1995), and *Arabidopsis* mutants with the weak *cdka;1* allele showed a dwarf phenotype with fewer and larger leaf cells (Dissmeyer *et al*., 2007). However, in both cases, plants underwent normal morphogenesis with normal developmental timing.

CDKBs are plant-specific CDKs, and their expression is restricted from the late S to the M phase (Porceddu *et al*., 2001). CDKB is further divided into 2 subgroups: CDKB1 and CDKB2. In *Arabidopsis*, there are 2 genes for each type of CDKB: *CDKB1;1* and *CDKB1;2* and *CDKB2;1* and *CDKB2;2* (Vandepoele *et al*., 2002). Transgenic plants overexpressing a dominant negative mutant of *CDKB1;1* produced less number of stomata with abnormal shape (Boudolf *et al*., 2004a); they also showed a higher level of DNA ploidy, demonstrating that CDKB1 is required to suppress endoreduplication (Boudolf *et al*., 2004b). On the other hand, the up- or downregulation of *CDKB2* caused severe defects in meristem functions. In overexpression or knock-down lines, the shoot apical meristem (SAM) contained considerably fewer and larger cells, and the strict organisation into 3 distinct cell layers was disrupted (Andersen *et al*., 2008). This indicates the differential role of CDKB2 in meristem organisation. C-type and E-type CDKs are assumed to regulate basal transcription, rather than cell division, by phosphorylating the CTD of RNA polymerase II, as mentioned above. Loss of CDKC;1 and CDKC;2 function resulted in altered leaf and flower growth (Cui *et al*., 2007). The *Arabidopsis cdke;1* mutants had defects in the specification of stamen and carpel identities and in the proper termination of stem cells in the floral meristem (Wang and Chen, 2004). Therefore, although CDKC and CDKE

do not act upon CDKs, they are involved in plant growth and development.

Plant CDKDs have both CDK and CTD kinase activities

The threonine residues within the T-loop are also conserved in plant CDKs (except CDKE). Joubés *et al*. (2000) classified plant CDK7 homologues into the CDKD group (Figure 0-4). The first plant CAK orthologue was identified in rice; it was a Cdc2-related protein kinase named R2 (Hata, 1991), thereafter renamed Orysa;CDKD;1. It contains an extended C-terminal region of 92 amino acids, which is not present in animal and yeast CAKs. Fabian-Marwedel *et al*. (2002) showed that in a tobacco protoplast system, this extended region contains a nuclear localization signal (NLS). When *Orysa;CDKD;1* was overexpressed in a *cak1/civ1*-deficient mutant of budding yeast, it partially suppressed the temperature sensitivity. In addition, immunoprecipitates of rice proteins with the anti-Orysa;CDKD;1 antibody phosphorylated the threonine residue (Thr-161) within the T-loop of rice CDKA;1 (Orysa;CDKA;1) and the *Arabidopsis* CTD (Yamaguchi *et al*., 1998). These data suggest that Orysa;CDKD;1 is a functional homologue of vertebrate CAKs.

There are three *CDKD* genes in the *Arabidopsis* genome: *Arath;CDKD;1*, *Arath;CDKD;2* and *Arath;CDKD;3*, which hereafter referred to as *CDKD;1*, *CDKD;2* and *CDKD;3*, respectively (Umeda, 2002; Shimotohno *et al*., 2003). With the exception of CDKD;2, both CDKD;1 and CDKD;3 have the C-terminal extension along with a significant sequence similarity to that of Orysa;CDKD;1. CDKD;1 and CDKD;3 displayed almost exclusive nuclear localization, whereas CDKD;2 localized in the cytoplasm and nuclei in *Arabidopsis* protoplasts and onion epidermal cells (Shimotohno *et al*., 2004). This suggests that the C-terminal region contains the NLS.

Enzyme-activity analysis demonstrated that CDKD;2 and CDKD;3 phosphorylated both human CDK2 and *Arabidopsis* CTD, whereas CDKD;1 lacks these kinase activities *in vitro* (Shimotohno *et al*., 2003; 2004) (Table 0-1). Interestingly, CDKD;2 and CDKD;3 differed in their preference for substrates. CDK2 kinase activity of CDKD;3 was higher than that of CDKD;2, whereas CDKD;2 had a higher CTD kinase activity than CDKD;3 (Shimotohno *et al*., 2004) (Table 0-1 and Figure 0-3).

In higher plants, cyclin H homologues have been isolated from poplar, rice and *Arabidopsis* (named Poptr;CYCH;1, Orysa;CYCH;1 and CYCH;1, respectively) (Yamaguchi *et al*., 2000; Shimotohno *et al*., 2004). In the cyclin box region, plant cyclin H shows approximately 60% similarity to human cyclin H. Although all *Arabidopsis* CDKDs interact with CYCH;1 in the yeast two-hybrid system, CDKD;1 showed a significantly lower interaction when compared with CDKD;2 and CDKD;3. The kinase activities of CDKD;2 and CDKD;3 on CDKs and CTD were markedly elevated on binding with CYCH;1 in insect cells (Shimotohno *et al*., 2004). These results suggest that plant cyclin H is a regulatory subunit of CAK, which positively controls the CDK and CTD kinase activities of CDKD, similar to that in vertebrates and fission yeasts.

MAT1 homologues in vertebrates and yeasts have two motifs: an N-terminal RING finger domain that plays a crucial role in basal transcription and the CTD phosphorylation process, and a central coiled-coil domain that is associated with XPD and XPB helicases, components of TFIIH complexes (Busso *et al*., 2000). The carboxyl terminus of MAT1 is also important for the association and activity of the vertebrate-type CAK complex (Busso *et al*., 2000). *Arabidopsis* and rice genomes encode putative MAT1 homologues (At4g30820 in *Arabidopsis*). However, it remains unknown whether these homologues function as MAT1.

In cultured *Arabidopsis* cells, CDKD;3 forms two distinct complexes with molecular masses >700 and 130 kD, respectively. The larger complex phosphorylates the CTD but not human CDK2, whereas the smaller complex showed a higher CDK2 kinase activity but low CTD kinase activity. Such biochemical features are similar to those of CDK7-like kinases from metazoa, which form a TFIIH complex >700 kD and a 180-kD complex consisting of the three CAK subunits (Devault *et al*., 1995; Schultz *et al*., 2000). In contrast, a majority of the CDKD;2 kinases form a complex with a molecular mass of approximately 200 kD; this complex phosphorylates the CTD substrate. A minor CDKD;2 complex >700 kD also displayed CTD kinase activity, while both complexes showed only trace levels of CDK2-kinase activities (Shimotohno *et al*., 2004). Immunoprecipitation studies showed that CYCH;1 formed a stable complex with CDKD;2 in suspension cells (Shimotohno *et al*., 2006).

Transcripts of *Orysa;CDKD;1* and *Orysa;CYCH;1* were accumulated in the S phase in partially synchronized rice suspension cells; the CTD kinase activity of Orysa;CDKD;1 was elevated in the G1 and S phases. Therefore, Orysa;CDKD;1 may control S phase entry and/or progression through DNA replication and activation of down-stream CDKs. To support this notion, the rice suspension cells overexpressing Orysa;CDKD;1 accelerated S phase progression and increased the ratio of the G2 phase cells (Fabian-Marwedel *et al*., 2002). In contrast, microarray analysis of *Arabidopsis* cell cycle regulators with suspension-cultured cells showed that *CDKD;2* and *CDKD;3* are constantly expressed throughout the cell cycle. Rather, *CDKD;3* was upregulated after sucrose starvation and resupply in suspension cells (Menges *et al*., 2005). This indicates that CDKD;3 may be involved in the activation of CDK activity during cell cycle re-entry.

CDKF;1 is a plant-specific type of CAK

Arath;CDKF;1, which hereafter referred to as *CDKF;1*, has been isolated as a suppressor of *cak1* mutation in budding yeasts (Umeda *et al*., 1998). Its amino acid sequence is related to those of vertebrate-type CAKs, but the similarities are restricted to the conserved kinase domains. Using database searches, homologues of CDKF;1 can be identified only in plant species, namely, Euphorbia (AF230740), rice (AK120969) and soybean (AY439095) with identities of 49.8%, 35.9% and 39.3%, respectively, but not in other kingdoms (Umeda *et al*., 2005). A unique feature of CDKF;1 among the other CAKs is that it carries an unusual insertion of 111 amino acids (amino acid position 178–288) between its kinase active site and phosphoregulatory site corresponding to the T-loop in CDKs. This unusual stretch is not essential for CDKF;1 activity. However, all the four plant species possess CDKF-related kinases carrying the unique insertion with a significant amino acid similarity. Thus, this region may be involved in the control of the interactions of CDKF with specific regulatory proteins or substrates acting in plant-specific signalling pathways.

Immunoprecipitates of an *Arabidopsis* protein extract with the anti-CDKF;1 antibody phosphorylated human CDK2 at the threonine residue within the T-loop and activated its histone H1 kinase activity. However, CDKF;1 did not phosphorylate the *Arabidopsis* CTD *in vitro* and was unable to interact with CYCH;1 (Umeda *et al*., 1998; Shimotohno *et al*., 2004). CTD kinase activities of *Arabidopsis* were separated from the total protein extract into the flow-through fraction by DEAE Sepharose and precipitated by $p13^{suc1}$-agarose (Umeda *et al*., 1998), whereas CDKF;1 was immunologically detected only in the DEAE Sepharose-bound fraction and was not associated with

$p13^{suc1}$. These results indicate that CDKF;1 has a CDK-activating kinase activity, but is distinct from vertebrate-type CAKs, including plant CDKDs, which exhibit both CDK and CTD kinase activities. The CDKF;1 protein, on the other hand, shows an apparent molecular mass of 62 kD on SDS-PAGE analysis, and it occurs in a 130-kDa protein complex with a high CAK activity in suspension cells (Shimotohno *et al*., 2004). This complex is also confirmed in Euphorbia, and it is revealed that the complex consist of homo-dimer or -trimer (Personal communication with Dr. Chao). Taking into consideration that autophosphorylation is required for the activation of CDKF;1 unlike other CDKs, homodimerization or homotrimerization may be essential for the activation of CDKF;1 (Chao *et al*., 2007).

Despite the low sequence similarity, CDKF;1 is functionally related to the budding yeast Cak1 (Fig 0-4). This is supported by recent biochemical studies on CAKs (Tsakraklides and Solomon, 2002). These studies showed that (1) Cak1 and CDKF;1 were insensitive to the protein kinase inhibitor 5'-fluorosulfonylbenzoyladenosine (FSBA), which covalently modifies the invariant lysine in protein kinases, including CDK2 and CDK7 (Solomon *et al*., 1993; Enke *et al*., 1999); (2) they exhibited a preference for cyclin-free CDK2 as the substrate; (3) they lack CTD kinase activities; and (4) they did not require a highly conserved lysine, which is located in the nucleotide binding pocket of CDK family proteins. On the other hand, sequence alignment together with a comparison of kinetic parameters suggested that CDKF;1 is included in the same family as vertebrate CDK7 rather than the budding yeast Cak1. This classification is supported by the following features: (1) Cak1 lacks the glycine-rich motif (GXGXXG), which stabilizes ATP in the binding pocket (Kaldis *et al*., 1996), whereas CDK7 and CDKF;1 contain this motif, and (2) the threonine residue within the T-loop is conserved

in CDK7 and CDKF;1, but not in Cak1. These discrepancies may reflect evolutional diversification of CAKs and the uniqueness of CDKF;1.

CDKF;1 is also closely related to the fission yeast Csk1 which functions as CAKAK in terms of enzyme activity. When *Arabidopsis* CAK cDNAs were introduced into a fission yeast strain that carried a disrupted *csk1*Δ gene, only transformants expressing *CDKF;1*, but not *CDKD*s, were able to suppress the temperature sensitivity. Moreover, an *in vitro* kinase assay demonstrated that CDKF;1 phosphorylated the conserved serine and threonine residues within the T-loops of CDKD;2 and CDKD;3, but not CDKD;1. In *Arabidopsis* root protoplasts, the CTD kinase activity of CDKD;2 was elevated depending on its T-loop phosphorylation by CDKF;1 (Shimotohno *et al.*, 2004). These results suggested that CDKF;1 is a CAKAK that modulates the activity of CDKD;2 and CDKD;3. Therefore, it is possible that CDKF;1 controls CDK activities and basal transcription in *Arabidopsis* (Figure 0-3 and Table 0-1).

In this thesis, I studied the functional roles of *Arabidopsis* CAKs *in planta* to reveal how the CDK activities and basal transcription are involved in plant development. In *Chapter I*, I describe that CDKF;1 plays a pivotal role during post-embryonic development, but CDKF;1 activity is dispensable for gametogenesis and embryogenesis. This raises the possibility that CDKD regulates the early developmental processes, thus in *Chapter II*, I observed the expression patterns of CAKs in gametophytes and during embryogenesis and analyzed the phenotypes of the *cdkd;1 cdkd;3* double mutants. In *Chapter III*, I describe that the molecular functions of CDKD;1 and CDKD;3 are divergent from that of CDKF;1. Based on these findings, I propose that distinct functions of plant CAKs, CDKD and CDKF, are determined by both the specific expression profiles and the unique characteristics in enzyme activities.

Table 0-1. *Arabidopsis* CDKs and their possible functions

Name	Function	Homologue in vertebrates
CDKA	Regulation of the progression through the G1/S and G2/M phases. Male gametogenesis, embryogenesis and post-embryonic development.	CDK1, CDK2
CDKB1	Regulation of the progression through the early G2/M. Stomata formation and photomorphogenesis.	None
CDKB2	Regulation of the progression through the late G2/M. Maintenance of meristem activities.	None
CDKC	Regulation of transcription elongation by activating RNA polymerase II. Leaf and flower development.	CDK9
CDKD	CDKD;1; Unknown. CDKD;2; The major function is to activate RNA polymerase II. The minor one is CDK-activation. CDKD;3; The major function is CDK-activation. The minor one is to activate RNA polymerase II.	CDK7
CDKE	Negative regulation of transcription by inhibiting RNA polymerase II. Flower development.	CDK8
CDKF	Activation of CDKs, including CDKD;2 and CDKD;3 *in vitro*.	None

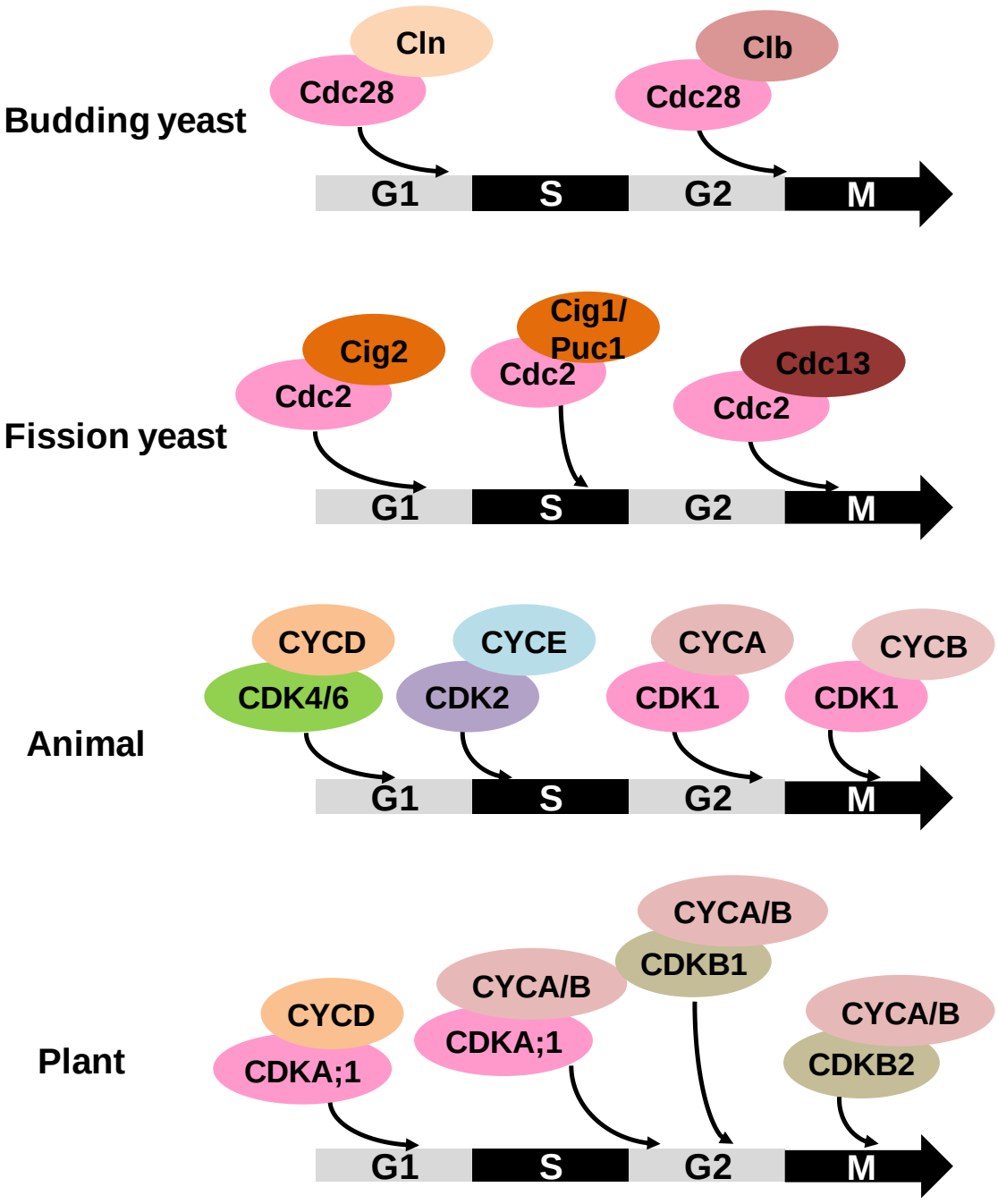

Figure 0-1. Schematic model of cell cycle regulation in yeasts, animals and plants.

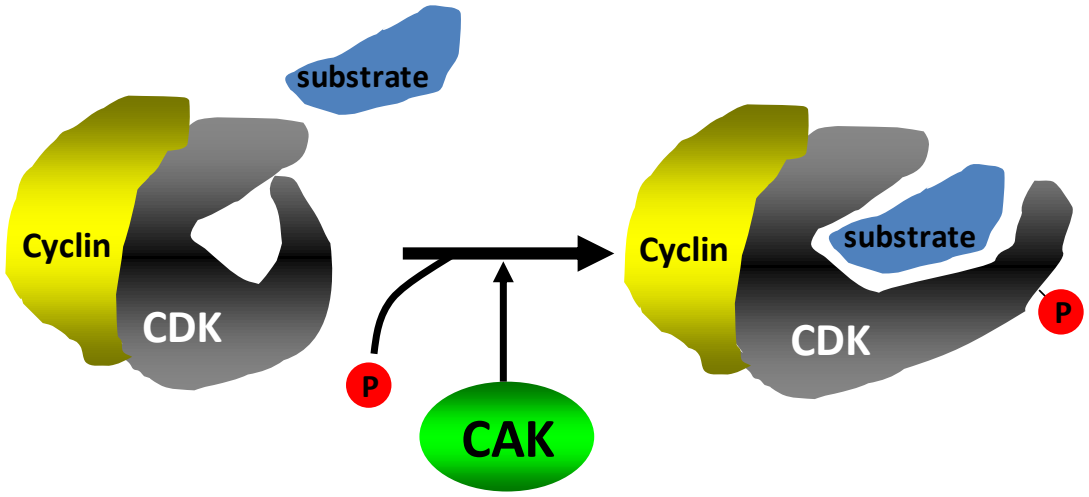

Figure 0-2. Activation mechanism of CDK by CAK.

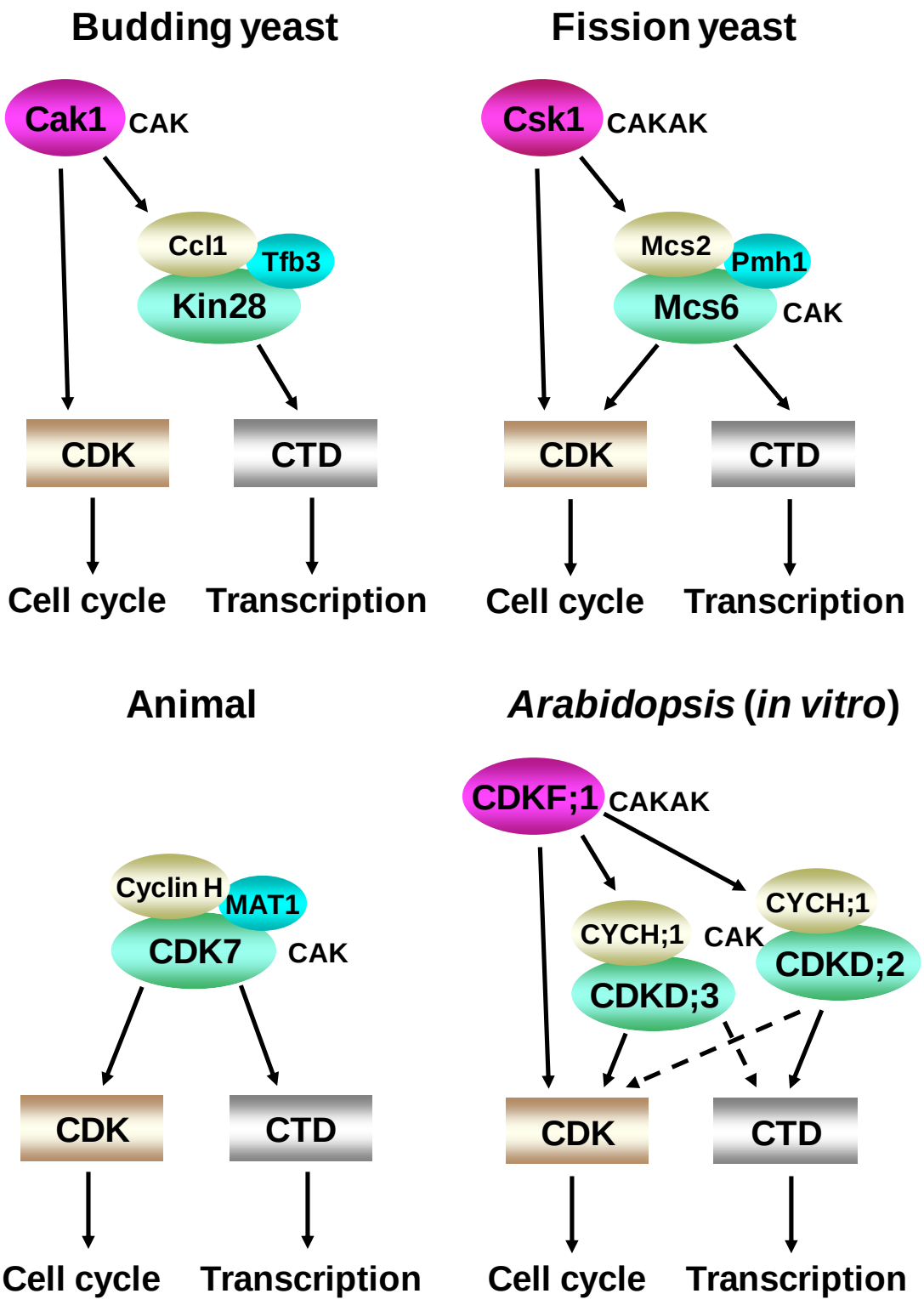

Figure 0-3. Schematic model of the CAK functions in yeasts, animals and *Arabidopsis*.

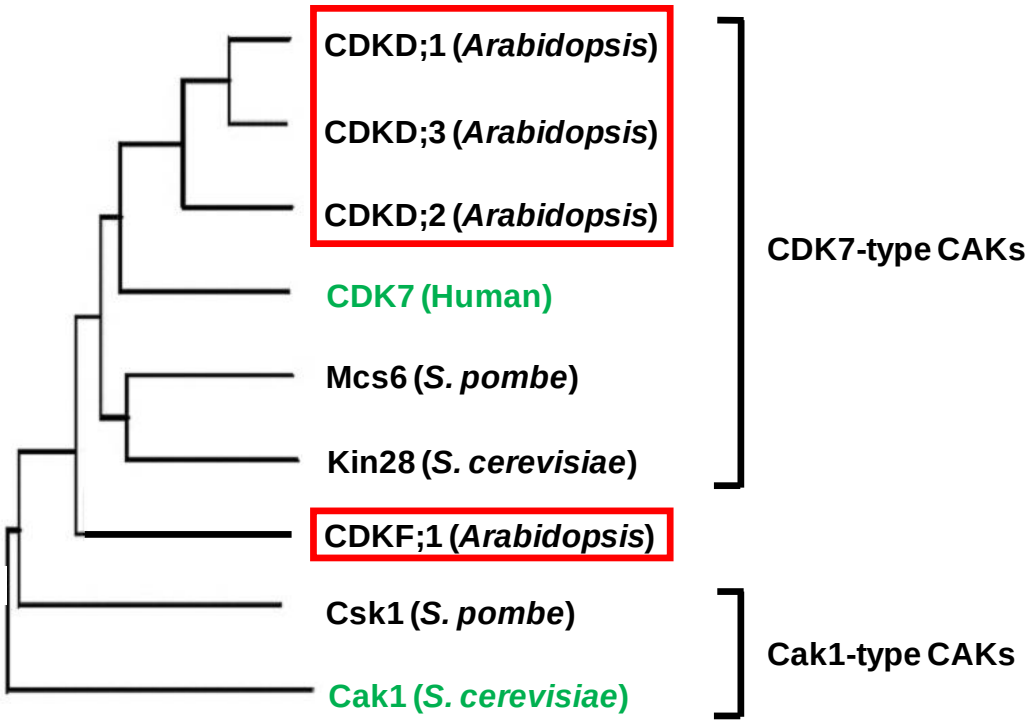

Figure 0-4. Phylogenetic tree of CAKs among yeasts, human and *Arabidopsis.*

The phylogenetic tree was constructed based on the amino acid sequences with the neighbor-joining method.

Chapter I

CDKF;1 controls the post-embryonic Arabidopsis development by stabilizing CDKD;2

<Abstract>

Cyclin-dependent kinases (CDKs) play an essential role in cell cycle regulation throughout life cycle of various organisms. Full activation of CDKs requires not only binding to cyclins but also phosphorylation of the T-loop domain. This phosphorylation is catalysed by CDK-activating kinases (CAKs). Plants have 2 distinct types of CAKs, namely, CDKD and CDKF; in *Arabidopsis*, CDKF;1 exhibits the highest CDK kinase activity *in vitro*. Previously, it was shown that CDKF;1 also functions in the activation of CDKD;2 and CDKD;3 by T-loop phosphorylation. Here, I isolated the knockout mutants of *CDKF;1* and showed that they had severe defects in cell division and cell elongation. No defect was observed during embryogenesis, suggesting that CDKF;1 function is primarily required for post-embryonic development. In the *cdkf;1* mutants, T-loop phosphorylation of CDKA;1, an orthologue of yeast Cdc2/Cdc28, was comparable to that in wild-type plants, and its kinase activity did not decrease. In contrast, the protein level and kinase activity of CDKD;2 were significantly reduced in the mutants. Substitution of threonine 168 with a nonphosphorylatable alanine residue made CDKD;2 unstable in *Arabidopsis* tissues. These results indicate that CDKF;1 is dispensable for CDKA;1 activation but is essential to maintain a steady-state level of CDKD;2, thereby suggesting a quantitative regulation of a vertebrate-type CAK in a plant-specific manner.

<Introduction>

In flowering plants, life cycle mainly consists of three processes; gametogenesis, embryogenesis and post-embryonic development. Gametogenesis is a process by which diploid or haploid precursor cells undergo cell division and differentiation to form mature haploid gametes. The male gamete is produced inside the pollen grain through the division of a generative cell into two sperm nuclei. Depending on the species, this can occur while the pollen forms in the anther or after pollination and growth of the pollen tube. The female gamete is produced inside the embryo sac of the ovule. After fertilization, the single-celled zygote undergoes a programmed pattern of cell division resulting in a mature embryo, which is called embryogenesis. The zygotic embryo is formed following double fertilisation of the ovule, giving rise to two distinct structures: the plant embryo and the endosperm which together go on to develop into a seed. The endosperm functions as an energy source of embryo. During embryogenesis, a basic body plan is established that consists of shoot meristem, cotyledons, hypocotyl, root, and root meristem along the apical–basal axis and a concentric arrangement of epidermis, subepidermal ground tissue, and central vascular cylinder along the radial axis. During post-embryonic development, plants continue to produce new leaves and roots from meristems, which is known as indeterminate growth. It is assumed that the regulation of cell cycle plays a pivotal role throughout all stages of these developmental processes.

As described in *General introduction*, cell cycle and transcription are controlled by the activities of CAKs. CDKD and CDKF were found to exhibit the CAK activity in rice, *Arabidopsis* and *Euphorbia* (Umeda *et al*., 1998; Yamaguchi *et al*., 1998; Chao *et al*., 2007). *Arabidopsis* has a single gene for CDKF, named CDKF;1,

which has been identified as a suppressor of the *cak1* mutation in budding yeast. It shows similarity to CDK7 only in restricted domains, and unlike vertebrate-type CAKs, it has CDK-activating kinase activity but no CTD kinase activity *in vitro*. Unlike other plant CDKs, binding to regulatory subunits, such as cyclins, is dispensable for the activation of CDKF;1, since the CDKF;1 protein, which was produced in *E.coli*, had the CAK activity on its own (Umeda *et al*., 1998; Chao *et al*., 2007). CDKF;1 exhibites a significant kinase activity towards CDKA;1 in *Arabidopsis* root protoplasts, and this activity is dependent on T-loop phosphorylation of CDKF;1 (Shimotohno *et al*., 2006). A striking feature is that CDKF;1 also phosphorylates CDKD;2 and CDKD;3 and functions as a CAKAK (Shimotohno *et al*., 2004; Umeda *et al*., 2005) (Figure 0-3 and Table 0-1).

Although it is still unknown how CAKs are involved in the plant development, there are several reports showing that events downstream of CAKs play significant roles during plant life cycle. The phenotypic analyses of the *Arabidopsis* mutants of *CDKA;1* and *Retinobalstoma-related protein* (*RBR*), which are the major regulators of cell cycle, revealed that the proper progression of cell cycle is essential for gametogenesis, embryogenesis and post-embryonic development (Hemerly *et al*., 1995; Ebel *et al*., 2004; Iwakawa *et al*., 2006; Nowack *et al*., 2006; Wyrzykowska *et al*., 2006; Dissmeyer *et al*., 2007; Johnston *et al*., 2008). Although it is poorly understood whether the TFIIH-dependent transcriptional regulation is essential for plant development, it has been reported that UVH6, a homologue of human XPD in the TFIIH complex, had an essential role in plant growth (Liu *et al*., 2003). Based on these observations, it is likely that CAK may affect various aspects of plant development via regulating cell cycle and/or transcription. In this chapter, I isolated and analyzed the knock-out mutants of

CDKF;1, and showed that they exhibited defects in cell division and cell expansion, leading to severe growth retardation in both shoots and roots. However, unlike CAKs in yeast and animals, the *cdkf;1* mutants did not show any defect in early developmental processes, including embryogenesis. In the mutants, the protein level and kinase activity of CDKD;2 were significantly reduced, while those of CDKA;1 did not change compared to that of wild-type plants. I propose that CDKF;1 plays an important role in post-embryonic development by regulating the protein stability of CDKD;2.

