Differential phosphorylation activities of CDK-activating kinases in *Arabidopsis thaliana*

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Abstract Activation of cyclin-dependent kinases (CDKs) requires phosphorylation of a threonine residue within the T-loop by a CDK-activating kinase (CAK). Here we isolated an *Arabidopsis* cDNA (*CAK4At*) whose predicted product shows a high similarity to vertebrate CDK7/p40^{MO15}. Northern blot analysis showed that expressions of the four *Arabidopsis* CAKs (*CAK1At–CAK4At*) were not dependent on cell division. CAK2At- and CAK4At-immunoprecipitates of *Arabidopsis* crude extract phosphorylated CDK and the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II with different preferences. These results suggest the existence of differential mechanisms in *Arabidopsis* that control CDK and CTD phosphorylation by multiple CAKs.

Key words: Cell cycle; Cyclin-dependent kinase; CDK-activating kinase; Transcription; *Arabidopsis thaliana*

1. Introduction

Cyclin-dependent kinases (CDKs) are a family of enzymes that coordinate cell cycle progression. For a fully active state, they require both association with regulatory subunits, cyclins, and phosphorylation on a conserved threonine residue located within the T-loop of the kinase subdomain VIII by CDK-activating kinases (CAKs) (for reviews, see [1,2]). CAKs of vertebrates are a heterotrimeric complex composed of a catalytic kinase subunit CDK7/p40^{MO15}, a regulatory subunit cyclin H, and an assembly factor MAT1 [3–5]. Vertebrate CAK also regulates basal transcription through phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II as a part of the general transcription factor IIH (TFIIH) [6–9]. In *Schizosaccharomyces pombe*, the homologues of CDK7 and cyclin H are Mcs6 and Mcs2, respectively, and as in the case of vertebrate CAKs, it has both Cdc2-activating kinase and CTD kinase activities in vitro [10–14]. Another single-subunit kinase Csk1 also phosphorylates Cdc2, but at this stage, it is not clear whether this phosphorylation activates the Cdc2 activity in vivo [14,15]. Rather, it has been reported that Csk1 functions as a CAK-activating kinase by phosphorylating Mcs6 [13,16].

In *Saccharomyces cerevisiae*, CAK and TFIIH-associated CTD kinase activities reside in two distinct proteins [17]. Kin28 is the closest structural relative to CDK7/p40^{MO15}. It forms a heterotrimeric complex like vertebrate CAKs and possesses CTD kinase activity as a subunit of TFIIH, but no CAK activity [18–20]. Instead, monomeric Cak1/Civ1 has been identified as the sole essential CAK in *S. cerevisiae*, although its amino acid sequence was found to be unrelated to those of vertebrate CAKs [21,22].

In plants, rice R2 was the first-identified CAK. It is closely related to CDK7/p40^{MO15} [23,24] and has both CDK and CTD kinase activities dependent on cyclin H [25]. We previously isolated an *Arabidopsis* cDNA encoding for a CAK homologue, *CAK1At*, as a suppressor of the CAK mutation in budding yeast, and showed that the similarity to vertebrate-type CAKs was restricted to several kinase domains [26]. CAK1At exhibited CDK kinase activity but no CTD kinase activity, which was separated biochemically in fractions with the ability to bind to yeast p13^{suc1} [26]. Thus, our results suggested the possible existence of other CAK-related proteins.

We have recently described two cDNAs coding for vertebrate-type CAKs, *CAK2At* and *CAK3At* [27]. Here we identified another gene for a CDK7/p40^{MO15} homologue in *Arabidopsis*. We also analyzed the transcript levels of each CAK-related gene in plant tissues and cell cultures. The results of kinase assays showed that CDK and CTD kinase activities might be differentially regulated by at least two distinct vertebrate-type CAKs in *Arabidopsis*.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana cell suspension culture (ecotype Columbia) was maintained as described previously [28]. At culture day 7, 15 ml of liquid culture was added to 35 ml of fresh medium, and cultivated in a 300 ml Erlenmeyer flask at 22°C in dark. Plants were grown at 23°C under continuous light conditions.

