Cyclin-dependent kinase-activating kinases CDKD;1 and CDKD;3 are essential for preserving mitotic activity in *Arabidopsis thaliana*

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Summary

For the full activation of cyclin-dependent kinases (CDKs), not only cyclin binding but also CDK phosphorylation is required. This activating phosphorylation is mediated by CDK-activating kinases (CAKs). *Arabidopsis* has four genes showing similarity to vertebrate-type CAKs, three *CDKD*s (*CDKD;1–CDKD;3*) and one *CDKF* (*CDKF;1*). We previously found that the *cdkf;1* mutant is defective in post-embryonic development even though the kinase activities of core CDKs remain unchanged relative to the wild-type. This raised a question about the involvement of CDKDs in CDK activation *in planta*. Here we report that the *cdkd;1 cdkd;3* double mutant showed gametophytic lethality. Most *cdkd;1-1 cdkd;3-1* pollen grains were defective in pollen mitosis I and II, producing one-cell or two-cell pollen grains that lacked fertilization ability. We also found that the double knockout of *CDKD;1* and *CDKD;3* caused arrest and/or delay in the progression of female gametogenesis at multiple steps. Our genetic analyses revealed that the functions of CDKF;1 and CDKD;1 or CDKD;3 do not overlap, either during gametophyte and embryo development or in post-embryonic development. Consistent with these analyses, *CDKF;1* expression in the *cdkd;1-1 cdkd;3-1* mutant could not rescue the gametophytic lethality. These results suggest that, in *Arabidopsis*, CDKD;1 and CDKD;3 function as CAKs controlling mitosis, whereas CDKF;1 plays a distinct role mainly in post-embryonic development. We propose that CDKD;1 and CDKD;3 phosphorylate and activate all core CDKs, CDKA, CDKB1 and CDKB2, thereby governing cell cycle progression throughout plant development.

Introduction

Double fertilization is one of the unique features of angiosperms. Two sperm cells are delivered through the pollen tube to the embryo sac; one fuses with the egg cell and generates the zygote, whereas the other fuses with the central cell, giving rise to the endosperm, which is required for embryo development and germination (for a review, see Dumas and Rogowsky, 2008). Precise control of cell division is prerequisite to proper development of both embryo and endosperm.

Gametophytes are produced through controlled mitotic divisions. In *Arabidopsis*, sporogenous initial cells, also called microspore or megaspore mother cells, undergo meiosis to produce a tetrad of microspores in the anther or four megaspores in the ovule (Liu and Qu, 2008). Microspores released from a tetrad by the action of callase further undergo an asymmetric cell division (pollen mitosis I, PM I) to form a bicellular pollen grain carrying two cells with distinct fates: the larger vegetative cell later forms a pollen tube nucleus, while the smaller generative cell undergoes a symmetric division (pollen mitosis II, PM II) to produce two sperm cells (Figure S1) (McCormick, 1993; 2004). The resultant three-celled pollen grain constitutes the male gametophyte. On the other hand, at the onset of female gametophyte development, only one of the four megaspores survives and undergoes three rounds of nuclear division and subsequent cellularization (Yang and Sundaresan, 2000; Drews and Yadegari, 2002). In *Arabidopsis*, megagametogenesis is divided into seven distinct stages, FG1 to FG7 (Figure S2) (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). First, the surviving megaspore undergoes mitosis to produce a binucleate cell (FG1 to FG2). Shortly thereafter, the two nuclei separate to the chalazal and micropylar poles, and a

vacuole is formed at the center (FG3). Two subsequent rounds of mitosis produce an eight-nucleate cell with four nuclei at each pole (FG4 to FG5). One nucleus from each pole (polar nuclei) then migrates toward the center, followed by cellularization that localizes three cells at each pole (FG5). The polar nuclei eventually fuse to produce the central cell (FG6), and finally the three antipodal cells undergo cell death, generating the female gametophyte comprising one haploid egg cell, two haploid synergid cells, and one diploid central cell (FG7) (Figure S2).

Cell division is regulated by the activity of cyclin-dependent kinases (CDKs). Based on amino acid sequence similarities, plant CDKs have been classified into eight types, CDKA to CDKG and the CDK-like kinase (CKL) (Joubés *et al*., 2000; Vandepoele *et al*., 2002; Takahashi and Umeda, 2014). Among them, CDKA and CDKB have a primary function in cell cycle control. CDKA is inferred as a functional ortholog of yeast CDC2/CDC28 and mammalian CDK1, and its activity is required for both G1-to-S and G2-to-M transitions. CDKB is a plant-specific CDK with two subtypes, CDKB1 and CDKB2, and its expression is restricted to the late S-to-M phase. Indeed, previous reports demonstrated that CDKB is involved in G2-to-M progression and suppresses endoreplication, in which cells replicate their chromosomes without mitosis (Boudolf *et al*., 2009). In *Arabidopsis*, the *cdka;1* knockout mutant undergoes PM I but not PM II during male gametogenesis, producing bicellular pollen grains with a single sperm-like cell and one vegetative cell (Iwakawa *et al*., 2006; Nowack *et al*., 2006). This suggests that, in *cdka;1*, CDKB has some activity in G1-to-S progression. Whereas *cdka;1* exhibits no obvious defects in megagametogenesis, the combination with knockout mutations in the two *CDKB1* genes caused lethality in both female and male gametogenesis (Nowack *et al*., 2012), indicating an overlapping function between A- and B-type CDKs during

female gametogenesis.

For full activation of CDKs, phosphorylation as well as cyclin binding is required; this phosphorylation is carried out at the conserved threonine residue within the T-loop by a CDK-activating kinase (CAK) (Umeda *et al*., 2005; Fisher, 2005; Harashima *et al*., 2007; Dissmeyer *et al*., 2007). Plants possess two types of CAKs, CDKD and CDKF, both of which exhibit CDK-activating kinase activities *in vitro* and in yeast cells (Umeda *et al*., 1998; Yamaguchi *et al*., 1998; Chao *et al*., 2007). CDKD is closely related to vertebrate-type CAKs in terms of amino acid sequence similarity and enzyme activity; *Arabidopsis* has three CDKDs (CDKD;1–CDKD;3), among which CDKD;2 and CDKD;3 display kinase activities toward not only CDKs but also the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II, suggesting that, like vertebrate-type CAKs, CDKD controls both CDK activation and basal transcription. The CDK-kinase activity of CDKD;3 is higher than that of CDKD;2, while CDKD;2 has higher CTD-kinase activity than CDKD;3 (Shimotohno *et al*., 2003). CDKD;1 expressed in insect cells exhibits neither CDK- nor CTD-kinase activity (Shimotohno *et al*., 2003). On the other hand, *Arabidopsis* has one CDKF, CDKF;1, which shows a lower similarity to vertebrate-type CAKs than CDKDs and exhibits kinase activity on CDK but not CTD (Shimotohno *et al*., 2004). A striking feature of CDKF;1 is that it phosphorylates and activates CDKD;2 and CDKD;3, thus functioning as a CAK-activating kinase (CAKAK) (Shimotohno *et al*., 2004; Umeda *et al*., 2005). However, in the *cdkf;1* knockout mutant, the kinase activities of CDKA, CDKB and CDKDs except CDKD;2 were the same as those in the wild-type (Takatsuka *et al*., 2009). Moreover, the *cdkf;1* mutant has fewer and smaller cells in leaves while the *cdka;1* mutant has fewer but larger cells (Dissmeyer *et al*., 2007), suggesting that CDKF;1 does not play a major role in CDK activation *in*

planta. Rather, in *cdkf;1*, the protein level and kinase activity of CDKD;2 were significantly reduced, indicating that CDKF;1 controls basal transcription by maintaining the steady-state level of CDKD;2 (Takatsuka *et al*., 2009).

As described above, CDKA and CDKB are involved in gametophyte development, while the *cdkf;1* mutant displays severe growth defects in post-embryonic development, but no obvious defect in seed development or germination (Takatsuka *et al*., 2009). This suggests that CDKD plays a major role in CDK activation during gametogenesis and embryogenesis. Here we investigated the phenotype of *cdkd* knockout mutants, and found that the *cdkd;1 cdkd;3* double mutant was gametophyte-lethal. This lethality was derived from impaired mitosis during both male and female gametogenesis. Our data showed that CDKF;1, which is dispensable in gametogenesis and embryogenesis, could not substitute for CDKD;3. We propose that CDKD;1 and CDKD;3 are the principal regulators of CDK activation, and are thus essential for cell cycle regulation throughout plant development.

Results

Expression analysis of CAKs in gametophytes and embryos

To investigate the role of CAKs during gametogenesis and embryogenesis, we first observed their expression patterns using 2-kb promoter regions fused to the β-glucuronidase (GUS) reporter gene. However, we could see no GUS signal for any of the CAKs, probably because the promoter regions lacked regulatory elements required for transcriptional activation. We therefore generated translational fusion constructs including the 2-kb promoter and full-length coding regions that were fused in-frame to GUS. As we reported previously, strong GUS signals were observed in young flowers of *pCDKF;1–CDKF;1::GUS*, while in mature flowers the GUS expression was mostly

restricted to stamen filaments and pedicels (Figure 1a) (Takatsuka *et al*., 2009). In *pCDKD;1–CDKD;1::GUS* and *pCDKD;2–CDKD;2::GUS*, GUS signals were detected in young flowers and in pistils of mature flowers; pedicels also showed signals for *CDKD;2*. *pCDKD;3–CDKD;3::GUS* displayed the same expression pattern as *pCDKD;2–CDKD;2::GUS*, but with a much higher expression level (Figure 1a).

We next observed expression in gametophytes. GUS signals for *CDKD;2* and *CDKD;3* were localized at the center of the pollen grain (Figure 1b), which probably represents an assemblage, called the male germ unit, consisting of the vegetative cell nucleus and two sperm cells (Lalanne and Twell, 2002). *pCDKD;1–CDKD;1::GUS* and *pCDKF;1–CDKF;1::GUS* showed GUS signals in whole pollen grains, with the strongest signal in the former being detected in the male germ unit (Figure 1b). Expression in ovules was observed for the three *CDKD*s but not for *CDKF;1* (Figure 1c), indicating that *CDKF:1* is dispensable for female gametogenesis.

