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Diverse phosphoregulatory mechanisms controlling cyclin-dependent kinase-activating kinases in *Arabidopsis*

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Summary

For the full activation of cyclin-dependent kinases (CDKs), not only cyclin binding but also phosphorylation of a threonine (Thr) residue within the T-loop is required. This phosphorylation is catalyzed by CDK-activating kinases (CAKs). In *Arabidopsis*, three CDKD genes (*CDKD;1–CDKD;3*) encode vertebrate-type CAK orthologues of which CDKD;2 exhibits high phosphorylation activity toward the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. Here, we show that CDKD;2 forms a stable complex with cyclin H and is downregulated by the phosphorylation of the ATP-binding site by WEE1 kinase. A knockout mutant of *CDKD;3*, which has a higher CDK kinase activity, displayed no defect in plant development. Instead, another type of CAK—CDKF;1—exhibited a significant activity toward CDKA;1 in *Arabidopsis* root protoplasts, and the activity was dependent on the T-loop phosphorylation of CDKF;1. We propose that two distinct types of CAK, namely, CDKF;1 and CDKD;2, play a major role in CDK and CTD phosphorylation, respectively, in *Arabidopsis*.

Introduction

Progression through the eukaryotic cell cycle is controlled by the activity of cyclin-dependent serine (Ser)/threonine (Thr) protein kinases (CDKs). CDKs are activated by binding to specific cyclin partners, and the activity of cyclin-CDK complexes is further regulated by the synthesis and degradation of cyclin subunits, binding to inhibitory proteins, and the phosphorylation of CDKs themselves (Morgan, 1997). CDK phosphorylation is mediated by two groups of protein kinases—CDK-activating kinases (CAKs) and WEE1 kinases. CAKs are responsible for activating the phosphorylation of conserved Thr residues within the T-loop (Kaldis, 1999). WEE1 kinases execute the inhibitory phosphorylation within the N-terminal ATP binding site and are counteracted by the action of Cdc25 phosphatases that are essential for dephosphorylating and activating CDKs to trigger mitosis (Featherstone and Russell, 1991; Kumagai and Dunphy, 1991; Nurse, 1990).

The catalytic subunit of vertebrate CAK is termed CDK7/p40^{MO15}, and its regulatory subunit is named cyclin H (Fisher and Morgan, 1994; Labbé *et al.*, 1994; Mäkelä *et al.*, 1994). In the presence of cyclin H, CDK7 activity is significantly stimulated (Fisher and Morgan, 1994). A third subunit that stabilizes the cyclin H-CDK7 complex is a RING finger protein named MAT1 (Devault *et al.*, 1995; Tassan *et al.*, 1995). In addition to CDKs, vertebrate CAK phosphorylates the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. The trimeric CDK7-cyclin H-MAT1 complex has been identified in the general transcription factor TFIIH that is involved in the initiation and elongation of transcription (Schwartz *et al.*, 2003;

Serizawa *et al*., 1995; Shiekhattar *et al*., 1995). In fission yeast, CAK consists of Mcs6/Crk1/Mop1, Mcs2, and Pmh1, which are closely related to CDK7, cyclin H, and MAT1, respectively, and phosphorylates both Cdc2 and the CTD (Bamps *et al*., 2004; Buck *et al*., 1995; Damagnez *et al*., 1995).

In budding yeast, a monomeric CAK, namely, Cak1p/Civ1p, has been shown to possess Cdc28p-activating kinase activity *in vivo* (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996). However, Cak1p has a very low sequence similarity to other CAKs and does not possess CTD kinase activity. The orthologue of CDK7 in budding yeast is Kin28p that is associated with TFIIH and phosphorylates the CTD (Cismowski *et al*., 1995; Feaver *et al*., 1994, 1996; Liu *et al*., 2004) but does not exhibit Cdc28p kinase activity. This indicates that CDK and CTD phosphorylations are controlled by distinct kinases in budding yeast. Kimmelman *et al*. (1999) have reported that Cak1p has another activity—to phosphorylate the T-loop of Kin28p and thereby stimulate its CTD kinase activity; this suggests that Cak1p is also involved in basal transcription.

Based on the primary structure, plant CDK-related proteins have been classified into six types, namely, CDKA–CDKF (Joubés *et al*., 2000; Vandepoele *et al*., 2002). Among them, CDKA is assumed to be an orthologue of Cdc2/Cdc28p and appears to function in both G1- to S-phase and G2- to M-phase progression (Hemerly *et al*., 1995). CDKD was assigned to the proteins that have a high similarity to vertebrate-type CAKs. The first plant CAK, namely, Orysa;CDKD;1, was identified in rice plants (Hata, 1991) and was shown to be closely related to mammalian CDK7 in terms of enzyme activity (Yamaguchi *et al*., 1998, 2000). There are three *CDKD* genes in *Arabidopsis*, namely,

Arath;CDKD;1, *Arath;CDKD;2*, and *Arath;CDKD;3* (originally named *CAK3At*, *CAK4At*, and *CAK2At*, respectively) (Shimotohno *et al.*, 2003; Umeda, 2002). Although both Arath;CDKD;2 and Arath;CDKD;3 (hereafter called CDKD;2 and CDKD;3, respectively) exhibited CDK and CTD kinase activities, Arath;CDKD;1 (hereafter called CDKD;1) showed neither activity (Shimotohno *et al.*, 2004). CDK2 kinase activity of CDKD;3 was extremely high as compared with that of CDKD;2, whereas CDKD;2 had higher CTD kinase activity than CDKD;3 (Shimotohno *et al.*, 2003). In insect cells, a cyclin H homologue, namely, Arath;CycH;1 (hereafter called CycH;1), bound and activated CDKD;2 and CDKD;3 but not CDKD;1.

A distinct type of CAK is encoded on the *CDKF* gene. In *Arabidopsis*, *Arath;CDKF;1* (originally designated as *CAK1At*) has been isolated as a suppressor of the *CAK* mutation in budding yeast (Umeda *et al.*, 1998). The amino acid sequence is related to those of vertebrate-type CAKs but similarities are restricted to the conserved kinase domains. Arath;CDKF;1 (hereafter called CDKF;1) phosphorylated and activated human CDK2 *in vitro* but did not phosphorylate the CTD and was unable to interact with CycH;1 (Shimotohno *et al.*, 2004; Umeda *et al.*, 1998). Recently, we revealed that CDKF;1 phosphorylates the T-loop of CDKD;2 and CDKD;3 and activates the CTD kinase activity of CDKD;2. Therefore, CDKF;1 functions as a CAK-activating kinase as well as a CAK (Shimotohno *et al.*, 2004; Umeda *et al.*, 2005). Here, we show that CDKF;1 exerted a high CAK activity in the absence of CycH;1 and required T-loop phosphorylation for the activity. In contrast, CDKD;2 was tightly associated with CycH;1 to display the CTD kinase activity, while it was negatively

regulated via phosphorylation of the ATP binding site by the WEE1 kinase. We propose a model of CDK and CTD phosphorylation by distinct CAKs in *Arabidopsis*.

