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The *Arabidopsis* Cks1At protein binds the cyclin-dependent kinases Cdc2aAt and Cdc2bAt

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Abstract In *Arabidopsis*, two cyclin-dependent kinases (CDK), Cdc2aAt and Cdc2bAt, have been described. Here, we have used the yeast two-hybrid system to identify *Arabidopsis* proteins interacting with Cdc2aAt. Three different clones were isolated, one of which encodes a Suc1/Cks1 homologue. The functionality of the *Arabidopsis* Suc1/Cks1 homologue, designed Cks1At, was demonstrated by its ability to rescue the temperature-sensitive *cdc2-L7* strain of fission yeast at low and intermediate expression levels. In contrast, high *cks1At* expression levels inhibited cell division in both mutant and wild-type yeast strains. Cks1At binds both Cdc2aAt and Cdc2bAt in vivo and in vitro. Furthermore, we demonstrate that the fission yeast Suc1 binds Cdc2aAt but only weakly Cdc2bAt, whereas the human CksHs1 associated exclusively with Cdc2aAt.

Key words: Cell cycle; Cks1At; Cyclin-dependent kinase; Yeast two-hybrid screening; *Arabidopsis thaliana*

1. Introduction

The control of cell cycle progression in eukaryotes is mainly exerted at two transition points: one in late G_1, before DNA synthesis, and one at the G_2/M boundary. Progression through these control points is mediated by cyclin-dependent protein kinase (CDK) complexes, containing a catalytic subunit of approximately 34 kDa encoded by a *cdk* gene, and a regulatory cyclin subunit [1].

The Suc1/Cks1 proteins represent another class of components of CDK complexes. The *suc1* and *cks1* genes were originally identified in fission yeast and budding yeast as suppressors of certain temperature-sensitive *cdc2/CDC28* alleles, respectively [2–4]. Mutant $p34^{cdc2}$ proteins suppressible by *suc1* overexpression were shown to have a reduced affinity for the Suc1 protein [5]. Homologues of *suc1/cks1* have since then been identified in a wide range of organisms, including human, *Drosophila*, and *Xenopus* [6–8]. The strong interaction of Suc1/Cks1 proteins with CDKs allows the purification of homologous CDKs from other species using affinity chromatography.

More than one decade after their initial discovery, the function of the *suc1/cks1* genes is still unclear. In yeasts, both *suc1* and *cks1* are essential genes, as was demonstrated by gene disruption [3,4]. Cells deleted for *suc1* show mitotic spindles of varying lengths and condensed chromosomes, typical for a late mitotic arrest. The presence of high cyclin levels suggests that this arrest is attributed to the inability to destroy the mitotic cyclins, which is a prerequisite to leave M phase [8,9].

In addition, the presence of high amounts of Suc1/Cks1 blocks cell cycle progression [3,8,10,11]. Analysis of *Xenopus* cell-free extracts indicates that the high Suc1/Cks1 levels inhibit the onset of mitosis by interfering with the dephosphorylation of the CDK Tyr^{15} residue by Cdc25 [8,11].

Recent crystallographic studies of the human Suc1/Cks1 homologue CksHs1 complexed with Cdk2 suggest that the Suc1/Cks1 protein may function as a docking factor for both positive and negative regulators of CDK complexes [12]. The cell cycle arrest observed by *suc1/cks1* deletion and overexpression may therefore be a consequence of the inability of the CDK complexes to associate with these regulators.

In *Arabidopsis*, two *cdk* genes have been isolated, *cdc2aAt* and *cdc2bAt* [13,14], of which the gene products share 56% amino acid identity. To search for proteins interacting with Cdc2aAt, we exploited a two-hybrid screen. Three different clones were identified, one being an *Arabidopsis* Suc1/Cks1 homologue. The *Arabidopsis cks1At* gene can suppress the temperature-sensitive *cdc2-L7* allele of fission yeast. Furthermore, we demonstrated that the Cks1At protein binds both *Arabidopsis* CDKs, in contrast to the fission yeast Suc1 and human CksHs1 homologues which show only weak or no affinity for Cdc2bAt, respectively.