<Results>

Expression pattern of CDKF;1

To understand the functional role of CDKF;1 in plant development, I first examined the expression pattern by using the β-glucuronidase (GUS) gene fused to the 2-kb promoter region of *CDKF;1*. However, no signal was detected in the transgenic plants, probably due to the lack of essential regulatory element(s) that promote(s) gene expression. Therefore, I generated another reporter line that expressed the CDKF;1-GUS fusion protein under the control of the *CDKF;1* promoter. As shown in Figures 1-1a and 1-1b, almost all tissues of seedlings were stained by incubation in a GUS-staining buffer for 4 h. This uniform expression pattern indicates that CDKF;1 may function not only in cell proliferation but also in cell expansion. To determine the tissues where CDKF;1 is highly expressed, I then incubated the samples for a shorter period. My results showed that actively dividing tissues were mainly stained, such as the apical meristems of shoots and roots, lateral root primordia, emerging lateral roots and vascular bundles (Figures 1-1c, 1-1d and 1-1i-k). The GUS expression was also observed in young flowers, especially in the stigma, pistil basal regions and vascular bundles of petals (Figures 1-1e and 1-1f). In anthers, the expression was restricted to pollen grains (Figure 1-1g). GUS expression in siliques was observed only in the basal region (Figure 1-1h).

Isolation of the* cdkf;1 *mutants

To reveal the *in vivo* function of CDKF;1, I identified *Arabidopsis* mutants from T-DNA insertion collections and named them *cdkf;1-1* and *cdkf;1-2*. These mutants have T-DNA insertions in the second exon of *CDKF;1* (Figure 1-2a). Both mutants exhibited the

same phenotype, and introduction of the genomic fragment containing the *CDKF;1* gene restored the phenotype of *cdkf;1-1* mutants (Figure 1-2b).

Next, I report the representative results obtained for the *cdkf;1-1* mutants. Total RNA was isolated from the *cdkf;1-1* mutants that were homozygous for T-DNA insertions and subjected to RT-PCR (Figure 1-2c). I could amplify the cDNA upstream of the T-DNA insertion site but not downstream of or encompassing the T-DNA insertion site. Immunoblotting analysis with the anti-CDKF;1 antibody that recognises the carboxy-terminal peptide did not detect intact CDKF;1 in the *cdkf;1-1* mutants (Figure 1-2c). This indicates that the mutants might produce transcripts with a truncation at the 3'-terminus. Next, I conducted rapid amplification of cDNA ends (3'-RACE) and isolated the full-length cDNA produced in the *cdkf;1-1* mutants. The cDNA, designated m*CDKF;1*, consisted of a partial *CDKF;1* lacking the C-terminal portion, 5 bp of an unknown sequence and 47 bp of the T-DNA end including the left border (Figure 1-3a). The open reading frame (ORF) that ended at the stop codon within the T-DNA encodes N-terminal 379 amino acids of CDKF;1, followed by 16 amino acids of unrelated peptide (Figure 1-3b). To examine the functionality of m*CDKF;1*, I expressed it in budding yeast GF2351 cells that carry a temperature-sensitive mutation in the *Cak1* gene. As shown in Figure 1-3c, the full-length *CDKF;1* could rescue temperature sensitivity, as reported previously (Umeda *et al*., 1998), but the cells expressing m*CDKF;1* did not grow at 37°C. This suggests that the *cdkf;1-1* mutants do not produce an active enzyme and thus are knockout mutants.

The* cdkf;1-1 *mutants are defective in both cell division and cell elongation

Homozygous *cdkf;1-1* mutants did not generate inflorescence under growth conditions

used in my study. Hence, I analysed the homozygous mutants segregated from *CDKF;1/–* plant seeds. They grew slowly and produced smaller, wavy leaves with abnormal serration (Figures 1-4a and 1-4b). In my thesis for master degree, I reported that these small leaves were due to the defects in both cell division and cell expansion. However, whether the shoot apical meristem is normally formed has not been examined. Sections of shoot apices indicated that the shoot apical meristem occupied only a limited area in the mutants (Figure 1-4c). I also found that under dark conditions, hypocotyl elongation was severely inhibited in the *cdkf;1-1* mutants (Figures 1-5a and 1-5b). Epidermal cells in the mutant hypocotyl were not becoming longer in the dark, indicating an inhibition of cell elongation (Figure 1-5c). GUS expression in the *pCDKF;1-CDKF;1::GUS* seedlings was not observed in the hypocotyl under light conditions, but it was detected in the upper portion of hypocotyls grown in the dark (Figure 1-5d). This suggests that CDKF;1 is required for hypocotyl cell elongation in response to dark conditions.

Cell division but not cell differentiation is inhibited in the cdkf;1-1 roots

Previously, I revealed that root growth of the *cdkf;1-1* mutants was retarded, and it stopped around 4 days after germination (DAG) (Takatsuka, thesis for master degree). As a result, the mutants produced primary roots with lengths of 2–3 mm and fewer lateral roots, which started to elongate when the primary roots stopped growing (Figure 1-6a) To monitor cell proliferation, I introduced *pCYCB1;1-CYCB1;1::GUS*—a G2/M phase marker—into the *cdkf;1-1* mutants. As shown in Figure 1-6b, the patchy pattern of GUS signals almost disappeared in the root meristem of 4 DAG mutants, suggesting that CDKF;1 is prerequisite to maintain cell proliferation during the post-embryonic

development of roots.

Aberrant root growth is often accompanied by a failure to maintain cell differentiation. Therefore, I analysed the expression pattern of several cell type-specific markers in the *cdkf;1-1* mutants. *SCARECROW* (*SCR*) is a marker gene for the endodermis, the cortex/endodermal initial cells and the quiescent centre (QC) (Di Laurenzio *et al*., 1996). As shown in Figure 1-6c, *SCR* expression did not change in the *cdkf;1-1* mutants, indicating that there was no defect in the radial patterning of roots. The expression patterns of the QC marker QC25, QC46 and QC184 were almost the same, but the area of GUS-stained cells was slightly larger in the mutants (Figure 1-6d). These results indicate that CDKF;1 is not involved in cell differentiation, but it plays an essential role in cell division during root development.

The* cdkf;1-1*mutants show suppression of phytohormone-induced cell proliferation

It is well known that many developmental processes of plants are regulated by phytohormones, such as auxin, cytokinin, gibberellin, ethylene, and so on. Among them, auxin and cytokinin are known to promote cell division in a cooperative manner (John *et al*., 1993). In the previous reports, commitment of both hormones to cell cycle progression has been suggested. *Arabidopsis* CDKA;1 has an auxin-responsive element (AuxRE) in its promoter region, and auxin regulates the transcription of *CDKA;1* (Hemerly *et al*., 1993; Chung and Parish, 1995). Moreover, it was reported that auxin stabilizes the CYCD-CDKA;1 complexes (Harashima *et al*., 2007). Cytokinin is known to elevate the transcription of not only *CDKA;1* but also *CYCD;3*, a G1/S cyclin (Hemerly *et al*., 1993; Riou-Khamlichi *et al*., 1999). Moreover, overexpression of *CYCD;3* bypassed cytokinin-requirement during the calli maintainance, indicating the

CYCD;3 function downstream of cytokinin signaling (Riou-Khamlichi *et al*., 1999). These reports indicate that both auxin and cytokinin regulate cell division via controlling cell cycle regulators. Hence, I examined how the *cdkf;1-1* mutants respond to these hormonal stimuli.

Hypoctyl is an inactive tissue in terms of cell division under normal conditions. However, exposure to auxin and cytokinin in an adequate balance of concentration makes explants dedifferentiate, and they start to form calli (Inoue *et al*., 2001). First, I examined the callus formation in the *cdkf;1-1* mutants on a callus-inducing medium (CIM) (Ozawa *et al*., 1998). In the explants of wild-type hypocotyls, callus formation started around 12 days after explanting, while the mutant explants did not start cell proliferation untill 16 days, suggesting that promotion of cell division was retarded in the *cdkf;1-1* mutants (Figure 1-7a). After 28 days, calli from the mutatnts were much smaller than those from wild-type plants. This suggests that in the *cdkf;1-1* mutants, the competence for cell division in response to hormonal stimuli is not lost, but the activity of cell division is drastically reduced. I then examined the callus formation in various combinations of the ratio of auxin to cytokinin. As shown in Figure 1-7b, the *cdkf;1-1* mutants formed much smaller calli in all auxin and cytokinin combinations, suggesting that reduced activity of cell division, but not impaired perception of hormonal balance, is the cause of the retarded callus formation in the *cdkf;1-1* mutants.

No obvious defect is observed during embryogenesis of the* cdkf;1-1 *mutants

To examine whether not only post-embryonic development but also embryogenesis is affected in the *cdkf;1-1* mutants, I observed mature embryos in seeds from the heterozygous mutants. Microscopically, I could not discern *cdkf;1/–* embryos from

wild-type or *cdkf;1*/+ embryos. I then observed cotyledons of mature embryos in wild-type and mutant parental seeds, but again found no difference in cell number or cell area (Figure 1-8). Moreover, I previously reported that no defect in the number of columella cell layers and the radial patterning of cell files was observed in embryonic roots in seeds from *cdkf;1-1*/+ plants (Takatsuka, thesis for master degree). Taken together, these results suggest that embryogenesis is not aberrant in the *cdkf;1-1* mutants.

CDKA and CDKB activities are maintained in the cdkf;1-1 mutants

To verify which *Arabidopsis* CDK(s) are *in vivo* targets of CDKF;1, the protein level and kinase activity of CDKs were compared between wild-type plants and the *cdkf;1-1* mutants. First, I examined A- and B-type CDKs, which directly regulate cell cycle progression (Figure 1-9a). Unexpectedly, the activity of CDKA;1 was not reduced in the *cdkf;1-1* mutants. Immunoblotting with anti-CDKB antibodies showed that the protein levels of CDKB1 and CDKB2 were higher in the mutants, but there was no difference in their kinase activities. This indicates that the CDKB kinase activity per molecule might be lower in the mutants, but the total activity was comparable to that in wild-type plants. As described above, because wild-type and mutant seedlings showed differences in development, I could not rule out the possibility that these results might reflect a difference in the state of cell differentiation. Therefore, I also examined the protein levels and activities of above-mentioned CDKs in calli, which are undifferentiated masses of dividing cells that are more uniform than seedlings. The results were almost the same as seedlings, and the kinase activities of CDKA, CDKB1 and CDKB2 in the mutants were comparable to those in wild-type calli (Figure 1-9b).

Next, to examine further the T-loop phosphorylation of CDKs, I monitored the phosphorylation state of CDKA;1 by using anti-phospho-Cdc2 (Thr161) antibody. This antibody recognised a protein with the same molecular weight as that of CDKA;1, while the band disappeared in a protein extract preincubated with lambda protein phosphatase or depleted with anti-CDKA;1 antibody (Figure 1-9c). This result indicates that the antibody specifically recognised CDKA;1 phosphorylated within the T-loop. Immunoblotting analysis with this antibody revealed that the phosphorylation level of CDKA;1 in the *cdkf;1-1* mutants was comparable to that in wild-type plants (Figure 1-9d), suggesting that CDKA;1 may be phosphorylated and activated by CAK(s) other than CDKF;1 in the mutants. Anti-phospho-Cdc2 (Thr161) antibody did not recognise CDKB1 or CDKB2, thus I could not examine the phosphorylation state of CDKBs.

CDKF;1 controls the steady-state level of CDKD;2 by T-loop phosphorylation

I then investigated CDKD;2 and CDKD;3, which are also *in vitro* targets of CDKF;1 (Umeda *et al*., 1998; Shimotohno *et al*., 2004). In wild-type seedlings, I could detect CDKD;2 but not CDKD;3 in a protein extract (Figure 1-10a). Interestingly, the protein level of CDKD;2 significantly decreased in the *cdkf;1-1* mutants, and I detected only a trace level of kinase activity on the CTD substrate (Figure 1-10a). In calli, I again observed that the protein level of CDKD;2 but not that of CDKD;3 was reduced in the *cdkf;1-1* mutants (Figure 1-10b). Semi-quantitative and quantitative RT-PCR showed that the transcripts of both CDKD;2 and CDKD;3 were increased in the mutant seedlings, compared to those of wild-type (Figure 1-10c and 1-10d); this may indicate a compensation for the loss of CDKF;1. These results suggest that the translation efficiency or protein stability of CDKD;2 was reduced in the absence of CDKF;1.

To observe the accumulation of CDKD proteins in tissues, I generated transgenic plants expressing the CDKD::GUS fusion protein under the control of the *CDKD* promoter. In wild-type roots, the CDKD;2::GUS fusion protein was accumulated in the meristematic and elongation zones, but it disappeared in the root tips of the *cdkf;1-1* mutants (Figure 1-11a). In contrast, the CDKD;3::GUS remained in the root tips of the mutants, although the expression domains were restricted to the distal region because of the reduced meristem size (Figure 1-11a). Similar results were also obtained in lateral root primordia, young leaves and hypocotyls (Figure 1-11a). Note that GUS mRNA levels of all two types of transgenic plants slightly increased in the *cdkf;1-1* mutants compared to those in wild-type plants (Figure 1-11b). Although CDKD;1, the nearest homologue of CDKD;3, is not phosphorylated by CDKF;1 *in vitro* (Shimotohno *et al*., 2004), I could not rule out the possibility that CDKD;1 is an *in vivo* substrate of CDKF;1. Hence, I conducted a similar expression analysis for CDKD;1. The result showed that the CDKD;1::GUS fusion protein was normally accumulated in the *cdkf;1-1* mutants, and the transcripts were again up-regulated in the *cdkf;1-1* mutants (Figure 1-12b). These results indicate that CDKF;1 controls the steady-state level of CDKD;2 but not CDKD;3 nor CDKD;1 in *Arabidopsis* tissues.

To investigate whether CDKD;2 protein is actively degraded via the proteasome-dependent pathway, the above-mentioned CDKD;2::GUS plants were treated with MG132, a specific inhibitor of proteasome, in the presence of cyclohexamide that inhibits *de novo* protein synthesis of CDKD;2. In the *cdkf;1-1* roots, MG132 treatment did not restore the accumulation of CDKD;2::GUS fusion protein, suggesting that CDKD;2 is not targeted to proteasome in the absence of CDKF;1 (Figure 1-11c).

To examine whether the control of the CDKD;2 protein level was dependent on phosphorylation by CDKF;1, I substituted the conserved threonine residue (T168) with alanine within the T-loop in the *pCDKD;2-CDKD;2::GUS* construct and introduced it into the wild-type plants. As shown in Figure 1-13a, GUS expression was considerably weaker in the T168A variant than in the wild-type plants in both the shoots and roots. Five transgenic lines showed similar results, and the *CDKD;2::GUS* transcripts showed the same level of accumulation in the wild-type plants and the T168A mutant (Figure 1-13b). These results suggest that T-loop phosphorylation by CDKF;1 is required to maintain the steady-state level of the CDKD;2 protein. When GFP-fudfsed T168A mutant protein was transiently expressed in onion epidermal cells, it was localized in the cytoplasm and nucleus as in the case of wild-type protein (Shimotohno *et al.*, 2004) (Figure1-13c). This suggests that phosphorylation by CDKF;1 does not affect the subcellular localization of CDKD;2 protein.

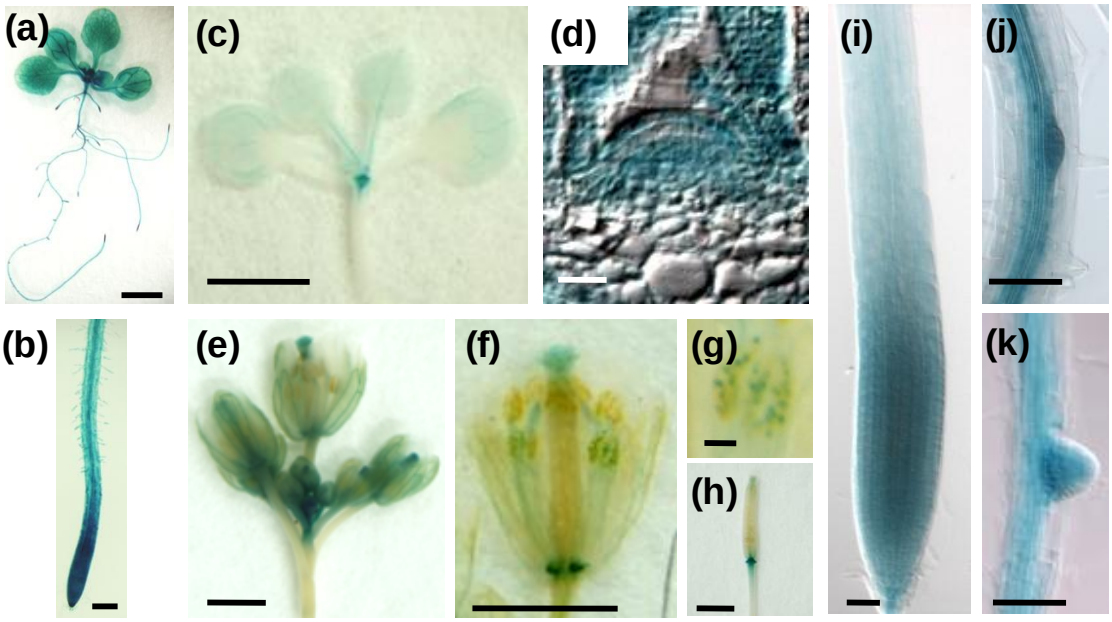

Figure 1-1. Spatial expression pattern of CDKF;1.

GUS staining of transgenic plants harbouring *pCDKF;1-CDKF;1::GUS*. (a, b) GUS staining of 12-day-old seedlings and 8-day-old roots, respectively, for 4 h. Bar = 5mm (a), 100 µm (b). (c) GUS staining of 12-day-old seedlings for 1 h. Bar = 1 mm. (d) Shoot apical meristem. Bar = 20 µm. (e) Flowers. Bar = 1 mm. (f) Magnified image of a flower. Bar = 1 mm. (g) Anther. Bar = 100 µm. (h) Young silique. Bar = 1 mm. (i) Primary root. Bar = 50 µm. (j) Lateral root primordium. Bar = 100 µm. (k) Emerging lateral root. Bar = 100 µm.

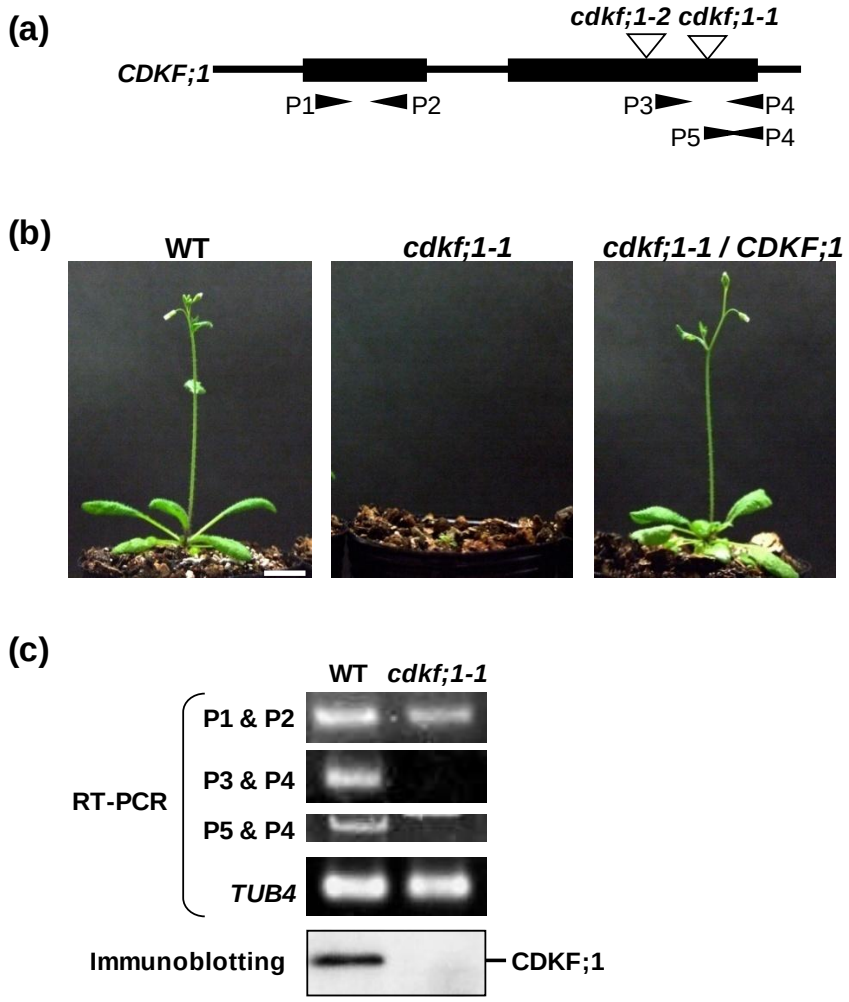

Figure 1-2. Isolation and complementation of the *cdkf;1* mutants.

(a) A schematic diagram of the *CDKF;1* gene. Exons and introns are indicated by black boxes and solid bars, respectively. Open triangles represent the T-DNA insertion sites. Black arrowheads indicate primers used for RT-PCR. (b) Complementation of the *cdkf;1-1* mutants with the *CDKF;1* genomic fragment. *Arabidopsis* plants (32 DAG) are shown. Bar = 1 cm. (c) Expression analysis of the *cdkf;1-1* mutant. RT-PCR was conducted with total RNA from whole seedlings (10 DAG) of wild-type plants or the mutants by using the indicated sets of primers. The amplified cDNAs were stained with ethidium bromide. Twenty micrograms of total protein was subjected to immunoblotting with anti-CDKF;1 antibody.

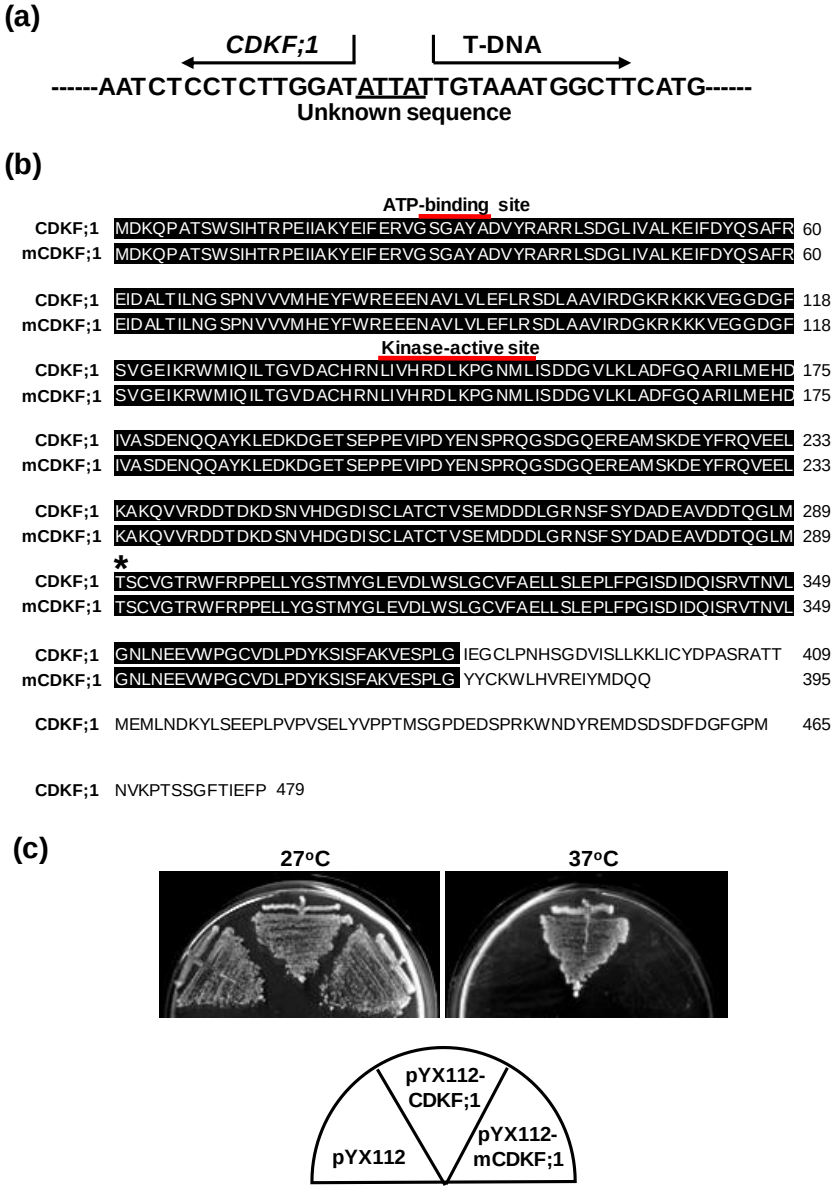

Figure 1-3. Characterization of the *cdkf;1* mutants.

(a) Junction sequences of the T-DNA insertion site. Five unknown nucleotides are inserted between the sequences of *CDKF;1* and the T-DNA left border. (b) Alignment of amino acid sequences of CDKF;1 and mCDKF;1. The asterisk indicates the conserved threonine residue within the T-loop. (c) Complementation of the *S. cerevisiae* mutant GF2351. Transformants carrying each plasmid were incubated at 27°C or 37°C for 4 days.

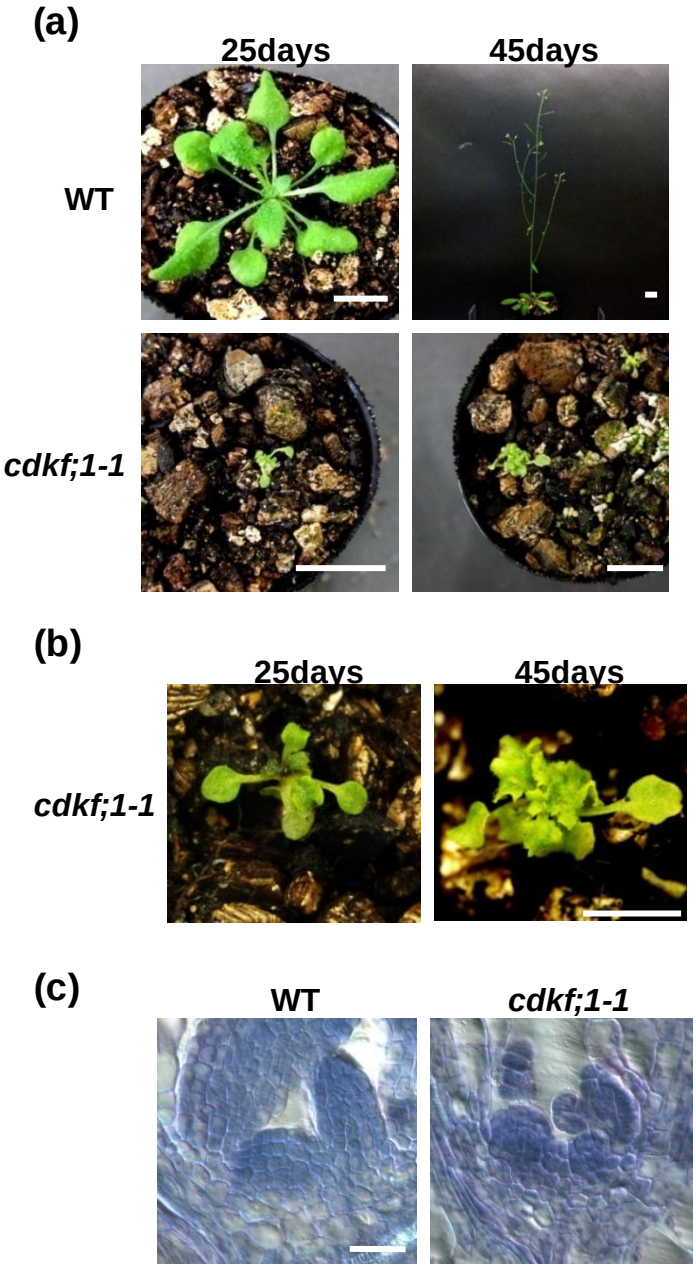

Figure 1-4. Shoot phenotypes of the *cdkf;1-1* mutants.

(a) 25-day-old and 45-day-old plants of wild-type and the *cdkf;1-1* mutants. Bars = 1 cm. (b) Magnified images of 25-day-old and 45-day-old plants of the *cdkf;1-1* mutants. Bar = 5 mm. (c) Shoot apices of 7-day-old seedlings stained with toluidine blue. Bar = 5 μm.

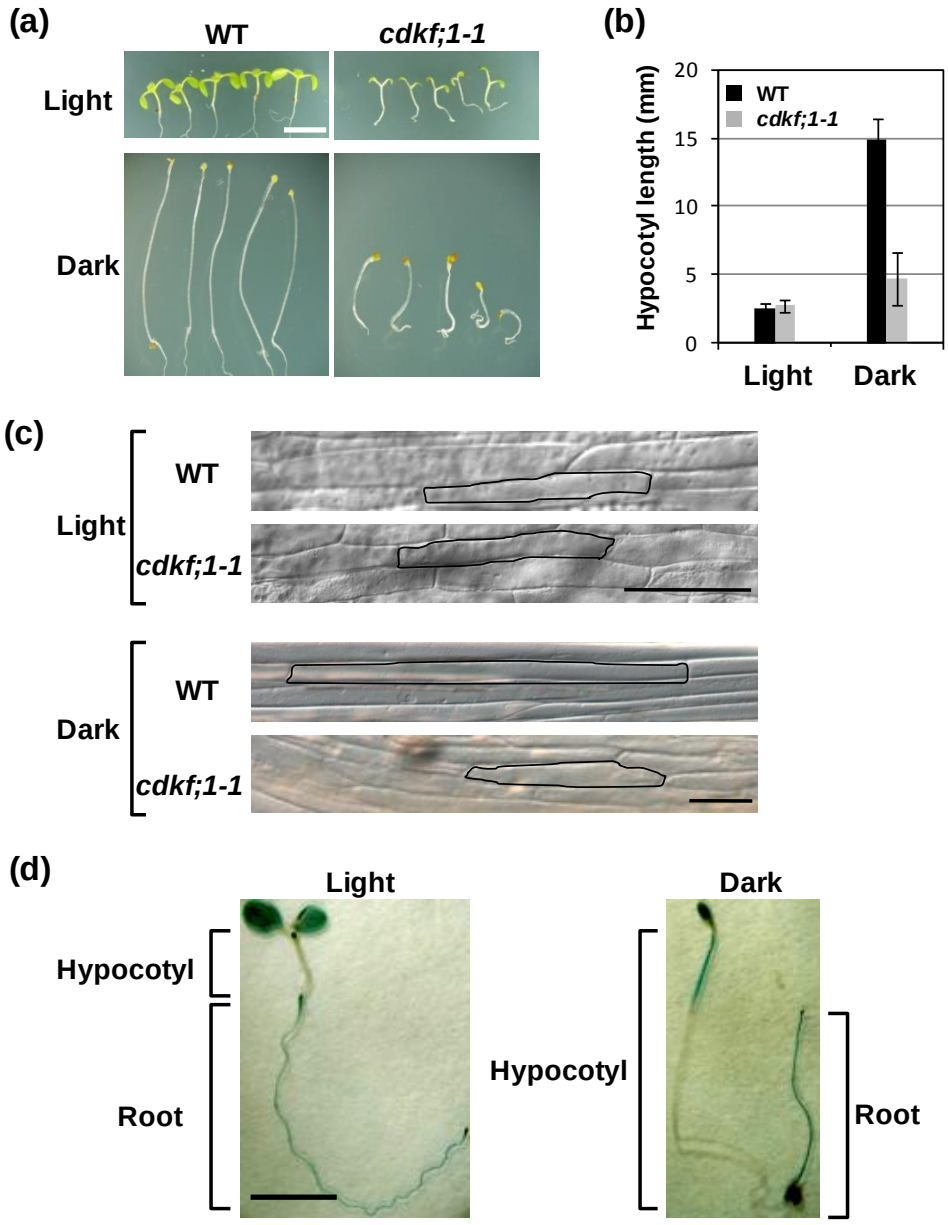

Figure 1-5. Inhibition of hypocotyl elongation in the *cdkf;1-1* mutants.
(a) 8-day-old seedlings of wild-type plants and the *cdkf;1-1* mutants grown under light or dark conditions. Bar = 5 mm. (b) Hypocotyl length of wild-type plants (black bars) and the *cdkf;1-1* mutants (grey bars) shown in (a). (c) Hypocotyl epidermal cells of wild-type plants and the *cdkf;1-1* mutants shown in (a). Bar = 100 μm. (d) GUS staining of 8-day-old seedlings harbouring *pCDKF;1-CDKF;1::GUS*. Bar = 5 mm.