Results

CycH;1 forms active kinase complexes with CDKDs in plant cells

We have recently reported that *Arabidopsis* CDKD;2 and CDKD;3 interacted with CycH;1 in yeast and insect cells; however, their interactions in plant cells remained unknown (Shimotohno *et al*., 2004). To identify CycH;1-CDKD complexes, we prepared a specific antibody against histidine (His)-tagged CycH;1. Immunoblot analyses performed using an *Arabidopsis* crude extract showed that three bands of 37, 39, and 40 kDa were detected (Fig. 1a). Depletion of the antibody from the antiserum by incubation with nickel-nitriloacetic acid (Ni-NTA) agarose carrying His-CycH;1 resulted in the disappearance of all bands on the blots (Fig. 1a). CycH;1 produced in yeast cells showed the same mobility on SDS-PAGE as the 37 kDa protein (Fig. 1a) suggesting that it might represent the intrinsic CycH;1. In plants, specific cross-reactions were observed in roots but were barely detected in shoots (Fig. 1a).

The immunoprecipitation of an *Arabidopsis* crude extract with the anti-CycH;1 antibody recovered all three proteins of 37, 39, and 40 kDa; however, the 37 kDa protein was most efficiently precipitated (Fig. 1b). The same immunoprecipitates contained CDKD;2 but not CDKF;1 (Fig. 1b). CDKD;3 in the CycH;1 immunoprecipitates was faintly detectable. Immunoprecipitation with the anti-CDKD antibodies showed that CycH;1 was coprecipitated with CDKD;2 but less efficiently

with CDKD;3 (Fig. 1b). These results indicate that **at least 37 kDa** CycH;1 forms a stable complex with CDKD;2 in plant cells.

In the *Arabidopsis* cell culture, CDKD;2 forms a major complex with a molecular mass of ~200 kDa, whereas CDKD;3 is included in two complexes of ~130 kDa and ~700 kDa, respectively (Shimotohno *et al.*, 2004; Fig. 1c). Here, we fractionated the total protein by Sephacryl S300 gel exclusion chromatography and immunoblotted each fraction with the anti-CycH;1 antibody. The 37 kDa protein was detected in a range from 50 to 250 kDa (fraction No. 56–66) that overlaps with the fractions including CDKD;2 and CDKD;3 (Fig. 1c). In contrast, the 39 kDa protein was included in fraction No. 50–62 (120 to 480 kDa) that corresponded well to those of CDKD;2 (Fig. 1c), suggesting that it may specifically bind CDKD;2. This is consistent with the result that the 39 kDa protein was included in the immunoprecipitate with the anti-CDKD;2 antibody (Fig. 1b). A kinase assay showed that the CycH;1 complex in fraction No. 50–66 exhibited kinase activity toward the glutathione S-transferase (GST) fusion of *Arabidopsis* RNA polymerase II CTD (Fig. 1c) but not toward the GST-fused human CDK2 (data not shown); this indicates that both the 37 kDa and 39 kDa proteins are associated with the CTD kinase activity. The nature of the 40 kDa protein remains unknown; it formed a larger complex than the 37 kDa or 39 kDa protein.

The kinase activity associated with CycH;1 was further investigated in a heterologous system by using tobacco BY2 cells. CycH;1 fused to green fluorescent protein (GFP) was expressed under the control of an estrogen-regulated promoter that responds to a derivative of estrogen, namely, β-estradiol (Zuo *et al.*, 2000).

Immunoprecipitation and immunoblotting with the anti-GFP antibody allowed detection of a 64 kDa protein that was also recognized by the anti-CycH;1 antibody (Fig. 2a), suggesting that the anti-GFP antibody specifically precipitated CycH;1-GFP. The immunoprecipitates displayed high CDK2 and CTD kinase activities (Fig. 2b), indicating that CycH;1-GFP bound and activated tobacco CAK(s). In cell cultures treated with 1 μM β-estradiol for 32 h, the GFP fluorescence was detected in both the cytoplasm and the nucleus (Fig. 2c). When transformed BY2 cells were synchronized with aphidicolin, the distribution of the GFP fluorescence did not vary throughout the cell cycle (data not shown). The transient expression of GFP-fused CycH;1 in onion (*Allium cepa*) epidermal cells showed a similar localization in the cytoplasm and nuclei (Fig. 2d).

The WEE1 kinase downregulates CDKDs by tyrosine phosphorylation

The amino acid sequences of all A- and B-type CDKs in *Arabidopsis* possess conserved Thr and tyrosine (Tyr) residues in the ATP-binding site (Fig. 3a); these residues may be the targets for phosphorylation by the WEE1 kinase. However, we found that CDKDs also have the typical Thr/Tyr residues (Thr21 and Tyr22 on CDKD;1; Thr23 and Tyr24 on CDKD;2; and Thr22 and Tyr23 on CDKD;3), whereas they are not conserved in vertebrate CAKs (Fig. 3a). This raised an interesting question—are plant CDKDs controlled by the WEE1 kinase?

As CDK phosphorylation by WEE1 has not been demonstrated in plants, we first tested whether *Arabidopsis* WEE1 could phosphorylate CDKA;1 *in vitro*. The

GST-fused WEE1 efficiently phosphorylated GST-CDKA;1 but not GST-CDKA;1 carrying a substitution of Tyr15 with phenylalanine (Phe) (Fig. 3b). No phosphorylation was observed with the control GST protein. This indicated that WEE1 could phosphorylate Tyr15 on monomeric CDKA;1. Similar assays were conducted using maltose binding protein (MBP)-fused CDKD;1, CDKD;2, and CDKD;3 as substrates. To exclude autophosphorylation, a lysine (Lys) residue in the catalytic cleft of each CDKD was changed to arginine (Arg). GST-WEE1 phosphorylated MBP-CDKD;2 and MBP-CDKD;3 but neither MBP-CDKD;1 nor the control MBP protein (Fig. 3b and c). The phosphorylation signal disappeared when Tyr24 of CDKD;2 or Tyr23 of CDKD;3 was substituted with Phe. These results suggested that the conserved Tyr residues of CDKD;2 and CDKD;3 are targeted by WEE1. CDKF;1 has Tyr32 in the corresponding region; however, the preceding Thr is missing (Fig. 3a). However, since CDKF;1 showed high autophosphorylation activity, we could not examine Tyr32 phosphorylation.

To test whether WEE1 inhibits CDKD activity in plant cells, we coexpressed c-myc and hemagglutinin (HA) epitope-tagged forms of WEE1 and CDKD, respectively, in *Arabidopsis* root protoplasts. Since CDKD;3 was not expressed in the protoplasts for unknown reasons, only CDKD;2 was assessed for CTD kinase activity by using immunoprecipitates with an anti-HA antibody. The enzyme activity was decreased by coexpression with myc-WEE1 (Fig. 3d, lane 2), and this result was reproducible, suggesting that WEE1 downregulated CDKD;2 in the protoplasts. Increasing the expression level of myc-WEE1 resulted in the lower accumulation of

HA-CDKD;2 (Fig. 3d, lane 3); thus, we failed to compare the kinase activities. GFP-fused WEE1 displayed nearly exclusive nuclear localization in *A. cepa* epidermal cells (Fig. 2d).

