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 D-type CDK genes (CDKD;1–CDKD;3) encode vertebrate-type CAK orthologues, of which CDKD;2 exhibits high phosphorylation activity towards 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 significant activity towards 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.

Keywords: cyclin-dependent kinase, CDK-activating kinase, cyclin H, WEE1, cell cycle, transcription.

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 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, which are essential for dephosphorylating and activating CDKs in order to trigger mitosis (Featherstone and Russell, 1991; Kumagai and Dunphy, 1991; Nurse, 1990).

The catalytic subunit of vertebrate CAK is termed CDK7/p40MO15, 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 Cap1p/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, Cap1p 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, which is associated with TFIIH and phosphorylates the CTD (Cismowski et al., 1995; Feaver et al., 1994, Feaver et al., 1997; 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 Cap1p has another activity: to phosphorylate the T-loop of Kin28p and thereby stimulate its CTD kinase activity. This suggests that Cap1p is also involved in basal transcription.

Based on the primary structure, plant CDK-related proteins have been classified into six types, namely, CDKA–CDKF (Joubès 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 Ori-sa;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: Arab;CDKD;1, Arab;CDKD;2 and Arab;CDKD;3 (originally named CAK3At, CAK4At and CAK2At, respectively) (Shimotohno et al., 2003; Umeda, 2002). Although both Arab;CDKD;2 and Arab;CDKD;3 (hereafter called CDKD;2 and CDKD;3, respectively) exhibited CDK and CTD kinase activities, Arab;CDKD;1 (hereafter called CDKD;1) showed neither CDK nor CTD kinase activity (Shimotohno et al., 2004). CDKD2 kinase activity of CDKD;3 was extremely high 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 CDFK gene. In Arabidopsis, Arab;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. Arab;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, whereas 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 (Figure 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 (Figure 1a). CycH;1 produced in yeast cells showed the same mobility on SDS-PAGE as the 37-kDa protein (Figure 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 (Figure 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 the most efficiently precipitated (Figure 1b). The same immunoprecipitates contained CDKD;2 but not CDKF;1 (Figure 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 (Figure 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 and ~700 kDa, respectively (Shimotohno et al., 2004; Figure 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

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detected in a range from 50 to 250 kDa (fraction No. 56–66), which overlaps with the fractions including CDKD;2 and CDKD;3 (Figure 1c). In contrast, the 39-kDa protein was included in fraction no. 50–62 (120–480 kDa), which corresponded well to those of CDKD;2 (Figure 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 (Figure 1b). A kinase assay showed that the CycH;1 complex in fraction No. 50–66 exhibited kinase activity towards the glutathione-S-transferase (GST)-CTD as the substrate. Arrowheads indicate the elution positions of marker proteins with their molecular masses.

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 (Figure 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 in the catalytic cleft of each CDKD (Figure 3a); these residues are not conserved in vertebrate CAKs (Figure 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-CDK;2 carrying a substitution of Tyr15 with phenylalanine (Phe) (Figure 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 (Figure 3b,c). The phosphorylation signal disappeared when either 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 (Figure 3a). However, as CDKF;1 showed high autophosphorylation activity, we could not examine Tyr32 phosphorylation.

To test whether WEE1 inhibits CDKD activity in plant cells, we co-expressed c-myc and hemagglutinin (HA) epitope-tagged forms of WEE1 and CDKD, respectively, in Arabidopsis root protoplasts. As CDKD;3 was not expressed in the protoplasts for unknown reasons, only CDKD;2 was assessed for CTD kinase activity by using immunoprecipitates

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 17β-estradiol for 32 h after release from the aphidicolin block. Protein extracts (15 μg) from cells expressing either green fluorescent protein (GFP) alone (lanes 1, 3 and 5) or CycH;1-GFP (lanes 2, 4 and 6) were 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 either the anti-CycH;1 (lanes 3 and 4) or the anti-GFP (lanes 5 and 6) antibody, as indicated.
(b) CDK and CTD kinase activities of CycH;1-GFP. Protein extracts (200 μg) from cells either expressing GFP alone (lane 1) or CycH;1-GFP (lane 2) were immunoprecipitated with the anti-GFP antibody, and the immunoprecipitates were subjected to a kinase assay by using either 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 (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.

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 (either wild-type (WT) or Y15F mutant) was incubated with 10 ng of either glutathione-S-transferase (GST) or GST-WEE1 in the presence of [γ-32P]ATP. Either GST or maltose binding protein (MBP) alone were used as controls.
(c) Phosphorylation of CDKD;2 and CDKD;3 by WEE1. MBP-CDKDs were reacted with 10 ng of either 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 co-expressed with either an empty vector pMESHI (lane 1) or with 10 μg (lane 2) or 50 μg (lane 3) of pMESHI-WEE1. Total protein (10 μg) was immunoblotted with the anti-CDKD;2 antibody. Protein extract (100 μg) was immunoprecipitated with the anti-hemagglutinin (anti-HA) antibody, followed by a phosphorylation reaction using GST-CTD as a substrate.
with an anti-HA antibody. The enzyme activity was decreased by co-expression with myc-WEE1 (Figure 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 (Figure 3d, lane 3); thus, we failed to compare the kinase activities. GFP-fused WEE1 displayed nearly exclusive nuclear localization in A. cepa epidermal cells (Figure 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 second exon of CDKD;1 and the third exon of CDKD;3, as shown in Figure 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). 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 (Figure 4b). RT-PCR with the CDKD;3 mutant (hereafter called cdkd;3-1) showed that neither the cDNA upstream nor downstream to the T-DNA insertion site was amplified (Figure 4b). Both mutants showed no developmental defects throughout the life cycle under normal growth conditions (Figure 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 co-expressed 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 Figure 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 co-expressed with HA-CDKA;1 in root protoplasts, HA-CDKA;1 did not exhibit kinase activity at all (Figure 5a). We then expressed either 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 (Figure 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; Kremler et al., 2006). 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 the