The expression level of *CDKF;1* was very low throughout embryogenesis; at the late embryo stage, GUS signal was detected only in the hypocotyl, in which cells are not actively dividing (Figure 2a) (Gendreau *et al*., 1997). This suggests that *CDKF;1* is not principally involved in cell cycle regulation during embryogenesis, which is consistent with our previous observation that the *cdkf;1* mutant exhibits only post-embryonic defects (Takatsuka *et al*., 2009). Whereas the expression level of *CDKD;2* was very low at the globular and torpedo stages, *pCDKD;1–CDKD;1::GUS* and *pCDKD;3–CDKD;3::GUS* showed relatively higher GUS signals during early embryogenesis, and in dividing tissues, such as root and shoot meristems and vascular bundles, at the later stage (Figure 2a and b). These results suggest that *CDKD;1* and *CDKD;3* are engaged in the control of cell division during embryogenesis. During endosperm development, nuclear divisions occur

at the globular stage in the absence of cellularization (Berger, 1999); we also observed GUS signals in the endosperm at the globular stage. As shown in Figure 2c, *CDKD;1* and *CDKD;3* showed GUS expression in the endosperm, with higher signals for *CDKD;3*, whereas we could not detect any such signal for *CDKD;2* or *CDKF;1*. These results suggest that *CDKD;1* and *CDKD;3* participate in endosperm development as well as embryogenesis.

cdkd;1 ***and*** **cdkd;3** ***double mutation is gametophyte-lethal***

We previously showed that CDKD;2 has a lower CDK-kinase activity than CDKD;3 *in vitro* (Shimotohno *et al*., 2004). Moreover, in the *cdkf;1* mutant, protein levels and kinase activities of CDKA;1 and CDKBs did not decrease relative to the wild-type although the steady-state level of CDKD;2 protein was reduced (Takatsuka *et al*., 2009). These results suggested that CDKD;2 does not have a major role in CDK activation. On the other hand, CDKD;1 produced in insect cells did not display CDK or CTD kinase activities (Shimotohno *et al*., 2003), but, as described above, CDKD;1 is highly expressed during gametogenesis and embryogenesis. Hence, in this study, we focused on *in vivo* functions of CDKD;1 and CDKD;3. We previously reported that single-knockout mutants of *CDKD;1* and *CDKD;3* (Figure 3a) exhibited no obvious phenotype (Shimotohno *et al*., 2006). However, no progeny with both mutations was obtained by crossing, and we therefore observed siliques of plants with heterozygous *cdkd;1-1* and homozygous *cdkd;3-1* mutations (*cdkd;1-1/+ cdkd;3-1/-*). We found empty spaces in an alignment of mature seeds (Figure 3b); about 50% of the seeds were abortive and did not form embryo-like structures (Table 1 and Figure 3c). This phenotype was complemented by expression of *CDKD;3* under its own promoter (Figure 3b and Table 1). Since half of the seeds were

defective in *cdkd;1-1/+ cdkd;3-1/-* plants, we inferred that *cdkd;1-1* and *cdkd;3-1* double mutation was lethal in the gametophyte, but not in the embryo.

After double fertilization, at least three rounds of nuclear divisions normally occur in the wild-type endosperm before the first zygotic division; the zygote then starts cell division to form an embryo, while the endosperm nuclei continue to proliferate (Figure 3d). However, in young siliques of *cdkd;1-1/+ cdkd;3-1/-* plants, we found unfertilized ovules that were arrested at early stages of female gametogenesis, such as FG1 and FG2, and at FG7 (Figure 3e). This indicates that *CDKD;1* and *CDKD;3* are required for female gametophyte development. We also observed seeds containing a one- or two-cell-stage embryo with a reduced number of endosperm nuclei, but at very low frequencies (Figure 3f).

To further investigate the observed lethality, we conducted reciprocal crosses between the wild-type and *cdkd;1-1/+ cdkd;3-1/-* (Table 2). As technical controls, pistils of wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants were pollinated with pollen from wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants, respectively. When wild-type pistils were pollinated with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants, 33.8% of seeds were aborted. Similarly, 39.9% of seeds were aborted in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants pollinated with wild-type pollen. These results indicate that not all but a large percentage of male and female gametophytes fail to survive when they lack both *CDKD;1* and *CDKD;3*.

cdkd;1 *and* cdkd;3 *mutations impair pollen mitosis*

To test pollen viability, we conducted Alexander staining, which can distinguish viable and nonviable pollen grains. Pollen grains from *cdkd;1* or *cdkd;3* knockout mutants were stained similarly to those from the wild-type, and most pollen grains from *cdkd;1-1/+*

cdkd;3-1/- plants were also normally stained and did not show any morphological defect (Figure S3). We then observed the cells in each pollen. Wild-type pollen grains contain three cells, a vegetative cell and two smaller sperm cells (Figure S1 and Figure 4a); indeed, DAPI staining visualized three nuclei in nearly 90% of wild-type pollen grains collected at floral stage 13, when the bud opens and anthesis occurs (Irish, 2010) (Table 3 and Figure 4b). However, at the same floral stage, only 56.7% of pollen grains from *cdkd;1-1/+ cdkd;3-1/-* plants possessed three nuclei; 23.6% contained two, 7.4% contained one nucleus, and 12.3% did not have any nucleus that was clearly stained with DAPI (Table 3, Figure 4a and b). This indicates that most *cdkd;1-1 cdkd;3-1* pollen was defective in PM I and PM II, producing one-cell or two-cell pollen grains, while about 13% [(56.7 – 50) x 2 = 13.4%] of pollen grains with both *cdkd;1-1* and *cdkd;3-1* mutations completed two rounds of cell division. To examine whether *cdkd;1-1 cdkd;3-1* pollen is able to fertilize, we observed transmission of the mutant alleles in wild-type pistils pollinated with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants. The ratio of *cdkd;1-1/-* to *cdkd;1-1/+* in F1 plants was 17.5% (Table S1), which is close to the percentage (13.4%) of three-cell pollen grains with both *cdkd;1-1* and *cdkd;3-1* mutations as described above. This suggests that *cdkd;1-1 cdkd;3-1* pollen with three nuclei retains fertilization ability.

As mentioned above, 33.8% of seeds in wild-type siliques pollinated with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants were abortive (Table 2). Moreover, 38.0% of embryos were abortive at three days after pollination (DAP) in this crossing (n = 400), suggesting that *cdkd;1-1 cdkd;3-1* pollen with no more than two cells lacks fertilization ability. To further examine the defect in fertility, we observed abortive seeds produced by crossing. In wild-type pistils pollinated with wild-type pollen, an 8- or 16-cell embryo with many endosperm nuclei was formed at 3 DAP (Figure 4c). By contrast, when wild-type pistils

were pollinated with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants, 44% of the resultant abortive seeds contained one central cell and one egg cell, but no synergid cell, at 3 DAP (Figure 4c). It is known that one of the two synergid cells is physically disrupted by pollen penetration, and that the persisting one becomes difficult to identify at 2 DAP (Christensen *et al*., 2002). Therefore, it is probable that some of the *cdkd;1-1 cdkd;3-1* pollen tubes were attracted by synergid cells, but that a sperm-like cell could not fuse with the central cell or the egg cell. On the other hand, 56% of the abortive seeds were degenerated and shrunken, and we could not find any cell structure in these seeds by microscopic observation. It has been reported that unfertilized ovules became degenerated (Chaudhury *et al*., 1997), as observed in wild-type siliques pollinated with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants, suggesting that the above-mentioned degenerated phenotype of abortive seeds is the outcome of unsuccessful fertilization. These results support the idea that most of the *cdkd;1-1 cdkd;3-1* pollen grains harboring no more than two cells lack fertilization ability.

Nuclear division in female gametogenesis is defective in* cdkd;1 cdkd;3 *ovules

We next analyzed female gametophyte development in *cdkd;1-1/+ cdkd;3-1/-* plants. In wild-type plants, ovules had reached stages FG5 to FG7 when the flowers opened and the stamens nearly reached the pistils (Onodera *et al*., 2008) (Table 4 and Figure 5a). However, in *cdkd;1-1/+ cdkd;3-1/-*, some ovules were still at FG1 to FG4, and we found FG7 ovules having central cells with abnormal, shrunken nuclei (Table 4 and Figure 5b). When pistils of *cdkd;1-1/+ cdkd;3-1/-* plants were pollinated with wild-type pollen, resultant abortive seeds contained ovules at FG5 to FG7 (Table S2). Although we could not determine whether gametogenesis was delayed or arrested, these results indicate that *CDKD;1* and

CDKD;3 are essential for nuclear division in ovules. We sometimes found seeds containing a two-cell stage embryo with only two endosperm nuclei in self-pollinated siliques of *cdkd;1-1/+ cdkd;3-1/-* plants as well as in *cdkd;1-1/+ cdkd;3-1/-* siliques pollinated with wild-type pollen (Figure 5c), but not in wild-type pistils pollinated with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants. This indicates that such a phenotype is caused by defects on the female side, and that some *cdkd;1-1 cdkd;3-1* ovules can undergo fertilization but do not proceed with nuclear divisions in the endosperm.

CDKF;1 is not genetically related to CDKD;1 or CDKD;3

Although the *cdkf:1* knockout mutant exhibits defects only in post-embryonic development (Takatsuka *et al*., 2009), *CDKF;1* is expressed in pollen grains and during embryogenesis, as described above. Therefore, to examine whether CDKF;1 has a redundant function with CDKDs during gametogenesis and/or embryogenesis, we generated double knockout mutants of *CDKF;1* and either *CDKD;1* or *CDKD;3*. While *cdkd;1-1/- cdkf;1-1/+* and *cdkd;3-1/- cdkf;1-1/+* seedlings grew normally, the double mutants of *cdkd;1-1 cdkf;1-1* and *cdkd;3-1 cdkf;1-1* showed growth inhibition similar to that observed in the *cdkf;1-1* mutant (Figure 6a). In the root tip of *cdkd;1-1 cdkf;1-1* and *cdkd;3-1 cdkf;1-1*, the meristem size was reduced and the number of columella cell layers decreased, as observed in *cdkf;1-1* (Figure 6b and c). These results suggest that, although CDKF;1 is involved in post-embryonic development, its function is distinct from that of CDKD;1 or CDKD;3. Moreover, almost all seeds developed normally in siliques of *cdkd;1-1/- cdkf;1-1/+* and *cdkd;3-1/- cdkf;1-1/+* plants (Figure S4 and Table 5), implying that double knockout of *CDKF;1* and *CDKD;1* or *CDKD;3* does not lead to defects in gametogenesis or embryogenesis as seen in mutants lacking both *CDKD;1* and *CDKD;3*.

This suggests that the functions of CDKF;1 and CDKDs do not overlap either during gametophyte and embryo development or in post-embryonic development.