CDKF;1 is involved in CDK activation in plant cells

We identified *Arabidopsis* mutants of *CDKD;1* and *CDKD;3* from T-DNA insertion collections. T-DNAs were inserted into the 2nd exon of *CDKD;1* and the 3rd exon of *CDKD;3* as shown in Fig. 4a. Each mutant was backcrossed with wild-type plants three times, and a homozygous line containing a single T-DNA insertion was established by genomic Southern hybridization with a labeled T-DNA probe (data not shown). Reverse-transcription (RT)-PCR with the *CDKD;1* mutant (hereafter called *cdkd;1-1*) showed that the cDNA upstream to the T-DNA insertion site was amplified from the mRNA of shoots and roots; however, the downstream region was not amplified at all (Fig. 4b). RT-PCR with the *CDKD;3* mutant (hereafter called *cdkd;3-1*) showed that the cDNA neither upstream nor downstream to the T-DNA insertion site was amplified (Fig. 4b). Both mutants showed no developmental defect throughout the life cycle under normal growth conditions (Fig. 4c), suggesting that CDKD;1 and CDKD;3 do not play an essential role in plant development.

In *Arabidopsis*, CDKD;3 and CDKF;1 were assumed to be the major kinases that catalyze the T-loop phosphorylation of CDKs (Shimotohno *et al.*, 2003, 2004; Umeda *et al.*, 1998). However, as described above, CDKD;3 was not essential for plant growth, leading to the proposal that CDKF;1 is responsible for the majority of the CDK-kinase

activities in *Arabidopsis* cells. Previously, we reported that CDKF;1 phosphorylates and activates human CDK2 *in vitro* (Umeda *et al*., 1998); however, *in vivo* interaction with endogenous *Arabidopsis* CDKs remains unknown. Here, we coexpressed myc-CDKF;1 and HA-CDKA;1 in *Arabidopsis* root protoplasts, and the immunoprecipitates with the anti-HA antibody were subjected to a kinase assay. As shown in Fig. 5a, HA-CDKA;1 displayed histone H1-kinase activity only when myc-CDKF;1 was co-expressed, indicating that CDKA;1 required CDKF;1 to exert its enzyme activity.

CDKF;1 also has a conserved Thr residue (Thr290) within the T-loop (Umeda *et al*., 1998). This prompted us to examine whether T-loop phosphorylation is required for CDKF;1 activity. When myc-CDKF;1 carrying the substitution of Thr290 with alanine was coexpressed with HA-CDKA;1 in root protoplasts, HA-CDKA;1 did not exhibit kinase activity at all (Fig. 5a). We then expressed the wild-type or the T290A mutant of myc-CDKF;1 in root protoplasts, and its activity was tested by using GST-CDK2 as a substrate. The result showed that the T-loop mutation significantly reduced the kinase activity of myc-CDKF;1 (Fig. 5b). These results suggest that the phosphorylation of Thr290 on CDKF;1 is indispensable for CDK activation in plant cells.

Discussion

Our results showed that CycH;1 forms a stable complex with CDKD;2 in *Arabidopsis* cells. GFP-fused CycH;1 was localized in the cytoplasm and nuclei, similar to the localization of CDKD;2 (Shimotohno *et al*., 2004). This is in contrast to other organisms where cyclin H and CAK complexes are exclusively localized to the nucleus

(Jordan *et al.*, 1997; Kaldis, 1999; Krempler *et al.*, 2005). During seed germination in *Arabidopsis*, transcripts of *CycH;1* and *CDKD;2* accumulate before the activation of cell division in the root apex (Menges *et al.*, 2005). The first signs of germination are resumption of essential transcription, DNA repair, or other metabolic processes. Therefore, the CycH;1-CDKD;2 complex may be involved in transcription via CTD phosphorylation during early seed germination. We failed to observe a tight interaction between CycH;1 and CDKD;3. This was surprising because CycH;1 interacted with CDKD;3 in an yeast two-hybrid assay, and it enhanced the kinase activity in insect cells (Shimotohno *et al.*, 2004). The 37 kDa protein of CycH;1 formed a protein complex of ~100 kDa in gel filtration fractions (fraction No. 62–66) that contained a significant amount of CDKD;3 (Fig. 1c). **Moreover, the GFP-fused CycH;1, which was overexpressed in tobacco BY2 cells and immunoprecipitated with the anti-GFP antibody, displayed not only CTD but also CDK kinase activities, an indication that the CAK activity was efficiently recovered with the GFP tag.** It is probable that the CycH;1-CDKD;3 complex is not as stable as the CycH;1-CDKD;2 complex *in vivo*; **thus, it might be dissociated during immnoprecipitation with the anti-CycH;1 antibody, which might recognize the CycH;1-CDKD;3 complex less efficiently.**

The anti-CycH;1 antibody recognized three *Arabidopsis* proteins of 37, 39, and 40 kDa. The 37 kDa protein was considered as the intrinsic CycH;1 because the recombinant protein that was expressed in yeast cells showed the same mobility on SDS-PAGE. Immunoprecipitation experiments showed that both the 37 kDa and 39 kDa proteins bound CDKD;2. However, gel filtration chromatography resolved the 37

kDa protein in fractions that overlapped with those of CDKD;2 and CDKD;3, while the peak fraction (fraction No. 58–60) of the 39 kDa protein was almost the same as that of CDKD;2. This suggests that the 39 kDa protein may have a higher affinity to CDKD;2. In mammals, two kinases—cyclin C-CDK8 and CK2—have been identified that phosphorylate cyclin H and regulate its activity (Akoulitchev *et al*., 2000; Schneider *et al*., 2002). However, a protein phosphatase treatment caused no mobility shift of CycH;1 on SDS-PAGE (data not shown), suggesting that a protein modification other than phosphoryation may produce the CycH;1 variants (Krempler *et al*., 2005). **Note that it is still possible that the 39 kDa and 40 kDa bands are not due to CycH;1 isoforms but rather to other proteins which contain epitope(s) similar to that of CycH;1.**

The protein kinase WEE1 was described in fission yeast, where mutated cells showed a small cell (*wee*) phenotype due to premature entry into mitosis (Featherstone and Ressell, 1991; **Russell and Nurse, 1987**). WEE1 is encoded at a single locus in the *Arabidopsis* genome, and its overexpression in fission yeast caused cells to elongate without cell division (Sorrell *et al*., 2002). A similar result was also reported with a maize WEE1 homologue that was able to inhibit the activity of $p13^{suc1}$-adsorbed CDK from maize (Sun *et al*., 1999). However, the direct link between CDKs and WEE1 has not been demonstrated in plants. Here, we reported that *Arabidopsis* WEE1 phosphorylated a specific Tyr residue within the ATP-binding site of CDKA;1 *in vitro*, suggesting that it has the same specificity as yeast and vertebrate WEE1 (Featherstone and Russell, 1991; Gould and Nurse, 1989; Lundgren *et al*., 1991; Parker *et al*., 1992;

Parker and Piwnica-Worms, 1992). Since recombinant CDKA;1 was expressed in *Escherichia coli*, *Arabidopsis* WEE1 may have the ability to phosphorylate monomers of CDKA;1. To our knowledge, there is no report of WEE1 phosphorylating CDK monomers in fission yeast and vertebrates (Parker *et al*., 1992).