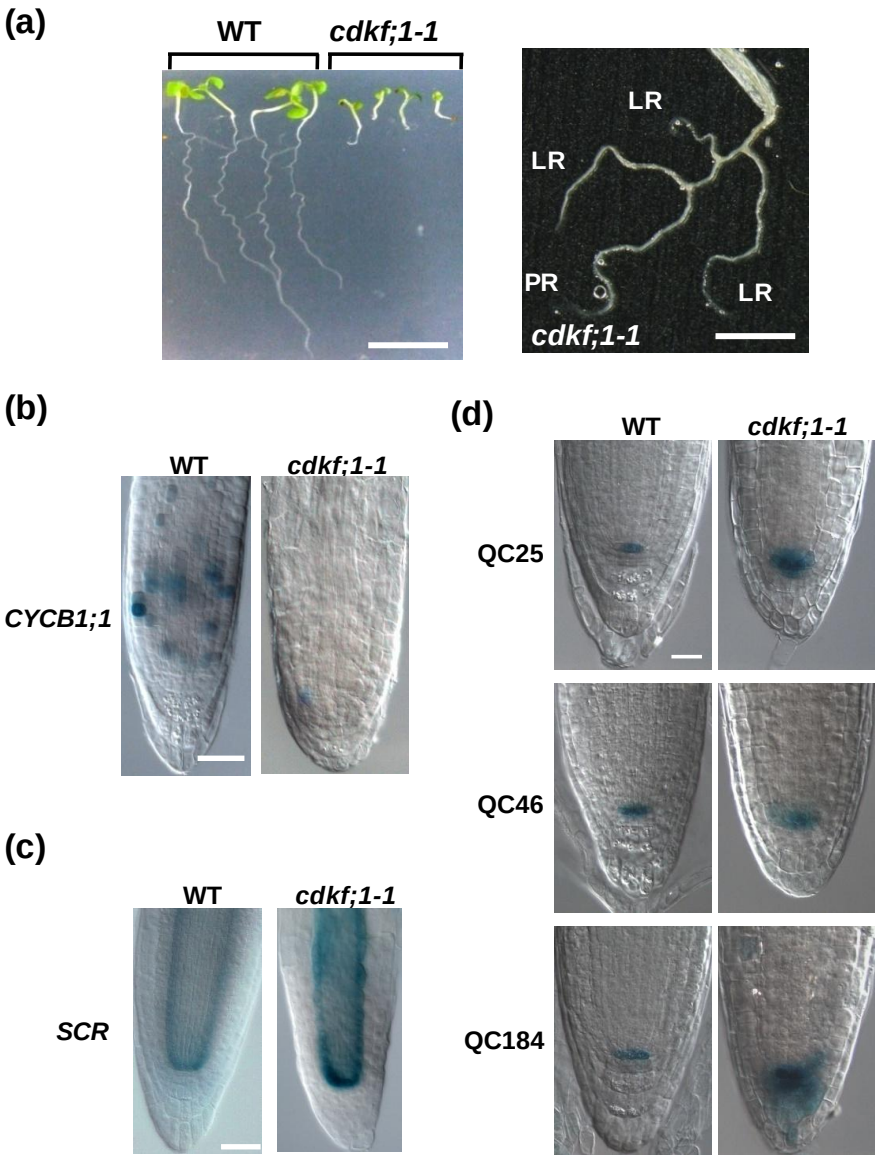

Figure 1-6. Inhibition of cell division in the *cdkf;1-1* roots.

(a) 8-day-old (left) and 27-day-old seedlings (right) of wild-type plants and *cdkf;1-1* mutants. PR, primary root; LR, lateral root. Bars = 1 cm (top) and 1 mm (bottom). (b) Expression pattern of *pCYCB1;1-CYCB1;1::GUS* in root tips of wild-type plants and the *cdkf;1-1* mutants (4 DAG). Bar = 50 µm. (c, d) Expression pattern of *pSCR-GUS* (c) and *QC25-GUS*, *QC46-GUS* and *QC184-GUS* (d) in wild-type plants and the *cdkf;1-1* mutants (4 DAG). Seedlings were GUS stained for 24 h (for *SCR*) or 18 h (for *QC25*, *QC46* and *QC184*). Bars = 25 µm.

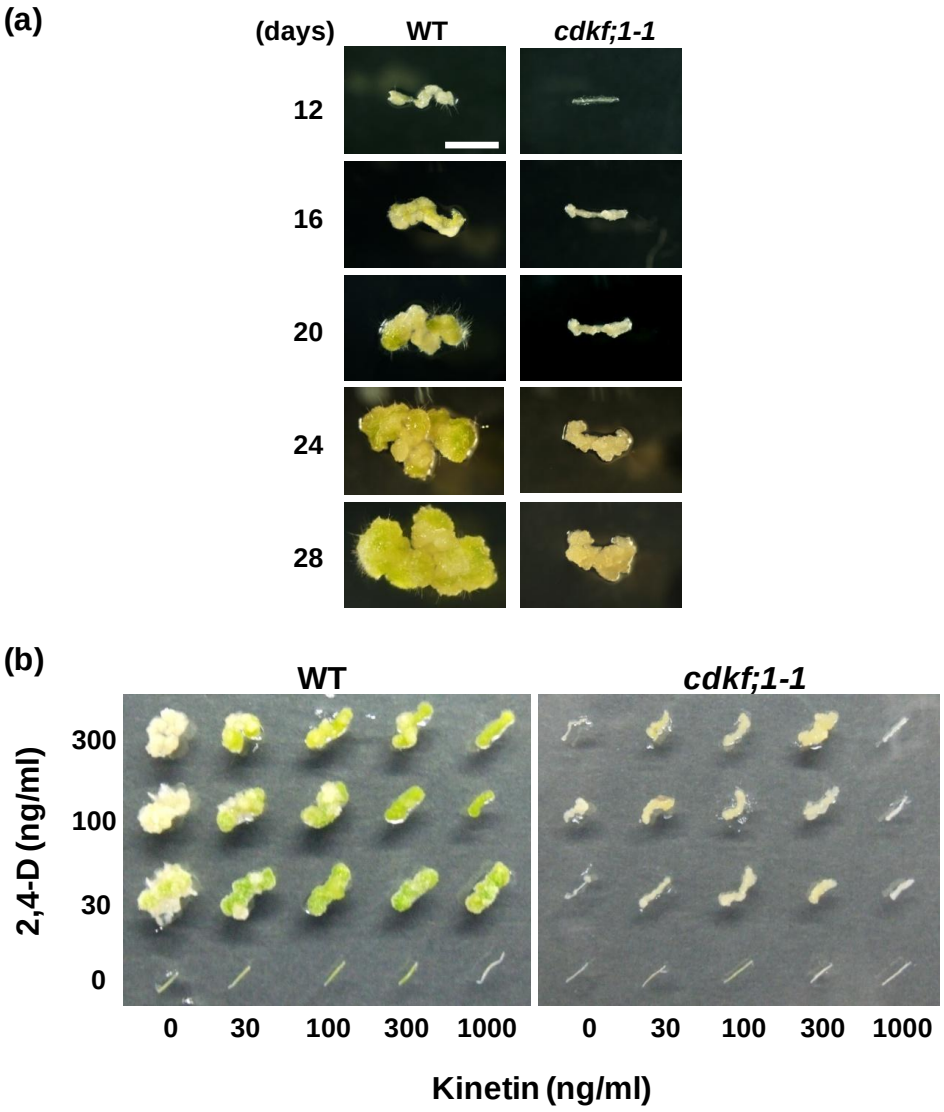

Figure 1-7. Retarded callus formation in the *cdkf;1-1* mutants

(a) Hypocotyl explants were cultured on a B5 medium containing 0.5 mg/l 2,4-dichlirophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. They were transferred onto a new medium by every 10 days. Bar = 5 mm. (b) Calli formed on a B5 medium containing various concentrations of 2,4-D and kinetin. They were transferred onto a new medium by every 10 days. Images were taken 30 days after explanting.

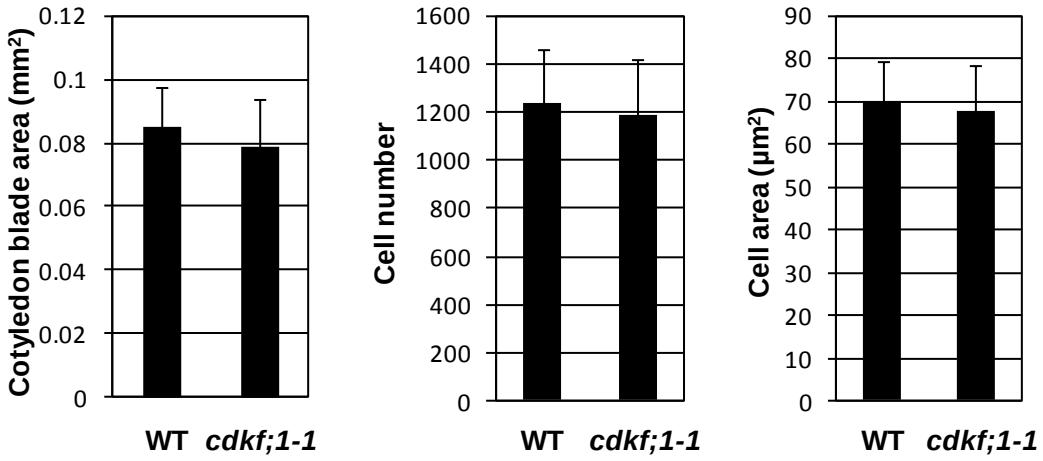

Figure 1-8. Embryogenesis is not defective in the *cdkf;1-1* mutants.

Blade area, cell number and cell area of cotyledons were measured with mature embryos of dry seeds obtained from wild-type plants and the heterozygous *cdkf;1-1* mutants. Data are presented as mean ± SE of 25 samples for wild-type plants and 89 samples for the *cdkf;1-1* mutants.

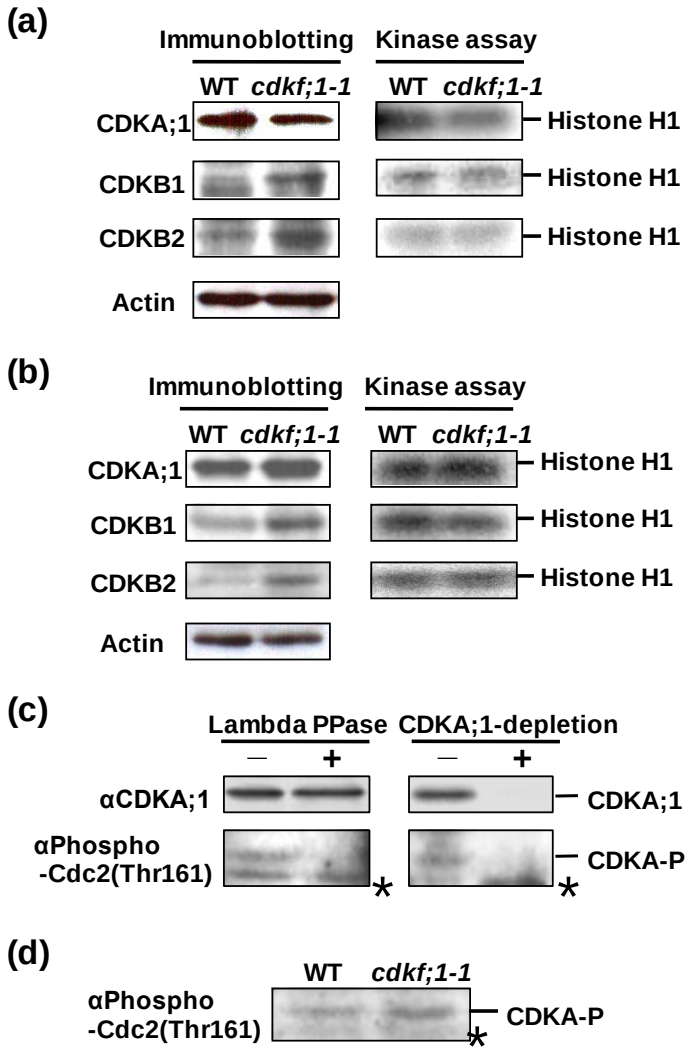

Figure 1-9. CDKA and CDKB activities are not reduced in the *cdkf;1-1* mutants.

(a, b) Protein levels and kinase activities of CDKA and CDKB in wild-type and the *cdkf;1-1* mutants. Protein extracts (10 μg for CDKA, 40 μg for CDKBs and 5 μg for actin) from 10-day-old seedlings (a) or calli (b) were subjected to immunoblotting with specific antibodies. A kinase assay was conducted with immunoprecipitates by using histone H1 as a substrate. (c) Detection of the phosphorylated form of CDKA;1. Protein extracts (60 μg) from calli were incubated with or without lambda protein phosphatase (left), or depleted with anti-CDKA;1 antibody (right). Then the samples were subjected to immunoblotting with anti-CDKA;1 or anti-phospho-Cdc2 (Thr161) antibody. CDKA-P indicates CDKA;1 phosphorylated within the T-loop. (d) Protein extracts (60 μg) from calli of wild-type plants and the *cdkf;1-1* mutants were subjected to immunoblotting with anti-phospho-Cdc2 (Thr161) antibody. Bands with an asterisk represent nonspecific cross-reactions with the antibody.

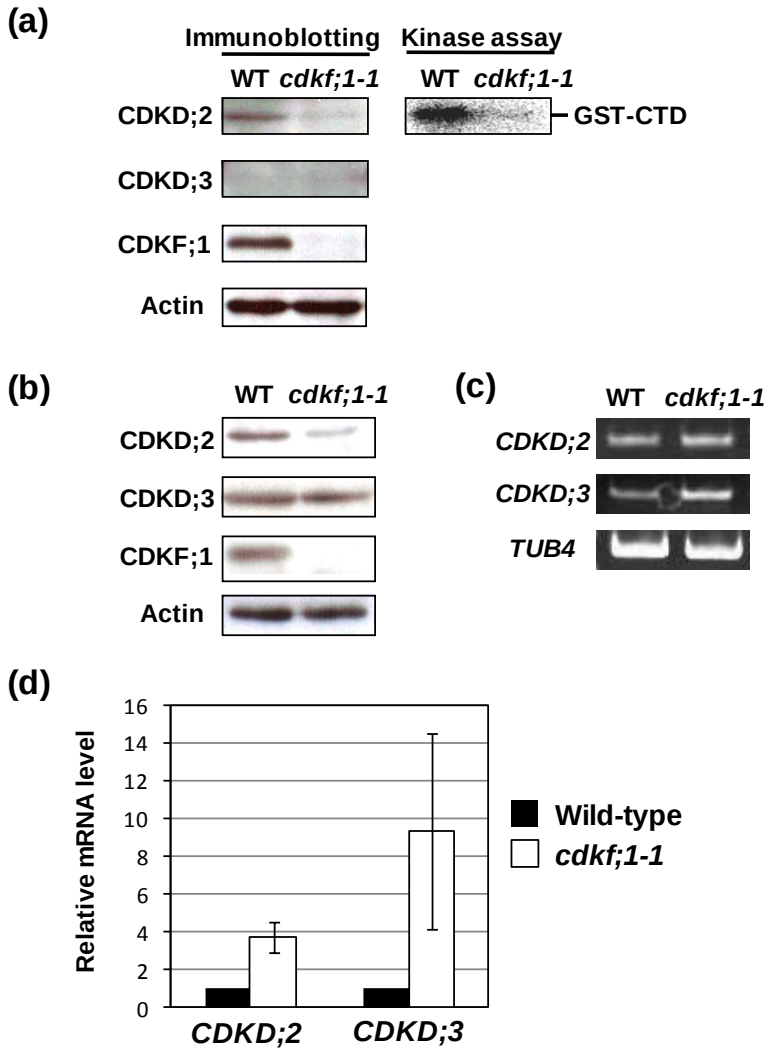

Figure 1-10. Protein level of CDKD;2 is reduced in the *cdkf;1-1* mutants.

(a) The protein levels and kinase activities of CDKD in wild-type plants and the *cdkf;1-1* mutants. Protein extracts (30 µg) from 10-day-old seedlings were subjected to immunoblotting with specific antibodies. A kinase assay was conducted with CDKD;2 immunoprecipitates by using GST-CTD as a substrate. (b) Protein levels of CDKD in calli of wild-type plants and the *cdkf;1-1* mutants. Protein extracts (30 µg) were immunoblotted with specific antibodies. (c) Semi-quantitative RT-PCR was conducted with total RNA from 10-day-old seedlings and the primer sets for *CDKD;2*, *CDKD;3* and *TUB4* as internal control. (d) Quantitative RT-PCR was conducted with the same RNA as that of (c). The expression levels of *CDKD;2* and *CDKD;3* were normalized to *ACT8* (*ACTIN 8*). Expression levels in the *cdkf;1-1* mutants are indicated as relative values when those in wild-type plants were set for 1. Data are presented as mean ± SE of 4 samples.

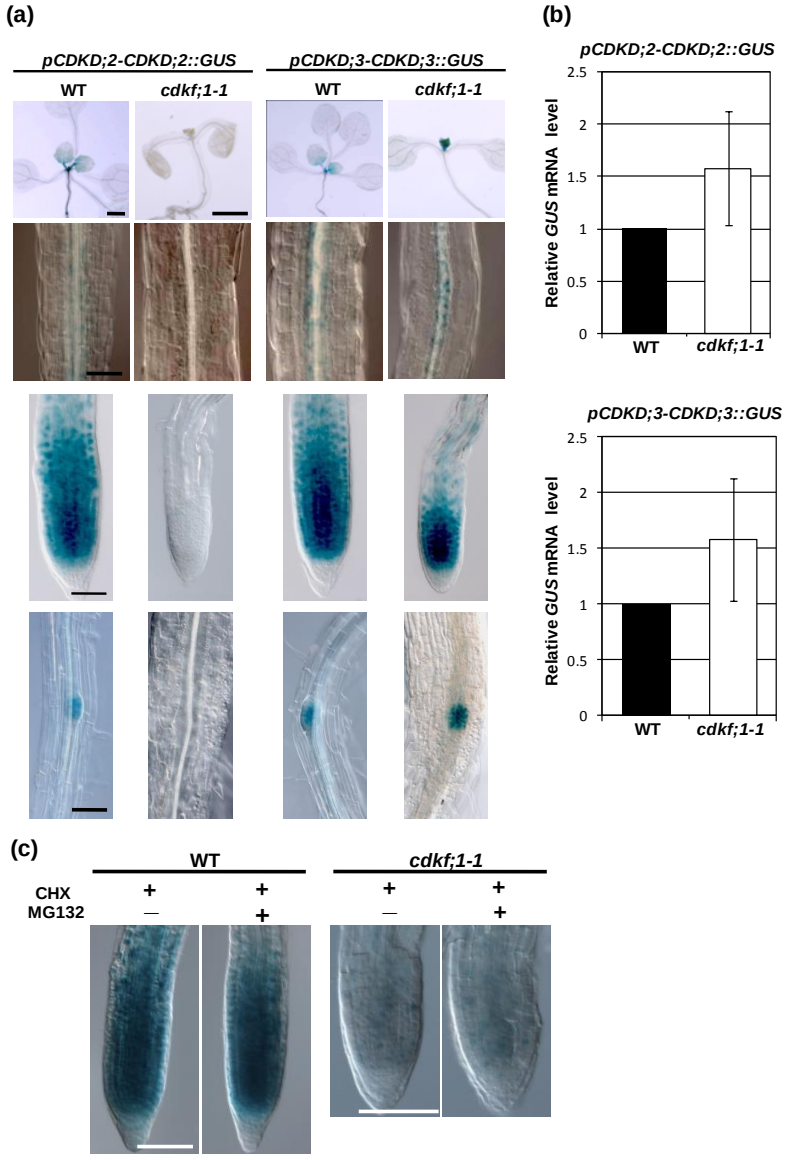

Figure 1-11. Decrease in the steady-state level of CDKD;2 in the *cdkf;1-1* mutants.

(a) GUS staining of transgenic plants harbouring *pCDKD;2-CDKD;2::GUS* or *pCDKD;3-CDKD;3::GUS*. Shoots of seedlings (top row), hypocotyls (second row), primary root tips (third row) and lateral root primordia (bottom row). Bar = 1 mm (top row) and 100 µm (second to bottom row). (b) Quantitative RT-PCR was conducted with total RNA from 10-day-old seedlings and the primer sets for the *GUS* gene. The *GUS* expression levels were normalized to *ACT8*. Expression levels in the *cdkf;1-1* mutants are indicated as relative values when those in wild-type plants were set for 1. Data are presented as mean ± SE of 5 transgenic lines. (c) 4-day-old wild-type plants or the *cdkf;1-1* mutants harbouring *pCDKD;2-CDKD;2::GUS* were incubated for 6 h in a liquid MSAR medium containing 100 µM cyclohexamide (CHX) with or without 100 µM MG132. Seedlings were fixed and GUS-stained for 2 h. Bars = 100 µm.

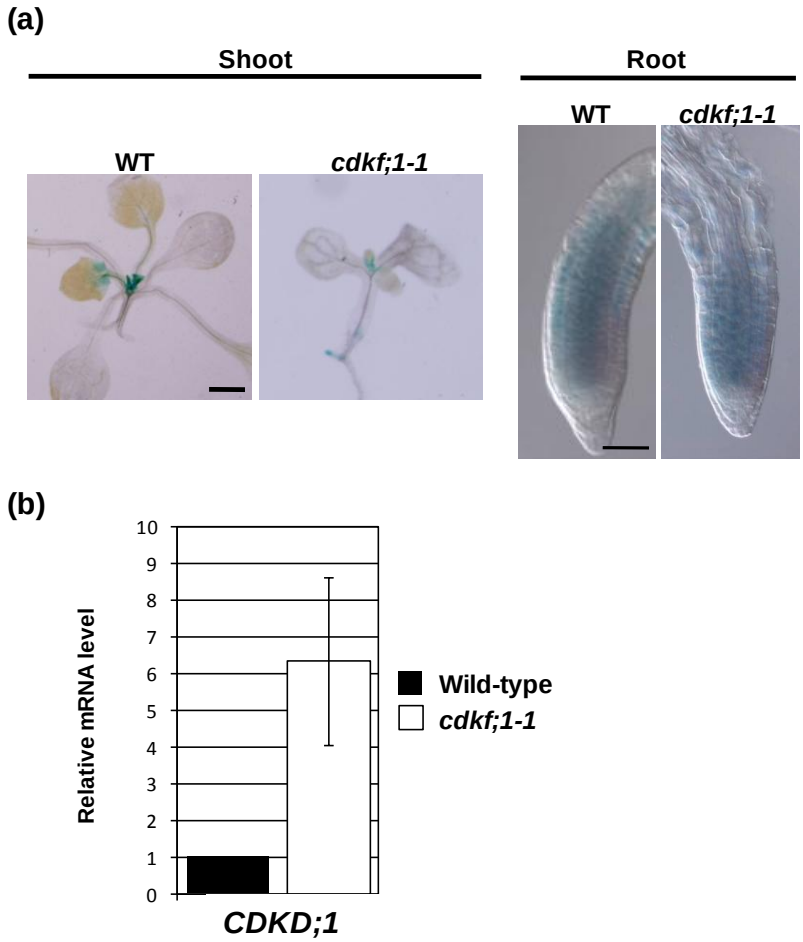

Figure 1-12. The stability of CDKD;1 protein is not reduced in the *cdkf;1-1* mutants.

(a) GUS staining of transgenic plants harbouring *pCDKD;1-CDKD;1::GUS*. Shoots of seedlings (left) and primary root tips (right). Bar = 1 mm (left) and 100 μm (right). (b) Quantitative RT-PCR was conducted with the same RNA as that of figure 1-10d. The expression level of *CDKD;1* was normalized to *ACT8*. Expression levels in the *cdkf;1-1* mutants are indicated as relative values when those in wild-type plants were set for 1. Data are presented as mean ± SE of 4 samples.

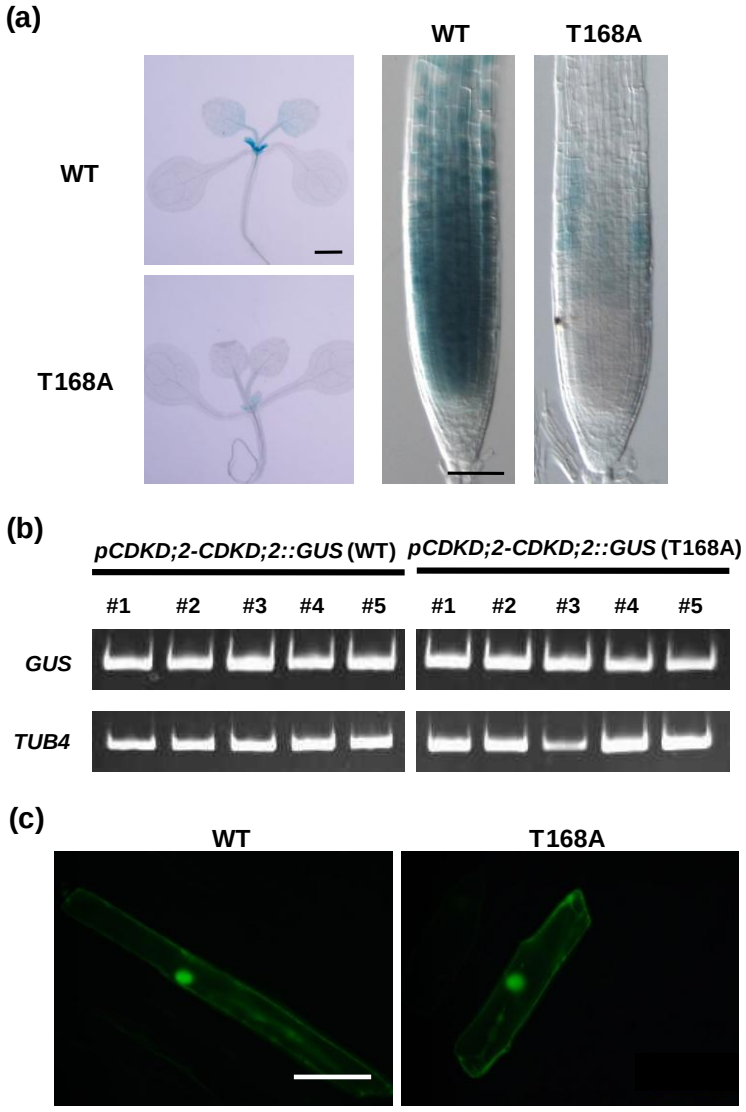

Figure 1-13. CDKD;2 with the T168A mutation is unstable in *Arabidopsis* tissues.

(a) GUS staining of transgenic plants harbouring *pCDKD;2-CDKD;2::GUS* (WT) or *pCDKD;2-CDKD;2::GUS* (T168A) in the wild-type background. Shoots (left) and primary roots (right) of 10-day-old and 4-day-old seedlings, respectively. Bar = 1 mm (left) and 100 µm (right). (b) RT-PCR was conducted with total RNA from 10-day-old seedlings of transgenic plants harbouring *pCDKD;2-CDKD;2::GUS* (WT) or *pCDKD;2-CDKD;2::GUS* (T168A) by using primers for *GUS* and *TUB4*. Five independent lines were examined for each transgenic plant. (c) CDKD;2-GFP (WT) or CDKD;2-GFP (T168A) was transiently expressed under the 35S promoter in onion epidermal cells. GFP fluorescence was observed with confocal microscopy. Bar = 200 µm.

<Discussion>

I showed that the *cdkf;1-1* mutants exhibited defects in cell division in both the shoots and roots. I also found that they were defective in cell elongation in dark-grown hypocotyls. These results indicate that the CDKF;1 function is not limited to cell cycle control, but it plays multiple roles in the process of cell growth. The CDKF;1::GUS fusion protein under the control of the *CDKF;1* promoter was expressed in various tissues with a higher accumulation in the meristems of shoots and roots, dark-grown hypocotyls, young flowers and pollen grains.

The *cdkf;1-1* mutants appeared to complete embryogenesis successfully, suggesting that CDKF;1 is primarily required for post-embryonic development. In seedlings, the kinase activity of CDKA;1 was almost the same as that in wild-type plants. This indicates that the characteristic phenotype of the *cdkf;1-1* mutants originated from disordered functions of factor(s) other than CDKA;1. In line with this assertion, it was noted that *cdka;1* null mutants failed to progress through the second mitotic division during male gametophyte development (Iwakawa *et al*., 2006; Nowack *et al*., 2006), while there seemed to be no defect in *cdkf;1-1* mutant pollen, as described in *Chapter III*. Moreover, transgenic plants overexpressing a dominant-negative type of CDKA;1 produced fewer but larger leaf cells (Hemerly *et al*., 1995), while both the cell number and cell size were reduced in the *cdkf;1-1* mutants. I found that CDKB1 and CDKB2 kinase activities per molecule were lower in the mutants, but the total CDKB activity was comparable to that in wild-type plants. Therefore, it is unlikely that a dysfunction of CDKB caused the *cdkf;1-1* mutant phenotype. I assume that CDKF;1 may have other unknown target(s) that function in regulating cell division and cell elongation during post-embryonic development.

CDKF;1 is able to suppress CAK mutation in budding and fission yeasts, and it phosphorylates and activates human CDK2 *in vitro* (Umeda *et al*., 1998; Shimotohno *et al*., 2004). Moreover, it was previously shown that CDKF;1 overexpression in *Arabidopsis* root protoplasts activates CDKA;1, suggesting that CDKF;1 is involved in activating endogenous CDKs *in vivo* (Shimotohno *et al*., 2006). However, in the present study, I found that CDKA;1 was not downregulated in the *cdkf;1-1* mutants. It is unlikely that CDKF;1 activates CDKA;1 only in specific tissues because CDKA;1 and CDKF;1 are expressed in largely overlapping tissues (Hemerly *et al*., 1993; this study). Mechanisms in which the lack of a particular cell cycle regulator is compensated by other proteins have been reported in animals. For instance, Aleem *et al*. (2005) showed that in *CDK2*$^{-/-}$ mice, CDK1 compensated for the loss of CDK2 function by forming an active CDK1-cyclin E complex. During mouse embryonic development, 3 D-type cyclins are expressed in mutually exclusive cell types. However, mice lacking 2 of the 3 D-type cyclins could develop normally until late gestation because the remaining cyclin was ubiquitously expressed in the whole embryo, thereby losing its tissue specificity and compensating for the loss of the other 2 D-type cyclins (Ciemerych *et al*., 2002). In the *cdkf;1-1* mutants, it is also likely that the redundant function of CDKDs might be sufficient for the activation of CDKA;1. Among the 3 CDKD homologues, CDKD;3 exhibited a higher suppression activity in the *S. cerevisiae* mutant GF2351 (Shimotohno *et al*., 2003). Although the transcript and protein levels of CDKD;3 were reported to be low in plant tissues (Shimotohno *et al*., 2003; this study), its activity might be sufficient to fully phosphorylate and activate CDKA;1, without the CDKF;1 function. This possibility is examined in *Chapter II*.

The protein level and kinase activity of CDKD;2 were significantly decreased

in the *cdkf;1-1* mutants. In addition, the expression of a nonphosphorylatable form (T168A) of CDKD;2 was reduced compared to that of the wild-type protein, regardless of the sustained level of transcripts. These results indicate the important role of T-loop phosphorylation in maintaining the steady-state level of CDKD;2. However, the proteasome inhibitor MG132 did not stabilize CDKD;2::GUS in the *cdkf;1* mutants, and CDKD;2 (T168A) fused to GFP was localized in the cytoplasm and nuclei in the same manner as the wild-type protein (Shimotohno *et al*., 2004; this study). Therefore, I assume that the T-loop phosphorylation by CDKF;1 controls the protein stability of CDKD;2 rather than protein degradation via the ubiquitin-proteasome pathway or a change in subcellular localization.