CDKF;1 *cannot rescue the gametophyte lethality of the* cdkd;1 cdkd;3 *double mutant*

To further examine the functional divergence between CDKF;1 and CDKDs, we conducted a promoter-swapping experiment, in which the *CDKF;1* coding region fused to *GUS* was expressed using the *CDKD;3* promoter (*D;3-F;1::GUS*; Figure 7a). We first confirmed the functionality of the CDKF;1::GUS fusion protein by expressing *GUS*-fused *CDKF;1* using the *CDKF;1* promoter (*F;1-F;1::GUS*; Figure 7a); it fully rescued the growth defect of the *cdkf;1-1* mutant (Figure 7b). We then examined the *CDKD;3* promoter activity in *D;3-F;1::GUS*. While the promoter of *CDKF;1*, but not of *CDKD;3*, is active in mature columella cells (Takatsuka *et al*., 2009), the GUS signal of *D;3-F;1::GUS* was absent in the columella (Figure 7c). Moreover, the GUS signal of *D;3-F;1::GUS* was observed in ovules, where the promoter of *CDKD;3*, but not of *CDKF;1*, is active (Figure 7d and Figure 1c). These results indicate that *D;3-F;1::GUS* produces functional CDKF;1::GUS protein in tissues where exogenous *CDKD;3* is expressed. Finally, we observed seed development in *cdkd;1-1/+ cdkd;3-1/-* plants with or without *D;3-F;1::GUS*. As shown in Table 5 and Figure 7e, almost the same percentage (44–48%) of seeds were abortive regardless of the expression of *D;3-F;1::GUS*, demonstrating that CDKF;1 cannot substitute for CDKD;1 or CDKD;3 during gametogenesis or embryogenesis.

Discussion

GUS-fused CDKD;1 and CDKD;3 expressed under their own promoters accumulated in

pollen grains and ovules. We also found that the combination of *cdkd;1-1* and *cdkd;3-1* mutations disrupted pollen mitosis. Previous reports showed that pollen grains from the *cdka;1* knockout mutant were defective in mitosis: they failed in PM II, but not PM I, producing one vegetative cell and one sperm-like cell (Iwakawa *et al*., 2006; Nowack *et al*., 2006). However, in *cdkd;1-1/+ cdkd;3-1/-* plants, we found pollen grains with only one vegetative cell, as well as others with one vegetative cell and one sperm-like cell, suggesting that pollen with both *cdkd;1* and *cdkd;3* mutations is defective in both PM I and PM II. Nowack *et al.* (2012) reported that pollen from the *cdka;1/+ cdkb1;1/- cdkb1;2/-* mutant displayed early germline arrest, and that 3% of pollen grains were unicellular, indicating an impairment in PM I. Therefore, it is likely that CDKD;1 and CDKD;3 control PM I by activating CDKB1 as well as CDKA;1. Since 7.4% of pollen grains from *cdkd;1-1/+ cdkd;3-1/-* plants were unicellular (Table 3), the defect in PM I is apparently severer in *cdkd;1-1 cdkd;3-1* than in *cdka;1 cdkb1;1 cdkb1;2*. Because plants have another type of CDK, CDKB2, that regulates G2-to-M progression (Menges *et al*., 2005), CDKD;1 and CDKD;3 may also control CDKB2 during pollen mitosis.

Arabidopsis mutants with defects in pollen mitosis vary in their fertilization ability. Chen *et al*. (2008) reported that pollen grains with defects in *MSI1*, which encodes a factor associating with the core subunits of chromatin assembly factor 1 (CAF1) complex, are impaired in PM II. However, when wild-type ovules were pollinated with *msi1* pollen, the sperm-like cell fused with either the egg cell or the central cell, generating seeds with either an embryo lacking endosperm or endosperm lacking an embryo. Another example is mutants of *DUO1* and *DUO3*, which encode an R2R3-type MYB transcription factor and a protein related to a cell-lineage regulator in gonadogenesis in *Caenorhabditis elegans*, respectively (Brownfield *et al*., 2009a; 2009b). Both *duo1* and

duo3 mutants produce a single sperm-like cell due to impaired pollen mitosis; however, fertilization never occurs with either the egg cell or the central cell because of defective differentiation of the sperm cell (Brownfield *et al*., 2009a; 2009b). When we pollinated wild-type pistils with pollen from *cdkd;1-1/+ cdkd;3-1/-* plants, we could not find any seeds with either embryo or endosperm, indicating that the single sperm-like cell produced in *cdkd;1 cdkd;3* pollen grains does not successfully fuse with the egg cell or the central cell. Therefore, as reported for *duo1* and *duo3*, the double knockout of *CDKD;1* and *CDKD;3* perturbs sperm cell differentiation during gametogenesis, causing failure in fertilization with both the egg cell and the central cell.

Liu *et al*. (2008) demonstrated that dysfunctions of the RING-type E3 ubiquitin ligases *RHF1a* and *RHF2a* caused male and female gametophyte lethality due to the accumulation of Kip-related protein 6 (KRP6), an inhibitor of CDK-cyclin complexes. In the *rhf1a rhf2a* double mutant, development of female gametophytes was arrested at early stages, making a good contrast with the *cdka;1* knockout mutant in which no defect in female gametogenesis is evident. This suggests that, in *rhf1a rhf2a*, accumulated KRP6 inhibits not only CDKA;1 but also other CDKs which have an essential role in female gametophyte development. This is consistent with the previous observation that triple mutations in *CDKA;1, CDKB1;1* and *CDKB1;2* caused defects in female gametogenesis; namely, ovules contained only one to four free nuclei, suggesting arrest of nuclear division before FG4 (Nowack *et al*., 2012). In this study, we revealed that the combination of *cdkd;1* and *cdkd;3* mutations also caused arrest and/or delay in the progression of female gametogenesis at multiple steps. Therefore, it is probable that CDKD;1 and CDKD;3 control both A- and B-type CDKs during female as well as male gametogenesis.

During endosperm development, expression was detected for *CDKD;1* and

CDKD;3, but not for *CDKD;2* or *CDKF;1* (Figure 2c) (Day *et al*., 2008). Siliques of *cdkd;1-1/+ cdkd;3-1/-* plants pollinated with wild-type pollen contained endosperm with fewer nuclei, and some central cells harbored distorted nuclei (Figure 5b). Proper development of the central cell is known to be essential for endosperm formation; for example, the deficiency of *AGL61*, which encodes a Type I MADS domain protein, leads to morphological defects of the central cell, causing a failure in endosperm development (Bemer *et al*., 2008; Steffen *et al*., 2008). It will therefore be of interest to examine whether the central cell is specified in *cdkd;1 cdkd;3*, and whether nuclear divisions are also impaired during endosperm development.

Hajheidari *et al*. (2012) reported that *cdkd;1 cdkd;2* and *cdkd;2 cdkd;3* double mutants showed dwarfism in post-embryonic development, but did not describe any gametophytic defect. By contrast, we could not obtain seedlings with both *cdkd;1* and *cdkd;3* mutations. We found that mitosis in female and male gametogenesis was severely defective when *cdkd;1* and *cdkd;3* mutations were combined. Taken together, these data indicate that CDKD;1 and CDKD;3 are essential CDK-activating kinases during gametogenesis, and even though CDKD;2 has some function during gametogenesis, it plays a distinct role from CDKD;1 or CDKD;3. In addition, our promoter-swapping experiment revealed that *CDKF;1* cannot substitute for *CDKD;1* or *CDKD;3*. During post-embryonic development, CDKF;1 does not have a pivotal role in activating CDKA;1 or CDKBs, but is instead involved in stabilization of CDKD;2 (Takatsuka *et al*., 2009). Since CDKD;2 has a higher kinase activity toward CTD than CDK (Shimotohno *et al*., 2004), CDKF;1 may principally control basal transcription by stabilizing CDKD;2. These results are consistent with the above-mentioned idea that CDKD;2 (and CDKF;1) has a distinct function from CDKD;1 or CDKD;3. We propose that, in *Arabidopsis*, CDKD;1

and CDKD;3 are the CAKs that phosphorylate and activate core CDKs controlling the cell cycle. We previously reported that CDKD;1 expressed in insect cells exhibits neither CDK- nor CTD-kinase activity (Shimotohno *et al*., 2003). However, our genetic data indicate that CDKD;1 has a redundant function with CDKD;3 during gametogenesis. This contradiction can be explained by the possibility that CDKD;1 requires some unknown regulator(s) for its enzyme activity which is, conversely, dispensable for CDKD;3. An important subject for future research will be to investigate functional similarity and divergence between these two core CAKs in plants.

In mammals, CDK7 functions as a CAK, exhibiting *in vitro* phosphorylation activity on all CDKs that directly regulate the cell cycle, such as CDK1, CDK2, CDK4 and CDK6 (for a review, see Fisher, 2005). On the other hand, some of the CDKs are phosphorylated and activated *in vivo* by kinases other than CDK7 (Larochelle *et al*., 1998; Bockstaele *et al.,* 2009): p42, for example, acts as a CAK for CDK2 (Liu *et al*., 2004). Plants possess two types of CDKs, CDKD and CDKF, both of which display sequence similarity to yeast and mammalian CAKs and exhibit kinase activities on human CDK2 *in vitro* (Shimotohno *et al*., 2004). However, as discussed above, CDKF;1 is not an essential CAK, whereas CDKD;1 and CDKD;3 are crucial for CDK activation. Are CDKD;1 and CDKD;3 redundant in terms of the control of cell division during plant development? Liu *et al*. (2004) proposed that a multiple-CAK system is beneficial for an elastic response to various conditions: one CAK may participate in maintaining CDK activity, for example, while other(s) may be engaged in the response to internal and/or external stimuli. The co-expression database ATTED-II (http://atted.jp/) shows that *CDKD;3* is closely associated with E2F target genes that include crucial regulators of G1-to-S progression. Indeed, the promoter region of *CDKD;3* has an E2F-binding sequence

(TTTCCCGG) (Vandepoele *et al*., 2005). E2F activity is inhibited by retinoblastoma-related (RBR) protein, which is inactivated by CDK phosphorylation in response to growth stimuli (Nakagami *et al*., 2002; Hirano *et al*., 2008). It is therefore probable that *Arabidopsis* CDKD;3 mediates between growth signals and the cell cycle, and adjusts cell division activity to suit various environmental conditions. Further studies will reveal how CDKD;3 activity is controlled in response to internal and external signals, and how its function differs from that of CDKD;1.

Experimental Procedures

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Col-0) was first grown at 23°C on a Murashige and Skoog agar plate (Murashige *et al*., 1962), and then transferred onto soil and grown under continuous light conditions. The *cdkf;1-1* mutant was grown on an MSAR agar plate (Koncz *et al*., 1990) under short-day conditions (16 h dark; 8 h light) (Takatsuka *et al*., 2009). The *cdkd;1-1*, *cdkd;3-1* and *cdkf;1-1* mutants were described previously (Shimotohno *et al*., 2006; Takatsuka *et al.*, 2009). When double mutants were generated, *cdkf;1-1* was identified using sulfadiazine, and *cdkd;1-1* and *cdkd;3-1* were tested by genomic PCR using primers described previously (Shimotohno *et al*., 2006; Takatsuka *et al.*, 2009). Transgenic plants expressing *pCDKD;2–CDKD;2::GUS*, *pCDKD;3–CDKD;3::GUS* and *pCDKF;1–CDKF;1::GUS* were described by Takatsuka *et al.* (2009).