Interestingly, WEE1 also phosphorylated CDKD;2 and CDKD;3 *in vitro*, and CDKD;2 kinase activity was inhibited by WEE1 in *Arabidopsis* root protoplasts. This is the first study to demonstrate that CAK activity is regulated by WEE1. *Arabidopsis* WEE1 is strongly S-phase regulated (Menges *et al*., 2005); thus, it is likely that CDKD activities may be downregulated in the S phase. Our mutational analyses revealed that WEE1 targeted the specific Tyr residues of CDKD;2 and CDKD;3. The Thr and Tyr residues within the ATP-binding site are conserved not only in *Arabidopsis* CDKDs but also in other plant CDKDs. Therefore, CDKD phosphorylation by WEE1 may be a common regulatory mechanism of plant CAKs. Although CDK7/p40^{MO15}-related proteins in vertebrates lack the Thr/Tyr motif, those in budding and fission yeasts possess conserved residues, namely, Thr17 and Tyr18 on Kin28p and Thr21 and Tyr22 on Mcs6. This suggests that they may be also controlled by WEE1 kinases. CDKD;1 also has the typical Thr/Tyr motif but was not phosphorylated by WEE1 in our assay. This distinct feature of CDKD;1 was noted in the T-loop phosphorylation; CDKF;1 phosphorylated CDKD;2 and CDKD;3 but not CDKD;1 regardless of the conserved Thr residue within the T-loop (Shimotohno *et al*., 2004).

We observed previously that *CDKD;3* and *CDKF;1* but neither *CDKD;1* nor *CDKD;2* were able to suppress the *cak1*^{ts} mutation of budding yeast (Shimotohno *et al*.,

2003). In fact, a significant level of CDK kinase activity was detected with CDKD;3 and CDKF;1 but not with CDKD;1 and CDKD;2 (Shimotohno *et al*., 2003, 2004; Umeda *et al*., 1998). Here, we demonstrated that a knockout mutant of *CDKD;3* showed no defect in plant development. **In contrast, a homozygous mutant of *CDKF;1* showed a severe defect under the same growth conditions (unpublished results).** Therefore, it is likely that CDKF;1 plays a major role in CDK phosphorylation and activation. The CDK kinase activity of recombinant CDKF;1 produced in insect cells was 10-fold higher than that of CDKD;3 (Shimotohno *et al*., 2004), thus supporting the above idea. Based on these results, we propose a model for CDK and CTD phosphorylation in *Arabidopsis*, that is, the T-loop of CDKs is phosphorylated by CDKF;1, and the CTD is phosphorylated by CycH;1-CDKD;2 that is negatively regulated by the WEE1 kinase (Fig. 6). **CycH;1-CDKD;2 also exhibits the CDK kinase activity, but its level is significantly lower than that of CDKF;1 (Shimotohno et al., 2003, 2004).** The manner in which different kinases are engaged in CDK and CTD phosphorylation is similar to that observed in budding yeast; Kin28p phosphorylates the CTD (Cismowski *et al*., 1995; Feaver *et al*., 1994), and Cak1p phosphorylates and activates Cdc28p (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996). Kimmelman *et al*. (1999) reported that Cak1p is also involved in basal transcription through Kin28p phophorylation. Similarly, *Arabidopsis* CDKF;1 phosphorylates Ser162 and Thr168 within the T-loop of CDKD;2 and activates its CTD kinase activity (Fig. 6) (Shimotohno *et al*., 2004; Umeda *et al*., 2005). Therefore, despite the low sequence similarity, CDKF;1 is functionally related to budding yeast

Cak1p. This is supported by recent biochemical studies on CAKs (Tsakraklides and Solomon, 2002); Cak1p and CDKF;1 displayed a preference for cyclin-free CDK substrates, were insensitive to the protein kinase inhibitor 5´-fluorosulfonylbenzoyladenosine (FSBA), and were insensitive to the mutation of a highly conserved Lys residue found in the nucleotide binding pocket. Moreover, Kaldis *et al*. (1998) revealed that Cak1p localized in both the nucleus and cytoplasm similar to CDKF;1. Conservation of these unusual properties in budding yeast and *Arabidopsis* may indicate shared evolutionary requirements in cell cycle regulation and transcription.

In *Arabidopsis* root protoplasts, CDKA;1 was active when it was coexpressed with CDKF;1. This is the first demonstration that a plant CDK requires CAK for its activity. Using database searches, CDKF;1 homologues were found only in plant species, namely, *Euphorbia*, rice, and soybean, but not in other kingdoms (Umeda *et al.*, 2005). All the four plant CDKFs contain the phosphoregulatory site within the T-loop. A mutation of Thr290 in the T-loop region of CDKF;1 dramatically reduced CAK activity, suggesting that the T-loop phosphorylation is essential for CDKF activity *in vivo* (Fig. 6). Therefore, the identification of an upstream kinase will be particularly interesting because it will link internal and/or external signals to cell cycle machineries that govern not only cell proliferation but also cell differentiation during plant development.

Experimental procedures

Plant material

Arabidopsis thaliana (ecotype Columbia) plants were grown at 23ºC under continuous light conditions. For the isolation of protoplasts, 50–70 root segments from 10-day-old seedlings grown on Murashige and Skoog (Sigma, St. Louis, MO) agar plates were used. An *Arabidopsis* cell suspension culture was maintained as described previously (Glab *et al*., 1994). Tobacco BY-2 cells were maintained in a modified Murashige and Skoog medium as described by Nagata *et al*. (1992). Total protein was extracted from the suspension cells 4 days after subculture (Magyar *et al*., 1997).