2. Materials and methods

2.1. Yeast two-hybrid system

Vectors and strains used were provided with the Matchmaker Two-Hybrid System (Clontech, Palo Alto, CA). Baits were constructed by inserting PCR fragments into the pGBT9 vector. The PCR fragments were created from the cDNAs using primers to incorporate *Eco*RI restriction enzyme sites. For *cdc2aAt* the primers 5′-CGAGATCTGAATTCATGGATCAGTA-3′ and 5′-CGAGATCTGAATTCCTAAGGCATGCC-3′ were used, for *cdc2bAt* the primers 5′-CGGATCCGAATTCATGGAGAACGAG-3′ and 5′-CGGATCCGAATTCTCAGAACTGAGA-3′. The PCR fragments were cut with *Eco*RI and cloned into the *Eco*RI site of pGBT9, resulting in the plasmids pGBTCDC2A and pGBTCDC2B. The GAL4 activation domain cDNA fusion library of 3-week-old vegetative *Arabidopsis* plants was obtained from Clontech.

For the screening a 1-l culture of the *Saccharomyces cerevisiae* strain HF7c (*MAT_a ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3 URA3::$GAL4_{17mers(3x)}$-$CyC1_{TATA}$-LacZ*) was cotransformed with

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Abbreviations: HB, homogenization buffer; MBP, maltose-binding protein; NCB, new column buffer; PBS, phosphate-buffered saline

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590 μg pGBTCDC2A, 1100 μg DNA of the library, and 40 mg salmon sperm carrier DNA using the lithium acetate method [15]. To estimate the number of independent cotransformants, 1/1000 of the transformation mix was plated on Leu^- and Trp^- medium. The rest of the transformation mix was plated on medium to select for histidine prototrophy (Trp^-, Leu^-, His^-). After 6 days of growth at 30°C, the colonies larger than 2 mm were streaked on histidine-lacking medium supplemented with 10 mM 3-amino-1,2,4-triazole (Sigma, St. Louis, MO). Colonies capable of growing under these conditions were tested for β-galactosidase activity as described [16].

Of the His^+ and $LacZ^+$ colonies the activation domain plasmids were isolated as described [17]. The pGAD10 inserts were PCR amplified using the primers 5′-ATACCACTACAATGGATG-3′ and 5′-AGTTGAAGTGAACTTGCGGG-3′. PCR fragments were digested with *Alu*I and fractionized on a 2% agarose gel. Plasmid DNA of which the inserts gave rise to different restriction patterns were electroporated into *Escherichia coli* XL1-Blue, and the DNA sequence of the inserts was determined. Extracted DNA was also used to retransform HF7c to test the specificity of the interaction.

A truncated form of Cks1At was generated by PCR using the primers 5′-GAGAGCCATGGGTCAGATCC-3′ and 5′-CCCTTCT-AGAGTTAGTTAAGAGGC-3′. The PCR fragment was cut with *Nco*I and *Xba*I and cloned blunt into the blunted *Eco*RI site of pGAD424, resulting in pGADCKSQ. Association with Cdc2aAt and Cdc2bAt was assayed by cotransformation of pGADCKSQ with pGBTCDC2A and pGBTCDC2B, respectively, as described above.

2.2. DNA blot hybridization

Total DNA from *Arabidopsis thaliana* ecotype Col-0 was prepared as described [18] and digested with *Bam*HI, *Dra*I, *Eco*RI, or *Hin*dIII. Restriction fragments were separated on a 0.8% agarose gel and transferred to nylon membranes (Hybond-N; Amersham). Hybridization was carried out using a random primed radiolabeled *cks1At* DNA probe in 3×SSC (1×SSC: 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0), 5×Denhardt's solution, 0.5% SDS, and 20 mg/ml denatured salmon sperm DNA at 60°C for 20 h. The filter was washed 3 times with 2×SSC, 0.1% SDS at 60°C for 15 min.

2.3. Preparation of Cks1At-Sepharose

For Cks1At expression and purification a fusion protein with the maltose-binding protein (MBP) was generated. The *cks1At*-coding region was PCR amplified using the primers 5′-GAGAGCCATGG-GTCAGATCC-3′ and 5′-CCAATACTCATAGATCTGTTGC-3′. The PCR fragment was cut with *Nco*I and *Bgl*II and cloned blunt into the blunted *Eco*RI site of pMAL-c2 (Biolabs, Beverly, MA) and the resulting vector, pMALSUC, was transformed into *E. coli* XL1-Blue. *E. coli* cells were grown until OD = 0.4 and the production of the fusion protein was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Cells were spun down, resuspended in new column buffer (NCB) containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, and were frozen. After thawing, cells were sonicated and spun, and the supernatant was loaded on an amylose resin column pre-equilibrated with NCB. Subsequently, the column was washed with 8 vol. of NCB. Next, the fusion protein was eluted with 3 vol. of NCB supplemented with 10 mM maltose. Cks1At was cleaved from MBP using 1% (w/w) factor Xa (Biolabs). The fragments were separated by size fractionation using a S200 Sepharose column using Beads Couple Buffer containing 0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl as running buffer. Purified Cks1At was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 5 mg/ml of gel according to the manufacturer's instructions. *Schizosaccharomyces pombe* Suc1-Sepharose beads were prepared as described [19].