2.2. Cloning of CAK4At cDNA

The *CAK4At* cDNA was amplified by PCR from an *Arabidopsis* cDNA library subcloned into the yeast expression vector pYX112 [26]. In the first PCR, the inner primers, F1 (5′-GAGTTACTGTTTGGGAGTCGACAGTATGGA-3′) and R1 (5′-CAGCCTGCAGCCCAAACATCAACTCCT-3′), were used to amplify 5′- and 3′-half of the *CAK4At* cDNA in combination with vector primers, the forward primer (5′-GGAGTTTAGTGAACTTGC-3′) and the reverse primer (5′-GGGATGTATCGGTCAGTC-3′), respectively. The obtained

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Abbreviations: CDK, cyclin-dependent kinase; CTD, carboxy-terminal domain; CAK, CDK-activating kinase; TFIIH, general transcription factor IIH; GST, glutathione *S*-transferase; MBP, maltose-binding protein

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fragments were purified and further used as templates for the second PCR with the two vector primers. Resultant PCR products were blunted and subcloned into the *Sma*I site of pBluescript II SK$^-$ (Stratagene, La Jolla, CA, USA). The phylogenetic tree of CAK proteins was constructed using the CLUSTAL V software package [29].

2.3. Complementation of yeast CAK mutations

For complementation test with budding yeast cells, the coding regions of *Arabidopsis CAK*s were amplified by PCR, blunt-ended and cloned into the blunt-ended *Eco*RI site of pYX112, which carried an ARS/CEN replicon, the triose-phosphate isomerase promoter, and the selectable marker *URA3*. The plasmids were introduced into *S. cerevisiae* strain GF2351 (*MAT*α, *civ1-4*, *ura3*, *leu2*, *trp1*, *lys2*, *ade2*, *ade3*) [22] by the lithium acetate method [30]. Total protein of yeast cells was extracted as described by Vitaly [31]. To express CAKs in fission yeast, the coding regions of *Arabidopsis CAK*s were cloned into the blunt-ended *Mcs*I site of pREP3, carrying an *ARS1* replicon, an *NMT1* promoter, and a selectable *LEU2* marker. The resultant plasmids were used to transform *S. pombe* strain JM1224 (*mcs6-13*, *cdc2-3w*, *cdc25-22*, *leu1-32*, *h*-) [11] or SP755 (*mcs2-75*, *cdc2-3w*, *cdc25-22*, *leu1-32*, *h*-) [32] by the lithium acetate method [33]. Transformants were cultured on a minimal medium in the presence or absence of 2.0 μM thiamine.

2.4. Northern blot hybridization

RNA extraction and Northern blot hybridization were conducted as described previously [25,34]. The carboxy-terminal portion of an open reading frame (ORF) plus 3′-untranslated region of each CAK cDNA was amplified by PCR and used as a gene-specific probe for hybridization. *CAK2At* probe extending from nucleotide no. 1132 of the cDNA (accession no. AB047274), *CAK3At* probe extending from nucleotide no. 1020 of the cDNA (accession no. AB047275), and *CAK4At* probe extending from nucleotide no. 976 of the cDNA (accession no. AB074116) were used. In the case of *CAK1At*, the whole coding region was used as probe. Probe DNA fragments were labeled with [α-^{32}P]dCTP using the *Bca*BEST™ Labeling Kit (TaKaRa, Kyoto, Japan).

2.5. Immunoblotting and kinase assays

Arabidopsis protein samples were extracted from suspension cells 4 days after subculture as described by Magyar et al. [35]. Polyclonal antibodies were raised in rabbits against a peptide corresponding to the carboxy-terminal 12 amino acids of CAK2At (^{379}SHLKRKLDLEFQ^{391}) or the carboxy-terminal 10 amino acids of CAK4At (^{339}RRVMGPEGFT^{348}). Antibodies were purified with HiTrap rProtein A FF affinity columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Anti-CAK1At antibody has been described previously [26]. Immunoblotting was conducted using an ECL Western Blotting Detection Kit (Amersham). Kinase assays were performed as described previously [26]. As substrates, fusion proteins glutathione *S*-transferase (GST)–CDK2 (with the K33R mutation), GST–CDK2 (with the T160A mutation) and GST–CTD were expressed and purified as described previously [26].