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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 a 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 quantity of CDKD;3 (Figure 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 activity, 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 immunoprecipitation 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 CDKD;2 kinase activity 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- 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, whereas 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 phosphorylation may produce the CycH;1 variants (Krempler et al., 2005). Note that it is still possible that the 39- and 40-kDa bands are not associated with CycH;1 isoforms, but rather with other proteins that 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 caused by premature entry into mitosis (Featherstone and Russell, 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 p13suc1-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 and Piwica-Worms, 1992; Parker et al., 1992). As 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 indicates 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 CDKs.
Therefore, CDKD phosphorylation by WEE1 may be a common regulatory mechanism of plant CAKs. Although CDK7/p40MO15-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 cak1Δ 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 (C.K. and M.U., unpublished data). 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: the T-loop of CDKs is phosphorylated by CDKF;1, and CTD is phosphorylated by CycH;1-CDKD;2, which is negatively regulated by the WEE1 kinase (Figure 6). CycH;1-CDKD;2 also exhibits 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 phosphoactivates 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 phosphorylation. Similarly, Arabidopsis CDKF;1 phosphoactivates Ser162 and Thr168 within the T-loop of CDKD;2 and activates its CTD kinase activity (Figure 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 was localized in both the nucleus and cytoplasm in a manner 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 co-expressed with CDKF;1. This clearly shows that a plant CDK requires CAK for its activity. Using database searches, CDKF;1 homologues were found only in plant species, namely Euphorbia, rice (Oryza sativa) and soybean (Glycine max), but not in other kingdoms (Umeda et al., 2005). All the four plant CDKF;1s 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 T-loop phosphorylation is essential for CDKF activity in vivo (Figure 6). Therefore, the identification of an upstream kinase will be particularly interesting because it will link internal and/or external signals to cell-cycle machinery that governs not only cell proliferation but also cell differentiation during plant development.
Experimental procedures

Plant material
Arabidopsis thaliana (ecotype Columbia) plants were grown at 23°C 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, USA) agar plates were used. An Arabidopsis cell suspension culture was maintained as described previously (Glab et al., 1994). Tobacco BY2 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-Institut für Züchtungsforschung (Rios et al., 2002) and The Salk Institute, respectively. Seed stocks numbers of cdkd;1-1 and cdkd;3-1 are MP18258 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'-CTGGGAATGGCAGAATCCAGGATC-3' and 5'-GTTCTGTGAGTGATCCGATTAGAG-3', were used for cdkd;1-1 and 5'-GAGA- TTTCTGCCCTGCTGGCGGCAAACAGGCAT-3' and 5'-CAGCCCAAAGAAATGTTGC-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) was used to produce WEE1-GFP, CycH;1-GFP (or cdkd;1-1 and GST-fused WEE1, respectively. The destination vectors between the entry clones and the destination vectors pDEST15 and pMESHI or the EcoRI site of pMENCHU (Ferrando et al., 2000, 2001) were cloned into the EcoRI site of pGEX-5X-1 (Amersham Biosciences, Piscataway, NJ, USA) and either the BamHI/Sal or the EcoRI site of pMAL-c2X (New England BioLabs, Beverly, MA, USA) to produce GST-CDKD;1, MBP-CDKD;2 and MBP-CDKD;3, respectively. The other constructs have been described previously (Shimotohno et al., 2004).

Protein expressions in Escherichia coli
pGEX and pDEST15 vectors were transformed into the E. coli strain of either BL21 or BL21-Al (Invitrogen), respectively. The transformants were grown at 27°C to an OD600 of 0.6–0.8, followed by induction with either 0.2 mM isopropyl l-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 OD600 of 0.4–0.6, followed by induction with 0.4 mM IPTG, allowed to continue culturing for a further 5 h, and then were purified with amyllose resin (New England Biolabs). His-tagged CycH;1 was expressed in E. coli BL21-Al cells, as described above, and purified using a nickel-Sepharose column (Qiagen, Hilden, Germany).

Protein expressions in plant cells
Agrobacterium-mediated transformation of tobacco BY2 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 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 x 10⁶ cells) were transfected with 50 µg of plasmid DNA and incubated at 22°C 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, Hercules, CA, USA).

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 pRotein 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 Biosciences). Phosphatase treatment was performed with 200 U of lambda protein phosphatase (New England Biolabs) in a buffer (50 mM Tris-HCl, 0.1 mM Na₂EDTA, 5 mM DTT, 0.01% polyoxy-
ethylene lauryl ether, pH 7.5) at 30°C for 1 h. The kinase assay was performed as described previously (Shimotohno et al., 2004; Umeda et al., 1998). Immunoprecipitation was conducted either with specific antibodies or with the anti-HA (12CA) monoclonal antibody (Roche, Indianapolis, IN, USA), an anti-c-myc monoclonal antibody (Berkeley Antibody Company, Richmond, CA, USA) or an anti-GFP polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) as described by Umeda et al. (1998). Fractionation of Arabidopsis protein extracts by Sepharch Y300 gel exclusion chromatography was performed as described previously (Shimotohno et al., 2004). Eclution profiles of proteins in LMW and HMW gel filtration calibration kits (Amersham Biosciences) were used for estimations of molecular mass.

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