2.4. Cks1At-binding and kinase assay

Protein extracts were prepared from 2-day-old cell suspensions of *A. thaliana* Col-0 in homogenization buffer (HB) containing 50 mM Tris-HCl (pH 7.2), 60 mM β-glycerophosphate, 15 mM nitrophenyl phosphate, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM vanadate, 50 mM NaF, 20 μg/ml leupeptin, 20 μg/ml aprotenin, 20 μg/ml soybean trypsin inhibitor, 100 μM benzamidine, 1 mM phenylmethylsulfonylfluoride, and 0.1% Triton X-100. In a total volume of 300 μl of HB, 150 mg of protein was loaded on 30 μl of 50% (v/v) Cks1At–Sepharose or control Sepharose beads and incubated on a rotating wheel for 2 h at 4°C. Beads were washed 3 times with Beads Buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM NaF, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotenin, 10 μg/ml soybean trypsin inhibitor, 100 μM benzamidine, and 1 mM phenylmethylsulfonylfluoride. Beads were resuspended in 25 μl of SDS-loading buffer and boiled. The supernatant was separated on a 12.5% SDS-PAGE gel and electroblotted on nitrocellulose membrane (Hybond-C^+; Amersham). Filters were blocked overnight with 2% milk in phosphate-buffered saline (PBS), washed 3 times with PBS, probed for 2 h with specific antibodies for Cdc2aAt (1:5000 dilution) or Cdc2bAt (1:2500 dilution) in PBS containing 0.5% Tween-20 and 1% albumin, washed for 1 h with PBS with 0.5% Tween-20, incubated for 2 h with peroxidase-conjugated secondary antibody (Amersham), and washed for 1 h with PBS containing 0.5% Tween-20. Protein detection was done by the chemoluminescent procedure (Pierce, Rockford, IL). Kinase assay was performed as described [19], using 30 μl of 50% (v/v) Cks1At–Sepharose or control Sepharose beads and 150 μg protein extract in a total volume of 300 μl of HB.

2.5. Cks1At expression in fission yeast

The *cks1At* gene was cloned under the control of the thiamine repressible *nmt1* promoter [20]. The *cks1At*-coding region was amplified by PCR as described above (Preparation of Cks1–Sepharose), and cut with *Nco*I and *Bgl*II. The pREP81CKS plasmid was obtained by cloning the PCR fragment blunt into the blunted *Nde*I site of pREP81; pREP41CKS was obtained by cloning the PCR fragment blunt into the blunted *Nde*I and *Bam*HI sites of pREP41. pREP3CKS was obtained by cloning the PCR fragment into the *Nco*I and *Bam*HI sites of BNRP3, which is a pREP3 derivative [19]. Plasmids were transformed into fission yeast strains *972 leu1-32 h*$^-$ (wild type) and *cdc2-L7 leu1-32 h*$^-$ using the lithium acetate method [15]. Transformants were plated on minimal medium with or without 5 mg/ml thiamine [21], and incubated at 28°C. To analyze the ability of *cks1At* to rescue *cdc2-L7* cells, plates were incubated at 35°C. Cells were fixed in 70% ethanol and analyzed by light microscopy.