2.6. Depletion of specific antibodies from antiserum

For preparation of maltose-binding protein (MBP)-fusion proteins, the ORFs of *CAK1At*, *CAK2At*, *CAK3At* and *CAK4At* were amplified by PCR with primers that included recognition sequences for specific restriction enzymes. After digestion with appropriate enzymes, the amplified fragments were ligated to the pMALC2 vector (New England Biolabs, Beverly, MA, USA) at the sites of *Sal*I, *Eco*RI, *Eco*RI and *Bam*HI/*Sal*I, respectively. The resultant plasmids were then introduced into *Escherichia coli* strain BL21. MBP-fusion proteins were purified with amylose resin (New England Biolabs) according to the protocol supplied by the manufacturer. For depletion of specific antibodies from the antiserum, MBP–CAK2At or MBP–CAK4At was bound to amylose resin, and the matrix was mixed with antiserum and incubated for 4 h at 4°C. After centrifugation, the supernatant was collected and used as depleted antiserum.

3. Results and discussion

3.1. Structural features of vertebrate-type CAKs of Arabidopsis

We have recently isolated two *Arabidopsis* cDNAs coding for vertebrate-type CAKs, named *CAK2At* and *CAK3At* [27]. By using the information of the genomic sequence of *Arabidopsis*, we identified another gene, designated *CAK4At*, whose predicted product has a high homology to CDK7/p40^{MO15}. Two cDNAs with a poly A stretch were isolated by PCR from the *Arabidopsis* cDNA library, and each had the same sequence with different lengths at the 5′- and 3′-ends. These clones contained an ORF of 348 amino acid with a calculated molecular mass of 37 kDa (Fig. 1A). Several in-frame stop codons were found in the 5′-untranslated region, indicating that these clones coded the full-length cDNA. According to the nomenclature proposed by Vandepoele et al. [36], CAK1At, CAK2At, CAK3At and CAK4At correspond to CDKF;1, CDKD;3, CDKD;1 and CDKD;2, respectively.

The deduced amino acid sequence of CAK4At showed the highest homology of 72% to rice R2, and 56% homology to human CDK7 (Fig. 1B). The other vertebrate-type CAKs, CAK2At and CAK3At, shared 70% identity with CAK4At, as shown in Fig. 1A. Rice R2 and *Arabidopsis* CAK2At and CAK3At, but not CAK4At, contained an extended carboxy-terminal region of 60–70 amino acids, which is not present in vertebrate and fission yeast CAKs (Fig. 1A; [24]). As reported by Umeda et al. [26], the CAK similarity of CAK1At is limited to the conserved kinase domains. Therefore, CAK1At is distinct from the other three CAKs in the phylogenetic tree (Fig. 1B). To our knowledge, multiple occurrences of CDK7/p40^{MO15} homologues have not been reported so far in other organisms.

3.2. Transcript levels of CAKs in plant tissues and suspension culture

To investigate the spatial expression patterns of *Arabidopsis* CAKs, we performed Northern blot analysis using total RNA isolated from various organs and culture cell suspensions. Probes specific to each CAK were designed as described in Section 2. As shown in Fig. 2A, significant levels of *CAK1At* transcripts were detected in all organs including differentiated tissues, and the highest expression was observed in culture cell suspensions, as reported previously [26]. Expressions of *CAK2At* and *CAK4At* were prominent in actively dividing cells in suspension cultures, but very low in plant tissues. In contrast, *CAK3At* transcripts were detected at lower levels even in culture cell suspensions (Fig. 2A).

We then analyzed the transcript levels of each CAK at different time points after initial dilutions of the suspension culture (Fig. 2B). As shown in Fig. 2C, the transcript amount of *CAK1At* was maintained at a high level even after cessation of cell division (day 8–9). Furthermore, the mRNA levels of the other vertebrate-type CAKs also remained constant until the early stationary phase (Fig. 2C), suggesting that their expression was not dependent on cell division. A similar expression profile has been also described for *Arabidopsis CDKA;1* [37].