Identification of T-DNA insertion mutants

The T-DNA insertion mutants of *CDKD;1* and *CDKD;3* were isolated from the collections of the Max-Planck-Institute für Züchtungsforschung (**Rios *et al*., 2002**) and The Salk Institute, respectively. Seed stock numbers of *cdkd;1-1* and *cdkd;3-1* are MPI8258 and SALK_120536, respectively. 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´ for *cdkd;3-1*. The nucleotide sequences of the amplified fragments were determined to identify the T-DNA insertion site. Each line was backcrossed with wild-type plants three times. The Titanium One-Step RT-PCR kit (BD Biosciences Clontech, San Diego, CA) was used for

RT-PCR with total RNA from shoots and roots of 21-day-old seedlings. In a total reaction volume of 10 μl, 0.1 μg of RNA and the following primers were used: 5´-TTGCTTGTGATCCGATTAGAGAGTT-3´ (P1) and 5´-CATCACCAGGCGAAAGATAGAGATT-3´ (P2) or 5´-CCAAACAACTTGTTGATAGGACCCA-3´ (P3) and 5´-TAGACAGAAAGAATGATTCAAACTA-3´ (P4) for *cdkd;1-1*; 5´-CGAATTCGTCGACATGCCGGAGCAGCC-3´ (P1) and 5´-GAACTCAAAGACAAGATGCA-3´ (P2) or 5´-AGTGGCCGGATTTAACAAAG-3´ (P3) and 5´-TCCTTGTCAACATGAGACTT-3´ (P4) for *cdkd;3-1*. As a control, *TUB4* cDNA was amplified by using primers 5´-CTCTGTGCATCAGCTTGTCGAAAACG-3´ and 5´-CCGAGGGAGCCATTGACAACATCTT-3´. The PCR conditions were 1 cycle at 50ºC for 60 min and at 94ºC for 5 min; 35 cycles at 94ºC for 30 sec, at 65ºC for 30 sec, and at 68ºC for 1 min; and 1 cycle at 68ºC for 2 min.

Plasmid construction

The coding regions of *WEE1* and *CycH;1* were amplified by PCR and cloned into the GATEWAY entry vector pENTR/D-TOPO (Invitrogen, San Diego, CA). Recombination reactions were performed between the entry clones and the destination vectors pDEST15 and pDEST17 by using LR Clonase (Invitrogen) to produce His-tagged CycH;1 and GST-fused WEE1, respectively. The destination vector pYES-DEST52 (Invitrogen) was used to express CycH;1 in *Saccharomyces cerevisiae*

strain YPH500. Yeast protein extracts were prepared as described (Vitaly, 2000). For transient expression in *A. cepa* epidermal cells, the destination vectors pGWB5 or pGWB6 (gifts from Dr. Nakagawa) were used to produce WEE1-GFP, CycH;1-GFP (or GFP-CycH;1), respectively. To express CycH;1-GFP in an estrogen-inducible manner, the *CycH;1-GFP* fragment with the *Xho*I site at the N-terminal end and the *Spe*I site at the C-terminal end was PCR-amplified and cloned into the *Xho*I/*Spe*I sites of pER8 (Zuo *et al.*, 2000). For expression in *Arabidopsis* root protoplasts, cDNAs of *WEE1*, *CDKF;1*, and *CDKA;1* were cloned into the *Sal*I site of pMESHI or the *Eco*RI site of pMENCHU (Ferrando *et al.*, 2000, 2001) to produce myc-WEE1, myc-CDKF;1, and HA-CDKA;1, respectively. Nucleotide substitutions were introduced using a Mutan-Super Express Km kit (TaKaRa). cDNAs of *CDKA;1*, *CDKD;2*, and *CDKD;3* were cloned into the *Eco*RI site of pGEX-5X-1 (Amersham Biosciences, Piscataway, NJ) and the *Bam*HI/*Sal*I or *Eco*RI site of pMAL-c2X (New England BioLabs, Beverly, MA) to produce GST-CDKA;1, MBP-CDKD;2, and MBP-CDKD;3, respectively. The other constructs have been described previously (Shimotohno *et al.*, 2004).

Protein expressions in E. coli

pGEX and pDEST15 vectors were transformed into the *E. coli* strain BL21 or BL21-AI (Invitrogen), respectively. The transformants were grown at 27°C to an OD_{600} of 0.6–0.8, followed by induction with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) or 0.2% L-arabinose, and allowed to continue culturing for 4–6 h. GST-fusion proteins were purified with glutathione-Sepharose 4B (Amersham Biosciences). The purification of

GST-fused human CDK2 (carrying the K33R mutation) and *Arabidopsis* CTD has been described previously (Poon *et al.*, 1993; Umeda *et al.*, 1998). MBP-fused proteins were expressed in *E. coli* BL21 cells; these cells were grown to an OD_{600} of 0.4–0.6, followed by induction with 0.4 mM IPTG, allowed to continue culturing for a further 5 h, and were then purified with amylose resin (New England Biolabs). His-tagged CycH;1 was expressed in *E. coli* BL21-AI cells as described above and purified using a nickel-Sepharose column (Qiagen, Hilden, Germany).

Protein expressions in plant cells

Agrobacterium-mediated transformation of tobacco BY-2 cells was performed as described by Ito *et al.* (1998). For synchronization, a 7-day-old culture was diluted 1:9, mixed with 5 $mg \cdot L^{-1}$ aphidicolin, and cultured for 24 h. Aphidicolin was then removed by washing the cells with the fresh medium to restart the cell cycle. In order to express CycH;1-GFP, 1 μM β-estradiol was added to the culture just after release from the aphidicolin block. The preparation and transfection of *Arabidopsis* root protoplasts has been described previously (Abel and Theologis, 1994). Protoplasts (2×10^5 cells) were transfected with 50 μg of plasmid DNA and incubated at 22ºC for 12 h under continuous illumination. Transient expression in *A. cepa* epidermal cells was conducted as described by Shimotohno *et al.* (2004). The GFP fluorescence was observed with a confocal laser scanning microscope system (MicroRadiance MR/AG-2; Bio-Rad).

Immunoblotting and kinase assay

A polyclonal antibody was raised in rabbits against the His-tagged CycH;1 produced in *E. coli*. The antiserum was purified with a HiTrap rProtein A FF affinity column (Amersham Biosciences) and then with a HiTrap N-hydroxysuccinimide (NHS)-activated HP affinity column (Amersham Biosciences) that covalently bound His-CycH;1. Depletion of the antibody was performed with His-CycH;1 as described by Shimotohno *et al*. (2003). Immunoblotting was conducted by using an ECL Western Blotting Detection kit (Amersham Biotech). Phosphatase treatment was performed with 200 units of lambda protein phosphatase (New England Biolabs) in a buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% polyoxyethylene lauryl ether, pH 7.5) at 30ºC for 1 h. The kinase assay was performed as described previously (Shimotohno *et al*., 2004; Umeda *et al*., 1998). Immunoprecipitation was conducted with specific antibodies or with the anti-HA (12CA) monoclonal antibody (Roche, Indianapolis, IN), an anti-c-myc monoclonal antibody (Berkeley Antibody Company, Richmond, CA), or an anti-GFP polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) **as described by Umeda *et al.* (1998).** Fractionation of *Arabidopsis* protein extracts by Sephacryl S300 gel exclusion chromatography was performed as described previously (Shimotohno *et al.*, 2004). Elution profiles of proteins in LMW and HMW gel filtration calibration kits (Amersham Biosciences) were used for estimations of molecular mass.