Plasmid construction for plant transformation

To make *pCDKD;1–CDKD;1::GUS*, a genomic fragment of *CDKD;1* from 1697 bp upstream of the start codon to 1 bp before the stop codon was amplified by PCR and

cloned into the GATEWAY entry vector pDONR221 (Invitrogen). A recombination reaction was conducted between the entry clone and the Gateway destination vector pGWB3 (Nakagawa *et al*., 2007) using LR clonase (Invitrogen), generating a *GUS*-fusion gene. For the complementation test of *cdkd;1-1/+ cdkd;3-1/-*, *pCDKD;3–CDKD;3* was constructed by cloning a genomic fragment of *CDKD;3* from 2011 bp upstream of the start codon to 2018 bp downstream of the stop codon into pGWB3. For the promoter-swapping experiment, promoter fragments of *CDKF;1*, *CDKD;1* and *CDKD;3* from 2032, 1697 and 2000 bp upstream of the start codon, respectively, to 1 bp before the start codon were amplified and cloned into the entry vector pDONR P4-P1R (Invitrogen). The coding regions of *CDKF;1*, *CDKD;1* and *CDKD;3* from the start codon to 1 bp before the stop codon were amplified and cloned into the entry vector pDONR221 (Invitrogen). The resultant entry clones carrying promoter or coding regions were reacted with the destination vector R4pGWB533 (Nakagawa *et al*., 2008) using LR clonase, generating fusion gene comprising a promoter, a coding region and the *GUS* gene.

Microscopy

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 overnight in chloral hydrate solution (71% chloral hydrate and 11% glycerol).

Alexander staining was performed to test pollen viability (Alexander, 1969; Ravi *et al*., 2008). When anthers were about to dehisce, they were collected and dissected in Alexander staining solution (9.5% ethanol, 0.1 mg/ml Malachite Green, 25% 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, pH 2.3). The samples were then transferred to a drop of Alexander staining solution on a glass slide, and gently pressed with a coverslip so that the staining solution penetrated the anthers. After incubation at 37°C for 6 h, images were acquired under bright-field microscopy.

Nuclei in pollen grains were visualized 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 (pH 7.0), 1 mM EDTA, 0.1% Triton X-100, 0.4 μg/ml DAPI (4',6-diamidino-2-phenylindole)]. After brief mixing and centrifugation, pelleted pollen grains were transferred onto glass slides. Microscopic observation was performed using an LSM510 confocal laser scanning microscope system (Zeiss, Thornwood, NY, USA) with UV excitation. To observe female gametophyte development, sepals, petals and stamens were removed from collected flowers, and the pistils were fixed and cleared and subjected to microscopic analyses. At the early phase of floral stage 13, when flowers were opening and the stamens nearly reached the pistils (Bowman, 1994), female gametogenesis progressed into stages FG5 to FG7 as described by Yu *et al*. (2005).

GUS staining

For GUS staining, siliques and pollen were collected from flowers at floral stage 13. Tissues were fixed in 90% acetone for 15 min on ice, washed with GUS buffer [100 mM sodium phosphate (pH 7.0), 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-indolyl-β-D-glucuronide). The samples were then degassed for 45 min, and incubated at 37°C overnight.

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Supporting Information

Figure S1. Male gametogenesis in *Arabidopsis*.

Figure S2. Female gametogenesis in *Arabidopsis*.

Figure S3. Examination of pollen viability.

Figure S4. Seed formation in *cdkd;1 cdkf;1* and *cdkd;3 cdkf;1*.

Table S1. Transmission efficiency of *cdkd;1-1* and *cdkd;3-1* mutations from pollen.

Table S2. Ovule number in *cdkd;1 cdkd;3* pistils pollinated with wild-type pollens.

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Table 1. Number of seeds produced in *cdkd;1-1/+ cdkd;3-1/-* plants

Genotype	Normal seeds (%)	Abortive seeds (%)
WT	222 (97.8)	5 (2.2)
cdkd;1-1/+ cdkd;3-1/-	311 (50.2)	302 (49.8)
cdkd;1-1/+ cdkd;3-1/- with *pCDKD;3-CDKD;3*	215 (91.9)	19 (8.1)

Seeds containing no embryo at the stage when normal seeds reach maturity were defined as abortive seeds.

Table 2. Number of seeds produced by reciprocal crosses between wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants

Parental genotype (Female x Male)	Normal seeds (%)	Abortive seeds (%)
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 3. Percentage of tri-, bi- and mononucleate and degenerated pollen grains produced in wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants

Genotype	Three nuclei	Two nuclei	One 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%

These data are also shown in Figure 4b. n =106 (WT) and 203 (*cdkd;1-1/+ cdkd;3-1/-*).

Table 4. Number of ovules produced in wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants

Genotype	Ovules at distinct stages during female gametogenesis					Degenerated ovules
	FG1	FG2	FG3	FG4	FG5-7	
WT	1	0	0	0	347	3
cdkd;1-1/+ cdkd;3-1/-	30	45	8	5	471	56

Table 5. Number of seeds produced in *cdkd;1-1/- cdkf;1-1/+*, *cdkd;3-1/- cdkf;1-1/+* and *cdkd;1-1/+ cdkd;3-1/-* plants expressing *D;3-F;1::GUS*

Genotype	Normal seeds (%)	Abortive seeds (%)
WT	171 (96.8)	6 (3.2)
cdkf;1-1/+	163 (91.6)	15 (8.4)
cdkd;1-1/- cdkf;1-1/+	194 (92.8)	15 (7.2)
cdkd;3-1/- cdkf;1-1/+	175 (97.8)	4 (2.2)
cdkd;1-1/+ cdkd;3-1/-	83 (55.7)	66 (44.3)
cdkd;1-1/+ cdkd;3-1/- with *D;3-F;1::GUS*	64 (52.0)	59 (48.0)

Figure legends

Figure 1. Expression patterns of CAKs in flowers and gametophytes.

GUS staining of transgenic plants harboring *pCDKF;1–CDKF;1::GUS*, *pCDKD;1–CDKD;1::GUS*, *pCDKD;2–CDKD;2::GUS* or *pCDKD;3–CDKD;3::GUS*. (a) Flowers. (b) Mature pollen. (c) Siliques (left) and ovules (right). Bars = 1 mm (a), 50 μm (b), 1 mm (c, left) and 50 μm (c, right).

Figure 2. Expression patterns of CAKs in embryo and endosperm.

GUS staining of transgenic plants harboring *pCDKF;1–CDKF;1::GUS*, *pCDKD;1–CDKD;1::GUS*, *pCDKD;2–CDKD;2::GUS* or *pCDKD;3–CDKD;3::GUS*. (a) Embryos at different stages. (b) Enlargement of the regions around the shoot apical meristem (SAM) and the root apical meristem (RAM) in late embryos. (c) Ovules with embryo at the globular stage. Red arrowheads indicate embryos. Bars = 50 μm (a, globular stage), 100 μm (a, heart stage to late embryo) and 100 μm (b, c).

Figure 3. Gametophyte-lethal phenotype of the *cdkd;1 cdkd;3* double mutant.

(a) T-DNA insertion sites in *cdkd;1-1* and *cdkd;3-1*. Exons are indicated by black boxes. (b) Seeds in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants with or without complementation by the genomic fragment of *CDKD;3*. (c) Seed abortion observed in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants. (d) Early embryo and endosperm development in wild-type ovules. Before the first zygotic division, at least three rounds of nuclear divisions occur in the endosperm (left). After mitosis starts in the embryo, endosperm nuclei continue to divide (middle and right). Black arrowheads indicate the zygote and embryos. (e) Unfertilized ovules at stages FG1, FG2 and FG7 observed in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants.

Open arrowheads indicate nuclei. (f) Ovules with decreased number of endosperm nuclei observed in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants. Red arrowheads indicate endosperm nuclei. Bars = 1 mm (b), 100 μm (c) and 10 μm (d-f).

Figure 4. Disturbed pollen mitosis in *cdkd;1 cdkd;3*.

(a) DAPI staining of mature pollen from anthers of wild-type and *cdkd;1/+ cdkd;3/-* plants. Tri-, bi- and mononucleate and degenerated pollen grains are shown. (b) Percentage of tri-, bi- and mononucleate and degenerated pollen grains. n = 106 (wild-type) and 203 (*cdkd;1/+ cdkd;3/-*). (c) Three-DAP ovules of wild-type plants pollinated with pollen from wild-type (left) or *cdkd;1-1/+ cdkd;3-1/-* (right) plants. Black and red arrowheads indicate nuclei of the central cell and the egg cell, respectively. Bars = 5 μm (a) and 50 μm (c).

Figure 5. Defects in female gametogenesis in *cdkd;1 cdkd;3*.

(a) Wild-type ovules when flowers have opened and stamens nearly reached pistils. Two ovules at stages FG5 and FG7 are shown. PN, polar nucleus (black arrowheads); ECN, egg cell nucleus (black arrows); SCN, synergid cell nucleus (white arrowheads); CCN, central cell nucleus (white arrows). (b) Ovules of *cdkd;1/+ cdkd;3/-* plants. Ovules at FG1 to FG7 were observed when flowers had opened and stamens nearly reached pistils. Black and open arrowheads indicate nuclei and a central cell with a distorted nucleus, respectively. (c) Aberrant endosperm development in a 3-DAP ovule of a *cdkd;1/+ cdkd;3/-* plant pollinated with wild-type pollen. Black and white arrowheads indicate an embryo and endosperm nuclei, respectively. Bars = 20 μm.

Figure 6. Phenotypes of *cdkd;1 cdkf;1* and *cdkd;3 cdkf;1*.

(a) Four-day-old seedlings of *cdkf;1-1/+*, *cdkf;1-1/-*, *cdkd;1-1/- cdkf;1-1/+*, *cdkd;1-1/- cdkf;1-1/-*, *cdkd;3-1/- cdkf;1-1/+*, and *cdkd;3-1/- cdkf;1-1/-*. (b) Root tips of 4-day-old seedlings. Arrowheads indicate the quiescent center (lower) and the first elongated cell in the cortex cell file (upper). (c) Columella cell layers of 4-day-old seedlings. Arrowheads indicate the quiescent center (white) and columella cell layers including the initial cell layer (black). Bars = 5 mm (a) and 50 μm (b, c).

Figure 7. *CDKF;1* cannot complement *cdkd;1 cdkd;3*.