3.3. Complementation of yeast CAK mutants with Arabidopsis CAKs

To test the functionality of *Arabidopsis* CAK homologues, we introduced CAK cDNAs into *S. cerevisiae* GF2351, which carries a temperature-sensitive mutation of the *cak1*/*civ1* gene [22]. Expression of each CAK in yeast cells was confirmed by immunoblotting with anti-CAK antibodies (Fig. 3A). Note that the CAK2At antibody detected both CAK2At and CAK3At as described below. Transformants that expressed *CA-*

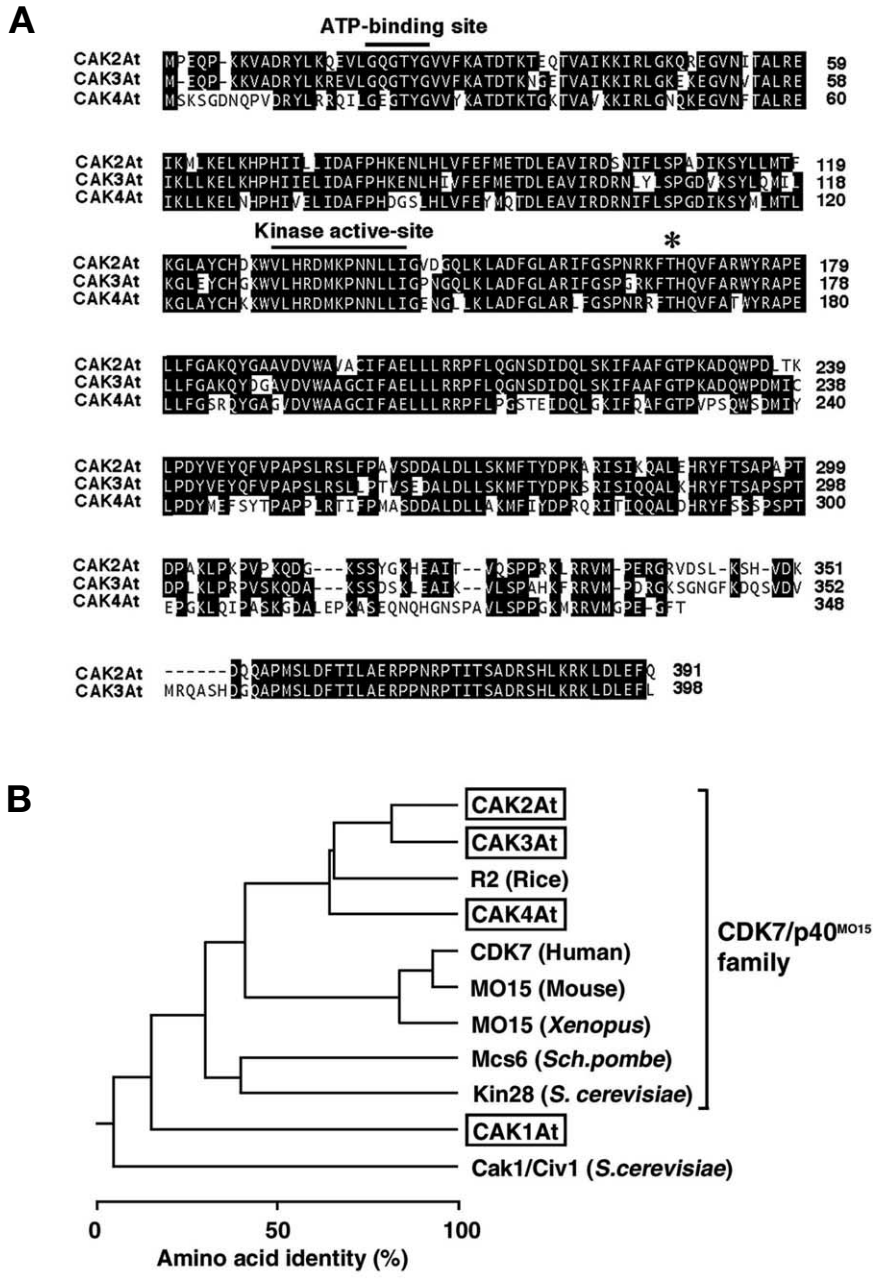

Fig. 1. Comparison of amino acid sequences of CAK-related proteins. A: Amino acid similarity among vertebrate-type CAKs of *Arabidopsis*. Amino acid sequences are aligned to yield the highest similarity to each other. Dashes represent gaps introduced to give maximal identity. Amino acids identical among more than two sequences are shown in white letters. Asterisk indicates the conserved threonine residue within the T-loop. B: Phylogenetic tree obtained by amino acid sequence comparison of CAK-related proteins.