Quantitative regulation of CAK components has also been described in other organisms. In animals, MAT1 is known to stabilize the CAK complex consisting of CDK7 and cyclin H (CYCH) (Devault *et al*., 1995; Fisher *et al*., 1995; Tassan *et al*., 1995). It was found that the loss of MAT1 resulted in the reduction of protein levels of CDK7 and CYCH, suggesting that MAT1 regulates the steady-state level of the CAK complex (Rossi *et al*., 2001). The CYCH-CDK7 dimer is also activated by the T-loop phosphorylation (Devault *et al*., 1995; Fisher *et al*., 1995; Tassan *et al*., 1995), but it remains unknown whether this phosphorylation is associated with the control of their protein levels. The amount of the budding yeast protein Kin28, an orthologue of CDK7, was reduced in a strain of *cak1* mutants, in which Kin28 was not subjected to activation by phosphorylation (Espinoza *et al*., 1998). Further, in the absence of a MAT1 orthologue, Tfb3, substitution of the conserved threonine residue within the T-loop with alanine led to a significant reduction in the Kin28 level (Kimmelman *et al*., 1999). These observations support my proposal that in *Arabidopsis*, the protein stability of

CDKD;2 depends on the T-loop phosphorylation by CDKF;1.

Shimotohno *et al*. (2004) reported that CDKF;1 phosphorylates not only CDKD;2 but also CDKD;3; however, the amount of CDKD;3 in calli was almost identical between the wild-type plants and *cdkf;1-1* mutants. CDKD;2 and CDKD;3 phosphorylate both CDK and CTD, albeit with different preferences: the CDK kinase activity of CDKD;3 is higher than that of CDKD;2, whereas CDKD;2 has higher CTD kinase activity than CDKD;3 (Shimotohno *et al*., 2003). Therefore, my results indicate that CDKF;1 controls basal transcription by regulating the steady-state level of CDKD;2 rather than CDKD;3 and that some phenotypes observed in the *cdkf;1-1* mutants might be caused by transcription defects. For unknown reasons, I was unable to overexpress a constitutive-active form (T168D) of CDKD;2 in the *cdkf;1-1* mutants; thus, to test the contribution of CDKD;2 function in the *cdkf;1* mutants, I may need to use a different approach. In *Drosophila*, the expression of a dominant-negative form of CDK7 severely delayed the onset of zygotic transcription during early embryogenesis, but did not alter the timing of the first 13 embryonic nuclear cycles, which occur without *de novo* transcription (Leclerc *et al*., 2000). This result indicated a major function of CDK7 in the transcriptional control in animal cells. Recently, *Arabidopsis* mutants of cyclin T have been shown to exhibit embryonic lethality (Cui *et al*., 2007). Plant cyclin T is a binding partner of CDKC, an orthologue of mammalian CDK9 that functions as a CTD kinase (Fülöp *et al*., 2005). Therefore, this report suggests an essential role of CTD kinases in early developmental processes. In budding yeast, Cak1p activates CDK9 orthologues, namely, Bur1 and Ctk1, via the phosphorylation of conserved threonine residues within the T-loop (Ostapenko *et al*., 2005; Yao *et al*., 2002), suggesting that *Arabidopsis* CDKF;1 may also control basal transcription through the phosphorylation

of CDKC and CDKD;2. Studies using loss-of-function mutants of CDKC and CDKD and their associated cyclins are expected to reveal the indispensable function of CTD kinases during plant development.

In yeast and animals, the CDK activity is modified by the WEE1 kinase, which phosphorylates the ATP-binding site and inhibits activity (Featherstone and Russell, 1991; Parker and Piwnica-Worms, 1992). However, in *Arabidopsis*, the knockout mutants of *WEE1* showed no obvious phenotype when grown under normal growth conditions (De Schutter *et al*., 2007). This suggests that unlike in yeast and animals, WEE1 is not a critical CDK modifier in plants. In contrast, in the current study, I showed that CDKF has a crucial role in the post-embryonic development of *Arabidopsis*. A dissection of the regulatory mechanisms underlying the CAK activity and the identification of novel substrates of plant-specific CDKF will reveal how cell cycle regulation is integrated into developmental control in cooperation with various signalling pathways and transcriptional networks.

<Meterials & methods>

Plant material

Arabidopsis thaliana (ecotype Col-0) was first grown at 23°C on an MSAR agar plate (Koncz *et al*., 1990) under short-day conditions (dark, 16 h; light, 8 h); it was then transferred onto soil and grown under continuous light conditions. Calli were generated from dark-grown hypocotyls (Kono *et al*., 2006).

Identification of T-DNA insertion mutants of CDKF;1

The T-DNA insertion mutants of *CDKF;1* were isolated from the collections of GABI-Kat and the Salk Institute; the seed stock numbers of *cdkf;1-1* and *cdkf;1-2* are GABI-Kat 315A10 and SALK_148336, respectively. The insertions were examined by genomic PCR with Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) by using a set of primers that hybridise to the T-DNA and *CDKF;1*: 5' -AACAACCGGCGACCAGTTGGAGTAT-3' and 5' -CCCATTTGGACGTGAATGTAGACAC-3' were used for *cdkf;1-1*, and 5' -AACAACCGGCGACCAGTTGGAGTAT-3' and 5' -GGATTTTCGCCTGCTGGGGCAAACCAGCGT-3' were used for *cdkf;1-2*. Each line was backcrossed 3 times with wild-type plants. The 3' end of the *CDKF;1* transcripts produced in the mutants was identified with a 3' RACE System (Invitrogen, San Diego, CA, USA). The primers used for PCR amplification were as follows: the adapter primer and the abridged universal amplification primer, 5' -GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3' and 5' -GGCCACGCGTCGACTAGTAC-3', respectively, and the *CDKF;1*-specific primer, 5' -ACGAATACTTCTGGCGTG-3'. For the subsequent nested-PCR, the following 3

primers for the ORF of *CDKF;1* were used: 5'-GGATACTCATGGAGC-3', 5' -GCTTCAGATGAAAACCAACAAGCTTAC-3' and 5' -GATGGAGAAACCAGCGAACCACCAG-3'. The PCR products were purified, and their nucleotide sequences were determined.

RT-PCR

cDNA was synthesised with total RNA by using Superscript II reverse transcriptase (Invitrogen). In a total reaction volume of 20 µl, 1–2 µg of RNA was used. For PCR reactions, Ex Taq DNA polymerase (TaKaRa) and the primers listed in Table 1-1 were used. The PCR conditions were as follows: 1 cycle at 94°C for 2 min; 25–35 cycles at 98°C for 10 s, at 55°C for 30 s and at 72°C for 1 min; and 1 cycle at 72°C for 7 min. Quantitative RT-PCR was conducted with the LightCycler system (Roche, Basel, Switzerland) by using SYBR Premix Ex Taq (TaKaRa). The PCR conditions were as follows: 1 cycle at 95°C for 5 min; 45 cycles at 95°C for 10 s, at 60°C for 10 s and at 72°C for 10 s.

Yeast complementation test

m*CDKF;1* was PCR-amplified from the cDNA prepared from the *cdkf;1-1* mutants and cloned into the *Eco*RI site of pYX112. A complementation test was conducted with the budding yeast strain GF2351 (*MATa, civ1-4, ura3, leu2, trp1, lys2, ade2* and *ade3*) (Thuret *et al*., 1996). Transformants were incubated on a minimal medium lacking uracil at 27°C or 37°C for 4 days.

Plasmid construction for plant transformation

For a complementation test of the *cdkf;1* mutants, the genomic fragment of *CDKF;1* was amplified from 2032 bp upstream of the start codon to 1032 bp downstream of the stop codon; it was cloned into the GATEWAY entry vector pENTR/D-TOPO (Invitrogen). A recombination reaction was performed between the entry clone and the destination vector pGWB1 (Nakagawa *et al.*, 2007) by using LR Clonase (Invitrogen). For expression analysis, the genomic fragments of *CDKF;1*, *CDKD;1*, *CDKD;2* and *CDKD;3* were amplified from 2032, 1697, 2011 and 2000 bp upstream of the start codon, respectively, to the amino acids just before the stop codon. These fragments were cloned into pENTR/D-TOPO, and an LR reaction was performed with the destination vector pGWB3 (Nakagawa *et al.*, 2007) to be in frame with the GUS gene. The T168A mutation in CDKD;2 was introduced using a GeneTailor™ Site-Directed Mutagenesis System (Invitrogen).

Microscopy observations

To observe the structure of shoot apical meristems, 7-day-old seedlings grown on MSAR media were prepared. Where the tissue is to be sectioned, the seedlings were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formalin) at 4°C overnight, and dehydrated with ethanol series. For the complete dehydration, samples were finally soaked in 100% ethanol overnight. To substitute Technovit for ethanol, 1/2 volumes of ethanol was removed and 1/6 volumes of Technovit solution [0.5g hardener I in 50 ml Technovit 7100 (Heraeus Kulzer, Germany)] was added, then incubated for 20 min with gentle rocking. Subsequently, 1/3 volumes of Technovit solution was added and incubated similarly; 1/2 volumes of the mixture of ethanol and Technovit solution was removed and 1/2 volumes of Technovit solution was added for similar incubation; all

the solution was removed, and Technovit solution was added for similar incubation. Finally, all the solution was removed, and Technovit solution was added again for further incubation for overnight with gentle rocking. Substituted samples were soaked in the Technovit solution with 1/14 volumes of hardener II, and hardened. Then the samples were cut at 8 μm for longitudinal sections, and observed with microscopy. Roots were mounted on a glass slide in a chloral hydrate solution. Embryos obtained from dry seeds were soaked in water for at least 1 h and subsequently fixed and cleared as described previously (Malamy and Benfey, 1997). Epidermal cells of hypocotyls grown under dark or light condition were observed after fixation by FAA. GUS staining was conducted as described by Umeda *et al*. (2000). Prepared samples were observed under a light microscope equipped with Nomarski optics. Transient expression in onion epidermal cells was conducted by particle bombardment. One microgram of pUC-CDKD;2 (WT) or pUC-CDKD;2 (T168A) plasmid was coated onto 16 μl gold particles with 16 μl of 2.5 M $CaCl_2$ and 3 μl of 0.1 M spermidine. After that, the particles were washed with 70% ethanlol and 100% ethanol, sequentially. Suspended particles in 100% ethanol were spread on the macrocarriers. Macro carriers were set to the particle gun, and the particles were shot to onion epidermal cells according to the manufacture's protocol (Bio-Rad, Alfred Nobel Drive Hercules, CA, USA). Microscopic observation was performed using a confocal laser scanning microscope system LSM510 (Zeiss, Thornwood, NY, USA) with an argon ion laser (488 nm).

Immunoblotting and kinase assay

Immunoblotting was performed using an ECL Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK). Specific antibodies of *Arabidopsis* CDKs were as

described previously (Umeda *et al*., 1998; Kono *et al*., 2003; Shimotohno *et al*., 2003). A polyclonal anti-CDKB1 antibody was raised against the carboxy-terminal FDSLDKSQF peptide of CDKB1;1. Commercial antibodies used in this thesis are anti-actin antibody (MP Biomedicals, Morgan Irvine, CA, USA) and anti-phospho-Cdc2 (Thr161) antibody (Cell Signaling Technology, Danvers, MA, USA). For phosphatase treatment, 50 µg of protein extract was incubated with 200 U of lambda protein phosphatase (New England Biolabs, MA, USA) at 30°C for 1h. For kinase assay, 40 µg of protein extract was immunoprecipitated and subjected to kinase assay by using histone H1 (Roche) or GST-CTD as a substrate (Umeda *et al*., 1998).

Table 1-1. Primers used for RT-PCR.

Name	Sequence (5' > 3')
CDKD;2 forward	GATATTAAGTCTTATATGTTG
CDKD;2 reverse	CTCAGGCGCTCTGTACCATGTAG
CDKD;3 forward	AAGCCAAATAACTTGTTAAT
CDKD;3 reverse	AAGCTTTGTTAAATCCGGCC
CDKF;1 forward	AAGAAGGTAGAAGGAGGGGATGGAT
CDKF;1 reverse	CATTTCACTAACAGTGCACGTTGCA
TUB4 forward	AGAGGTTGACGAGCAGATGA
TUB4 reverse	CCTCTTCTTCCTCCTCGTAC
CDKD;1 forward (Real-time)	GGCAAATGGGTTCTGCAC
CDKD;1 reverse (Real-time)	GCTGTCCATTGGGTCCTATC
CDKD;2 forward (Real-time)	AAGCCACCGACACAAAGACT
CDKD;2 reverse (Real-time)	GTAGCTTGATTTCTCTTAAAGCTGTG
CDKD;3 forward (Real-time)	AAGCAGGCTCTAGAACACAGGTA
CDKD;3 reverse (Real-time)	GGCTTTGGGAGCTTAGCC
ACT8 forward (Real-time)	CTAAACTAAAGAGACATCGTTTCCA
ACT8 forward (Real-time)	GTTTTTATCCGAGTTTGAAGAGGCT
GUS forward (Real-time)	TTAACTATGCCGGAATCCATCGC
GUS reverse (Real-time)	AACGCTGACATCACCATTGGC
GUS forward (RT-PCR)	ACTGAACTGGCAGACTATCC
GUS forward (RT-PCR)	ACGATGCCATGTTCATCTGC
P1	ACGAATACTTCTGGCGTG
P2	ACTGCTTCGTAGCATCG
P3	GTTAGTGAAATGGATGATGATCTCGG
P4	CACTACTGGTAGGCTTTACATTCATGGGT
P5	GAGGATTCTCCGAGAAAGTGGAATG

Chapter II

CDKD;1 and CDKD;3 cooperatively control gametogenesis in Arabidopsis

<Abstract>

In *Chatpter I*, I indicated the possibility that CAK(s) other than CDKF;1 regulate(s) gametogenesis and embryogenesis of plants. To identify which CAK(s) is involved in, I observed the expression patterns of CAKs in gametophytes and during embryogenesis. I found that CDKD;1 and CDKD;3 were highly expressed in both gametophytes and embryos, whereas CDKF;1 and CDKD;2 were poorly expressed. Then, I analyzed the physiological functions of CDKD;1 and CDKD;3 in gametogenesis by using knock-out mutants. While each single mutant showed no obvious phenotype, the *cdkd;1-1 cdkd;3-1* double mutants were gametophyte-lethal. Reciprocal crossing experiment revealed that CDKD;1 and CDKD;3 were required for both male and female gametogenesis. During male gametogenesis, pollen mitosis was inhibited in the double mutants. Aberrant pollen grains of the *cdkd;1-1 cdkd;3-1* double mutants could not fertilize normally, suggesting that disturbed pollen mitosis resulted in non-functional pollen. I also found that nuclear divisions were arrested or delayed during female gametogenesis. Resultant abnormal ovules could not be fertilized successfully. Based on these results, I concluded that CDKD;1 and CDKD;3 control cell cycle progression during gametogenesis in a cooperative manner.

<Introduction>

The alternation between diploid and haploid generations is fundamental in the life cycles of both animals and plants. The meiotic cell cycle is common between animals and, but in animals the products of meiosis are gametes, whereas for most plants, subsequent mitotic cell cycles are needed for gamete formation. However, the regulatory mechanism of mitotic cell cycle progression during gametophyte development is still largely unknown.

In *Arabidopsis*, the diploid sporogenous initial cells, also called microspore mother cells or megaspore mother cells, undergo meiosis to produce a tetrad of microspores in the anther, or to produce four megaspores in the ovule. The microspores, freed from the tetrad by the action of callase, further undergo an asymmetric mitosis (pollen mitosis I, PM I) to form bi-cellular pollen composed of two cells with distinct fates (Figure 2-1). The larger cell, the vegetative cell, does not divide again and later forms a pollen tube. The smaller cell, the generative cell, undergoes a symmetric mitosis (pollen mitosis II, PM II) to produce two sperm cells (McCormick, 1993, 2004) (Figure 2-1). The three-celled pollen grain is called the male gametophyte.

For female gametophyte development, only one functional megaspore out of the four megaspores survives, and it undergoes three rounds of mitotic divisions and subsequent cellularizations to produce a seven-celled mature embryo sac, also termed the female gametophyte (Yang and Sundaresan, 2000; Drews and Yadegari, 2002) (Figure 2-2). Megagametogenesis in wild-type *Arabidopsis* has been described (Misra, 1962; Poliakova, 1964; Webb and Gunning, 1990; Mansfield *et al*., 1991; Murgia *et al*., 1993; Webb and Gunning, 1994; Schneitz *et al*., 1995; Christensen *et al*., 1997) and has been divided into seven morphologically distinct stages that are depicted in Figure 2-2.

First, the surviving megaspore (stage FG1) undergoes mitosis to produce a two-nucleate cell (stage FG2). Shortly thereafter, the two nuclei separate to the chalazal and micropylar poles and a vacuole is formed at the center (stage FG3). The developing female gametophyte then undergoes second (stage FG4) and third rounds of mitosis. This results in an eight-nucleate cell with four nuclei at each pole separated by a large central vacuole (beginning of stage FG5). Next, one nucleus from each pole (the polar nuclei) migrates toward the female gametophyte's center and the embryo sac becomes cellularized. The polar nuclei eventually fuse and the female gametophyte consists of seven cells and seven nuclei (stage FG6). At the final developmental step, the three antipodal cells undergo cell death. Thus, the mature *Arabidopsis* female gametophyte (stage FG7) consists of one haploid egg cell, two haploid synergid cells, and one diploid central cell.

A unique feature of plant fertilization is double fertilization. During the double fertilization process, sperm cells are delivered through the pollen tube to the embryo sac. One sperm cell fuses with the egg cell and generates the zygote, whereas the second sperm cell fuses with the central cell, giving rise to the endosperm (Figure 2-3). The endosperm supports the developing embryo (for review, see Dumas and Rogowsky, 2008).

Many mutants defective in gametophyte development, especially, many meiotic and mitotic cell cycle mutants in *Arabidopsis* male and female gametophyte development were identified through both forward and reverse genetics approaches. However, the functions of core cell cycle regulators, such as CDKs and cyclins, in gametogenesis largely remain unknown while information of their roles in post-embryonic development has been accumulating. To my knowledge, CDKA;1, a

critical regulator of the cell cycle in plants, is the sole CDK whose function in gametogenesis has been characterized. In the null mutant of *CDKA;1*, the transmission rate from the male side was severely reduced (Iwakawa *et al*., 2006; Nowack *et al*., 2006). The *cdka;1* mutant produced mature bicellular pollen grains, consisting of a single sperm-like cell and a vegetative cell, due to a failure in PMII. These findings demonstrate that the CDK activities are prerequisite for cell cycle progression during gametogenesis. As described in *General introduction*, CAK is indispensable for the full activation of CDK, implying that some CAKs may be engaged in CDK activation during gametogenesis. To identify such CAK(s), I compared the expression patterns of CAKs in gametophytes and embryos. Since the knockout mutants of CDKF;1 showed no defect in the gametogenesis and embryogenesis (*Chapter I*), here I conducted the phenotypical analyses of the knock-out mutants of CDKD;1 and CDKD;3, which also exhibit a significant level of CAK activity in yeast cells and *in vitro*.

<Results>

Expression analysis of CAKs in gametophytes and embryos

To identify the CAK(s) which function(s) in gametogenesis and embryogenesis, I compared the expression patterns among the four CAKs by using transgenic plants described in *Chapter I*. First, I observed the expression patterns in flowers, reproductive organs of flowering plants (Figure 2-4). As described in *Chapter I*, the strong GUS expression of CDKF;1::GUS was observed in young flowers, especially in the stigma, pistil basal regions and vascular bundles of petals. GUS expression in siliques was observed only in the basal region. In mature flowers, the GUS expression was mainly restricted to stamen filaments and pedicels (Figure 2-4). The expression of CDKD;1::GUS was restricted to young flowers, and pistils and anthers in mature flowers. Its expression level in flowers was higher than that in seedlings (Figure 2-4 and Figure 1-12). CDKD;2::GUS was expressed in a similar manner to that of CDKD;1, with the exception of the expression in pedicels of CDKD;2::GUS plants. However, the expression level of CDKD;2 was lower than that observed during post-embryonic development (Figure 2-4 and Figure1-11). Although CDKD;3::GUS showed the same expression pattern as that of CDKD;2::GUS, the expression level was much higher (Figure 2-4). These results indicate that CDKD;1 and CDKD;3 are the CAKs which show a higher expression in reproductive organs.

Next, I observed the expression patterns of CAKs in gametophytes in more detail. Although all four CAKs were expressed in mature pollen grains, the expression patterns were slightly different. CDKF;1::GUS expression was observed in whole pollen grains which consist of one generative cell and two sperm cells (Figure 2-5a). In contrast, the expressions of CDKD;2::GUS and CDKD;3::GUS were observed only in

spots within pollen grains (Figure 2-5a). The spots may indicate functional assemblages called male germ unit which contains the nucleus of a vegetative cell and two sperm cells (Lalanne and Twell, 2002). CDKD;1::GUS was expressed in whole pollen grains, but the highest expression was detected in the male germ units (Figure 2-5a). These results suggest that CDKD and CDKF may have different functions in male gametogenesis. All three CDKDs but not CDKF;1 were expressed in ovules (Figure 2-5b), suggesting that only CDKD is engaged in female gametogenesis.

As shown in *Chapter I*, CDKF;1 function is not prerequisite for embryogenesis. This raises two possibilities. One is that the molecular function of CDKF;1 is not necessary for embryogenesis. The other one is that CDKF;1 is not expressed during embryogenesis. To discern these two possibilities and identify CAK(s) regulating cell division during embryogenesis, I observed the expression patterns of CAKs in embryos. As shown in Figure 2-6a, CDKF;1::GUS was poorly expressed throughout embryogenesis. At the late embryo stage, CDKF;1::GUS was expressed only in hypocotyls, in which cells are not actively dividing as far as I observed with the mitotic CYCB1::GUS marker. These results suggest that CDKF;1 is not involved in cell cycle progression during embryogenesis. CDKD;2::GUS, which is stabilized by CDKF;1 as shown in *Chapter I*, also showed a low expression during embryogenesis, suggesting that the CDKF;1-CDKD;2 pathway may not play a significant role during embryogenesis. In contrast, both CDKD;1::GUS and CDKD;3::GUS were highly expressed throughout early embryogenesis and in dividing tissues during late embryogenesis, such as apical meristems of roots and shoots and vascular bundles (Figure 2-6a, b). These results suggest that CDKD;1 and CDKD;3 may play a role in cell division during embryogenesis. In addition, CDKD;3::GUS was observed in the

endosperm in which nuclear divisions and following cellularization occur, whereas CDKF;1::GUS was not expressed (Figure 2-6c). CDKD;2::GUS was not expressed in the endosperm (data not shown). Although CDKD;1::GUS was not observed in the endosperm under my experimental conditions, Day *et al*. (2008) classified *CDKD;1* into a group of genes which were highly expressed in the endosperm, based on the transcriptome analysis of endosperm. Taken together, these results suggest that CDKD;1 and CDKD;3 may play a significant role in embryonic cell divisions.

Isolation of the cdkd;1-1 cdkd;3-1 *double mutants*

Shimotohno *et al*. (2006) previously isolated the single knock-out mutants of *CDKD;1* and *CDKD;3* (Figure 2-7a). Although each single mutant showed no obvious phenotype, I failed to obtain the *cdkd;1-1 cdkd;3-1* double mutants. Therefore, I observed plants with the heterozygous *cdkd;1* mutation and the homozygous *cdkd;3* mutation, which are hereafter referred to as the *cdkd;1-1/+ cdkd;3-1/-* plants. In siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants, empty spaces were found in the alignment of mature seeds (Figure 2-8a), suggesting the lethality of the *cdkd;1-1 cdkd;3-1* double mutants during gametogenesis and/or embryogenesis. Expression of CDKD;3::GUS under the control of the *CDKD;3* promoter restored the phenotype of the *cdkd;1-1 cdkd;3-1* double mutants. This indicates that the knock-out of *CDKD;1* and *CDKD;3* was the cause of the phenotypes observed in the *cdkd;1-1/+ cdkd;3-1/-* plants (Figure 2-7b). Furthermore, this also suggests that CDKD;3::GUS fusion protein was functional and the promoter region of *CDKD;3* was enough for the proper expression pattern of *CDKD;3*.

The cdkd;1-1 cdkd;3-1 *double mutants show gametophyte-lethality*

As mentioned above, empty spaces were observed in siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants, whereas siliques of the wild-type plants were filled with mature seeds (Figure 2-8a), suggesting the embyo- or gametophyte-lethality of the *cdkd;1-1 cdkd;3-1* double mutants. Abortive seeds resided in the empty spaces of the *cdkd;1-1/+ cdkd;3-1/-* siliques and no embryo-like structures were formed (Figure 2-8b). The mature siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants contained ~50 % abortive seeds, whereas almost all the seeds were normally developed in wild-type plants (Table 2-1). These results suggested the gametophyte-lethality of the *cdkd;1-1 cdkd;3-1* double mutants.

The impaired seed development observed in the *cdkd;1-1/+ cdkd;3-1/-* plants might be owing to secondary or tertiary effects of the mutations. Thus, to identify the primary effect of the mutations, the phenotypes of the double mutants were first observed in younger siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants. After double fertilization, at least three rounds of nuclear divisions normally occur in the endosperm before the first zygotic division. After that, the zygote starts cell division to form an embryo, while endosperm nuclei continue to proliferate (Figure 2-9a). In the young siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants, I found ovules that were arrested at the early stages of female gametogenesis, such as FG1 and FG2, and those that remained unfertilized at FG7 stage (Figure 2-9b). Occasionally, I found seeds containing one cell- or two cell-stage embryo with a reduced number of endosperm nuclei (Figure 2-9c). This indicates the engagement of CDKD;1 and CDKD;3 in the central cell development, as discussed later.

CDKD;1 and CDKD;3 are prerequisite to both male and female gametogenesis

To examine whether the observed lethality was due to the defect in male and/or female gametogenesis, I conducted reciprocal crosses between wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants (Table 2-2). As an experimental control, pollen grains from wild-type or *cdkd;1-1/+ cdkd;3-1/-* plants were pollinated to pistils of wild-type or *cdkd;1-1/+ cdkd;3-1/-* plants, respectively. The ratios of abortive seeds from these control crosses were almost the same as those from self-pollinated siliques, indicating that no technical problem needs to be considered in the following results of reciprocal crosses. Pollens from *cdkd;1-1/+ cdkd;3-1/-* plants pollinated to wild-type stigma caused 33.8 % of seed abortion, suggesting that not all but a significant fraction of *cdkd;1-1 cdkd;3-1* pollens was lethal in male gametophyte. Similarly, 39.9 % abortive seeds were observed in sliques of *cdkd;1-1/+ cdkd;3-1/-* plants fertilized with wild-type pollens. This indicates that a large portion of the *cdkd;1-1 cdkd;3-1* female gametophytes were lethal. Taken together, these results suggest that CDKD;1 and CDKD;3 cooperatively play not necessarily essential but significant roles in both male and female gametogenesis.

Pollen mitosis is inhibited in the* cdkd;1-1 cdkd;3-1 *double mutants

Male gametophyte-lethality of the *cdkd;1-1 cdkd;3-1* double mutants might be due to the reduced pollen viability. Hence, I conducted Alexander-staining to distinguish between viable and nonviable pollens (Figure 2-10). Pollen grains from the *cdkd;1* homozygous mutants, the *cdkd;3* homozygous mutants and the *cdkf;1* heterozygous mutants were viable as well as those from wild-type plants. In the *cdkd;1-1/+ cdkd;3-1/-* plants, likewise, almost all the pollen grains were viable, suggesting that pollen viability was not affected in the *cdkd;1-1 cdkd;3-1* double mutants. In addition, pollen morphology of the *cdkd;1-1/+ cdkd;3-1/-* plants was normal (Figure 2-11). These

results raised the possibility that pollen mitosis, rather than viability or morphology, was affected by the mutations.

A mature pollen comprises three nuclei, namely a large vegetative cell and two small sperm cells, the actual gametes (Figure 2-1 and Figure 2-12a). I examined whether the *cdkd;1-1 cdkd;3-1* pollen completed pollen mitosis by DAPI-staining. As shown in Table 2-3 and Figure 2-12b, nearly 90% of mature pollens from wild-type plants contained three nuclei, indicating that they reached maturity in the floral stage 13 when I collected pollens. On the other hand, those with three nuclei was reduced at the same stage of the *cdkd;1-1/+ cdkd;3-1/-* flowers (Table 2-3 and Figure 2-12b). Namely, 7.4 % of pollens contained one nucleus, probably due to a failure of PMI (Table 2-3 and Figure 2-12a, b). 23.6 % of pollens contained two nuclei consisting of one vegetative cell and one sperm-like cell, suggesting a failure of PMII (Table 2-3 and Figure 2-12a, b). Furthermore, 12.3 % of pollens from the *cdkd;1-1/+ cdkd;3-1/-* anthers contained no nucleus (Table 2-3 and Figure 2-12a and b). Abnormal pollens without nucleus might be generated by developmental arrest and degeneration of pollens, since such abnormalities were rarely observed in wild-type pollens (Figure 2-12b). These results suggest that a significant fraction of the *cdkd;1-1 cdkd;3-1* pollens was defective in the progression of both PMI and PMII, whereas two pollen mitoses completed successfully in 6.7% of the *cdkd;1 -1 cdkd;3-1* pollens. This was consistent with the result of reciprocal crosses showing that not all but a significant fraction of *cdkd;1-1 cdkd;3-1* pollens was lethal in male gametophyte. Taken together, these results suggest that CDKD;1 and CDKD;3 cooperatively regulate the cell cycle progression during pollen mitosis.

To examine the functionality of mutant pollens during fertilization, pollen grains from the *cdkd;1-1/+ cdkd;3-1/-* plants were pollinated to wild-type pistils. In the

wild-type siliques fertilized with wild-type pollens, eight or sixteen cell embryos with a lot of endosperm nuclei were generated at 3 DAP, indicating that double fertilization normally occurred (Table 2-4 and Figure 2-13). In contrast, 41.7 % of total seeds were abortive in wild-type siliques fertilized with pollens from the *cdkd;1-1/+ cdkd;3-1/-* plants (Table 2-4). Considering that 43.3% pollens from the *cdkd;1-1/+ cdkd;3-1/-* plants had less than 2 nuclei or were generated (Table 2-3 and Figure 2-12b), all of the abortive pollens including those with one sperm-like cell and one vegetative cell were unlikely to fertilize wild-type ovules successfully. Abortive seeds contained no embryo and can be classified into two groups; namely 'unfertilized' and 'degenerated'. Unfertilized ones have intact egg cell nucleus and central cell nucleus after pollination. 44 % of abortive seeds contained both the central cell and the egg cell, but no visible synergid cell (Table 2-4, unfertilized). It is known that one of synergid cells is physically disrupted by pollen penetration and persistent one becomes difficult to see at 2 DAP (Christensen *et al*., 2002). Thus, it is likely that the *cdkd;1-1 cdkd;3-1* pollens were normally attracted but their sperm-like cells could not successfully fertilize the central cell and the egg cell (Figure 2-13). On the other hand, 56% of abortive seeds were degenerated and shrunk (Table 2-4). In degenerated seeds, cellular structures of central cells, egg cells and so on are severely disrupted and cannot be microscopically identified. This might be the terminal phenotype of wild-type ovules that could not be fertilized successfully by the *cdkd;1-1 cdkd;3-1* pollen. To confirm that a failure of fertilization led to seed degeneration, I emasculated the stamens in wild-type flowers. Observation of ovules showed that unfertilized ovules were degenerated like wild-type ovules which were not fertilized by the *cdkd;1-1 cdkd;3-1* pollen (data not shown).

Nuclear divisions in female gametogenesis are inhibited in the* cdkd;1-1 cdkd;3-1 *double mutants

The results of reciprocal crosses suggested that the *cdkd;1-1 cdkd;3-1* double mutants are impaired in female gametogenesis. Hence, I analyzed the development of the female gametophyte in the *cdkd;1-1/+ cdkd;3-1/-* plants. Female gametogenesis of wild-type plants proceeds normally from the one-nucleate stage FG1 to FG7 (see Figures 2-2). Ovule development in wild-type pistils was primarily synchronous at the same flowering stage when flowers were opening and stamens nearly reached pistils, spanning at stage 5 to 7 (Table 2-5 and Figure 2-14a). On the other hand, ovule development was arrested at the early stages, such as FG1, FG2, FG3 and FG4, in the pistils of the *cdkd;1-1/+ cdkd;3-1/-* plants (Table 2-5 and Figure 2-14b). This result implies that CDKD;1 and CDKD;3 are required for nuclear divisions during early female gametogenesis.