(a) Schematic diagrams of GUS-fusion genes. Yellow and orange boxes indicate the promoter regions and exons of, respectively, *CDKD;3* and *CDKF;1*, and blue boxes indicate the *GUS* gene. Lines represent introns. (b) Complementation of *cdkf;1-1* by expression of *pCDKF;1-CDKF;1::GUS* (*F;1-F;1::GUS*). Ten-day-old seedlings are shown. (c, d) GUS staining of transgenic plants expressing *F;1-F;1::GUS*, *pCDKD;3-CDKD;3::GUS* (*D;3-D;3::*GUS) and *pCDKD;3-CDKF;1::GUS* (*D;3-F;1::GUS*). Root tips of 4-day-old seedlings (c) and pistils just before pollination (d) are shown. (e) Cleared mature siliques of wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants, and of *cdkd;1-1/+ cdkd;3-1/-* harboring *D;3-F;1::GUS*. Bars = 5 mm (b), 50 μm (c) and 1 mm (d, e).

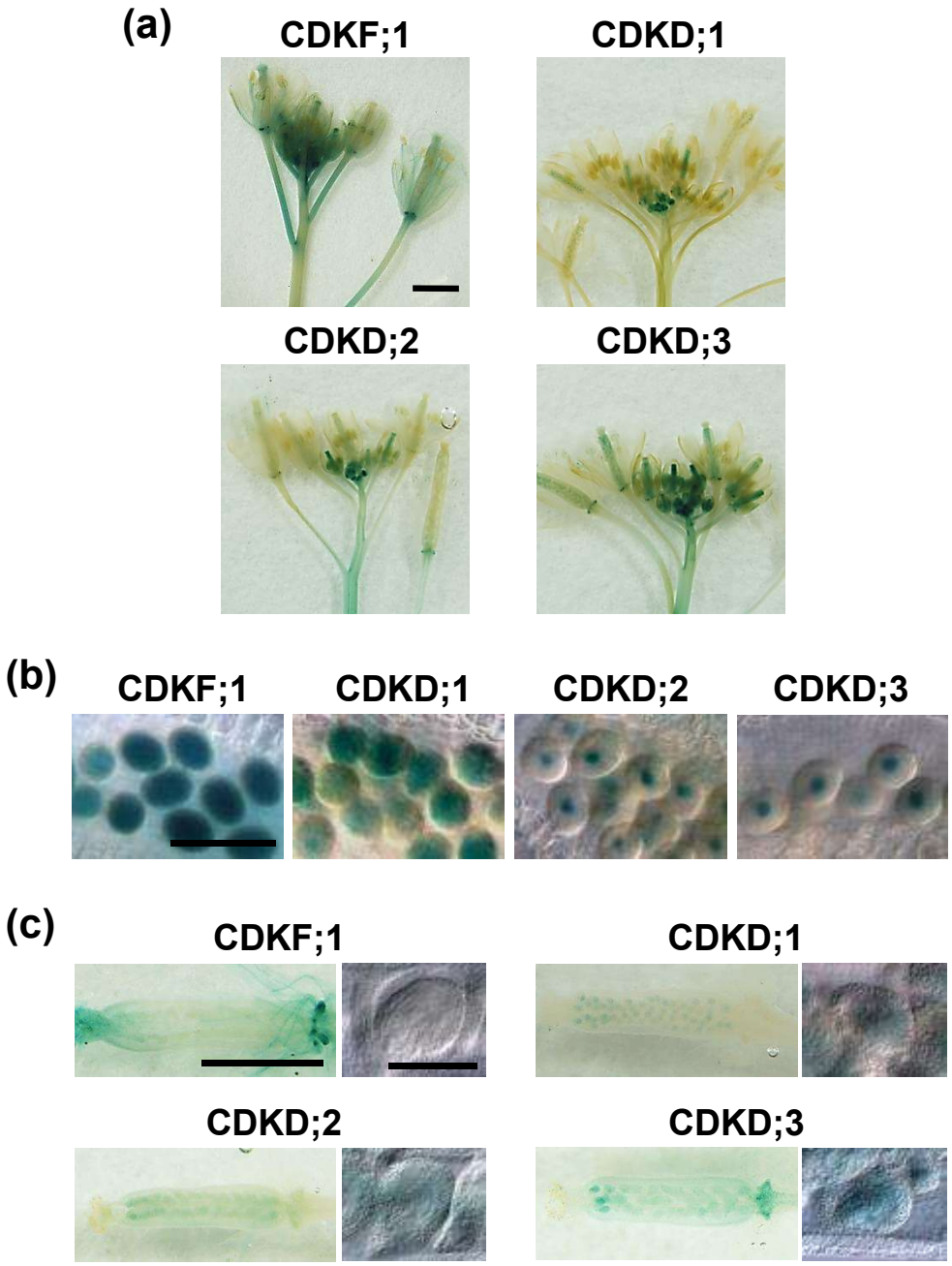


Figure 1. Expression patterns of CAKs in flowers and gametophytes.
GUS staining of transgenic plants harboring *pCDKF;1–CDKF;1::GUS*, *pCDKD;1–CDKD;1::GUS, pCDKD;2–CDKD;2::GUS or pCDKD;3–CDKD;3::GUS*. (a) Flowers. (b) Mature pollen. (c) Siliques (left) and ovules (right). Bars = 1 mm (a), 50 μm (b), 1 mm (c, left) and 50 μm (c, right).

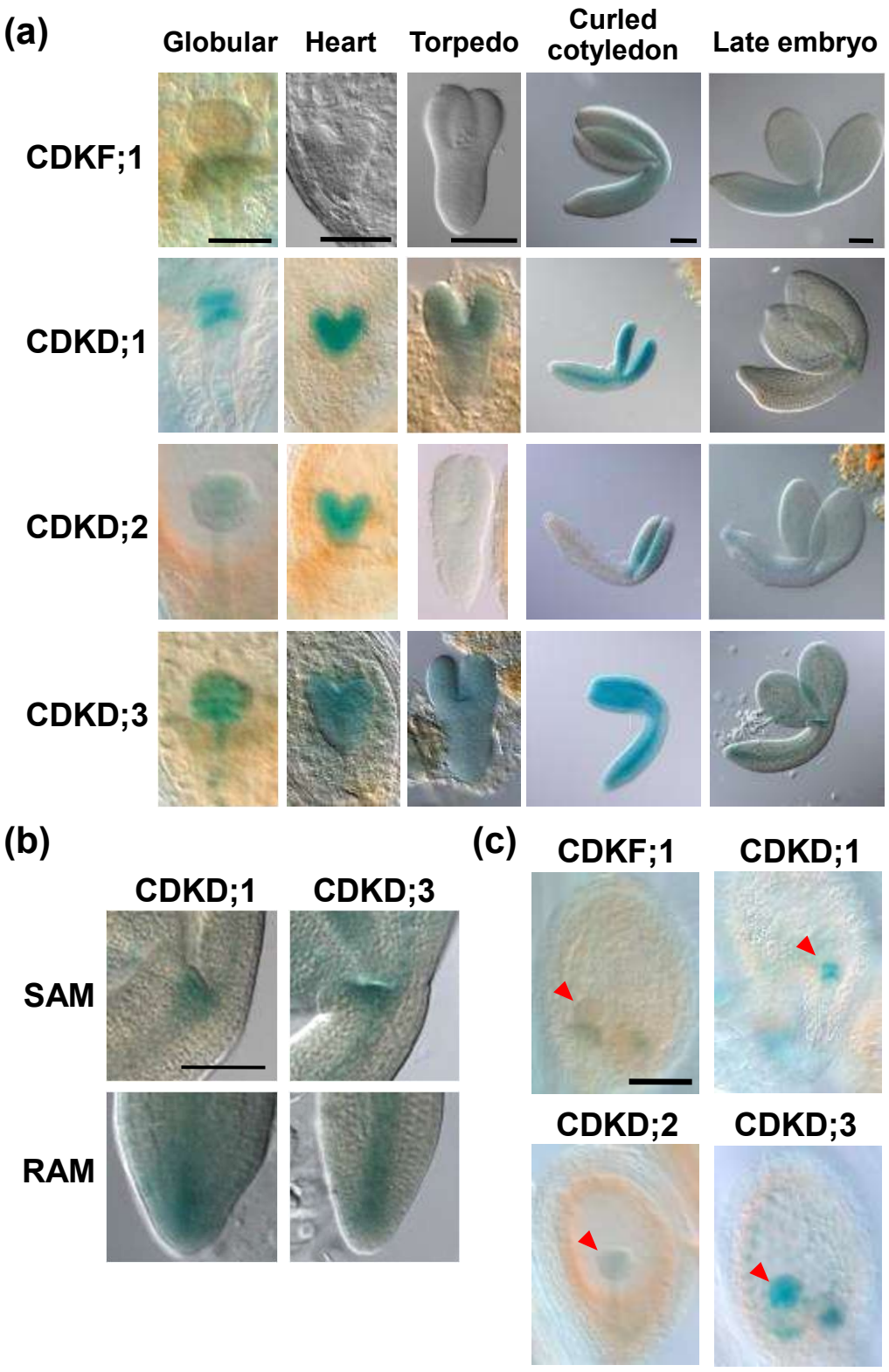
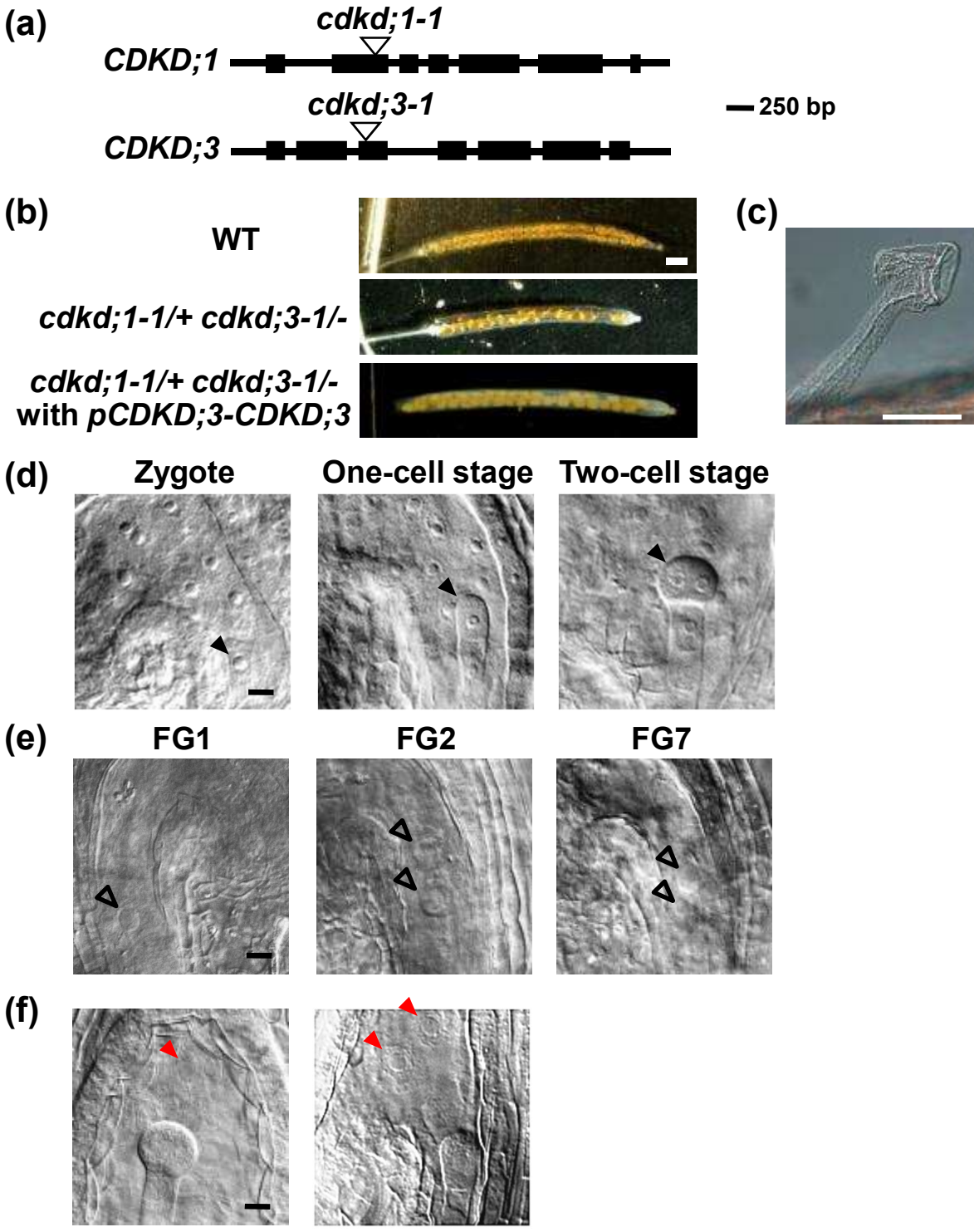