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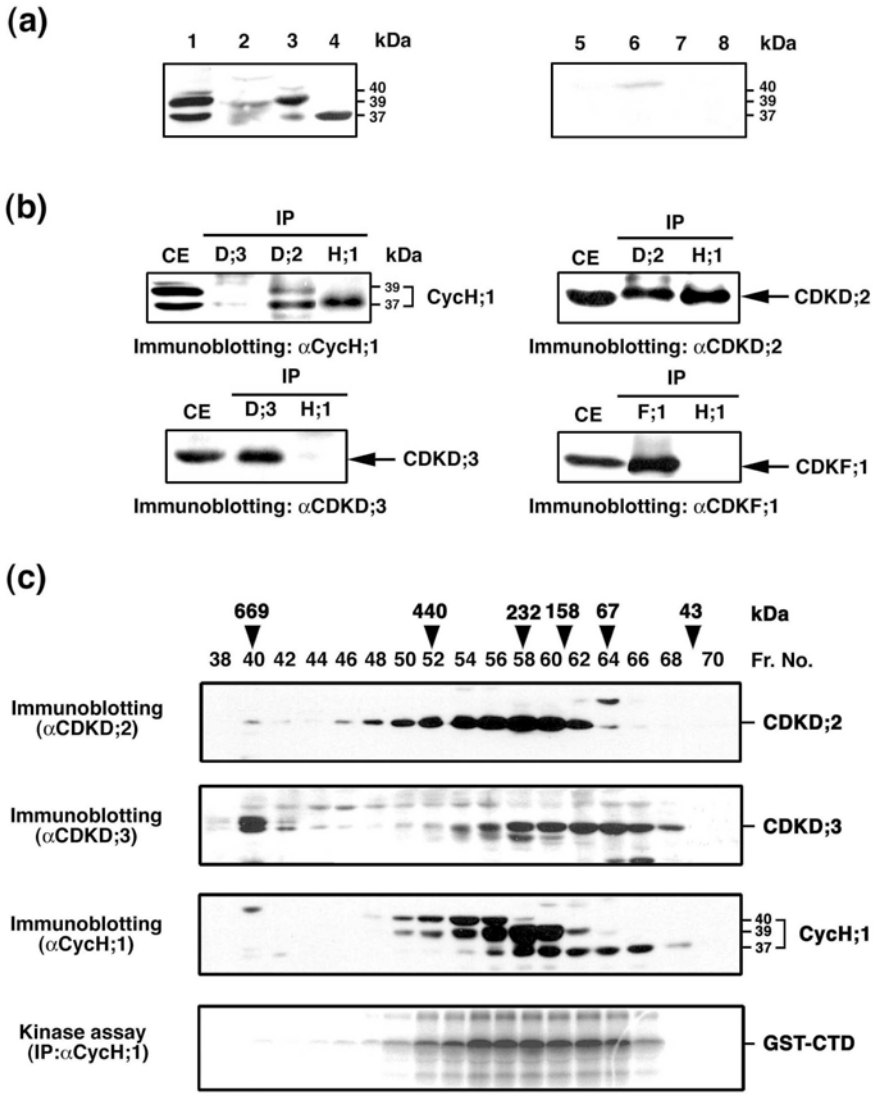

Figure 1. The CycH;1 complexes in *Arabidopsis*.

(a) Immunoblotting of CycH;1. Thirty micrograms of protein extracts from *Arabidopsis* suspension cells (lanes 1 and 5), shoots (lanes 2 and 6) and roots (lanes 3 and 7) of seedlings, and from yeast cells overexpressing CycH;1 (lanes 4 and 8) was immunoblotted with the anti-CycH;1 antibody (lanes 1–4) or with the antiserum depleted with nickel sepharose resin carrying His-CycH;1 (lanes 5–8). (b) *In vivo* interaction between CycH;1 and CDKDs or CDKF;1. Two hundred micrograms of protein extracts from suspension cells was immunoprecipitated with antibodies recognizing CDKD;2 (D;2), CDKD;3 (D;3), CDKF;1 (F;1), or CycH;1 (H;1); and the immunoprecipitates (IP) were immunoblotted with the indicated antibody. As a control, 15 μg of crude extract (CE) was loaded on an SDS-PAGE gel. (c) CAK complexes in suspension cells. Forty milligrams of protein extracts from suspension cells was fractionated using Sephacryl S300 gel exclusion chromatography, and 15 μl of each fraction was immunoblotted with antibodies recognizing CDKD;2, CDKD;3, or CycH;1. One hundred and fifty microliters of each fraction was immnoprecipitated with the anti-CycH;1 antibody and assayed for kinase activity by using glutathione S-transferase (GST)-CTD as the substrate. Arrowheads indicate the elution positions of marker proteins with their molecular masses.

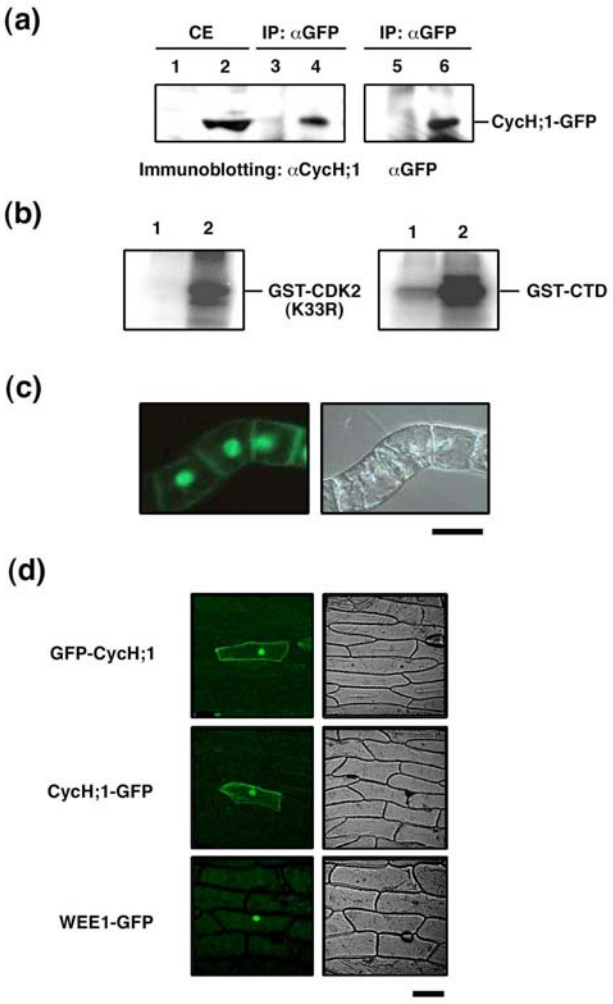

Figure 2. Expression of CycH;1-GFP in tobacco BY2 and onion epidermal cells.