K1At or *CAK2At* were able to grow at 34°C, whereas those carrying *CAK3At* or *CAK4At* cDNA failed to complement the CAK mutation (Fig. 3B). At a higher temperature of 36°C, only *CAK1At*-expressing cells could survive (Fig. 3B), suggesting that *CAK1At* has a higher suppression activity than *CAK2At*. When we expressed CAK cDNAs under the control of thiamine-repressible *NMT1* promoter in *S. pombe*, *CAK1At* and *CAK2At* again complemented the *mcs6* or *mcs2* mutation (data not shown).

Interestingly, only *CAK2At* could complement the mutants although the three vertebrate-type CAKs showed high homology. Hermand et al. [15] have recently reported that the mutation of the *cak1/civ1* gene of *S. cerevisiae* was rescued by co-expression of *Mcs6* and *Mcs2*, but not by that of either *Mcs6* or *Mcs2*. Moreover, we previously showed that the suppression activity of rice R2 in the budding yeast mutant was enhanced by co-expression with cyclin H [25]. Therefore, the difference in the suppression activity of *Arabidopsis* CAKs may reflect the capability of binding to yeast cyclin H homologue in yeast cells. Our preliminary results showed that CAK1At, but not the other CAKs, was active when produced in *E. coli* cells (unpublished data), suggesting that CAK1At might not require cyclin H for its activation in yeast cells, thus resulting in a high suppression activity.