Occasionally, I found central cells with the abnormal shrunk nuclei at the FG7 stage in the *cdkd;1-1/+ cdkd;3-1/-* plants, suggesting the requirement of CDKD;1 and CDKD;3 for the late female gametogenesis (Figure 2-14b). The ratio of arrested ovules at the early stages to total ovules was lower than that of abortive seeds to total seeds in mature siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants pollinated with wild-type plants (Table 2-2 and Table 2-6). This also raised the possibility that development of some ovules might be arrested at the later stages, FG5 ~ FG7. To examine the fertilization ability of ovules at the later stages in the *cdkd;1-1/+ cdkd;3-1/-* plants, I tested whether the *cdkd;1 cdkd;3* ovules at the late stages could be successfully fertilized by wild-type pollen. Wild-type ovules fertilized by wild-type pollens gave rise to embryos after pollination, whereas a fraction of *cdkd;1-1/+ cdkd;3-1/-* ovules fertilized by wild-type

pollens remained at stages FG5 ~ FG7 (Figure 2-15 and Table 2-6). This result suggests that development of some *cdkd;1-1 cdkd;3-1* ovules was delayed or arrested at the late stages of female gametogenesis. Taking into consideration that some ovules remained at FG1 and FG2 even after pollination (Table 2-6), it is likely that at least a fraction of ovules were arrested at early stages of female gametogenesis. However, it is difficult to discern whether the ovule development was delayed or arrested after the FG5 stage,. More detailed time lapse analysis during female gametogenesis will be required to answer this question. Additionally, seeds containing two cell stage embryo with two endosperm nucleus were formed in pistils of the *cdkd;1-1/+ cdkd;3-1/-* plants fertilized by wild-type pollens (Figure 2-16). As mentioned above, seeds, which showed the same phenotype, were also observed in self-pollinated siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants, but not in wild-type pistils fertilized by *cdkd;1-1/+ cdkd;3-1/-* pollens. These results suggest that defects in the female side caused such a phenotype.

Table 2-1. Number and percentage of normal and abortive seeds in the *cdkd;1-1/+ cdkd;3-1/-* plants.

Genotype	Normal (%)	Abortive (%)
WT	222 (97.8 %)	5 (2.2 %)
cdkd;1-1/+ cdkd;3-1/-	311 (50.2 %)	302 (49.8 %)

Abortive seeds were defined as seeds containing no embryo at the stage when normal seeds reached maturity.

Table 2-2. Reciprocal crosses between wild-type plants and *cdkd;1-1/+ cdkd;3-1/-* plants.

Parental genotypes (Female x Male)	Normal (%)	Abortive (%)
WT selfed	765 (92.2 %)	65 (7.8 %)
WT x WT	131 (89.1 %)	16 (10.9 %)
cdkd;1-1/+ cdkd;3-1/- selfed	353 (53.4 %)	308 (46.7 %)
cdkd;1-1/+ cdkd;3-1/- x *cdkd;1-1/+ cdkd;3-1/-*	102 (50.5 %)	100 (49.5 %)
WT x *cdkd;1-1/+ cdkd;3-1/-*	738 (66.2 %)	376 (33.8 %)
cdkd;1-1/+ cdkd;3-1/- x WT	776 (60.1 %)	515 (39.9 %)

Table 2-3. Percentage of tri-, bi,- and mono-nucleate pollens and degenerated pollens produced from wild-type or *cdkd;1-1/+ cdkd;3-1* plants.

Genotype	3 nuclei (%)	2 nuclei (%)	1 nucleus (%)	Degenerated (%)
WT	88.7	6.6	1.9	2.8
cdkd;1-1/+ cdkd;3-1/-	56.7	23.6	7.4	12.3

Number of pollens used for counting was 106 for wild type and 203 for *cdkd;1/+ cdkd;3/-*. A graph based on this result is depicted in Figure 2-12b.

Table 2-4. Number and percentage of seeds produced from wild-type ovules that were fertilized by wild-type or *cdkd;1-1cdkd;3-1* pollens.

Parental genotypes (Female x Male)	Normal (%)	Abortive (%)	(Unfertilized	Degenerated)
WT x WT	115 (935 %)	8 (6.5%)	(4	4)
WT x *cdkd;1-1/+ cdkd;3-1/-*	140 (58.3%)	100 (41.7%)	(44	56)

Abortive seeds are those containing no embryo. They are classified into two groups, i.e., ‘unfertilized’ and ‘degenerated’. ‘Unfertilized’ represents ovules containing an intact egg cell and a central cell at 3DAP. ‘Degenerated’ represents shrunk seeds, in which central or egg cells could not be identified.

Table 2-5. Number of ovules arrested at different stages during female gametogenesis.

Genotype	FG1	FG2	FG3	FG4	FG5~7	Degenerated
WT	1	0	0	0	347	3
cdkd;1-1/+ cdkd;3-1/-	30	45	8	5	471	56

'Degenerated' indicates ovules whose structure was severely disrupted due to developmental impairment.

Table 2-6. Number of seeds produced from wild-type or *cdkd;1-1 cdkd;3-1* ovules that were fertilized by wild-type pollens.

Parental genotypes (Female x Male)	Normal	Abortive	(FG1	2	3	4	5	6~7	Degenerated)
WT x WT	115 (93.5%)	8 (6.5%)	(0	0	0	0	0	0	8)
cdkd;1-1/+ cdkd;3-1/- x WT	138 (54.1%)	117 (46.1%)	(5	5	0	0	37	24	46)

Abortive seeds were classified into two groups. One group consists of ovules whose development was arrested at different FG stages (FG1 to 7), and the other one is ovules with disrupted morphological structures (Degenerated).

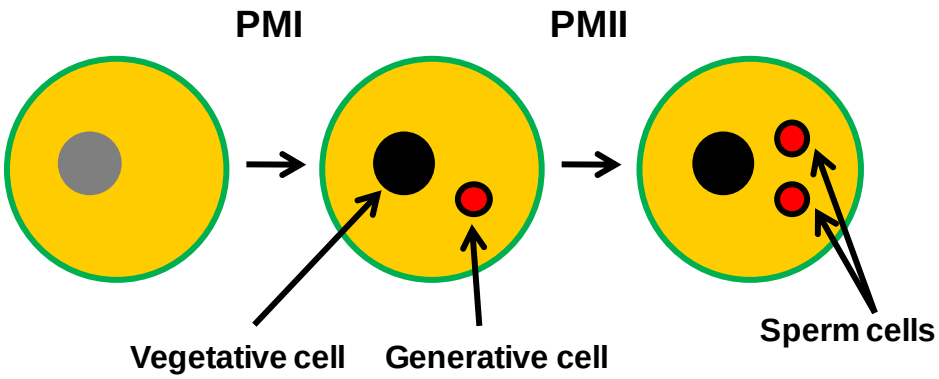

Figure 2-1. A schematic model of male gametogenesis in *Arabidopsis*.

Uninucleate cell divides asymmetrically to produce vegetative cell and generative cell in pollen mitosis I (PMI). Then, generative cell divides symmetrically to give rise to two sperm cells in pollen mitosis II (PMII).

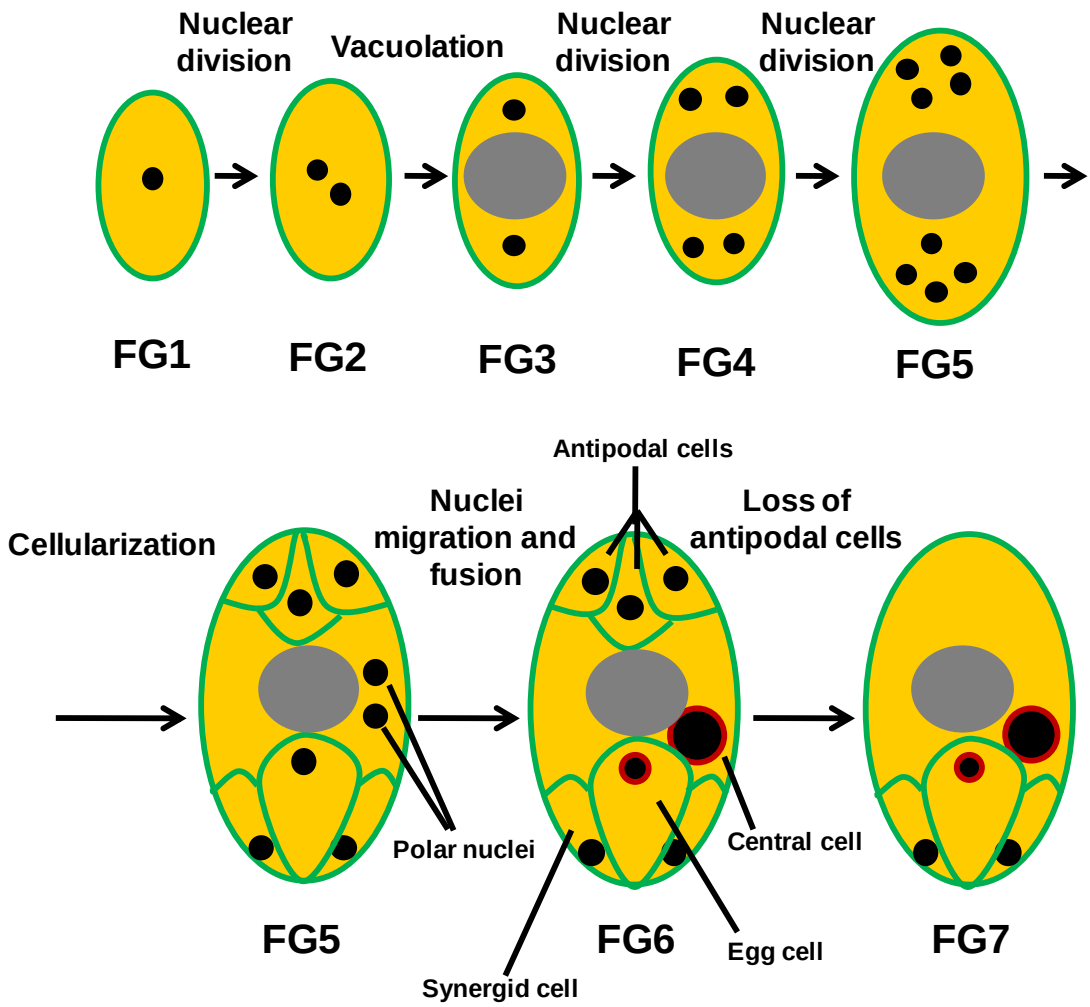

Figure 2-2. A schematic model of female gametogenesis in *Arabidopsis.*

After three rounds of nuclear divisions without cytokinesis, cellularization occurs. Two nuclei fuse and differentiate into central cell at FG6. At the last stage, antipodal cells disappear. Black circles indicate nuclei. Gray circles indicate central vacuole.

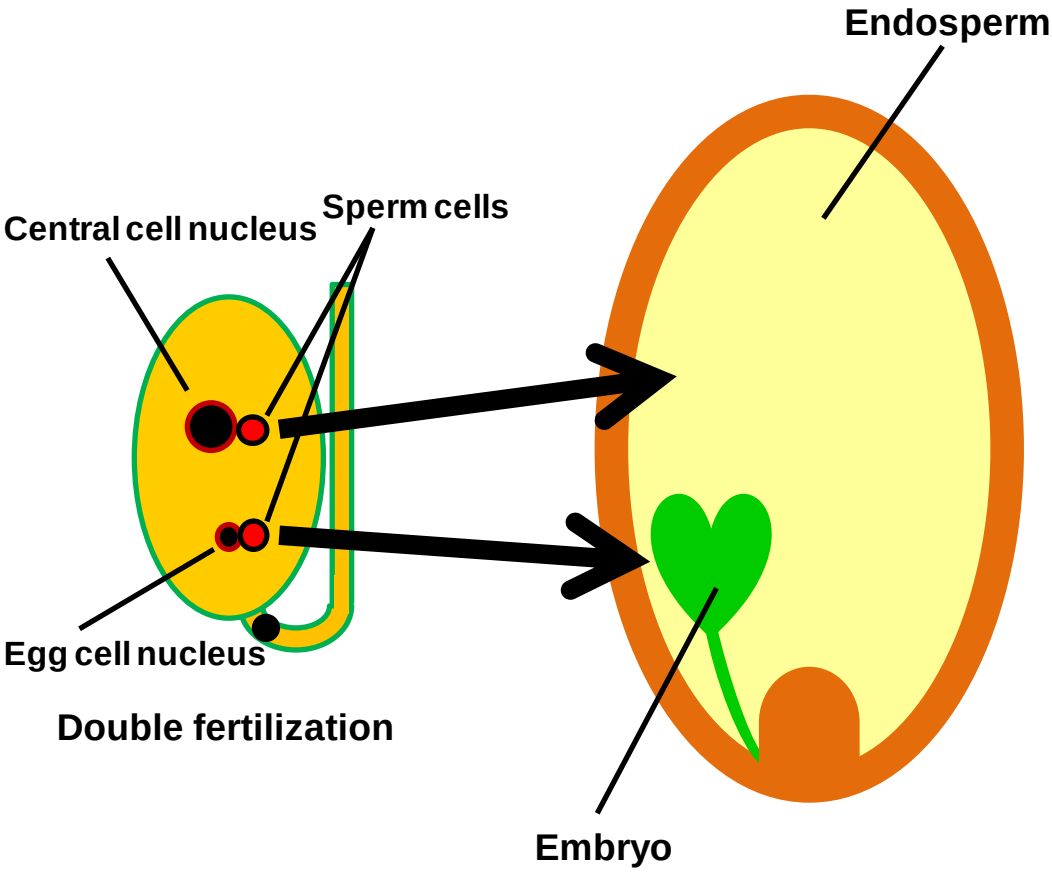

Figure 2-3. A schematic model of double fertilization and the development of embryo and endosperm in *Arabidopsis*.

Two sperm cells are delivered through the pollen tube to the embryo sac during the double fertilization process. One sperm cell fuses with the egg cell and generates the zygote, whereas the second sperm cell fuses with the central cell, giving rise to the endosperm.

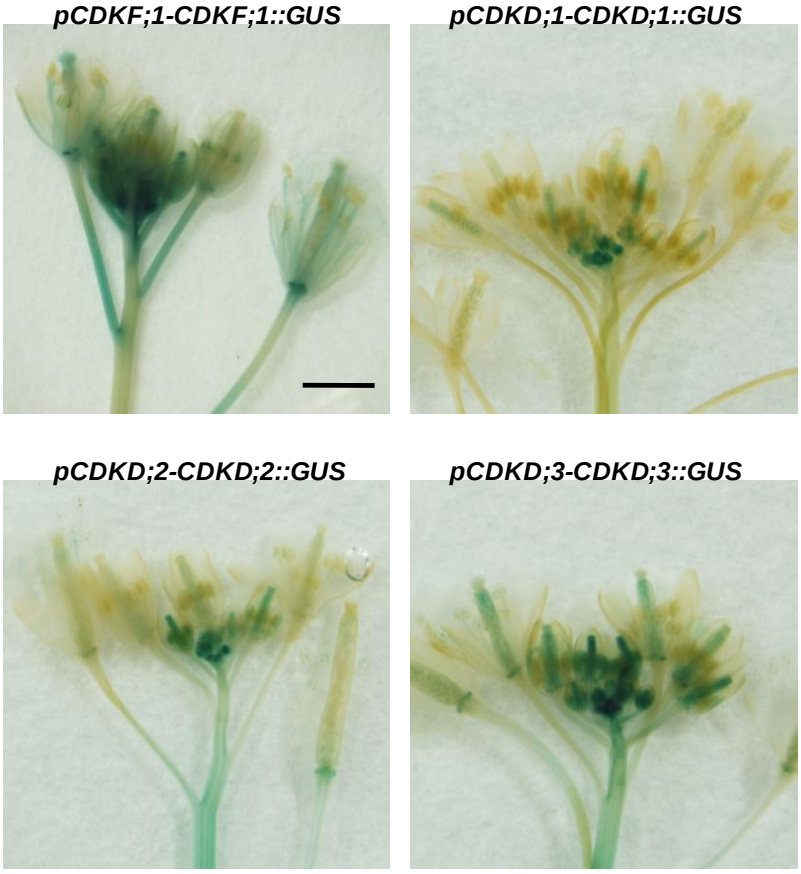

Figure 2-4. Expression patterns of CAKs in flowers.
GUS-staining of the transgenic plants harboring *pCDKF;1-CDKF;1::GUS*, *pCDKD;1-CDKD;1::GUS*, *pCDKD;2-CDKD;2::GUS* or *pCDKD;3-CDKD;3::GUS*, which are described in *Chapter I*. Bar = 1 mm.

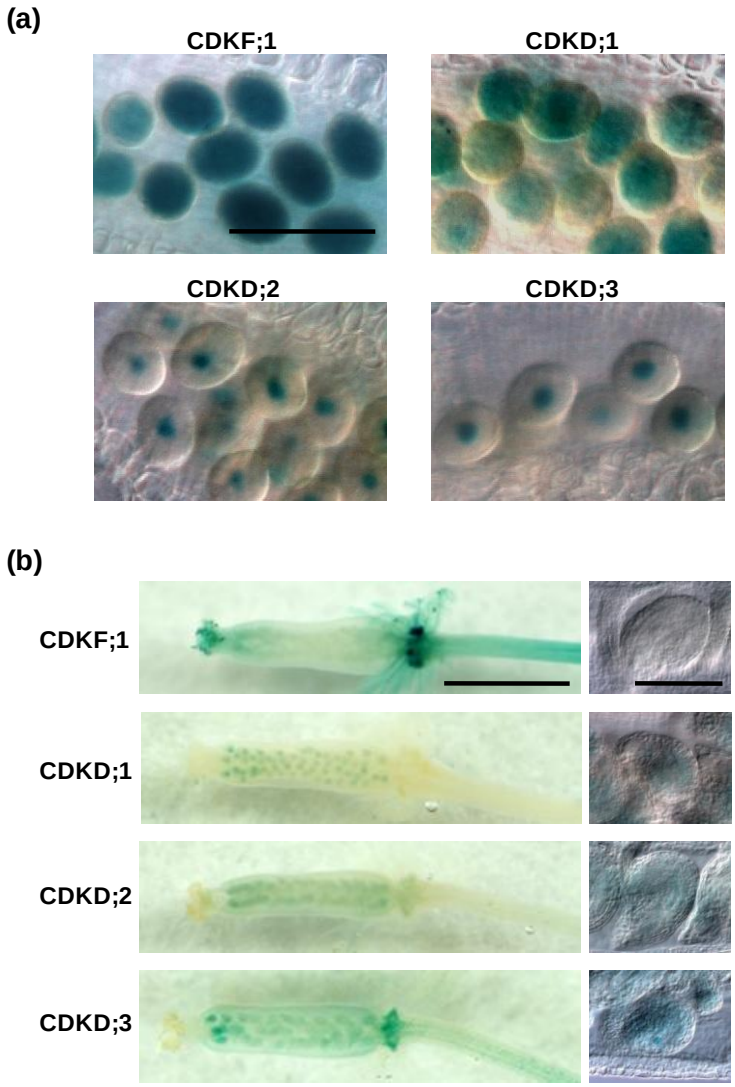

Figure 2-5. Expression patterns of CAKs in male and female gametophytes.
GUS-staining of the same transgenic plants as those in Figure 2-4. (a) Mature pollens just before pollination. Bar = 50 μm. (b) Siliques just before or after pollination (left) and ovules (right). Bars = 1 mm (left) and 50 μm (right).

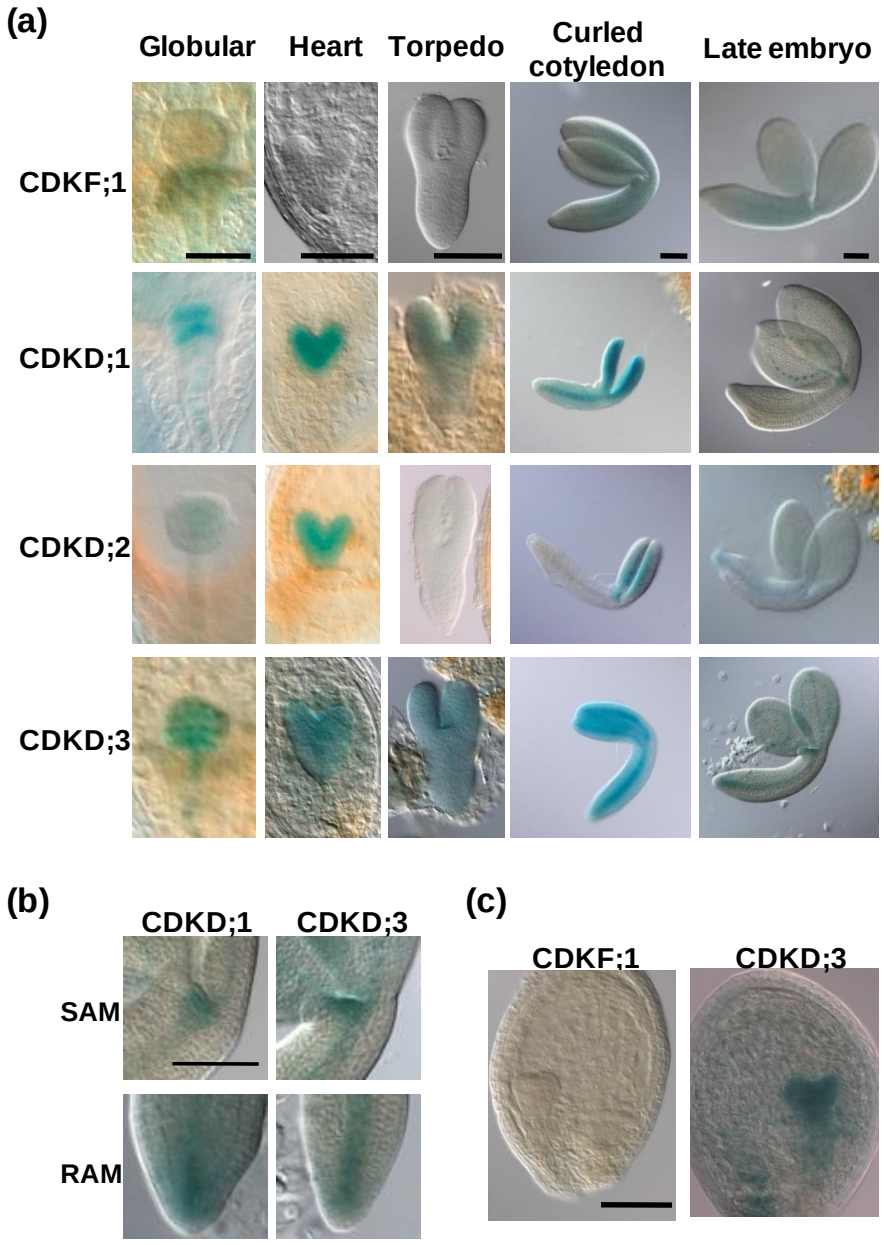

Figure 2-6. Expression patterns of CAKs in embryo and endosperm.
GUS-staining of the same transgenic plants as those in Figure 2-4. (a) Embryos at various developmental stages. Bars = 50 μm (Globular stage) and 100 μm (Heart stage to late embryo). (b) Accumulation of CDKD;1::GUS and CDKD;3::GUS in the shoot apical meristem (SAM) and the root apical meristem (RAM) of late embryos. Bar = 100 μm. (c) Endosperm at the heart stage. Bar = 100 μm.

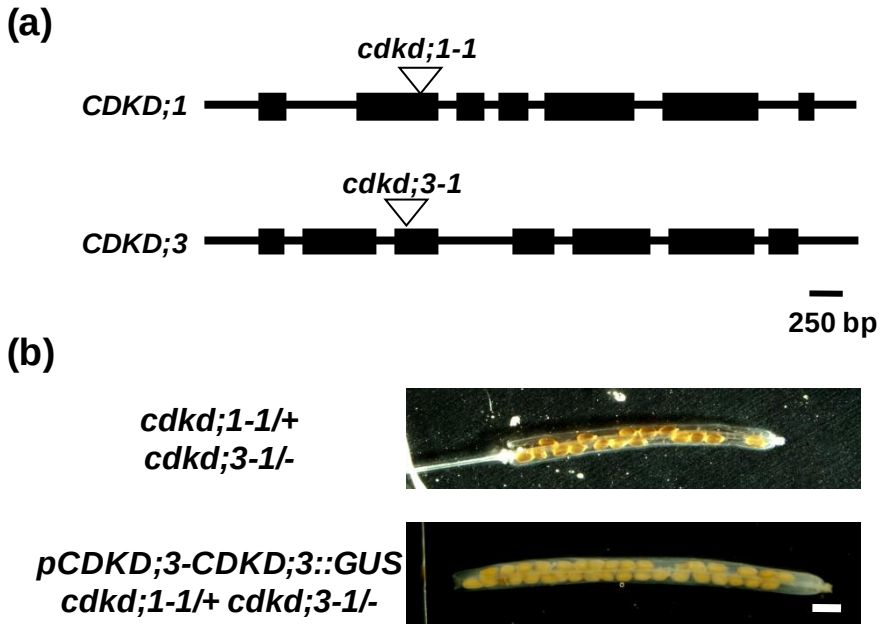

Figure 2-7. Isolation of the *cdkd;1 cdkd;3* double mutants.

(a) Schematic diagrams of the *CDKD;1* and *CDKD;3* genes. Exons and introns are indicated by black boxes and solid bars, respectively. Open triangles represent the T-DNA insertion sites. (b) Complementation of the *cdkd;1-1 cdkd;3-1* double mutants by the genomic fragment of *CDKD;3* fused to *GUS*. Bar = 1 mm.

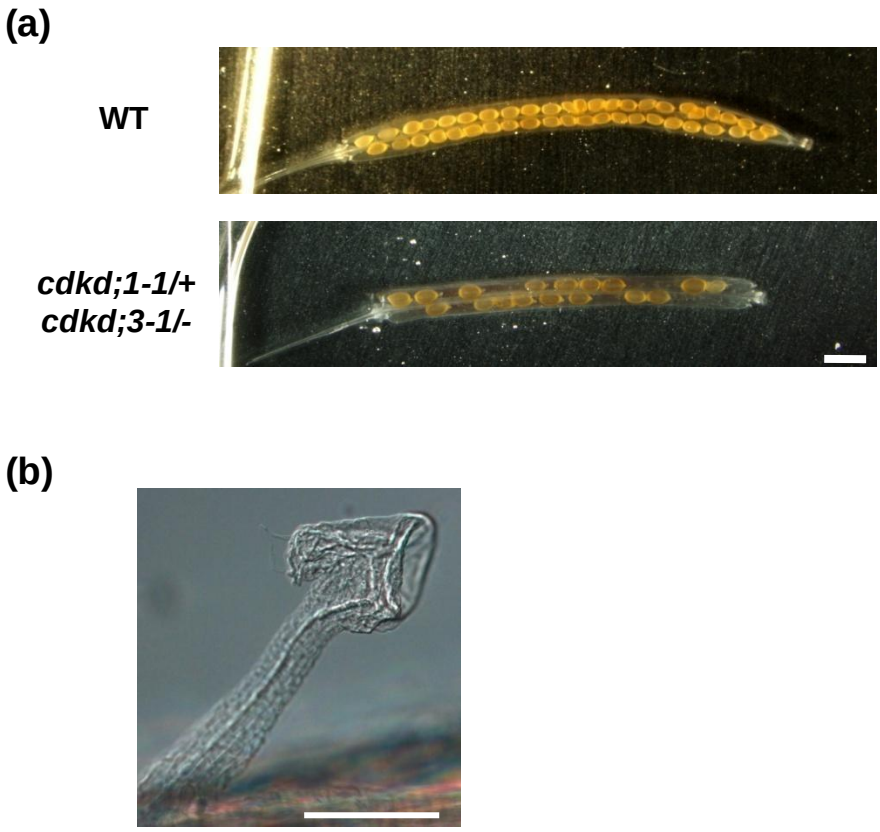

Figure 2-8. The gametophyte-lethal phenotype of the *cdkd;1 cdkd;3* double mutants.
(a) Cleared mature siliques of wild-type and *cdkd;1-1/- cdkd;3-1/-* plants. Bar = 1 mm.
(b) Abortive seeds were observed in empty spaces of the *cdkd;1-1/+ cdkd;3-1/-* siliques. Bar = 100 μm.

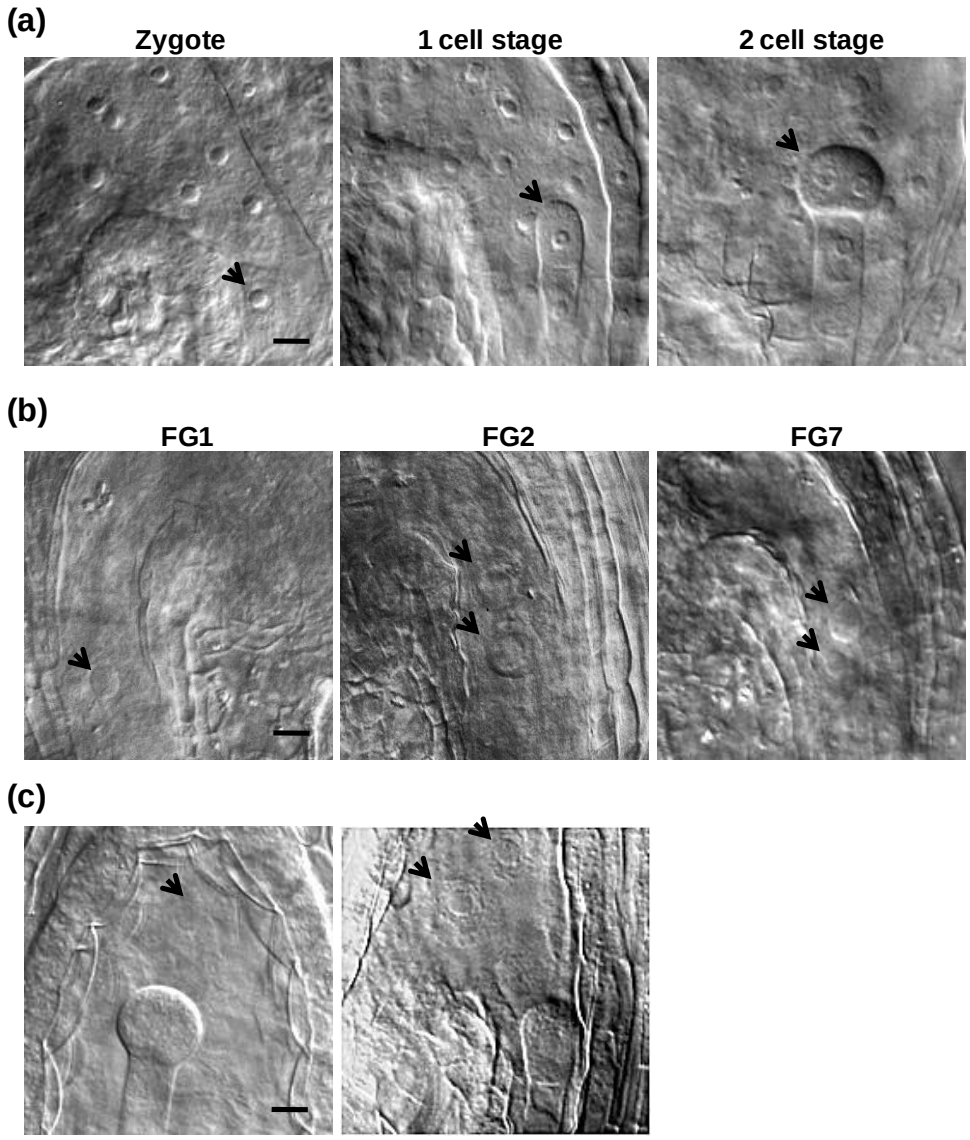

Figure 2-9. Abnormalities observed in ovules of the *cdkd;1-1/+ cdkd;3-1/-* plants after pollination.