Figure 2. Expression patterns of CAKs in embryo and endosperm.
GUS staining of transgenic plants harboring *pCDKF;1–CDKF;1::GUS*, *pCDKD;1–CDKD;1::GUS*, *pCDKD;2–CDKD;2::GUS* or *pCDKD;3–CDKD;3::GUS*. (a) Embryos at different stages. (b) Enlargement of the regions around the shoot apical meristem (SAM) and the root apical meristem (RAM) in late embryos. (c) Ovules with embryo at the globular stage. Red arrowheads indicate embryo. Bars = 50 μm (a, globular stage), 100 μm (a, heart stage to late embryo) and 100 μm (b, c).



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(a) T-DNA insertion sites in *cdkd;1-1* and *cdkd;3-1*. Exons are indicated by black boxes. (b) Seeds in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants with or without complementation by the genomic fragment of *CDKD;3*. (c) Seed abortion observed in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants. (d) Early embryo and endosperm development in wild-type ovules. Before the first zygotic division, at least three rounds of nuclear divisions occur in the endosperm (left). After mitosis starts in the embryo, endosperm nuclei continue to divide (middle and right). Black arrowheads indicate the zygote and embryos. (e) Unfertilized ovules at stages FG1, FG2 and FG7 observed in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants. Open arrowheads indicate nuclei. (f) Ovules with decreased number of endosperm nuclei observed in siliques of *cdkd;1-1/+ cdkd;3-1/-* plants. Red arrowheads indicate endosperm nuclei. Bars = 1 mm (b), 100 µm (c) and 10 µm (d-f).

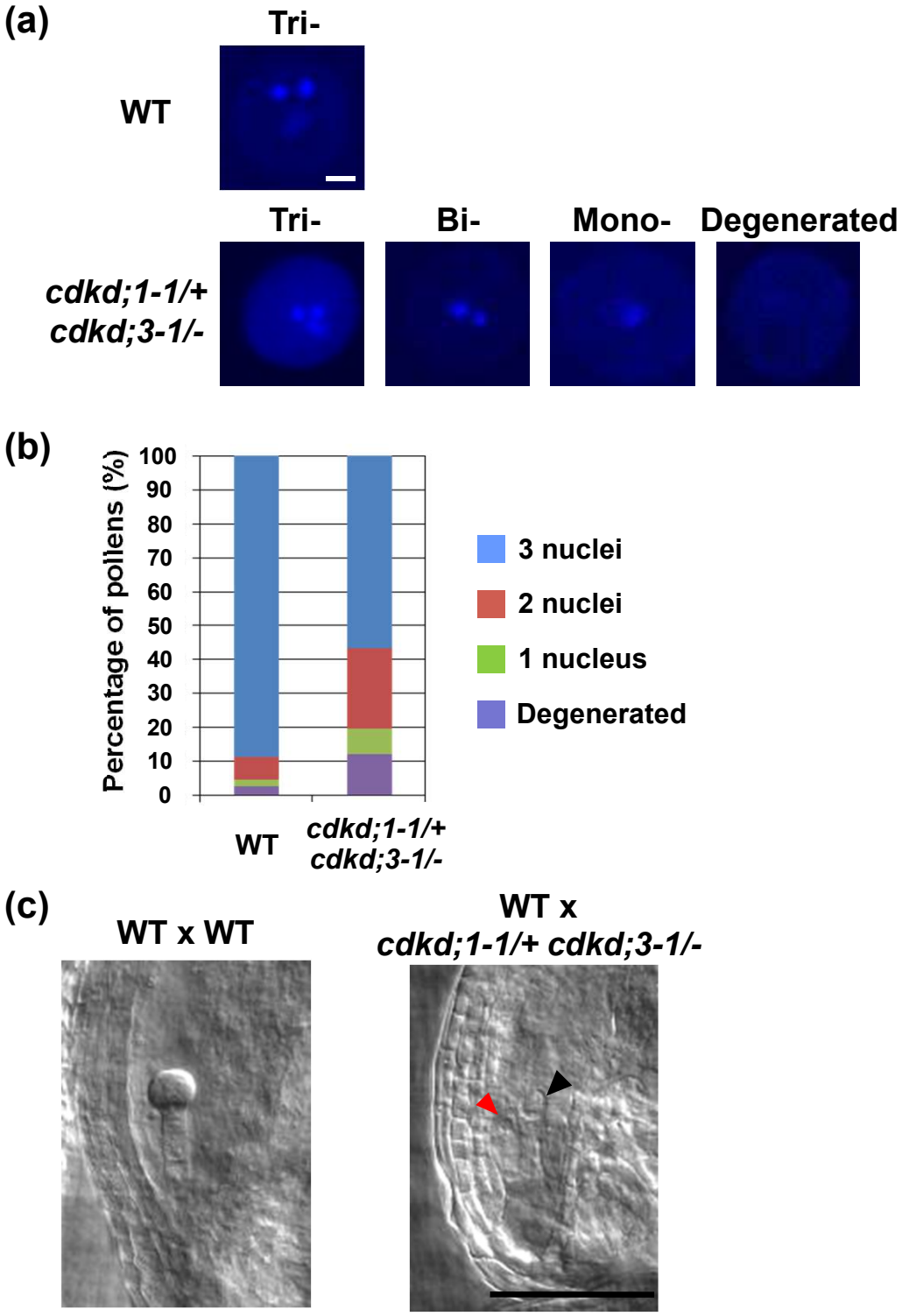

Figure 4. Disturbed pollen mitosis in *cdkd;1 cdkd;3*.
(a) DAPI staining of mature pollen from anthers of wild-type and *cdkd;1/+ cdkd;3/-* plants. Tri-, bi- and mononucleate and degenerated pollen grains are shown. (b) Percentage of tri-, bi- and mononucleate and degenerated pollen grains. n = 106 (wild-type) and 203 (*cdkd;1/+ cdkd;3/-*). (c) Three-DAP ovules of wild-type plants pollinated with pollen from wild-type (left) or *cdkd;1-1/+ cdkd;3-1/-* (right) plants. Black and red arrowheads indicate nuclei of the central cell and the egg cell, respectively. Bars = 5 µm (a) and 50 µm (c).

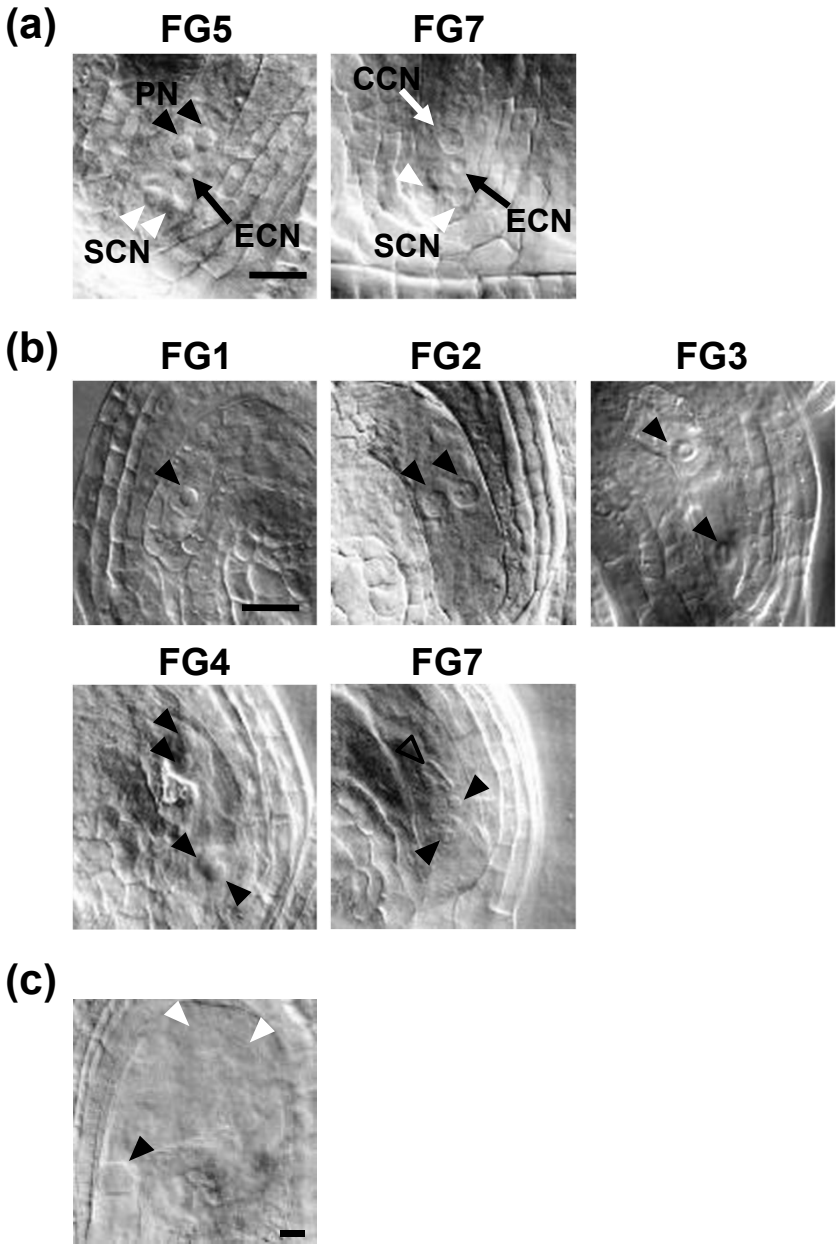

Figure 5. Defects in female gametogenesis in *cdkd;1 cdkd;3*.
(a) Wild-type ovules when flowers have opened and stamens nearly reached pistils. Two ovules at stages FG5 and FG7 are shown. PN, polar nucleus (black arrowheads); ECN, egg cell nucleus (black arrows); SCN, synergid cell nucleus (white arrowheads); CCN, central cell nucleus (white arrows). (b) Ovules of *cdkd;1/+ cdkd;3/-* plants. Ovules at FG1 to FG7 were observed when flowers had opened and stamens nearly reached pistils. Black and open arrowheads indicate nuclei and a central cell with a distorted nucleus, respectively. (c) Aberrant endosperm development in a 3-DAP ovule of a *cdkd;1/+ cdkd;3/-* plant pollinated with wild-type pollen. Black and white arrowheads indicate an embryo and endosperm nuclei, respectively. Bars = 20 μm.