(a) Immunological detection of CycH;1-GFP in tobacco BY2 cells. Transgenic BY2 cells were synchronized with aphidicolin and then treated with 1 μM β-estradiol for 32 h after release from the aphidicolin block. Fifteen micrograms of protein extracts from cells expressing green fluorescent protein (GFP) alone (lanes 1, 3, and 5) or CycH;1-GFP (lanes 2, 4, and 6) was immunoblotted with the anti-CycH;1 antibody (lanes 1 and 2), and 200 μg of protein extracts was immunoprecipitated with the anti-GFP antibody and detected with the anti-CycH;1 (lanes 3 and 4) or anti-GFP (lanes 5 and 6) antibody as indicated. (b) CDK and CTD kinase activities of CycH;1-GFP. Two hundred micrograms of protein extracts from cells expressing GFP alone (lane 1) or CycH;1-GFP (lane 2) was immunoprecipitated with the anti-GFP antibody, and the immunoprecipitates were subjected to a kinase assay by using GST-CDK2 (K33R) or GST-CTD as the substrate. To exclude the possibility of autophosphorylation, the lysine residue in the catalytic domain of CDK2 was substituted with arginine (K33R). (c) Subcellular localization of CycH;1-GFP in tobacco BY2 cells. Fluorescent microscopic images of CycH;1-GFP (left) and the corresponding differential interference contrast (DIC) images (right) are shown. Bar = 40 μm. (d) Subcellular localization of GFP-fused CycH;1 and WEE1 in onion epidermal cells. Fluorescent (left) and bright-field (right) images of cells are shown. CycH;1 fused to GFP at the amino terminus and the carboxy terminus were tested. Bar = 100 μm.

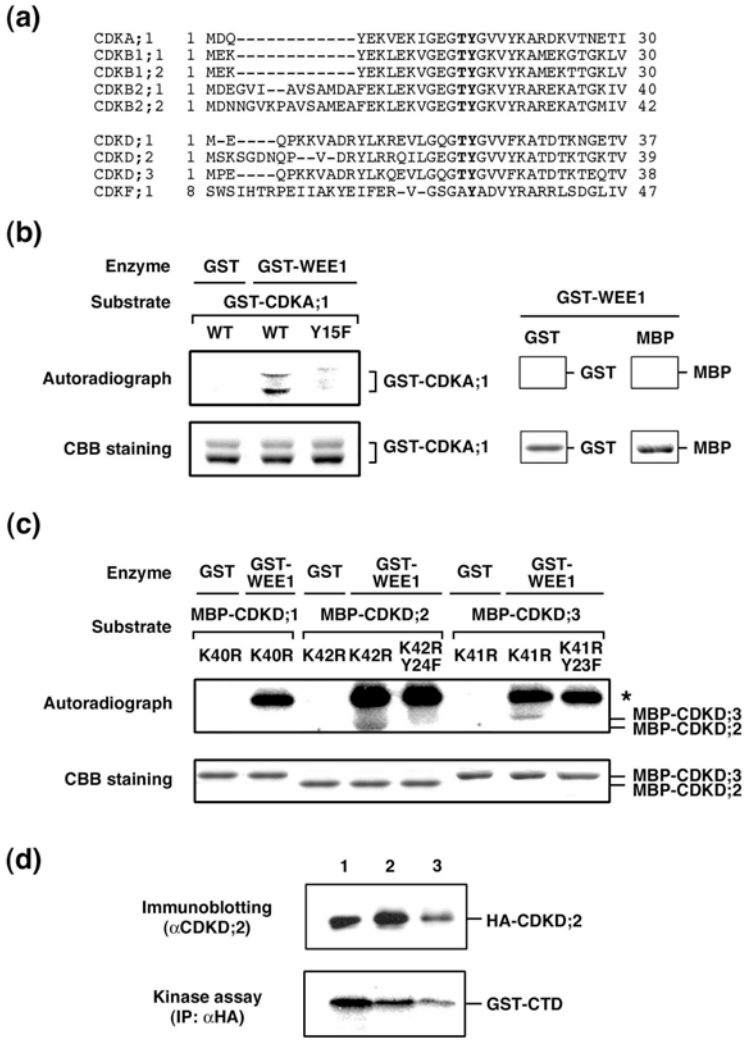

Figure 3. *Arabidopsis* WEE1 phosphorylates CDKDs as well as CDKA;1.

(a) Alignment of amino-terminal amino acids of *Arabidopsis* CDKs and CAKs. The conserved threonine (T) and tyrosine (Y) residues in the ATP-binding site are indicated by bold letters. Numbers indicate amino acid positions. Dashes represent gaps introduced to give maximal identity. (b) Phosphorylation of CDKA;1 by WEE1. GST-CDKA;1 (wild-type (WT) or Y15F mutant) was incubated with 10 ng of glutathione S-transferase (GST) or GST-WEE1 in the presence of [γ-^{32}P] ATP. GST or maltose binding protein (MBP) alone was used as controls. (c) Phosphorylation of CDKD;2 and CDKD;3 by WEE1. MBP-CDKDs were reacted with 10 ng of GST or GST-WEE1. To exclude autophosphorylation of the substrates, each lysine (K) residue in the catalytic domain of CDKDs was substituted with arginine (R). To determine the phosphorylation sites, Y24 in CDKD;2 and Y23 in CDKD;3 were substituted with phenylalanine (F). An asterisk indicates the autophosphorylation signal of GST-WEE1. (d) WEE1 downregulates CDKD;2 in *Arabidopsis* root protoplasts. CDKD;2 was coexpressed with an empty vector pMESHI (lane 1) or with 10 μg (lane 2) or 50 μg (lane 3) of pMESHI-*WEE1*. Ten micrograms of total protein was immunoblotted with the anti-CDKD;2 antibody. One hundred micrograms of protein extract was immunoprecipitated with the anti-hemagglutinin (anti-HA) antibody, followed by a phosphorylation reaction using GST-CTD as a substrate.

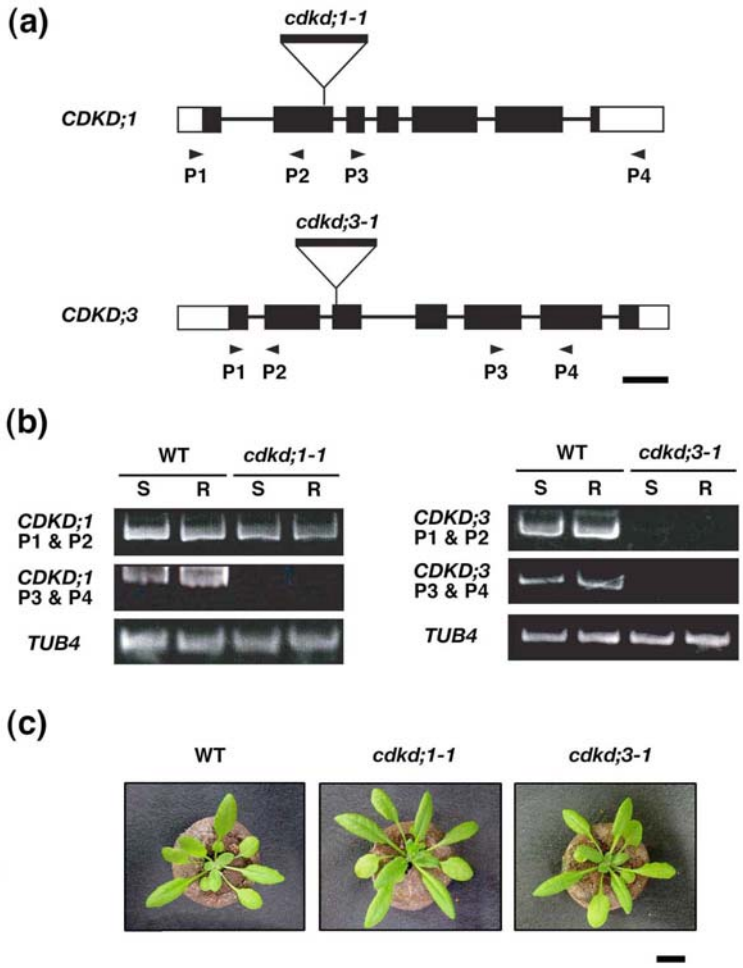

Figure 4. T-DNA insertion mutants of *CDKD;1* and *CDKD;3*.