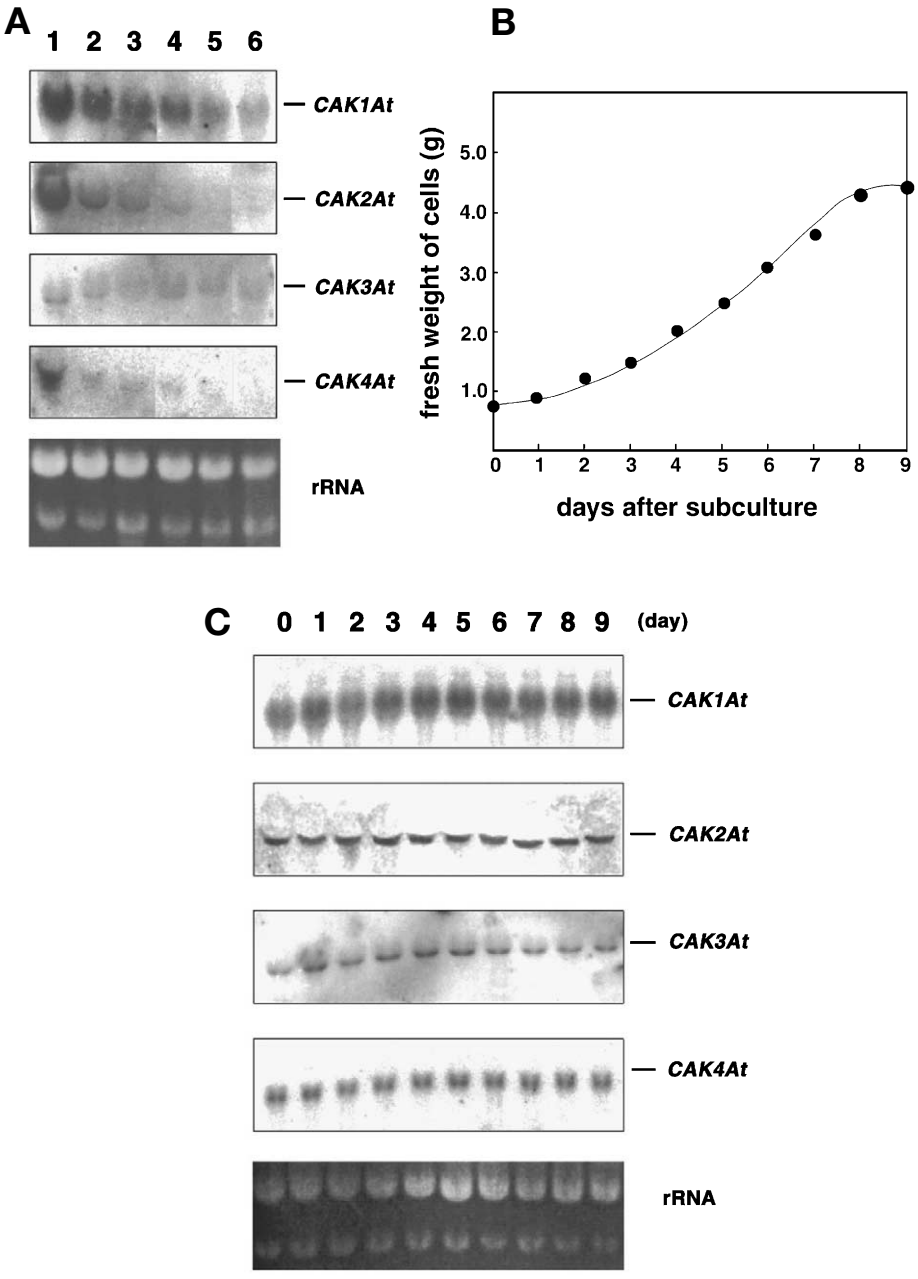

Fig. 2. mRNA accumulation of *Arabidopsis CAK*s. A: Transcript levels of *CAK*s in different *Arabidopsis* tissues. Total RNA samples (20 μg) were subjected to Northern blotting. Lane 1, suspension culture; lane 2, root; lane 3, shoot of seedling; lane 4, floral bud; lane 5, cauline leaf; lane 6, rosette. Ribosomal RNA (rRNA) visualized with ethidium bromide is shown as a control. B: Growth curve of *Arabidopsis* culture cell suspensions. Values of fresh weight of two independent cultures were averaged. C: Transcript levels in culture cell suspensions at different time points after subculture.

3.4. *Differential phosphorylation of CDK and CTD by Arabidopsis CAKs*

To investigate the enzyme activities of vertebrate-type CAKs, we prepared specific antibodies against the carboxy-terminal peptides of CAK2At and CAK4At. As shown in Fig. 4A, the CAK2At antibody detected both CAK2At and CAK3At fused to MBP. This was probably explained by the fact that CAK2At and CAK3At shared 11 of the 12 amino acids of the peptide antigen. In contrast, the CAK4At antibody specifically detected MBP–CAK4At (Fig. 4A). When the crude protein extracted from the culture cell suspension was subjected to immunoblotting, the CAK2At and CAK4At antibodies recognized proteins of 45.5 and 38.5 kDa, respectively (Fig. 4B). Depletion of these antibodies from the antiserum, by incubation with amylose resin carrying MBP–CAK2At or MBP–CAK4At, resulted in the disappearance of bands on the blots (Fig. 4B), indicating the specific recognition of CAK proteins by each antibody.

The crude extracts from *Arabidopsis* culture cell suspensions were immunoprecipitated with the CAK2At or CAK4At antibody, and immunoprecipitates were subjected to kinase assay. To exclude possible autophosphorylation, a kinase-inactive mutant of GST–CDK2, carrying a K33R replacement, was used as a substrate. As shown in Fig. 4C, both CAK2At-

and CAK4At-immunoprecipitates phosphorylated the GST–CDK2 substrate, but the activity was higher with the immunoprecipitates of CAK2At compared with CAK4At. The GST control protein was not phosphorylated by either of these immunoprecipitates (Fig. 4D). When GST–CDK2 with a T160A replacement within the T-loop was used as a substrate, no phosphorylation was observed (Fig. 4C), indicating that the immunoprecipitates specifically phosphorylated T160 of CDK2. We then used *Arabidopsis* CTD fused to GST as substrate in kinase assay. Again both immunoprecipitates phosphorylated GST–CTD, but the CAK4At-immunoprecipitate showed a higher activity than CAK2At (Fig. 4D). These results indicate that the kinase activity of CAK4At was higher against CTD compared to CDK2, whereas CDK2 was preferentially phosphorylated by CAK2At-immunoprecipitates.