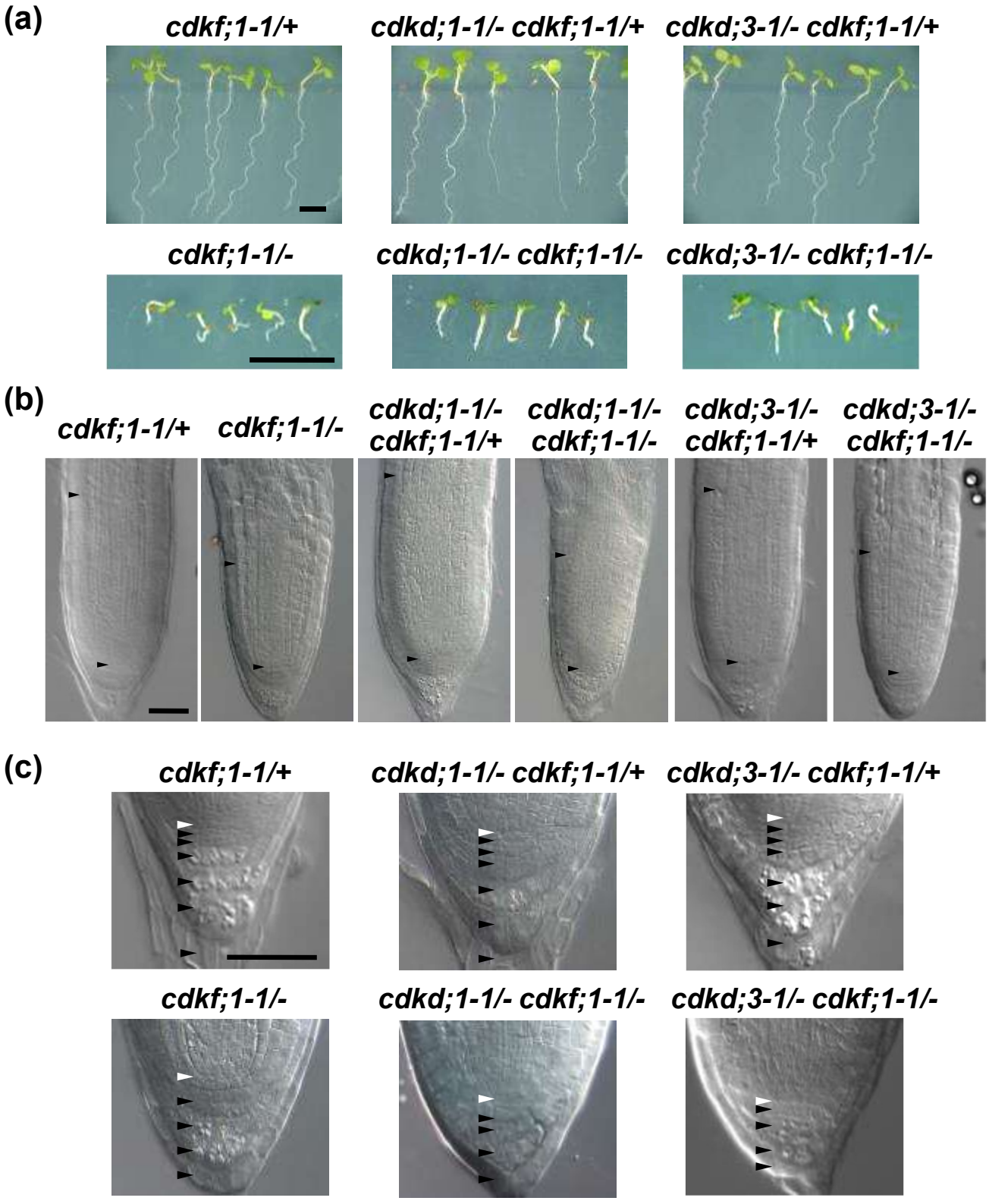

Figure 6. Phenotypes of *cdkd;1 cdkf;1* and *cdkd;3 cdkf;1*.
(a) Four-day-old seedlings of *cdkf;1-1/+*, *cdkf;1-1/-*, *cdkd;1-1/- cdkf;1-1/+*, *cdkd;1-1/- cdkf;1-1/-*, *cdkd;3-1/- cdkf;1-1/+*, and *cdkd;3-1/- cdkf;1-1/-*. (b) Root tips of 4-day-old seedlings. Arrowheads indicate the quiescent center (lower) and the first elongated cell in the cortex cell file (upper). (c) Columella cell layers of 4-day-old seedlings. Arrowheads indicate the quiescent center (white) and columella cell layers including the initial cell layer (black). Bars = 5 mm (a) and 50 μm (b, c).

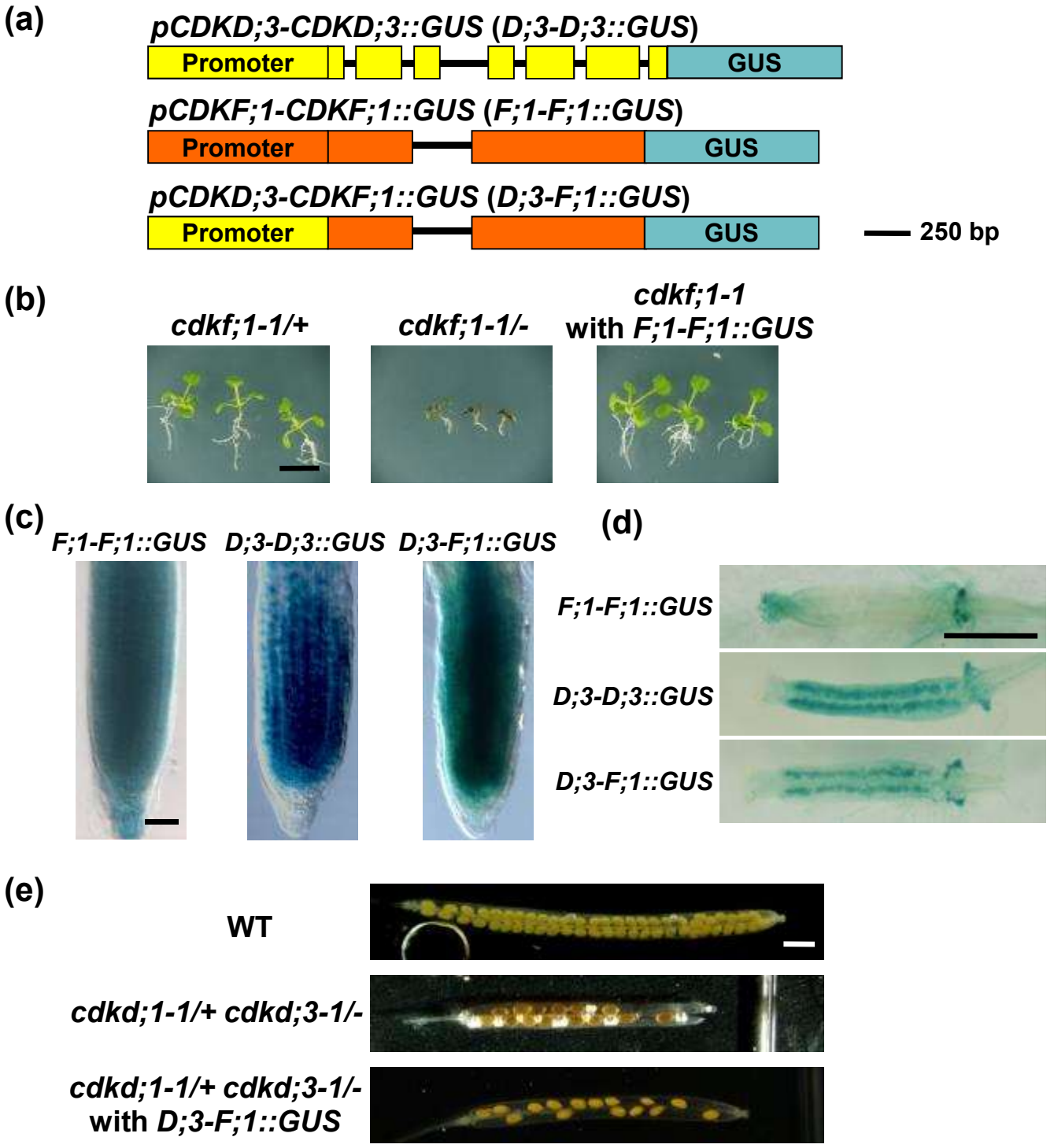

Figure 7. *CDKF;1* cannot complement *cdkd;1 cdkd;3*.
(a) Schematic diagrams of GUS-fusion genes. Yellow and orange boxes indicate the promoter regions and exons of, respectively, *CDKD;3* and *CDKF;1*, and blue boxes indicate the *GUS* gene. Lines represent introns. (b) Complementation of *cdkf;1-1* by expression of *pCDKF;1-CDKF;1::GUS* (*F;1-F;1::GUS*). Ten-day-old seedlings are shown. (c, d) GUS staining of transgenic plants expressing *F;1-F;1::GUS*, *pCDKD;3-CDKD;3::GUS* (*D;3-D;3::*GUS) and *pCDKD;3-CDKF;1::GUS* (*D;3-F;1::GUS*). Root tips of 4-day-old seedlings (c) and pistils just before pollination (d) are shown. (e) Cleared mature siliques of wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants, and of *cdkd;1-1/+ cdkd;3-1/-* harboring *D;3-F;1::GUS*. Bars = 5 mm (b), 50 μm (c) and 1 mm (d, e).