(a) Schematic diagrams of the *CDKD;1* and *CDKD;3* genes. Exons and introns are indicated by boxes and solid bars, respectively. Black and open boxes represent coding and noncoding regions, respectively, on which the T-DNA insertion sites are shown. Arrowheads indicate primers that were used for reverse-transcription polymerase chain reaction (RT-PCR). Bar = 1 kb. (b) Expression analysis of the mutants. RT-PCR was conducted with total RNA from shoots (S) and roots (R) of wild-type (WT) or mutant seedlings by using the indicated sets of primers. The amplified cDNAs were stained with ethidium bromide. *TUBULIN 4 (TUB4)* was used as a control. (c) Eighteen-day-old seedlings of WT and T-DNA insertion mutants. Bar = 1 cm.

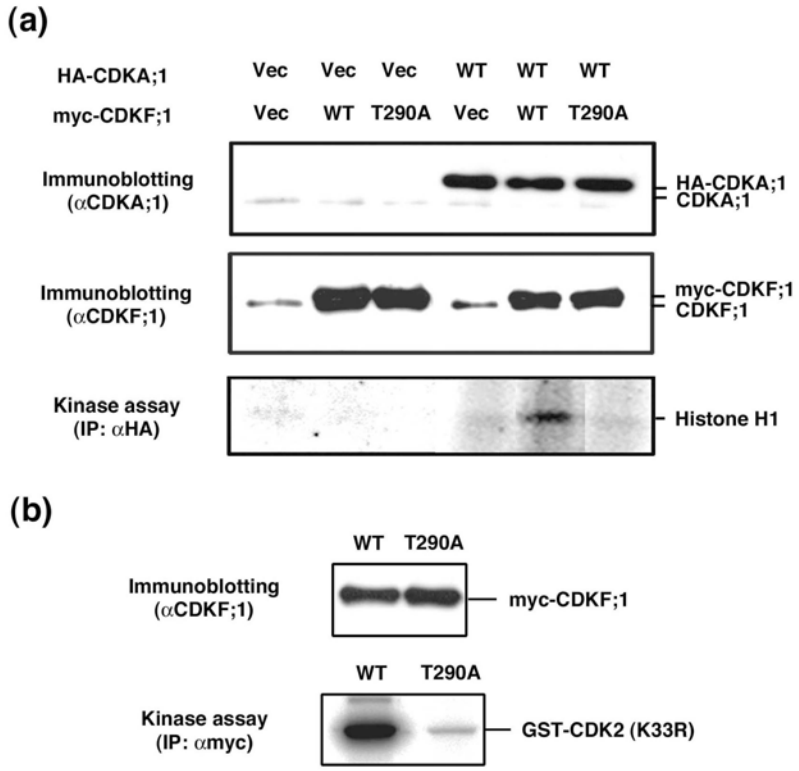

Figure 5. T-loop phosphorylation of CDKF;1 is indispensable for CAK activity.

(a) Histone H1 kinase activitiy of CDKA;1 in *Arabidopsis* root protoplasts. HA-CDKA;1 and/or myc-CDKF;1 (wild-type (WT) or T290A mutant) were expressed in root protoplasts, and 10 μg of crude extracts was immuoblotted with the anti-CDKA;1 or anti-CDKF;1 antibody. As a control, pMENCHU and/or pMESHI vectors (Vec) were introduced into root protoplasts. Endogenous CDKA;1 and CDKF;1 were also detected. One hundred micrograms of protein extracts was immunoprecipitated with the anti-hemagglutinin (anti-HA) antibody and assayed for histone H1 kinase activity. (b) Loss of CDKF;1 activity by T-loop mutation. WT or the T290A mutant of myc-CDKF;1 was expressed in *Arabidopsis* root protoplasts, and 5 μg of crude extracts was immunoblotted with the anti-CDKF;1 antibody. One hundred micrograms of crude extracts was immunoprecipitated with the anti-c-myc antibody, and the immunoprecipitates (IP) were subjected to a kinase assay in which glutathione S-transferase (GST)-CDK2 (K33R) was used as the substrate.

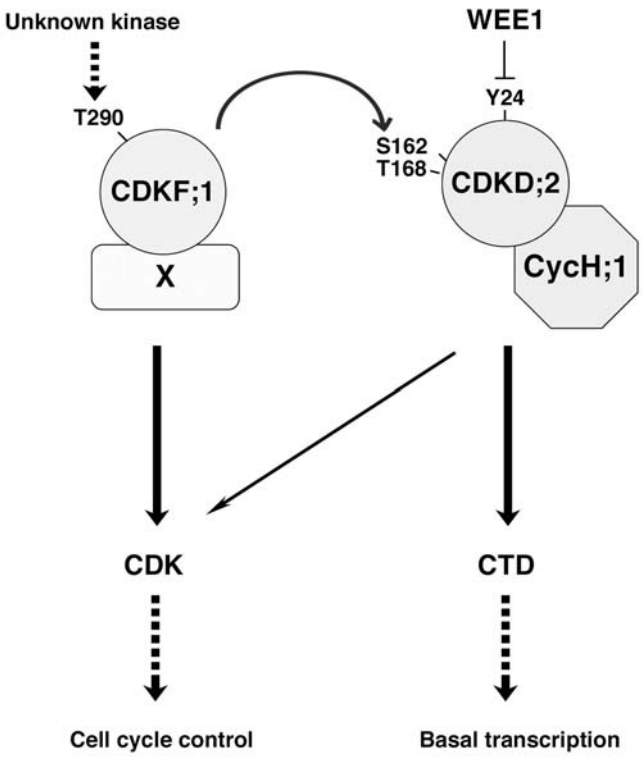

Figure 6. Phosphorylation of CDK and CTD in *Arabidopsis*.

CDKF;1 is activated by phosphorylation of T290 within the T-loop and exerts CDK kinase activity to control the cell cycle. CDKF;1 forms an active 130 kDa complex in *Arabidopsis*; hence, some unknown subunit(s) (X) may be included in the complex. Another activity of CDKF;1 is to phosphorylate and activate CDKD;2 that forms a stable complex with CycH;1 to display CTD kinase activity. **The CDK kinase activity of CycH;1-CDKD;2 is significantly lower than that of CDKF;1.** CDKD;2 is downregulated by WEE1 that phosphorylates Y24 in the ATP-binding site of CDKD;2.