The above data showed that vertebrate-type CAKs of *Arabidopsis* differentially phosphorylate CDK2 and CTD. Since the CAK2At antibody recognized recombinant CAK3At as well as CAK2At (Fig. 4A), we cannot separate the kinase activities of CAK2At and CAK3At by using immunoprecipitates as enzyme. When we prepared CAK proteins in insect cells and used them in phosphorylation reactions, CAK3At did not show any kinase activity whereas CAK1At, CAK2At and CAK4At displayed CDK and/or CTD kinase activities in vitro (unpublished data). Therefore, although CAK3At has a high identity (83%) with CAK2At at the amino acid level, it may be inactive, leading to inability to rescue the CAK mutations in yeasts. The results of kinase assay showed that CAK4At phosphorylated both CDK2 and CTD, but the activity was lower against CDK2 compared to CTD. The lower activity on CDK may account for the failure of CAK4At to complement the budding and fission yeast mutants. Hermand et al. [15] indicated that the synthetic lethality of the *mcs6-13 csk1*Δ strain of fission yeast primarily reflects impaired Cdc2 activation by CAK, and not CTD phosphorylation. In fact,

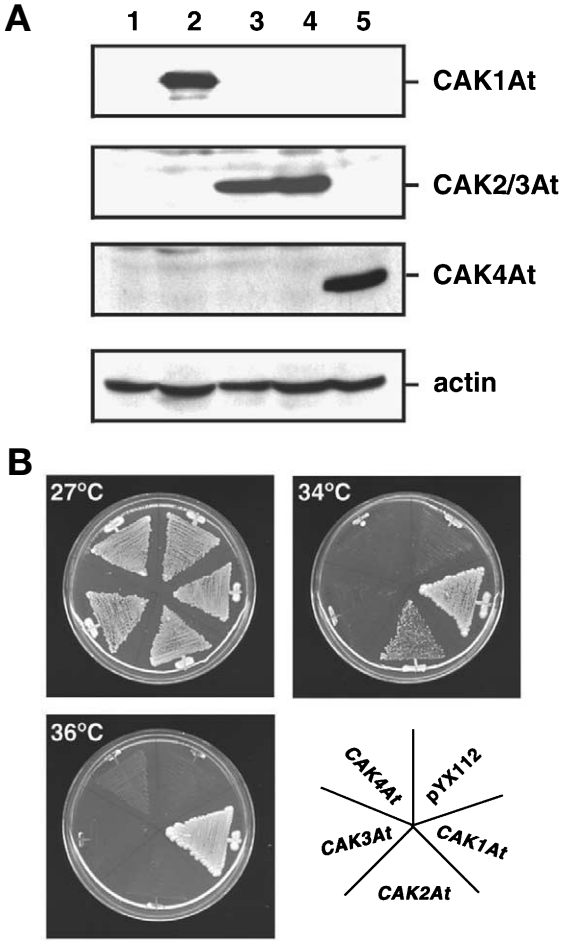

Fig. 3. Overexpression of *CAK1At* and *CAK2At* suppresses CAK mutations in budding yeast. A: Expression of *Arabidopsis* CAKs in *S. cerevisiae* mutant GF2351. Yeast cells carrying pYX112 (lane 1), pYX112-*CAK1At* (lane 2), pYX112-*CAK2At* (lane 3), pYX112-*CAK3At* (lane 4) or pYX112-*CAK4At* (lane 5) were grown at 27°C, and total protein extracted from the same amount of yeast cells was immunoblotted with the anti-CAK1At, -CAK2At or -CAK4At antibody. Anti-actin antibody (ICN Biomedicals, Inc.) was used as a control. B: Complementation of the *S. cerevisiae* mutant GF2351. Transformants carrying each plasmid were incubated at 27, 34 or 36°C for 4 days.