(a) Wild-type ovules. Before the first zygotic division, at least three rounds of nuclear divisions occur in the endosperm (left panel). After embryonic cell division starts, nuclear divisions continue in the endosperm, producing an embryo surrounded by numerous endosperm nuclei (middle and right panels). Black arrows indicate zygote and embryos. Bar = 10 µm. (b) Ovules arrested at the FG1 or FG2 stage observed in siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants (left and middle panels). FG7 embryo sacs that remained unfertilized were also observed (right panel). Black arrows indicate nuclei in female gametophytes. Bar = 10 µm. (c) Occasionally, seeds with decreased endosperm nuclei were observed in siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants. Black arrows indicate endosperm nuclei. Bar = 10 µm.

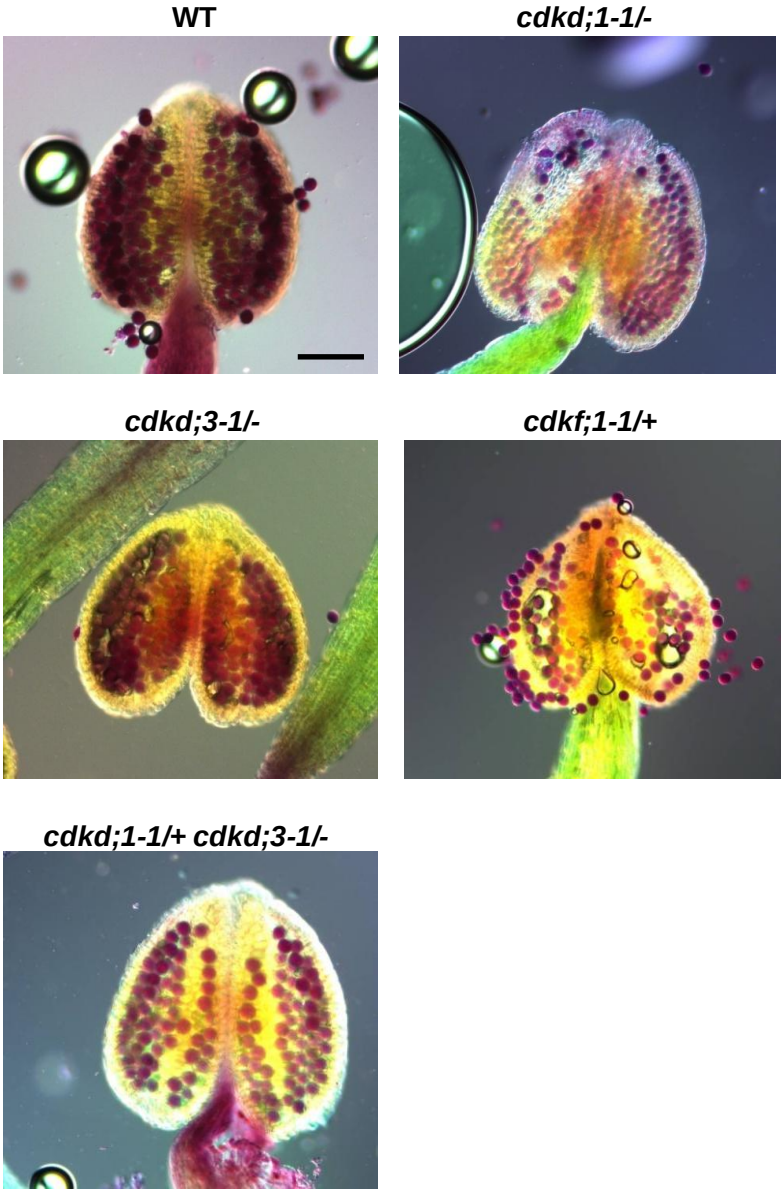

Figure 2-10. Alexander-staining of pollens of the *cdkd;1 cdkd;3* double mutants. Alexander-staining was conducted to distinguish between viable and nonviable pollen. Violet-stained pollen indicates viable pollen, and blue-stained pollen indicates nonviable pollen. Anthers just before dehiscence were stained. Bar = 100 μm.

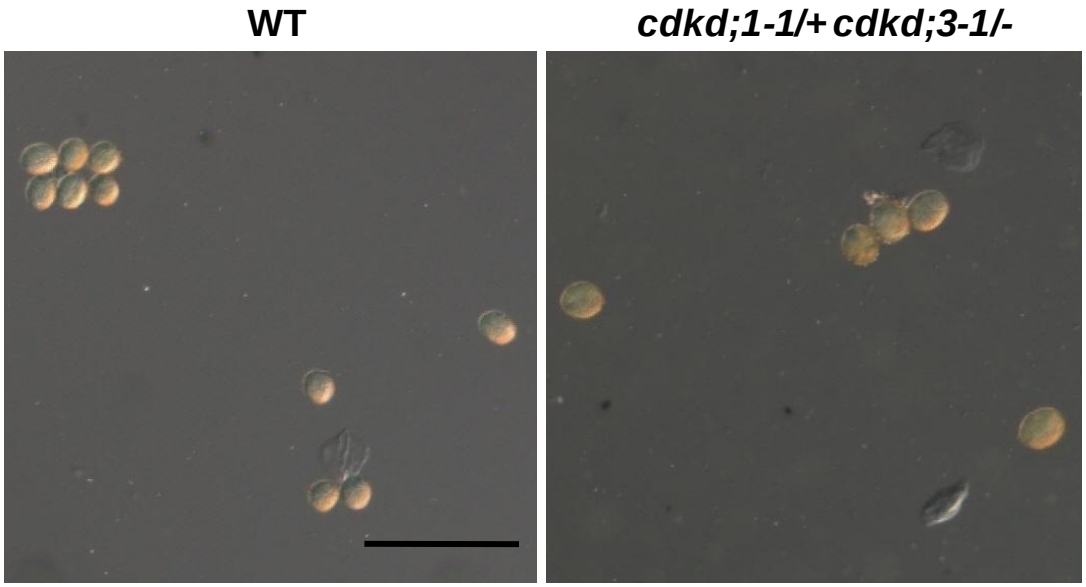

Figure 2-11. Pollen grains from the *cdkd;1-1/+ cdkd;3-1/-* plants.
Pollen grains from mature flowers of wild-plants or the *cdkd;1-1/+ cdkd;3-1/-* plants were observed with microscopy. Bar = 100 μm.

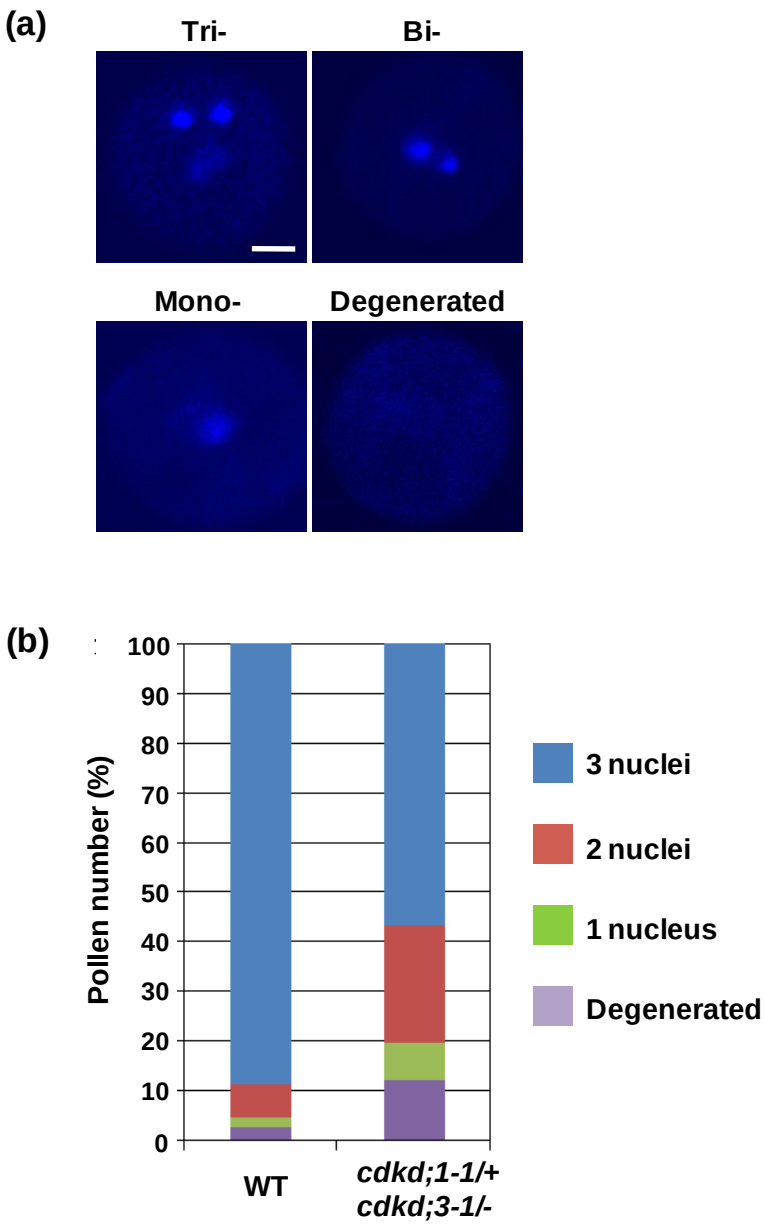

Figure 2-12. Distrurbed pollen mitosis in the *cdkd;1 cdkd;3* double mutants.
(a) DAPI-staining of mature pollens from anthers of wild-type or the *cdkd;1/+ cdkd;3/-* plants. A trinucleate (Tri-) pollen from wild-type and binucleate (Bi-) and mononucleate (Mono-) and degenerated pollens from the *cdkd;1/+ cdkd;3/-* plants are shown. Bar = 5 µm. (b) The ratioes of tri-, bi,- and mono-nucleate pollens and degenerated pollens are shown. Number of pollens used for counting was 106 for wild type and 203 for *cdkd;1/+ cdkd;3/-*.

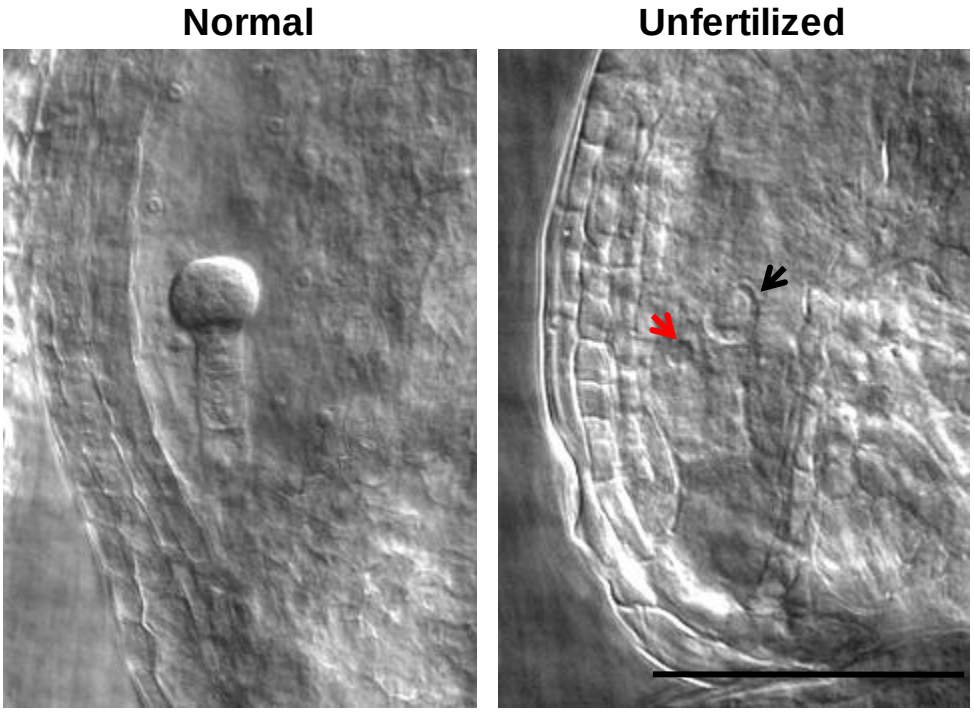

Figure 2-13. The *cdkd;1-1 cdkd;3-1* pollen cannot fertilize the wild-type ovules .
Ovules of wild-type plants fertilized by pollens from wild-type or the *cdkd;1-1/+ cdkd;3-1/-* plants were observed at 3 DAP. The black and red arrows indicate the central cell nucleus and the egg cell nucleus, respectively. Bars = 50 μm**.**

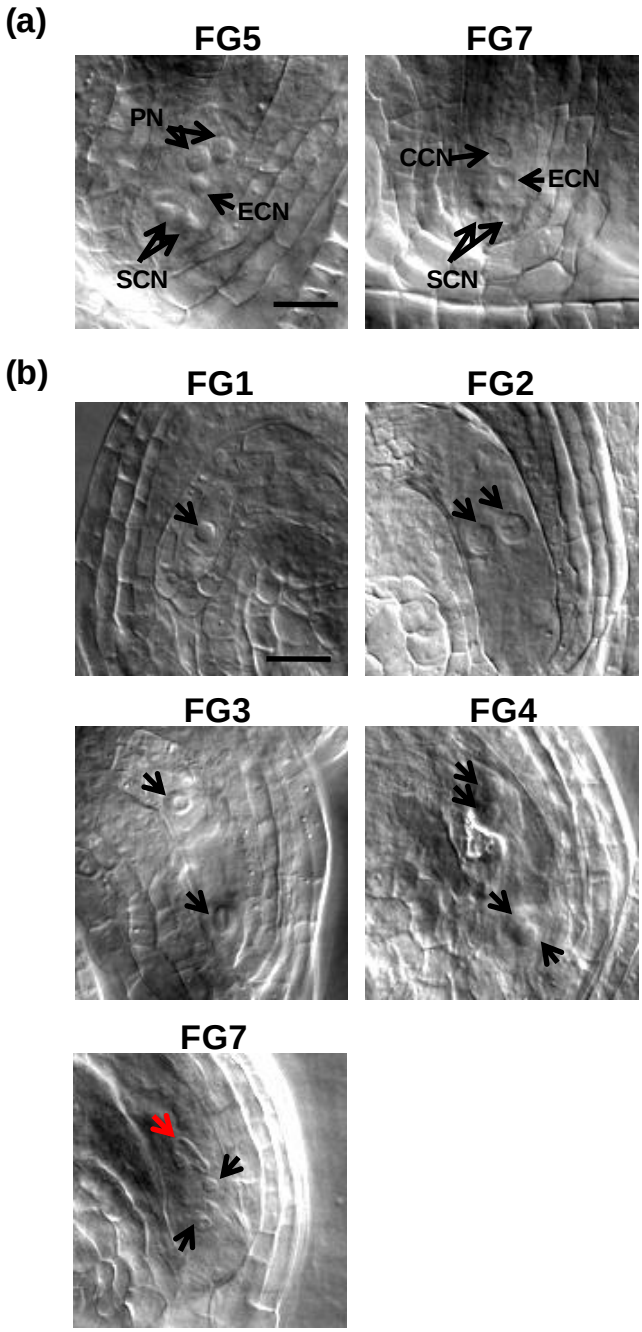

Figure 2-14. Abnormalities in female gametogenesis in the *cdkd;1-1 cdkd;3-1* double mutants.

(a) Almost all wild-type ovules progressed into the late stages of female gametogenesis, such as FG5 and FG7. PN, polar nuclei; ECN, egg cell nucleus; SCN, synergid cell nuclei; CCN, central cell nuleus. Bar = 20 μm. (b) Nuclear divisions during female gametogenesis were inhibited in the *cdkd;1 cdkd;3* double mutants. Black arrows indicate nuclei in embryo sacs. A red arrow indicates a central cell with a distorted nucleus at the FG7 stage in the *cdkd;1-1/+ cdkd;3-1/-* embryo sacs. Bar = 20 μm.

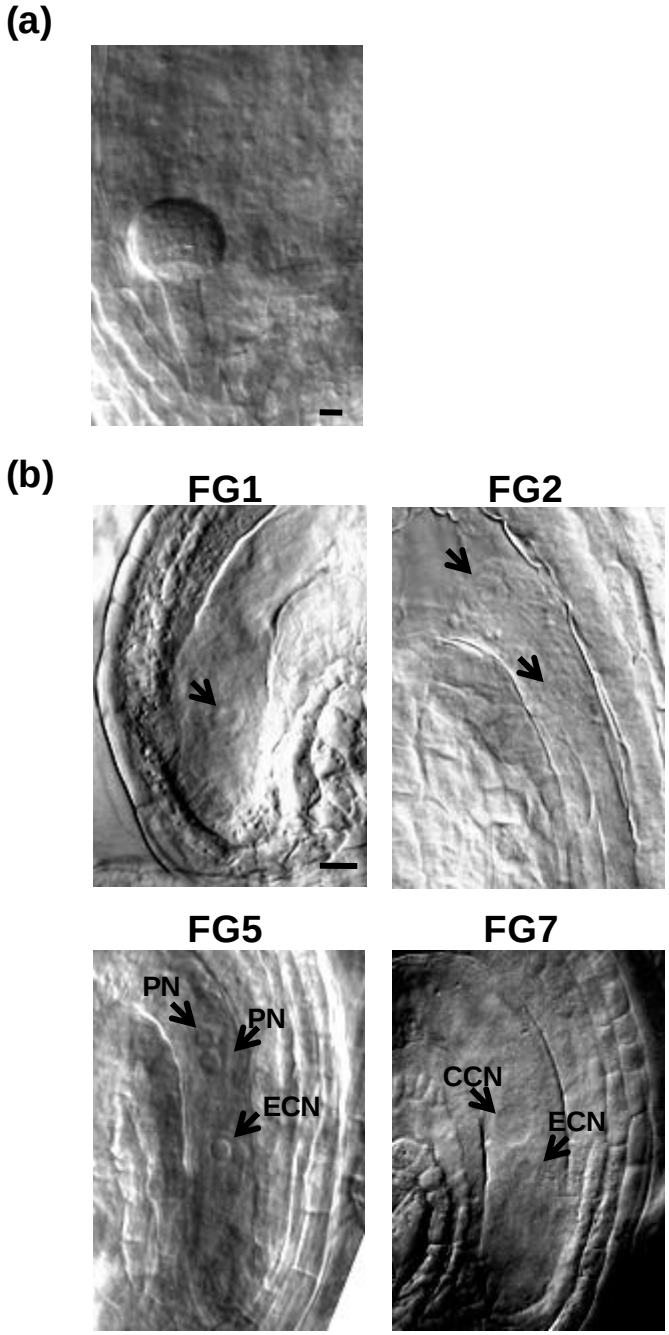

Figure 2-15. Abnormalities in ovules of the *cdkd;1-1/+ cdkd;3-1/-* plants fertilized by wild-type pollens.

(a) Wild-type ovules fertilized by wild-type pollens contain developing embryos. Bar = 10 μm. (b) Ovules observed in siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants fertilized by wild-type pollens. Ovules that were not successfully fertilized remained in different stages of female gametogenesis. Black arrows indicate nuclei in female gametophytes. PN, polar nuclei; ECN, egg cell nucleus; CCN, central cell nuleus.Bar = 10 μm.

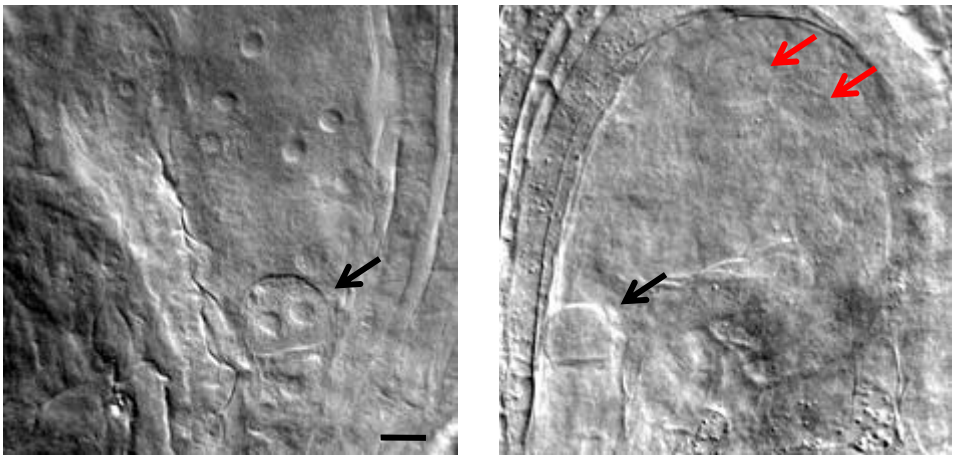

Figure 2-16. Aberrant endosperm development in the *cdkd;1-1/+ cdkd;3-1/-* ovules fertilized by wild-type pollens.

The normal seed containing an embryo and numerous endosperm nuclei (left), and the aberrant seed with only two endosperm nuclei (right). Black arrows indicate embryos. Red arrows indicate endosperm nuclei. Bar = 10 μm.

<Discussion>

In this chapter, I showed that the *cdkd;1-1 cdkd;3-1* double mutants exhibited defects in both pollen mitosis and nuclear divisions in female gametophytes. CDKD;1::GUS and CDKD;3::GUS under the control of their own promoters were highly expressed in both pollen and ovules, indicating important roles of CDKD;1 and CDKD;3 in gametogenesis. Moreover, they were also highly expressed during embryogenesis, suggesting that CDKD;1 and CDKD;3 may be the major CAKs that control gametogenesis and embryogenesis of *Arabidopsis*. In contrast, CDKF;1::GUS under the control of *CDKF;1* promoter was not expressed in ovules. This is consistent with the fact that the *cdkf;1-1* mutants exhibit no abnormality in female gametophytes. During embryogenesis, the expression level of CDKF;1::GUS was low and CDKD;2::GUS, whose stability is dependent on CDKF;1, was poorly expressed. These results suggest that the CDKF;1-CDKD;2 pathway may not be involved in cell cycle progression during embryogenesis. Exceptionally, CDKD;2 was expressed in ovules where CDKF;1::GUS expression was not detected. This implies that the stability of CDKD;2 protein is not dependent on CDKF;1 and CDKD;2 may be subjected to a different mode of stabilization and/or activation in female gametophytes.

I found disrupted pollen mitosis caused by the mutations of *cdkd;1-1* and *cdkd;3-1* pollen. Gametophytic mutants with various phenotypes in pollen development and function have been identified in *Arabidopsis* through genetic screening for segregation distortion and phenotypical screening for abnormal pollen morphology (Chen and McCormick, 1996; Bonhomme *et al*., 1998; Howden *et al*., 1998; Grini *et al*., 1999; Johnson and McCormick, 2001; Lalanne and Twell, 2002; Twell *et al*., 2002; Lalanne *et al*., 2004). Among them, several male gametophytic mutants with defects in

pollen mitosis have been characterized, such as *duo1* (*duo pollen1*), *duo3* (*duo pollen3*), *msi1* (*multicopy suppressor of ira1*) and *cdka;1* (Durbarry *et al*., 2005; Iwakawa *et al*., 2006; Nowack *et al*., 2006; Chen *et al*., 2008; Brownfield *et al*., 2009a, b). The *cdka;1* pollens fail to progress through PMII, producing one vegetative cell and only one sperm cell (Iwakawa et al., 2006; Nowack et al., 2006). However, the progression through PMI is not affected. In contrast, some of the *cdkd;1-1/+ cdkd;3-1/-* pollens possesed only one vegetative cell or one vegetative cell and one sperm-like cell, suggesting that they were impaired in the progression through PMI as well as PMII. The severer defect in pollen mitosis observed in the *cdkd;1-1 cdkd;3-1* double mutants indicates that CDKD;1 and CDKD;3 may control not only CDKA;1 but also other factor(s) that is involved in pollen mitosis. Although Nowack *et al*. (2006) argued that the *cdka;1* pollen underwent PMI by surplus CDKA;1 protein or remaining mRNA from premeiotic stages, the possibility that other CDKs, such as CDKB, may compensate for the loss of CDKA;1 cannot be eliminated. It is interesting to examine whether CDKD;1 and CDK;3 can phosphorylate and activate CDKB to promote the progression of PMI.

Another different phenotype between the *cdkd;1-1 cdkd;3-1* and the *cdka;1* pollens was found in the ability of fertilization. One sperm cell in the *cdka;1* pollen exclusively fertilizes the egg cell, while the endosperm develops without fertilization. Based on these results, Nowack *et al*. (2006) proposed a positive signal from the fertilized egg cell to initiate proliferation of the central cell. A similar observation has been reported in the *msi1* mutants of *Arabidopsis*. MSI1 is a factor associating with the two core subunits of chromatin assembly factor 1 complex (CAF1 complex), FAS1 and FAS2. Loss of function of FAS1 or FAS2 affects the S-G2 and the G2-M transitions in *Arabidopsis*, suggesting the involvement of CAF1 in cell cycle regulation. Chen *et al*.

(2008) reported that the *msi1* pollen failed to undergo PMII, producing abnormal pollens with one vegetative cell and only one sperm cell, the same as those of the *cdka;1* pollen. Crossing wild-type ovules with pollens from *msi1/+* plants produced seeds with endosperm but without embryo or those with embryo but without endosperm. However, when the wild-type ovules were pollinated with the *cdkd;1-1/+ cdkd;3-1/-* pollens, I could not find any seeds containing only embryo or endosperm. A similar phenotype has been reported in the *duo1* and *duo3* mutants (Brownfield *et al.*, 2009a, b). *DUO1* encodes a gene for R2R3 type MYB and is specifically expressed in the male gametophytes. Division of a generative cell is prevented in *DUO1*-defecient pollens, thus producing a single sperm-like cell and a vegetative cell. However, unlike the *cdka;1* and *msi1* pollens, the *duo1* single sperm-like cell never fertilizes the egg cell or the central cell. The *duo1* pollens lacked the expression of CYCB1;1, a mitotic cyclin interacting with A- and B-type CDKs. This suggests that DUO1 is involved in cell cycle progression during pollen mitosis. In addition, the expression levels of the sperm cell-specific markers are dramatically reduced in the *duo1* pollen, suggesting that gamete differentiation and function are severely impaired in *duo1*. In the null mutants of *DUO3*, a conserved gene for a cell lineage regulator of gonadogenesis in *Caenorhabditis elegans*, pollens contain an undifferentiated single sperm-like cell that cannot perform fertilization successfully. However, the *duo3* pollens, normally express CYCB1;1, indicating that DUO3 is engaged in sperm cell differentiation but not in cell cycle progression. Taken together, cell differentiation rather than cell cycle progression during PMII may be indispensable for subsequent fertilization. Although I have not tested whether the single sperm-like cell in the *cdkd;1 cdkd;3* pollen acquires the identity of sperm cell, it is likely that the mutations of *CDKD;1* and *CDKD;3* perturbed

sperm cell differentiation due to severe defects in pollen mitosis. It will be interesting to compare the differentiation state of the single sperm-like cells between the *cdkd;1-1 cdkd;3-1* double mutants and the *cdka;1* mutants.

I also revealed that CDKD;1 and CDKD;3 are required for female gametogenesis. Female gametogenesis was arrested or delayed at various stages in the *cdkd;1-1 cdkd;3-1* double mutants, suggesting that the activities of CDKD;1 and CDKD;3 are required throughout female gametogenesis. As mentioned in *Chapter I*, CDKA;1 must be activated by CAK(s) other than CDKF;1 in the *cdkf;1-1* mutants. Moreover, the pollen phenotype of the *cdkd;1-1 cdkd;3-1* double mutants was similar to that of the *cdka;1* mutants. This implies that CDKD;1 and CDKD;3 play a major role in activating CDKA;1, a central regulator of the cell cycle. However, it is still unclear how cell cycle-regulating CDKs are engaged in female gametogenesis. A previous report showed that the transmission of the null allele of *CDKA;1* through the male side was severely inhibited, whereas that through the female side was not affected. This suggests that development of the female gametophyte was not affected by the *cdka;1* mutation. Nevertheless, the requirement of CDKs for female gametogenesis has been recently suggested by Liu *et al*. (2008). They revealed that dysfunctions of *RHF1a* and *RHF2a*, RING-type E3 ligases, caused male and female gametophyte-lethality, due to the accumulation of Kip-related protein 6 (KRP6), an inhibitor of CDK-cyclin complexes. Similarly to the *cdka;1* mutants, the pollen development in the *rhf1a rhf2a* double mutants was blocked at PMII. Moreover, the development of female gametophytes was also arrested at the early stages, such as FG1 and FG2. A reduction of *KRP6* expression by RNAi largely rescued the gametophytic defects in *rhf1a rhf2a* double mutants, indicating the inhibitory role of KRP6 in both male and female gametogenesis.

Considering that the *cdka;1* mutants did not exhibit defects in female gametogenesis, Liu *et al*. (2008) proposed that KRP6 may have additional CDK targets other than CDKA;1. This hypothesis is consistent with the observation that all of the *Arabidopsis* KRP proteins, including KRP6, can bind to active complexes of CDKB-CYCD2 as well as CDKA;1-CYCD2 and inhibit the kinase activities (Nakai *et al*., 2006). The requirement of *CDKB* in female gametogenesis is also supported by a high expression during female gametogenesis (Swanson *et al*., 2005). To date, the function of CDKB in gametogenesis is absolutely unknown due to the lack of knockout mutants. Hence, isolation and characterization of loss-of-function mutants or knock-down mutants of *CDKB* will reveal the indispensable function of CDKs in female gametogeneis. It is also necessary to examine whether the expression of a constitutive active form of CDKA;1 or CDKB can suppress the female gametophyte-lethality of the *cdkd;1-1 cdkd;3-1* double mutants. On the other hand, I cannot eliminate the possibility that CDKD;1 and CDKD;3 control other unknown substrates other than CDKA and CDKB. To explore novel substrates for CDKD;1 and CDKD;3 will also give a hint how they control gametogenesis.

CDKD;1 and CDKD;3 are highly expressed in the endosperm (Day *et al*., 2008; this study), suggesting that they are also involved in endosperm development. The *cdkd;1-1/+ cdkd;3-1/-* plants fertilized by wild-type pollens produced fewer endosperm nuclei, but this phenotype would not be triggered in nuclear divisions during endosperm development, because wild-type alleles of both *CDKD;1* and *CDKD;3* genes were transmitted from the pollen into the endosperm nuclei. Therefore, the observed phenotype might be due to dysfunction of central cells during female gametogenesis. In favor of this view, I found abnormal central cells with distorted nucleus, as shown in

Figure 2-14b. Proper development of central cells is known to be essential for endosperm formation. Namely, the deficiency of AGL61, which encodes a Type I MADS domain protein exclusively expressing in the central cell and the early endosperm, leads to morphological defects of the central cell (Bemer *et al*., 2008; Steffen *et al*., 2008). When fertilized by wild-type pollens, *agl61* central cells fail to give rise to the endosperm, similarly to the severe phenotype of the *cdkd;1-1 cdkd;3-1* ovules fertilized by wild-type pollens. It is worthy to examine whether the central cell is specified successfully in the *cdkd;1-1 cdkd;3-1* double mutants.

In this chapter, I revealed that CAK activities are indispensable for mitosis during gametogenesis. Gamtophytes are usually generated through mitosis after meiotic programs, which are essential for eukaryotic sexual reproduction. As studied in animals, progression of not only mitosis but also meiosis during plant gametogenesis is assumed to require CDK activities. In fact, a weak *cdka;1* allele produces no pollen, and arrests the female gametophyte development right after or at meiosis. Moreover, *Arabidopsis* mutants of CYCA1;2, which is a binding partner of CDKA;1, showed defects in the progression of meiotic programs (Wang *et al*., 2004). Here I did not focus on meiosis, as the next step, it will be interesting to investigate how CAK activities are required for meiosis progression.

<Materials & methods>

Plant material

Arabidopsis thaliana (ecotype Col-0) was first grown at 23°C on Murashige and Skoog agar plate (Murashige *et al*., 1962) under continuous light conditions; it was then transferred onto soil and grown under continuous light conditions.