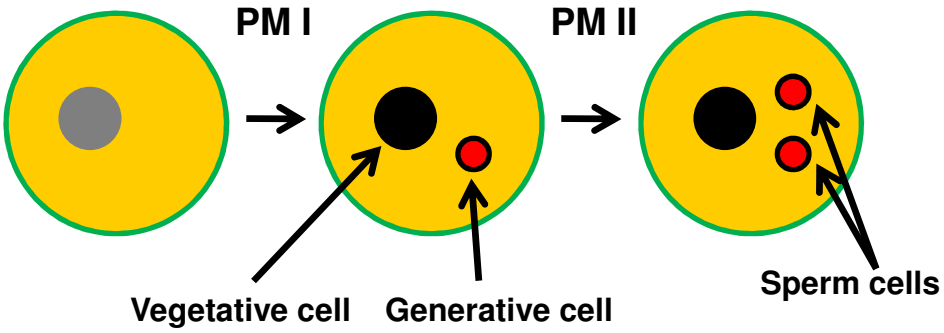

Figure S1. Male gametogenesis in *Arabidopsis.*
A vegetative cell divides asymmetrically to produce a daughter vegetative cell and a generative cell in pollen mitosis I (PM I). The generative cell then divides symmetrically to produce two sperm cells in pollen mitosis II (PM II).

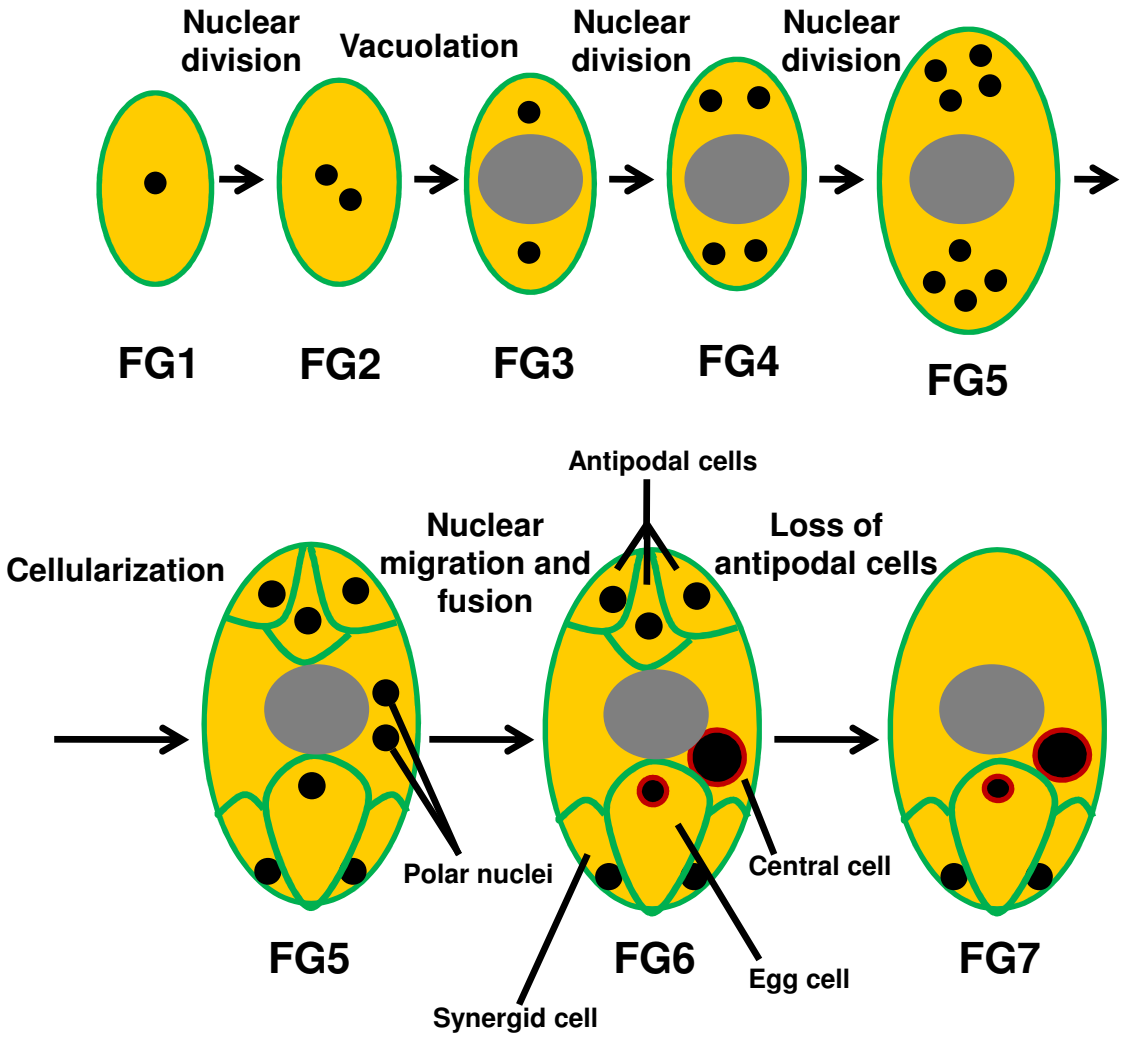

Figure S2. Female gametogenesis in *Arabidopsis*.
At FG6, the central cell is produced by fusion of two polar nuclei, followed by loss of three antipodal cells. Black and gray circles indicate nuclei and the central vacuole in the embryo sac, respectively.

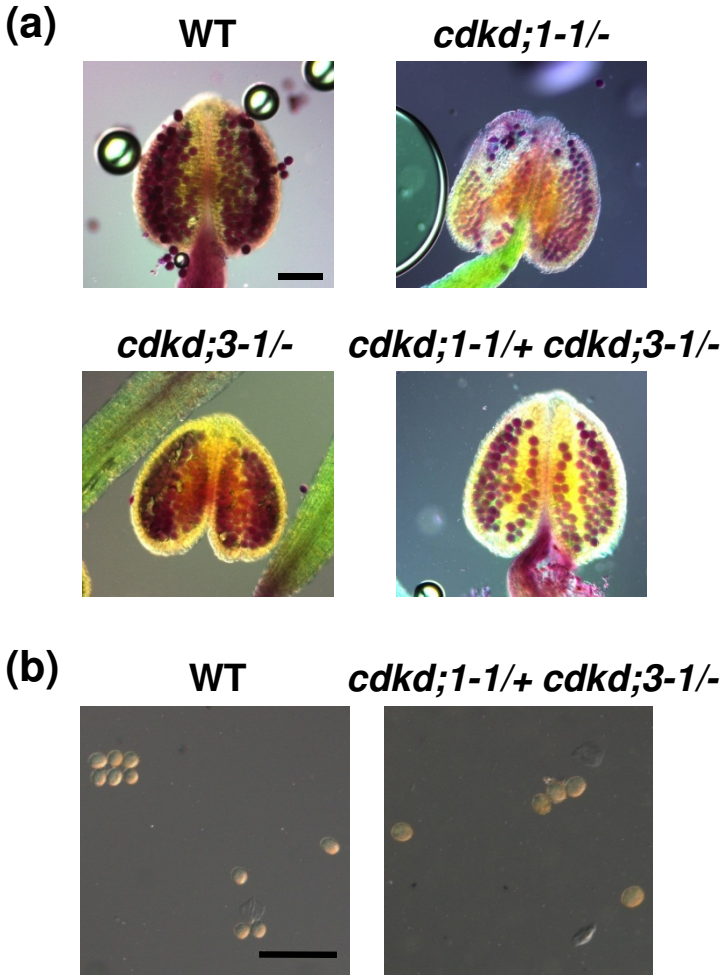

Figure S3. Viability of pollen from wild-type, *cdkd;1-1/-*, *cdkd;3-1/-*, and *cdkd;1-1/+ cdkd;3-1/-* plants.
(a) Alexander staining of pollen grains in anthers just before dehiscence. Violet- and blue-stained pollen grains are viable and nonviable, respectively. (b) Pollen grains from mature flowers of wild-type and *cdkd;1-1/+ cdkd;3-1/-* plants. Bars = 100 µm.

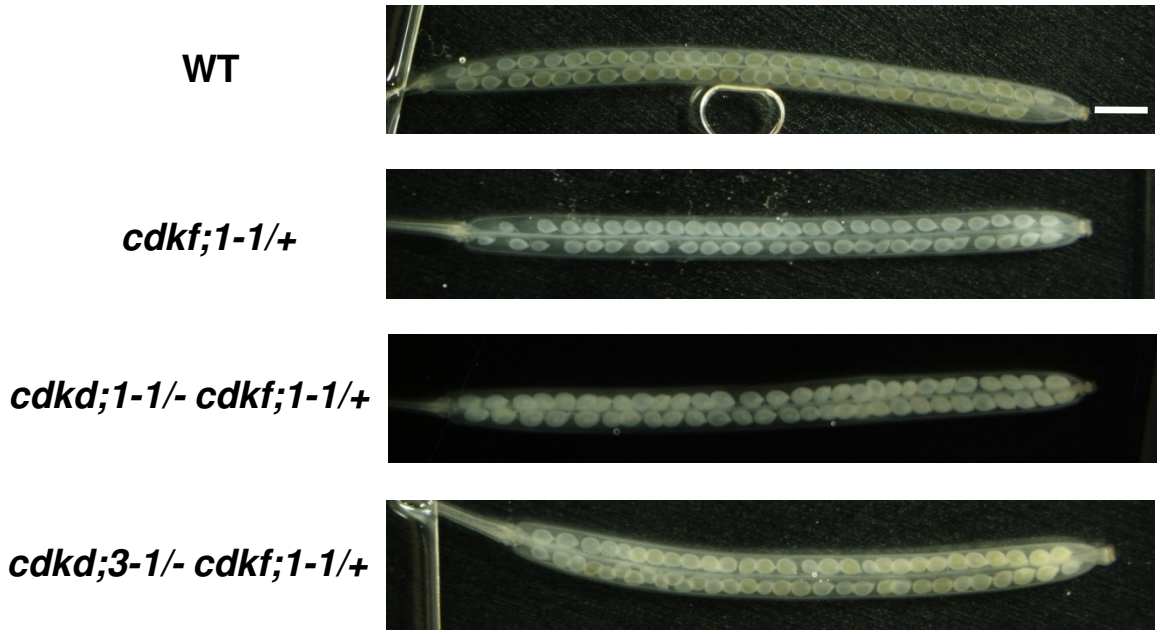

Figure S4. Seed formation in siliques of wild-type, *cdkf;1-1/+*, *cdkd;1-1/- cdkf;1-1/+*, and *cdkd;3-1/- cdkf;1-1/+* plants. Bar = 1 mm.