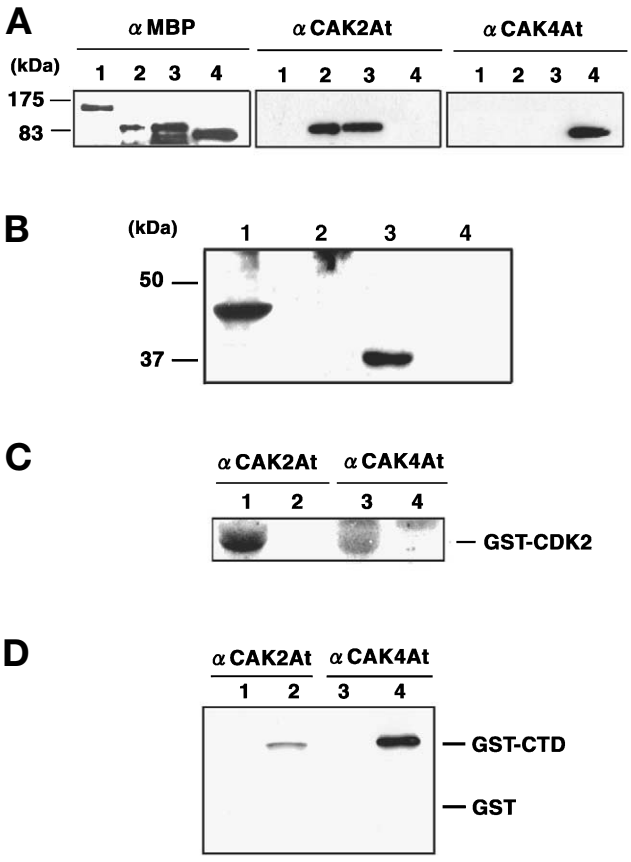

Fig. 4. Immunological detection of CAK2At and CAK4At and assay for CDK2 kinase and CTD kinase activities. A: Forty nanograms of CAK proteins fused to MBP were immunoblotted with the anti-MBP, anti-CAK2At or anti-CAK4At antibody. Lane 1, MBP–CAK1At; lane 2, MBP–CAK2At; lane 3, MBP–CAK3At; lane 4, MBP–CAK4At. B: Immunoblotting of total protein extract from *Arabidopsis* culture cell suspensions. Thirty micrograms of total protein was immunoblotted with anti-CAK2At (lane 1) or anti-CAK4At (lane 3) antibody, or with the antiserum depleted with amylose resin carrying MBP–CAK2At (lane 2) or MBP–CAK4At (lane 4), respectively. C: Immunoprecipitates of *Arabidopsis* proteins with the anti-CAK2At (lanes 1 and 2) or anti-CAK4At antibody (lanes 3 and 4) were assayed for CDK2 kinase activity, using GST–CDK2 (K33R) (lanes 1 and 3) or GST–CDK2 (T160A) (lanes 2 and 4) as substrates. D: Immunoprecipitates of *Arabidopsis* proteins with anti-CAK2At (lanes 1 and 2) or anti-CAK4At antibody (lanes 3 and 4) were assayed for CTD kinase activity, using GST (lanes 1 and 3) or GST–CTD (lanes 2 and 4) as substrates.

budding yeast Cak1/Civ1, which has CAK activity but no CTD kinase activity, suppressed *mcs6-13 csk1*Δ [15]. Therefore, the CAK mutation in fission yeast might be well suppressed by *Arabidopsis* CAK1At and CAK2At with higher CAK activity, but not by CAK4At with lower activity.

Why does *Arabidopsis* possess multiple CAK enzymes with different substrate preferences? It is unlikely that each has a cell-type-specific function because the data of Northern hybridization showed that expressions were not markedly different in various tissues. Multiple CAKs may have distinct functions in a cell to control CDK and RNA polymerase II. To answer these questions, characterization of other CAK components and analysis of knockout mutants are indispensable for further understanding of the regulatory mechanisms underlying cell cycle and basal transcription in plant cells.

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