Isolation of the cdkd;1-1 cdkd;3-1 double mutants

The T-DNA insertion mutants of *CDKD;1* and *CDKD;3* were isolated from the collections of the Max-Planck-Insititute für Züchtungsforschung and the Salk Institute; the seed stock numbers of *cdkd;1-1* and *cdkd;3-1* are MPI8258 and SALK_120536, respectively (Shimotohno *et al*., 2006). The insertions were examined by genomic PCR with Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) by using a set of primers that hybridize to the T-DNA and each *CDKD*: 5' -CTGGGAATGGCGAAATCAAGGCATC-3' and 5' -GTTGCTGATAGGTATCTAAAGCGAGAGGT-3' were used for *cdkd;1-1*, and 5' -GGATTTTCGCCTGCTGGGGCAAACCAGCGT-3' and 5' -CAGCCAAAGAAAGTTGCTGATAGGTATCTC-3' were used for *cdkd;3-1*. To amplify the genomic region encompassing T-DNA insertion sites, I used a set of primers as follows: 5'-GTTGCTGATAGGTATCTAAAGCGAGAGGT-3' and 5' -TGGCCGCTCAGCTAAGATGGTGAAA-3' for *CDKD;1*, and 5' -CAGCCAAAGAAAGTTGCTGATAGGTATCTC-3' and 5' -GTGATTGTTGGCCTGTTTGGAGGTC-3' for *CDKD;3*. To perform the complementation test, the destination vector pGWB3, in which the genomic fragment of *CDKD;3* was cloned, was introduced in the *cdkd;1-1/+ cdkd;3-1/-* plants (See Material

& methods in *Chapter I*), and the siliques of T1 plants were observed.

Microscopic observations

To observe seed development in siliques, green mature siliques were harvested and fixed in a solution of 90% ethanol and 10% acetic acid at 4°C overnight, hydrated through a graded series of ethanol and stored in water at 4°C. The samples were mounted on a glass slide and cleared with a chloral hydrate solution (71% chloral hydrate and 11% glycerol) during overnight incubation. The number of seeds was counted under this condition.

The morphology of mature pollens from anthers just before dehiscence was observed with the light microscope. Pollen viability was assessed using Alexander staining (Alexander, 1969; Ravi *et al*., 2008). Anthers that were about to dehisce were dissected in a drop of Alexander staining solution (9.5% ethanol, 0.1 mg/ml Malachite green, 25% of glycerol, 0.05 g /ml phenol, 0.05 g/ml chloral hydrate, 0.5 mg/ml acid fuchsin, 0.05 mg/ml Orange G and 2% acetic acid; the pH of the staining solution was adjusted to 2.3). The anthers were then transferred to a fresh slide containing a drop of Alexander staining solution. A coverslip was placed on the anthers and gently pressed so that the staining solution penetrated the anthers. The slides were then incubated at 37 °C for 6 h. Images were observed under bright field.

Nuclei in pollen grains were visualized essentially as described by Park *et al.* (1998) and Iwakawa *et al.* (2006). Open flowers were collected and immersed in a DAPI staining solution [100 mM sodium phosphate (pH7.0), 1 mM EDTA, 0.1% Triton X-100, 0.4 µg/ml)1 DAPI]. After brief mixing and centrifugation, pelleted pollen grains were transferred onto glass slides. Microscopic observation was performed using a

confocal laser scanning microscope system LSM510 (Zeiss, Thornwood, NY, USA) with UV excitation.

To observe the developing female gametophytes, flowers at the early phase of floral stage 13 were subjected to microscopic analyses. Floral stages were described by Bowman (1994). At the early phase of floral stage 13 when flower was opening and stamens nearly reached pistils, the female gametogenesis progressed into FG5 to FG7, as described by Yu *et al*. (2005). Sepals, petals and stamens were removed from collected flowers, and remained pistils were fixed and cleared (Malamy and Benfey, 1997).

GUS staining

For GUS staining, the transgenic plants harboring *pCDKF;1-CDKF;1::GUS*, *pCDKD;1-CDKD;1::GUS*, *pCDKD;2-CDKD;2::GUS* or *pCDKD;3-CDKD;3::GUS*, which are described in *Chapter I*, were used. Siliques and pollens were collected from flowers at floral stage 13. Seedlings were fixed in 90% acetone for 15 min on ice, washed with the GUS buffer [100 mM sodium phosphate buffer (pH7.0), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$], and immersed in the same buffer containing 0.5 mg/ml X-gluc (5-bromo-4-chloro-3-indoryl-β-D-glucuronide). Then, these samples were degassed for 45 min, and incubated at 37°C overnight.

Chapter III

Distinct functions of CAKs during Arabidopsis development

<Abstract>

In *Chapter I* and *Chapter II*, I revealed that the *cdkf;1-1* mutants had severe defects in cell division and cell elongation during post-embryonic development, whereas the *cdkd;1-1 cdkd;3-1* double mutants exhibited the defects in mitosis during gametogenesis. My data also suggested that these contrasting results might be due to the different expression profiles of CAKs. However, it is possible that the functional divergence of CAKs during plant development is caused by their different molecular functions, since *in vitro* analyses previously indicated that the four CAKs have partially overlapping but distinct enzyme activities (Shimotohno *et al*., 2004). Hence, I conducted the genetic analyses to compare the molecular functions between CDKD (CDKD;1 and CDKD;3) and CDKF during gametogenesis and post-embryonic development. Ectopic expression of CDKD under the control of *CDKF;1* promoter could not restore the severe phenotypes of the *cdkf;1-1* mutants. Furthermore, ectopically expressed CDKF;1 under the control of *CDKD;3* promoter could not rescue the gameophyte-lethal phenotypes of the *cdkd;1-1 cdkd;3-1* double mutants. The *cdkd;3-1 cdkf;1-1* double mutants showed no obvious phenotype during gametogenesis and exhibited the same phenotype as that of the *cdkf;1-1* mutants during post-embryonic development. These results suggest that the *in vivo* functions of CDKD (CDKD;1 and CDKD;3) and CDKF are diverged from each other and play an important role in plant development in a distinct manner.

<Introduction>

Unicellular organisms, such as budding yeasts and fission yeasts, require a single cyclin-dependent kinase Cdc28 and Cdc2, respectively, to drive cell division. In contrast, mammalian cells are thought to require the sequential activation of at least four different CDKs, CDK2, CDK3, CDK4 and CDK6, to drive cells through interphase (G1-S-G2), as well as CDK1 to proceed through mitosis (G2-M). Each CDK has been shown to exhibit the maximum activity at different stages of the cell cycle and have different substrates. CDK4 and CDK6, which bind to CYCD, show the highest activity at the early G1/S phase. The molecular function of CDK4 is to phosphorylate Rb and Smad3 and inhibit their antiproliferative functions. Rb is known as the sole substrate of CDK6 so far. CDK2–CYCE functions mainly at the late G1/S transition and its action is almost complementary to CDK4/6-CYCD. The CDK2-CYCE complex phosphorylates Rb and Smad3 similarly to CDK4 and CDK6. Other than Rb and Smad3, CDK2 phosphorylates p27 (an inhibitor of CDK), Skp2 (an E3 ubiquitin ligase of p27), and FOXO1 (an inducer of apoptosis) to promote G1/S progression (Montagnoli *et al*., 1999; Huang *et al*., 2006; Rodier *et al*., 2008). CDK3 also phosphorylates and inhibits Rb, but this phosphorylation event is restricted to the G0 exit (Ren and Rollins, 2004). CDK1 phosphorylates numerous substrates that facilitate important mitotic events during G2/M phase. In spite of such divergent functions of CDKs in different cell cycle phases, it has been reported that CDK1 is sufficient to drive the cell cycle in mammalian cells (Santamaría *et al*., 2007). Recent genetic evidence has proved that interphase CDKs are not essential for *in vivo* cell divisions. For instance, most laboratory strains of mice are deficient in CDK3 owing to a naturally occurring mutation (Ye *et al*., 2001). Moreover, CDK4 and CDK6 are not essential for prompting cells to exit from the quiescent state

(Malumbres *et al*., 2004). Likewise, cells lacking CDK2, a kinase that was previously assumed to be essential for driving the S phase, divide normally (Berthet *et al*., 2003; Ortega *et al*., 2003), and mouse embryonic fibroblasts (MEFs) lacking CDK4 and CDK2 can proliferate (Barrière *et al*., 2007). Mice with single or double CDK mutations showed proliferative defects, but only in specific cell types. Furthermore, mouse embryos lacking all interphase CDKs (CDK2, CDK3, CDK4 and CDK6) underwent organogenesis and developed to midgestation. In these embryos, CDK1 binds to all cyclins, resulting in the phosphorylation of Rb and the expression of genes that are regulated by E2F transcription factors. These results indicate that CDK1 is the sole essential CDK. Indeed, only mice lacking CDK1 drive impaired cell divisions during the very early stages of embryonic development, which indicates that CDK1 kinase activity cannot be compensated by interphase CDKs (Santamaría *et al*., 2007). These observations suggest that CDKs possess a high plasticity. In the absence of interphase CDK, CDK1 can interact with all cyclins and exhibit the kinase activities even in cell cycle phases at which CDK1 is originally inactive. These findings imply that the functional divergence of CDKs is guaranteed by both the biochemical features and the timing when CDKs exhibit the maximum activity by associating with phase-dependent cyclins.

As mentioned in *General introduction*, *Arabidopsis* four CAKs phosphorylate CDK and RNA polymerase II (RNAPII) with distinct preferences *in vitro* (Shimotohno *et al*., 2004), whereas the molecular function of CDKD;1 remains unknown. CDKD;2 has a major function in phosphorylation of RNAPII and a minor function in CDK-activation. Conversely, the major function of CDKD;3 is CDK-activation and the minor one is phosphorylation of RNAPII. CDKF;1 is exclusively involved in

phosphorylation and activation of CDKs, including CDKDs. Such partially overlapping biochemical features indicate that CAK functions may be redundant during plant development.

In *Chapter I* and *Chapter II*, I showed that CDKF;1 has an essential role in post-embryonic development bun not in embryogenesis and gametogenesis, whereas CDKD;1 and CDKD;3 are prerequisite for gametogenesis. I also found that CDKD;1 and CDKD;3, but not CDKF;1, are highly expressed during early development. On the other hand, Shimotohno *et al*. (2004) reported that CDKD;3 and CDKF;1 have partially overlapping but distinct biochemical features, as mentioned above. These findings raised two possibilities in terms of divergent functions of CDKD and CDKF during plant development. One is that the different expression profiles between CDKD and CDKF caused such a divergence in *in vivo* functions. The other possibility is that their molecular functions are so diverged to contribute to different aspects in plant development. To distinguish these two possibilities, I conducted genetical analyses by using chimeric CAK genes.

<Results>

Ectopic expression of CDKD::GUS under the control of* CDKF;1 *promoter cannot rescue the* cdkf;1 *phenotypes

To compare the functions between CDKD and CDKF in post-embryonic development, I examined whether ectopically expressed CDKD;1::GUS or CDKD;3::GUS under the control of *CDKF;1* promoter was able to rescue the severe phenotypes of the *cdkf;1-1* mutants. The plasmid constructs used for the promoter-swapping experiments are shown in Figure 3-1. In *Chapter I*, I showed that CDKF;1 expression under the control of *CDKF;1* promoter was able to suppress the phenotypes of the *cdkf;1-1* mutants, indicating that the cloned promoter region was enough to express a sufficient amount of *CDKF;1*. In addition, as shown in *Chapter II*, GUS-fused CDKD;3 protein is functional, thus the expression of CDKD;3::GUS under the control of *CDKF;1* promoter mimics expression of the functional CDKD;3 in tissues where *CDKF;1* transcripts usually accumulate. I did not examine the functionality of CDKD;1::GUS, but it might be functional, because CDKD;1 is highly homologous to CDKD;3 in amino acid sequences.

First, I tested whether the promoter swapping successfully works or not. I characterized differences in the expression patterns between CDKF;1::GUS and CDKD::GUS (CDKD;1::GUS, CDKD;3::GUS) under the control of their native promoters (Figure 3-2). In shoots, both CDKD::GUS and CDKF;1::GUS were strongly expressed in the apical meristems. However, the fluctuation of the expression level during leaf development was different between them. CDKF;1::GUS under the control of *CDKF;1* promoter (designated as *F;1-F;1::GUS*) was highly expressed in developing 3rd leaves, but its expression was also sustained in the vascular bundles of mature

cotyledons and 1st leaves. In contrast, the expressions of CDKD;1::GUS and CDKD;3::GUS under the control of their own promoters (designated as *D;1-D;1::GUS* and *D;3-D;3::GUS*, respectively) were detected in young 3rd leaves but not in vascular bundles of mature cotyledons and 1st leaves. The expressions of CDKD;1::GUS and CDKD;3::GUS under the control of *CDKF;1* promoter (designated as *F;1-D;1::GUS* and *F;1-D;3::GUS*, respectively) were detected not only in young 3rd leaves but also in the vascular bundles of mature cotyledons and 1st leaves (Figure 3-2). These results indicate that the expression patterns of *F;1-D;1::GUS* and *F;1-D;3::GUS* reflect that driven by the *CDKF;1* promoter in shoots. In roots, *F;1-F;1::GUS*, *D;1-D;1::GUS* and *D;3-D;3::GUS* were mainly expressed in apical meristem (Figure 3-3). *F;1-F;1::GUS*, but not *D;1-D;1::GUS* or *D;3-D;3::GUS*, was also expressed in the mature columella cells. Both *F;1-D;1::GUS* and *F;1-D;3::GUS* were expressed in mature columlla cells as well as in the meristematic region (Figure 3-3). These results indicate that the expression patterns of *F;1-D;1::GUS* and *F;1-D;3::GUS* mimic that of *F;1-F;1::GUS* in roots. Taken together, I concluded that promoters were successfully swapped between *CDKD* and *CDKF*.

I then observed transgenic lines of the *cdkf;1-1* mutants harbouring *F;1-D;1::GUS* or *F;1-D;3::GUS*. As negative controls, the non-transformed *cdkf;1-1* mutants and the *cdkf;1-1* mutants harbouring *D;1-D;1::GUS* or *D;1-D;3::GUS* were examined. As shown in Figure 3-4, the *cdkf;1-1* mutants harbouring *D;1-D;1::GUS* or *D;3-D;3::GUS* exhibited severe phenotypes at both 4 and 10 days after germination, such as a dwarfism, a retarded growth and a defect in root gravitropism, which are observed in the non-transformed *cdkf;1-1* mutants. This indicates that the expression of CDKD;1 or CDKD;3 under the control of their own promoters does not affect the

growth of the *cdkf;1-1* mutants. Previously, I reported that the size of the root meristem and the number of columella cell layers were reduced in the *cdkf;1-1* mutants (Takatsuka *et al*., 2009). These phenotypes were neither rescued by *F;1-D;1::GUS* and *F;1-D;3::GUS* (Figure 3-5a, b), and the growth defect was not suppressed (Figure 3-4). These results suggest that the not only the expression patterns but also the molecular functions of CDKD;1 and CDKD;3 are divergent from that of CDKF;1 during post-embryonic development.

The* cdkf;1 *phenotypes are not enhanced by the* cdkd;3 *mutation

To further analyze the relationship of molecular functions between CDKD and CDKF, I generated the double knock-out mutants of *CDKD;3* and *CDKF;1*. While the *cdkf;1-1/+* and *cdkd;3-1/- cdkf;1-1/+* seedlings grew normally, the seedlings of the *cdkd;3-1 cdkf;1-1* double mutants exhibited the growth inhibition similar to that observed in the *cdkf;1-1* mutants at both 4 and 10 days after germination (Figure 3-6a, b). In roots, the *cdkd;3-1 cdkf;1-1* mutants had a reduced size of meristem and reduced number of columella cell layers as observed in the *cdkf;1-1* mutants (Figure 3-7a, b). These results again suggest that the molecular function of CDKD;3 is divergent from that of CDKF;1 during post-embryonic development.

Ectopic expression of CDKF;1::GUS under the control of* CDKD;3 *promoter cannot rescue the lethal phenotype of the* cdkd;1 cdkd;3 *double mutants

Next, to examine whether the molecular function of CDKD;3 can be exchangeable with that of CDKF;1 during gametogenesis, I conducted a promoter swapping experiment in which the *CDKF;1* coding region fused to *GUS* was placed under the control of

CDKD;3 promoter and introduced into the *cdkd;1-1/+ cdkd;3-1/-* plants. Prior to this analysis, I tested the functionality of GUS-fused CDKF;1 protein. As shown in Figure 3-8a, the phenotypes of the *cdkf;1-1* mutants, which were described in detail in *Chapter I*, were fully complemented by expression of the CDKF;1::GUS fusion protein under the control of *CDKF;1* promoter (*F;1-F;1::GUS*), indicating the functionality of the CDKF;1::GUS fusion protein. Although *CDKF;1* promoter activity was detected in mature columella cells (Figure 3-8b), CDKF;1::GUS under the control of *CDKD;3* promoter (designated as *D;3-F;1::GUS*) was not expressed in the mature columella cells, which mimics the expression pattern of *D;3-D;3::GUS* (Figure 3-8b). Moreover, *D;3-F;1::GUS* was detected in the ovules where the *CDKF;1* promoter activity was absent (Figure 3-8c). These observations indicate that the transgenic plants harbouring *D;3-F;1::GUS* express the functional CDKF;1::GUS protein in a manner reflecting the *CDKD;3* promoter activity.

Then, I examined the seed development of transgenic lines of the *cdkd;1-1/+ cdkd;3-1/-* mutants harbouring *D;3-F;1::GUS*. As shown in Table 3-1 and Figure3-9, about a half of seeds in the siliques of the non-transformed *cdkd;1-1/+ cdkd;3-1/-* plants was abortive, which was described in *Chapter II*. Likewise, in the siliques of the *cdkd;1-1/+ cdkd;3-1/-* plants harbouring *D;3-F;1::GUS*, a half of seeds failed to develop. These results suggest that ectopically expressed CDKF;1::GUS under the control of *CDKD;3* promoter has no influence on the gametophyte-lethal phenotype of the *cdkd;1-1 cdkd;3-1* double mutants.

Seed development of the* cdkd;3 *and* cdkd;1 *mutants is not affected by the* cdkf;1 *mutation.

To further examine whether the molecular function of CDKD;3 overlaps with that of CDKF;1 during gametogenesis, I observed the seed development of the above-mentioned *cdkd;3-1 cdkf;1-1* double mutants. The siliques of wild-type plants and the *cdkf;1-1/+* plants, whose gametophyte development are not aberrant as described in *Chapter I* and *Chapter II*, were filled with developed seeds as expected (Figure 3-10 and Table 3-2). Also, in the siliques of the *cdkd;3-1/- cdkf;1-1/+* plants, almost all seeds developed normally (Figure 3-10 and Table 3-2). Additionally, seed development was not defective in the *cdkd;1-1/- cdkf;1-1/+* plants (Figure 3-10 and Table 3-2). These results demonstrate that both *cdkd;3-1 cdkf;1-1* and *cdkd;1-1 cdkf;1-1* double mutants can accomplish gametogenesis and embryogenesis, which is in contrast to the *cdkd;1-1 cdkd;3-1* double mutants. Therefore, I concluded that the molecular functions of CDKD;3 and CDKD;1 do not overlap with that of CDKF;1 during gametogenesis and embryogenesis.

Table 3-1. Seed development of the *cdkd;1-1/+ cdkd;3-1/-* plants harbouring *D;3-F;1::GUS*.

Parental genotypes	Normal seeds (%)	Abortive seeds (%)
WT	171 (97.2%)	5 (2.8%)
cdkd;1-1/+ cdkd;3-1/-	83 (55.7%)	66 (44.3%)
D;3-F;1::GUS cdkd;1-1/+ cdkd;3-1/-	64 (52.0%)	59 (48.0%)

Numbers of normal seeds and abortive were counted with cleared mature siliques from self-pollinated wild-type plants, the non-transformed *cdkd;1-1/+ cdkd;3-1/-* plants and the *cdkd;1-1/+ cdkd;3-1/-* plants harbouring *D;3-F;1::GUS*.

Table 3-2. Seed development of the *cdkd;3-1/- cdkf;1-1/+* plants and *cdkd;1-1/- cdkf;1-1/+* plants.

Parental genotypes	Normal seeds (%)	Abortive seeds (%)
WT	171 (96.8%)	6 (3.2%)
cdkf;1-1/+	163 (91.6%)	15 (8.4%)
cdkd;3-1/- cdkf;1-1/+	175 (97.8%)	4 (2.2%)
cdkd;1-1/- cdkf;1-1/+	194 (92.8%)	15 (7.2%)

Numbers of normal seeds and abortive were counted with cleared mature siliques from self-pollinated wild-type plants, the *cdkf;1-1/+* plants, *cdkd;3-1/- cdkf;1-1/+* plants and the *cdkd;1-1/- cdkf;1-1/+* plants.

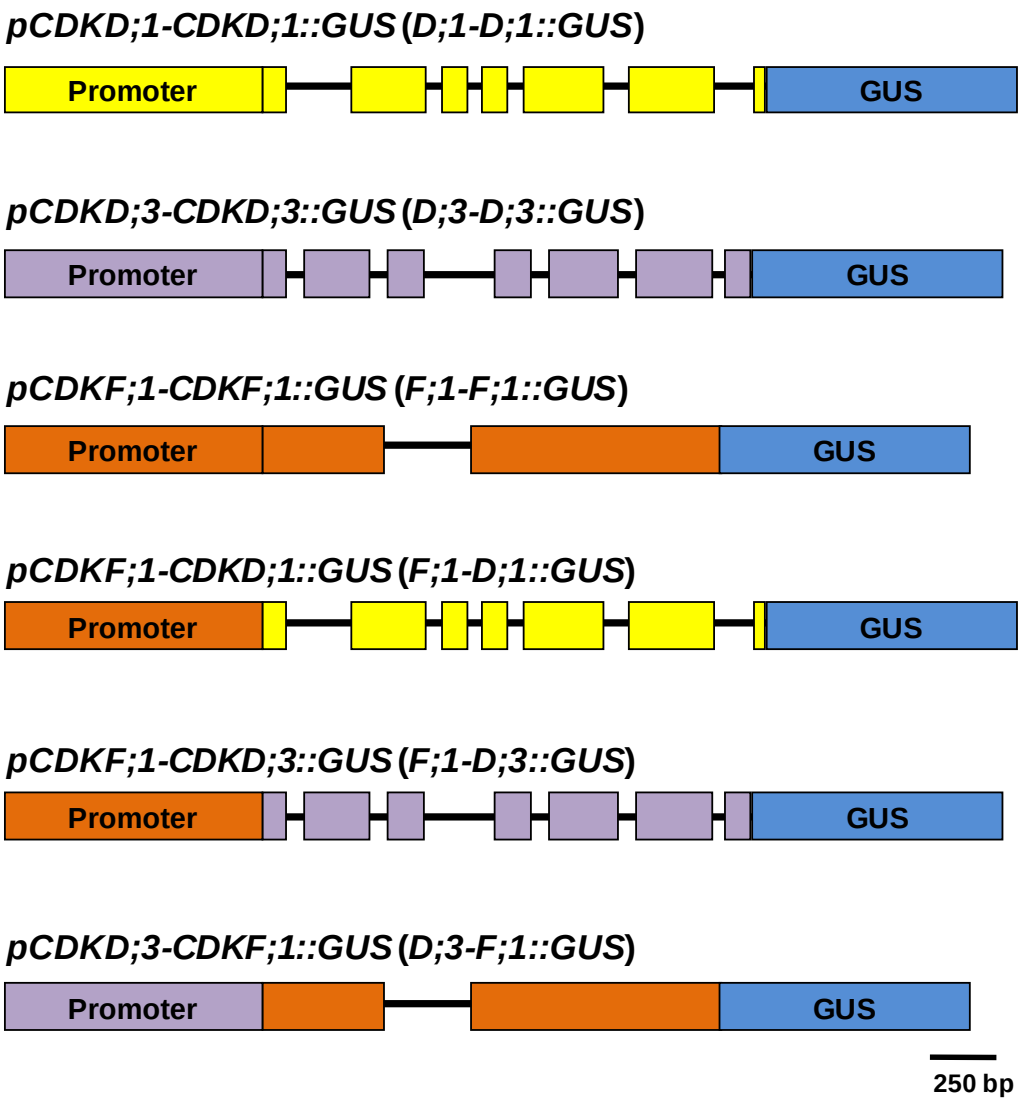

Figure 3-1. Schematic diagrams of plasmid constructs used for promoter swapping experiments.

Yellow boxes indicate the promoter regions and the exons of *CDKD;1* and *CDKD;3* genes. Orange boxes indicate the promoter region and the exons of *CDKF;1* gene. Blue boxes indicate the GUS genes. Solid bars indicate introns.

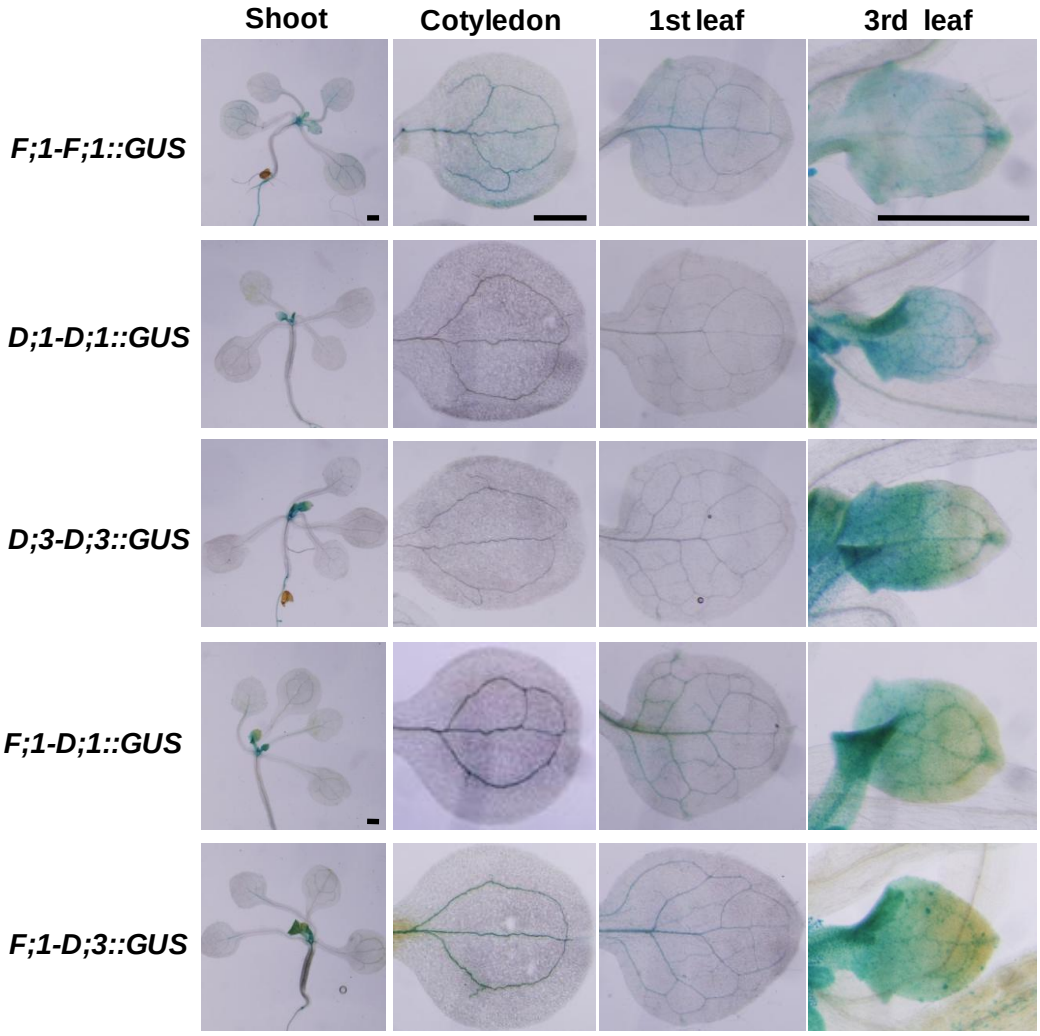

Figure 3-2. The expression patterns of CDKD::GUS under the control of *CDKF;1* promoter in shoots.

GUS staining of 10-day-old transgenic plants harbouring *pCDKF;1-CDKF;1::GUS* (*F;1-F;1::GUS*), *pCDKD;1-CDKD;1::GUS* (*D;1-D;1::GUS*), *pCDKD;3-CDKD;3::GUS* (*D;3-D;3::GUS*), *pCDKF;1-CDKD;1::GUS* (*F;1-D;1::GUS*) or *pCDKF;1-CDKD;3::GUS* (*F;1-D;3::GUS*). Whole seedlings (left panels), cotyledons (2nd panels from left), 1st leaves (3rd panels from left) and 3rd leaves (right panels). Bars = 0.5 mm.

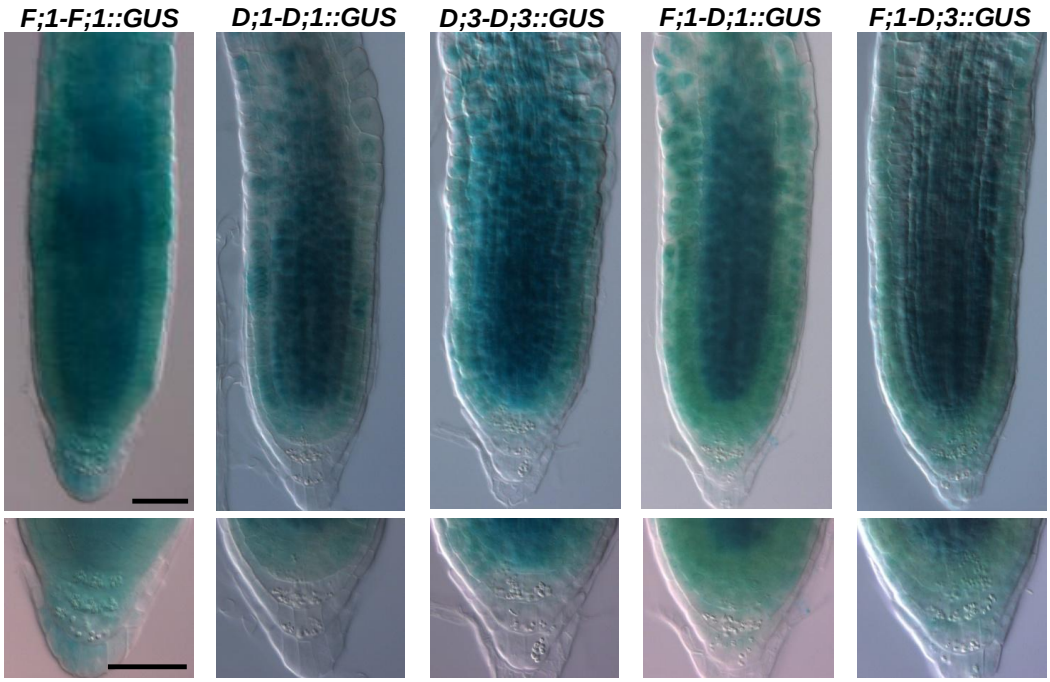

Figure 3-3. The expression patterns of CDKD::GUS under the control of *CDKF;1* promoter in roots.

GUS staining of 4-day-old transgenic plants harbouring *F;1-F;1::GUS*, *D;1-D;1::GUS*, *D;3-D;3::GUS*, *F;1-D;1::GUS* or *F;1-D;3::GUS*. Root tips (top row) and magnified distal root tips (bottom row). Bars = 50 μm.

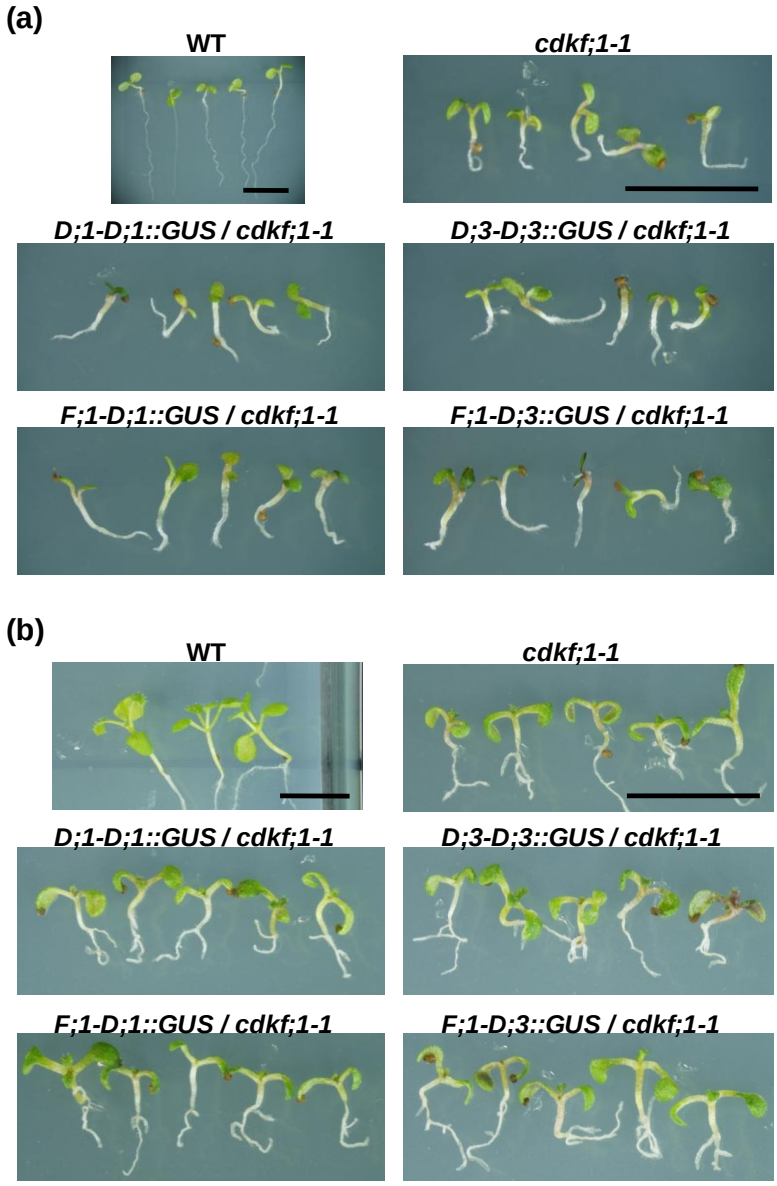

Figure 3-4. Lack of suppression activity of *F;1-D;1::GUS* or *F;1-D;3::GUS* in the *cdkf;1-1* mutants.

4-day-old (a) or 10-day-old (b) seedlings of wild-type plants, the non-transformed *cdkf;1-1* mutants, the *cdkf;1-1* mutants harbouring *D;1-D;1::GUS*, *D;3-D;3::GUS*, *F;1-D;1::GUS* or *F;1-D;3::GUS*. Bars = 5mm.

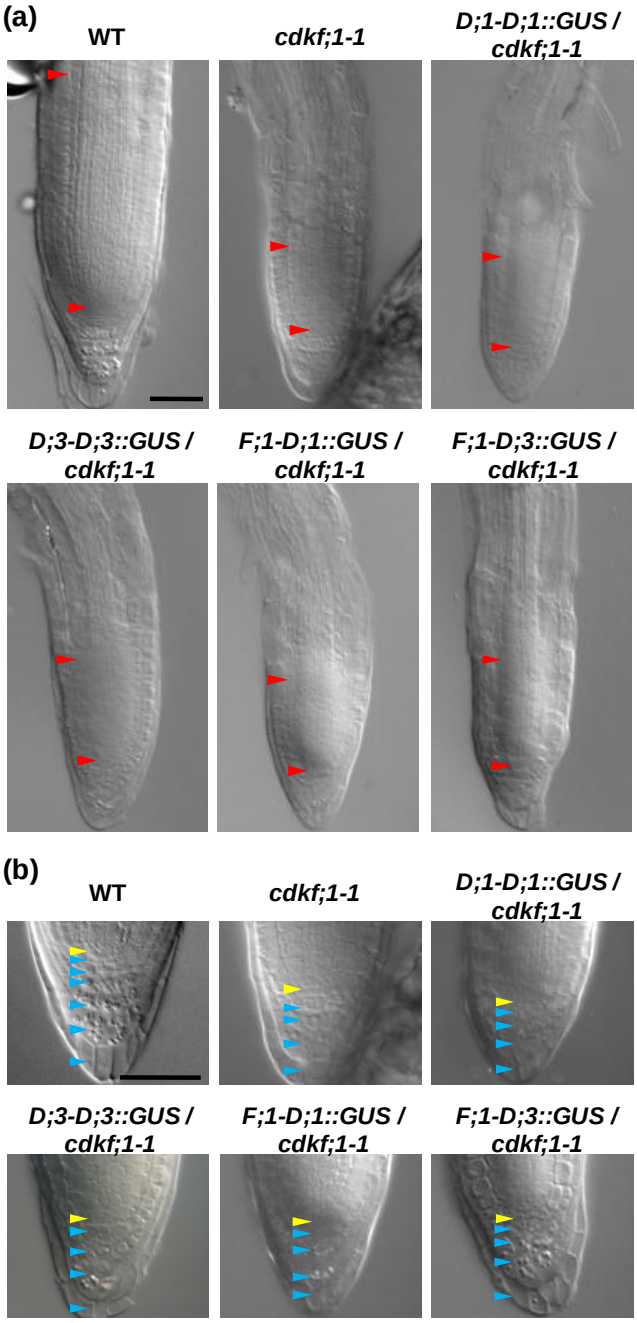

Figure 3-5. The root phenotypes of the *cdkf;1-1* mutants harbouring *F;1-D;1::GUS* or *F;1-D;3::GUS*.

(a) Primary roots of 4-day-old seedlings of wild-type plants, the *cdkf;1-1* mutants, the *cdkf;1-1* mutants harbouring *D;1-D;1::GUS*, *D;3-D;3::GUS*, *F;1-D;1::GUS* or *F;1-D;3::GUS*. Red arrowheads indicate the meristematic zone. Bar = 50 µm. (b) Root apices of 4-day-old seedlings of wild-type plants, the *cdkf;1-1* mutants, the *cdkf;1-1* mutants harbouring *D;1-D;1::GUS*, *D;3-D;3::GUS*, *F;1-D;1::GUS* or *F;1-D;3::GUS*. Yellow arrowheads indicate the quiescent centre and blue arrowheads indicate columella cells including initial cells. Bar = 50 µm.

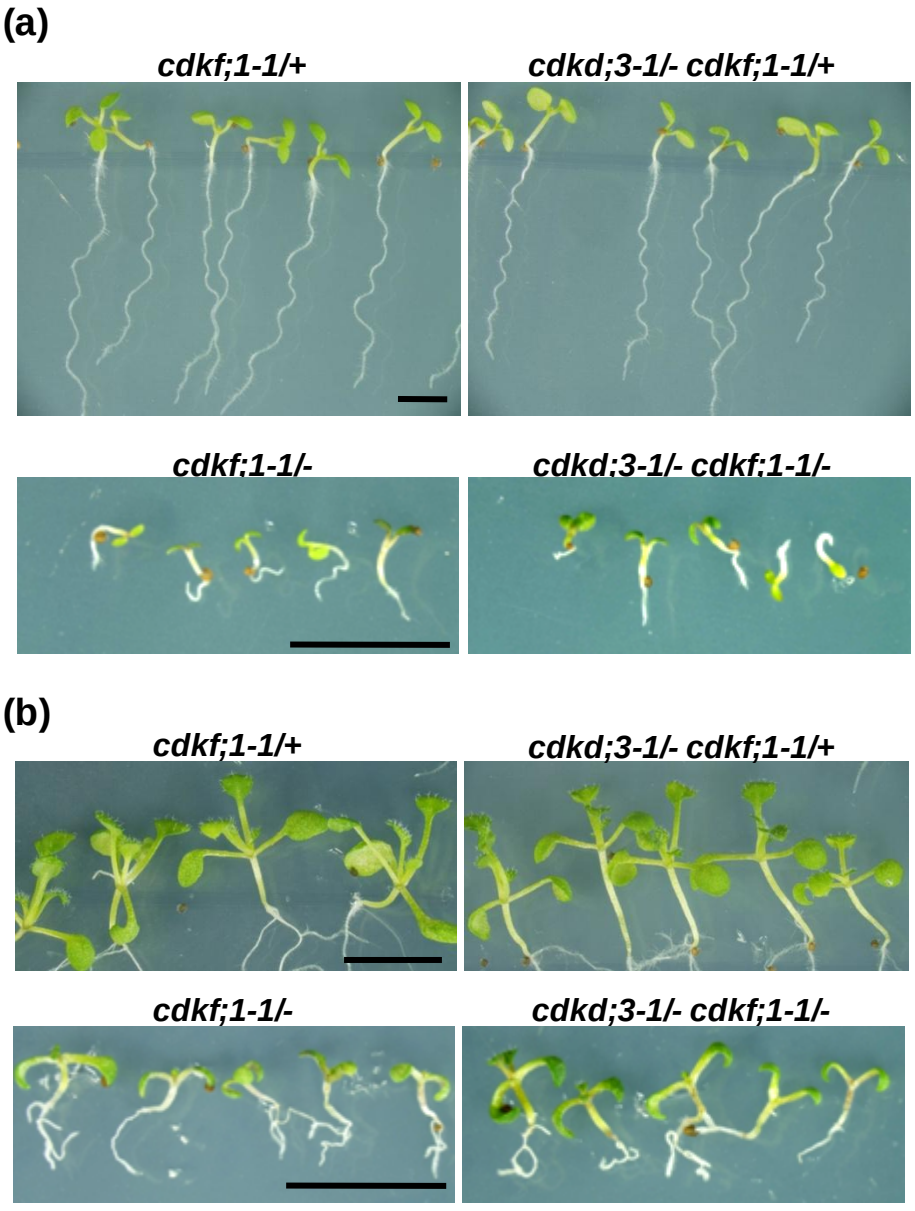

Figure 3-6. The phenotypes of *cdkd;3-1 cdkf;1-1* seedlings.
4-day-old (a) or 10-day-old (b) seedlings of the *cdkf;1-1/+* mutants, the *cdkd;3-1/- cdkf;1-1/+* mutants, the *cdkf;1-1/-* mutants and the *cdkd;3-1/- cdkf;1-1/-* mutants. Bars = 5 mm.

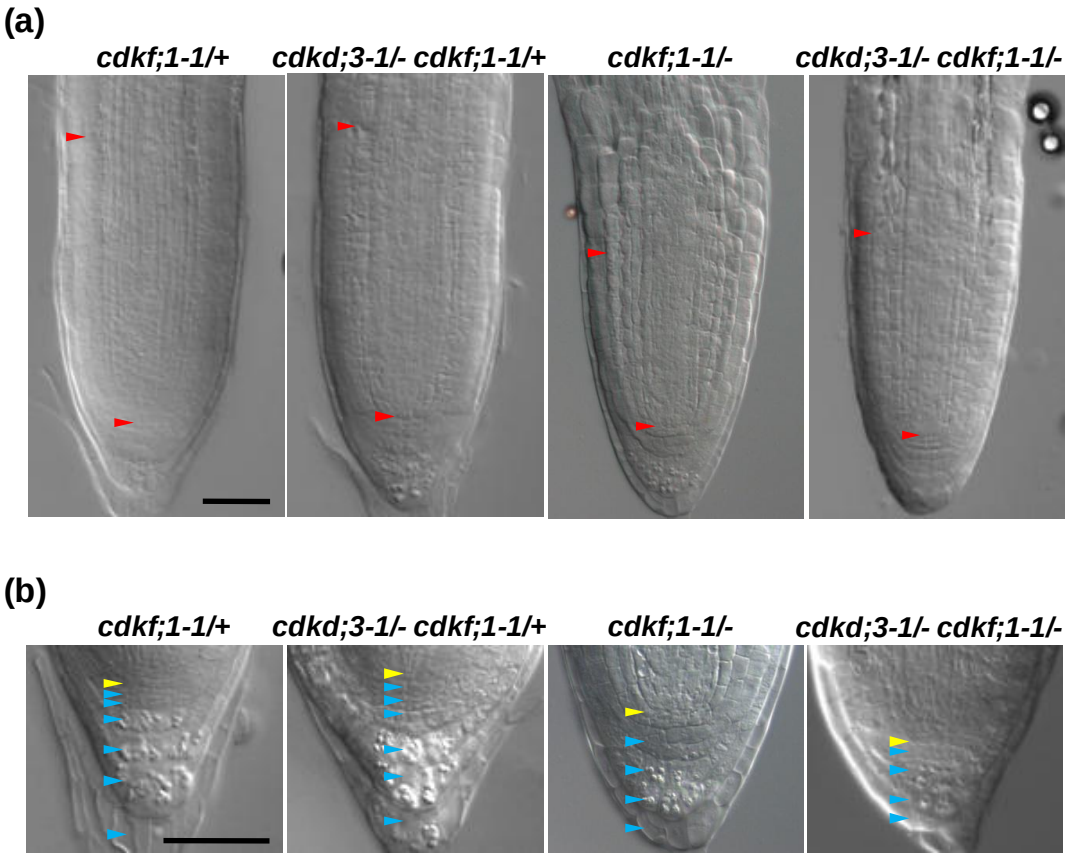

Figure 3-7. The root phenotypes of the *cdkd;3-1 cdkf;1-1* mutants.

(a) Primary roots of 4-day-old seedlings of the *cdkf;1-1/+* mutants, the *cdkd;3-1/- cdkf;1-1/+* mutants, the *cdkf;1-1/-* mutants and the *cdkd;3-1/- cdkf;1-1/-* mutants. Bar = 50 µm. (b) Root apices of 4-day-old seedlings of the *cdkf;1-1/+* mutants, the *cdkd;3-1/- cdkf;1-1/+* mutants, the *cdkf;1-1/-* mutants and the *cdkd;3-1/- cdkf;1-1/-* mutants. Yellow arrowheads indicate the quiescent centre and blue arrowheads indicate columella cells including initial cells. Bar = 50 µm.

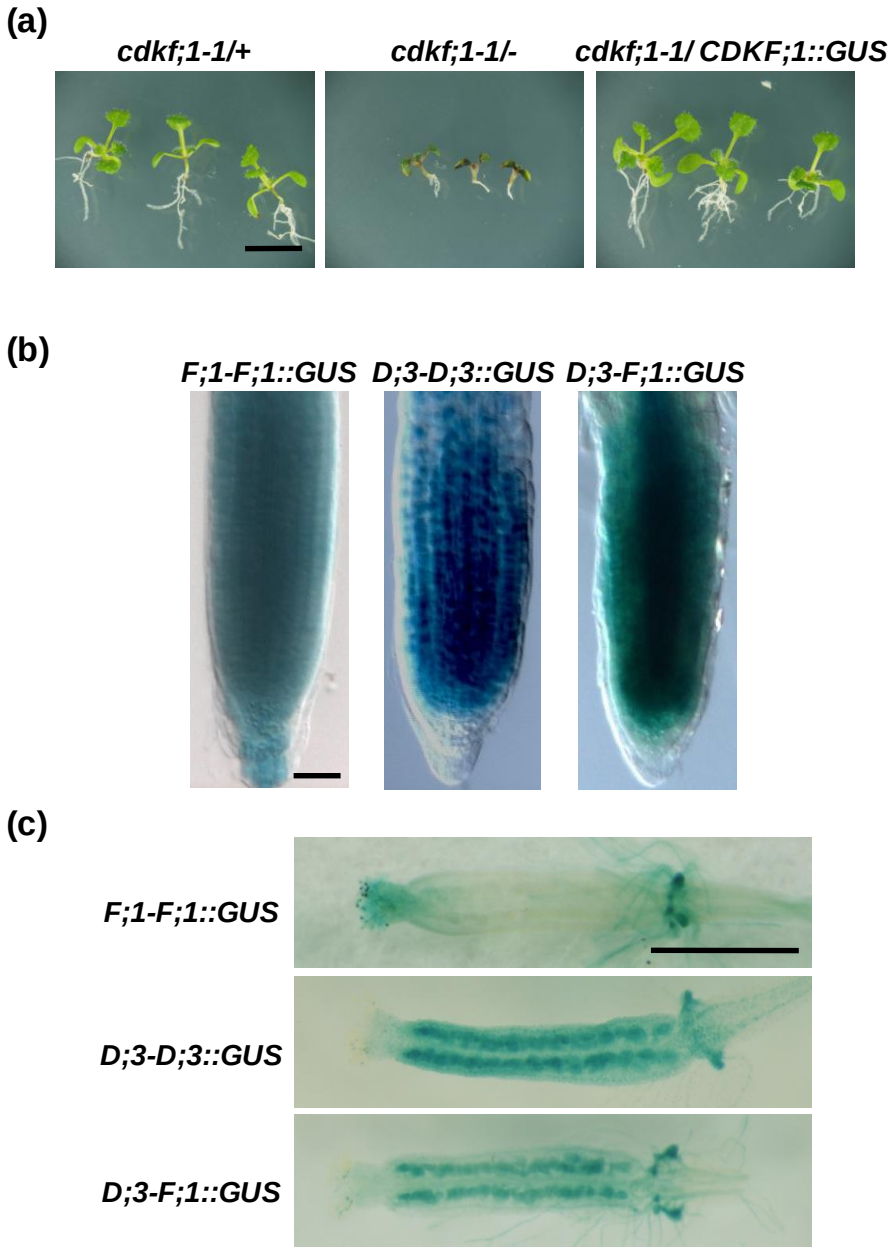

Figure 3-8. The expression patterns of CDKF;1::GUS under the control of *CDKD;3* promoter.

(a) Complementation of the c*dkf;1-1* phenotypes by the *CDKF;1* genomic fragment fused to *GUS*. 10-day-old seedlings grown on a medium containing sulfadiazine are shown. Bar = 5 mm. (b, c) The GUS staining of the transgenic plants harbouring *pCDKF;1-CDKF;1::GUS* (*F;1-F;1::GUS*), *pCDKD;3-CDKD;3::GUS* (*D;3-D;3::*GUS) or *pCDKD;3-CDKF;1::GUS* (*D;3-F;1::GUS*). Roots of 4-day-old seedlings (b) and pistils (c). Bars = 50 μm (b) and 1 mm (c).

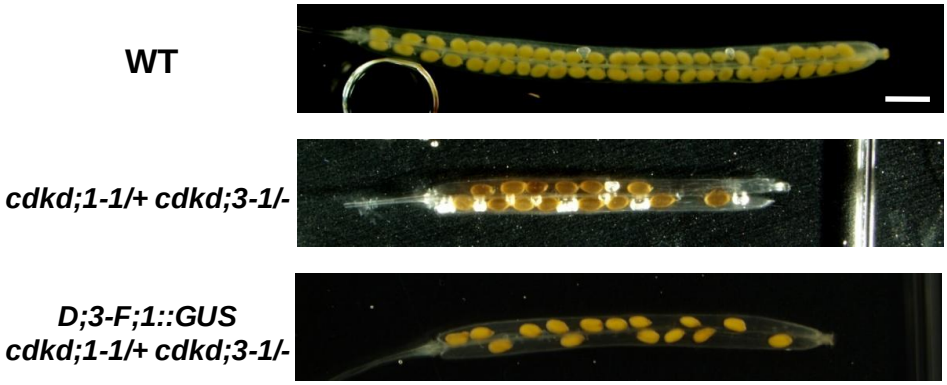

Figure 3-9. Lack of suppression activity of *D;3-F;1::GUS* in the *cdkd;1-1 cdkd;3-1* double mutants.

Cleared mature siliques of wild-type plants, the *cdkd;1-1/+ cdkd;3-1/-* plants and the *cdkd;1-1/+ cdkd;3-1/-* plants harbouring *D;3-F;1::GUS*. Bar = 1 mm.

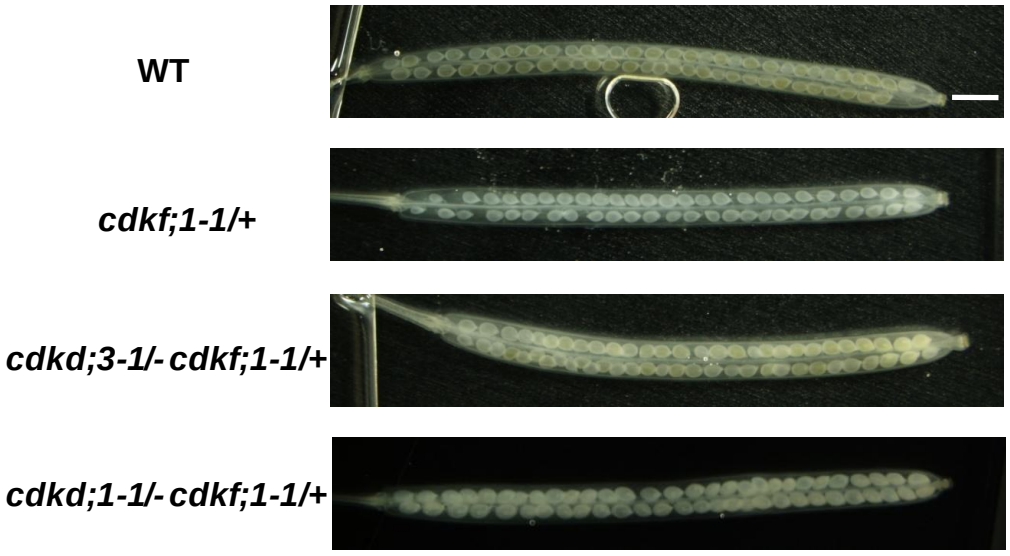

Figure 3-10. Normal seed development in the *cdkd;3-1 cdkf;1-1* and *cdkd;1-1 cdkf;1-1*. Cleared mature siliques of wild-type plants, the *cdkf;1-1/+* plants, the *cdkd;3-1/- cdkf;1-1/+* plants and the *cdkd;1-1/- cdkf;1-1/+* plants. Bar = 1 mm.

<Discussion>

Some spatial expression patterns are known to play a key role to have differentiated functions during plant development. *Arabidopsis* CCS52A, an activator of the anaphase promoting complex/cyclosome (APC/C) (Vanstraelen *et al*., 2009), has two isoforms, CCS52A1 and CCS52A2. Both are involved in meristem maintenance in the root tip, but with different expression profiles. CCS52A1 expressed in the elongation zone of roots stimulates endoreduplication and mitotic exit. In contrast, CCS52A2 is expressed in the distal region of the root meristem and controls the identity of the quiescent center (QC) cells and stem cell maintenance. Such different contributions to root development may indicate that CCS52A1 and CCS52A2 act on root meristem maintenance through different molecular mechanisms. However, the *CCS52A1* gene, which was introduced into the *ccs52a2* mutant under the control of the *CCS52A2* promoter, restored the root growth and cellular organization of the root meristem, suggesting that CCS52A1 and CCS52A2 proteins are functional homologues and that their divergent contributions to root development are derived from differences in their spatial expression patterns. In *Chapter II* and *Chapter III*, I showed that each CAK exhibits distinct expression patterns during the early and post-embryonic development. Such differences in expression priofiles may be also important for divergent functions of CAKs during plant development. However, my promoter-swapping experiments revealed that CAKs are not exchangeable in terms of molecular functions, thus CAK functions rely on their enzyme activities at least for CDKD;3 and CDKF;1. This assumption is supported by the findings that the activity of CDKD;2 but not CDKA;1 was down-regulated in the *cdkf;1-1* mutants, while the phenotypes of the *cdkd;1-1 cdkd;3-1* mutants partially overlaps with that of the *cdka;1* mutants. Therefore, I postulate that plants may have

one-to-one-like pairs between multiple CDKs and multiple CAKs, such as CDKF;1−CDKD;2 and CDKD;1 or CDKD;3−CDKA;1.

The activation of multiple CDKs by multiple CAKs has been barely suggested in other organisms. In yeasts, such a relationship is not required, since yeasts have only one CDK. As mentioned in *General introduction*, CDK7 had been widely recognized as the sole CAK in animals. Indeed, CDK7 can phosphorylate all the cell cycle-regulating CDKs, CDK1, CDK2, CDK4 and CDK6, *in vitro* (for review, see Fisher, 2005). However, some reports have challenged this concept. Larochelle *et al*. (1998) revealed that the CDK7 activity was required for the activation of CDK1 *in vivo*, whereas CDK2 might be activated by CAK(s) other than CDK7. Consistent with this, p42, a candidate CAK that may activate CDK2, was identified by Liu *et al*. (2004). However, due to a lack of extensive analyses of p42, its precise function largely remains unknown. CDK4 and its functional homologue CDK6 act as master integrators in G1/S progression. Recently, Bockstaele *et al* (2009) found that CDK4 might not be phosphorylated by CDK7 in intact cells but was more likely phosphorylated by another kinases, while CDK6 was efficiently phosphorylated by CDK7. Hence, one-to-one-like pairs between CDKs and CAKs may also exist in animals.

With respect to the question why animal cells need CAK(s) other than CDK7, Liu *et al*. (2004) argued that a multiple CAK system may be more flexible than a single CAK system under various conditions. For instance, one CAK may guarantee the constitutive housekeeping CAK activity, whereas the other CAKs may be engaged in the growth control in response to internal or external stimuli. Such a flexible CDK-activation mechanism may be also favorable for plant development. So far, I do not have a direct evidence that CDKD;1 and CDKD;3 activate the core plant CDKs,

CDKA and CDKB. However, it is very likely that CDKD;1 and CDKD;3, which are specifically expressed in actively dividing tissues, are the major CAKs controlling these CDKs. Moreover, the co-expression database ATTED-II (http://atted.jp/) shows that *CDKD;3* is very close to E2F-target genes (i.e., G1/S promoting genes). This suggests that CDKD;3-mediated CDK activation may be controlled by E2F, whose transcriptional activity responds to growth stimuli through the CYCD−RBR pathway as described in *General introduction*. In contrast, CDKF;1 showed more ubiquitous and constitutive expression pattern *in planta* and during the cell cycle (Takatsuka *et al.*, 2009). This would be suitable for the stabilization of CDKD;2, which is also constantly accumulated during the cell cycle (Ohno *et al.*, unpublished data).

In this thesis, I revealed that (i) CDKF;1 regulates post-embryonic development by the stabilization of CDKD;2 protein (*Chapter I*), (ii) CDKD;1 and CDKD;3 control gametogenesis in a cooperative manner (*Chapter II*), (iii) each CAK exhibits different expression pattern during plant development (*Chapter II*) and (iv) the molecular functions of CDKD;1 and CDKD;3 are divergent from that of CDKF;1 (*Chapter III*). Based on these results, I propose a model depicted in Figure 3-11. The CDKF;1 function is required for post-embryonic development of plants by stabilizing CDKD;2 protein but is not involved in gametogenesis and embryogenesis. Instead, CDKD;1 and CDKD;3 play significant roles in mitosis during gametogenesis and possibly embryogenesis. The CDKD;1/3 − CDKA;1 pathway may also control post-embryonic development together with CDKF;1−CDKD;2, but this hypothesis requires further investigations. The CDK-activation mechanisms, which I revealed in this study, are the first example in eukaryotes showing multiple CAKs play important roles in distinct developmental stages. Such divergent mechanisms may reflect the

plasticity of plants under various environmental conditions.

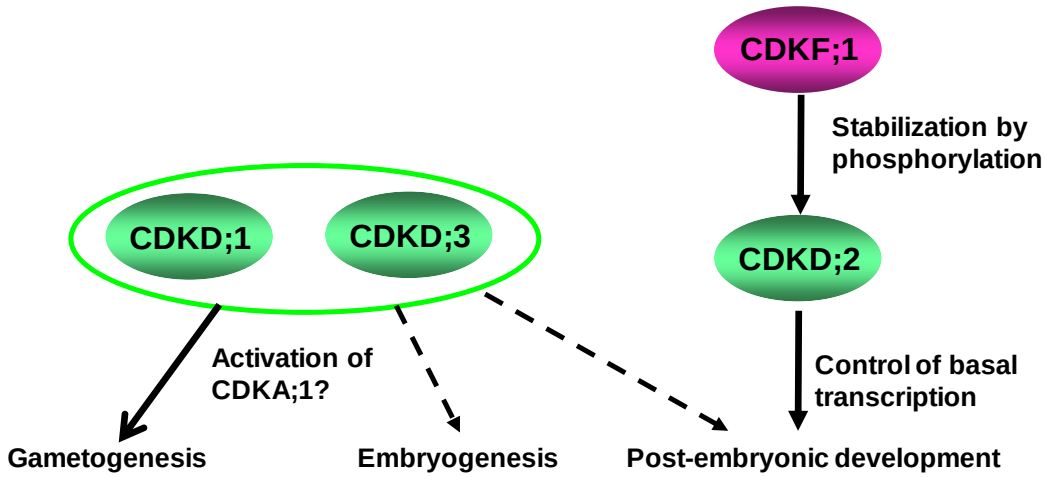

Figure 3-11. Proposed model of regulation of plant development by CAKs.

<Materials & methods>

Plant material

The seedlings of the *cdkf;1-1* mutants were grown at 23°C on an MSAR agar plate (Koncz *et al*., 1990) under short-day conditions (dark, 16 h; light, 8 h) for growth analysis. For the observation of siliques, plants germinated on an MS agar plate (Murashige *et al*., 1962), were grown on the soil under continuous light conditions.

Plasmid construction for plant transformation

For promoter-swapping experiments, I used the Hybrid Gateway Binary Vector that Nakagawa *et al*. (2008) developed. The entry clones carrying promoter fragments were constructed by the BP reaction using pDONR P4-P1R and an attB4-promoter-attB1 fragment prepared by adapter PCR. The promoter fragments of *CDKF;1*, *CDKD;1* and *CDKD;3* were amplified from 2032, 1697 and 2000 bp upstream of the start codon, respectively, to the 1 bp just before the start codon. The entry clones carrying ORFs were constructed by the BP reaction using pDONR221 and the attB1-cDNA-attB2 fragment prepared by adapter PCR. The ORFs of *CDKF;1*, *CDKD;1* and *CDKD;3* including introns were amplified from the start codon to 1 bp just before stop codon by genomic PCR. The promoter and ORF entry clones were reacted with the R4pGWB533 vector by using the LR clonase to form the promoter-ORF construct fused to *GUS* at the C-terminus. The resultant plamids, R4pGWB533-*F;1-D;1::GUS*, R4pGWB533-*F;1-D;3::GUS* and R4pGWB533-*D;3-F;1::GUS*, were introduced into the *cdkf;1-1/+* plants or the *cdkd;1-1/+ cdkd;3-1/-* plants using *Agrobacterium* strain GV3101. Hygromycin-resistant T1 or T2 progenies harboring the T-DNA were subjected to the analyses.

Isolation of the cdkd;3-1 cdkf;1-1 and cdkd;1-1 cdkf;1-1 double mutants

After crossing the *cdkd;3-1* or *cdkd;1-1* mutants with the *cdkf;1-1/+* mutants, T-DNA insertions in the *CDKF;1* gene were examined by selection with sulfadiazine sodium salt, and those in *CDKD;3* or *CDKD;1* were tested by genomic PCR using a set of primers that hybridize to the T-DNA and each *CDKD* 5'-GGATTTTCGCCTGCTGGGGCAAACCAGCGT-3' and 5'-CAGCCAAAGAAAGTTGCTGATAGGTATCTC-3' for *cdkd;3-1*, and 5' -CTGGGAATGGCGAAATCAAGGCATC-3' and 5' -GTTGCTGATAGGTATCTAAAGCGAGAGGT-3' were used for *cdkd;1-1*. In the F2 generation, the *cdkd;3-1/- cdkf;1-1/+* or *cdkd;1-1/- cdkf;1/+* plants were screened by the above-mentioned genomic PCR. Observations were conducted using the F3 plants.

Microscopic observation

Roots were mounted on a glass slide in a chloral hydrate solution, as described in *Chapter I*. Observation of green mature siliques and GUS staining were conducted as described in *Chapter II*.

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