3. Results

3.1. Two-hybrid screen using Cdc2aAt as bait

To identify Cdc2aAt-interacting proteins we used a two-hybrid system based on GAL4 recognition sites to regulate the expression of both *his3* and *lacZ* reporter genes [22,23]. The pGBTCDC2A vector, encoding a fusion protein between the C-terminus of the GAL4 DNA-binding domain and Cdc2aAt, was constructed by cloning the full-length coding region of *cdc2aAt* into the pGBT9 vector (see Section 2). For the screening, a GAL4 activation domain cDNA fusion library was used, constructed with RNA isolated from 3-week-old *Arabidopsis* vegetative tissues. The pGBTCDC2A plasmid was cotransformed with the library into the yeast HF7c reporter strain. A total of 10^7 independent cotransformants were screened for their ability to grow on histidine-free medium. A 6-day incubation at 30°C yielded 800 colonies. These 800 colonies were then tested for their ability to grow on medium without histidine in the presence of 10 mM 3-amino-1,2,4-triazole, reducing the number of positives to 235. Next, these colonies were tested for activation of the *lacZ* gene, and 143 turned out to be both His^+ and $LacZ^+$. After DNA preparation and restriction fragment analysis of all 143 positive clones, three different types of genes were identified.

The majority of cDNA clones (139) contained a small open reading frame coding for a protein of 87 amino acids, with a calculated molecular mass of 10.5 kDa. This gene was represented by at least two independent clones, as indicated by the varying length of their 5′- and 3′-untranslated regions. The longest clone contained a 5′-untranslated region of 15 bp and a 3′-untranslated region of 180 bp. A BLAST data base

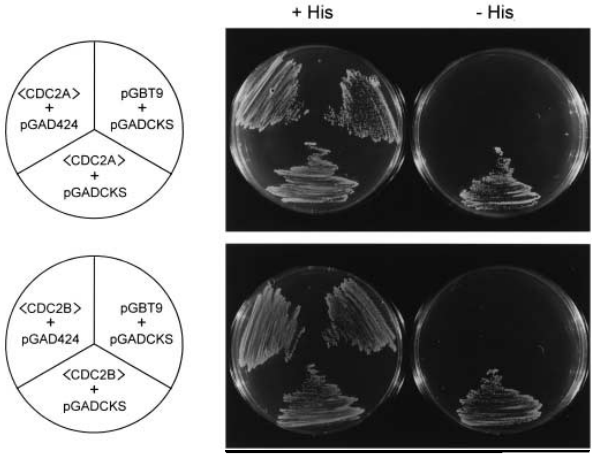

Fig. 1. Specific interaction of Cks1At with Cdc2aAt (top) and Cdc2bAt (bottom). Yeast HF7c cells were transformed with a plasmid expressing the GAL4 DNA-binding domain–Cdc2aAt (<CDC2A>) or –Cdc2bAt (<CDC2B>) fusion, together with the pGADCKS plasmid which encodes the GAL4 activation domain–Cks1At fusion. As controls <CDC2A> or <CDC2B> were cotransformed with a plasmid (pGAD424) encoding the GAL4 activation domain only; and pGADCKS was transformed with a plasmid (pGBT9) encoding the GAL4 DNA-binding domain only. Transformants were streaked on plates with (+His) or without (−His) histidine. Reconstitution of GAL4 activity restores the ability to grow on −His medium.

search revealed that this clone (called pGADCKS) encoded a Suc1/Cks1 homologue. The gene was designated *cks1At*, for **Cdk**-associating **s**ubunit from **A**rabidopsis **t**haliana.

The specificity of the interaction between Cdc2aAt and Cks1At was verified by the retransformation of yeast with pGBTCDC2A and pGADCKS. As controls, pGBTCDC2A was cotransformed with a vector containing only the GAL4 activation domain (pGAD424) whereas pGADCKS was cotransformed with a plasmid encoding the GAL4-binding domain only (pGBT9). Fig. 1 shows transformants plated on medium with or without histidine. Only transformants containing both pGBTCDC2A and pGADCKS were able to grow in the absence of histidine (Fig. 1, upper panel).

3.2. Characterization of the cks1At gene

Fig. 2 shows the deduced amino acid sequence of *cks1At* in alignment with Suc1/Cks1 homologues from yeasts, human, *Drosophila melanogaster*, *Xenopus laevis*, and *Patella vulgata*. Within the highly conserved region of 69 amino acids (from Gln^{3} to Leu^{71}), Cks1At has a high amino acid identity with all non-yeast homologues (68–70%). The lack of the 9-residue insertion (from Tyr^{55} to Leu^{63} in fission yeast) in Cks1At results in an identity of 48.7% and 50.0% with Suc1 of fission yeast and with Cks1 of budding yeast, respectively. In contrast to other non-yeast homologues, the Cks1At protein bears a C-terminal extension of 10–14 amino acids rich in glutamine residues. The need for this C-terminal domain for the interaction with Cdc2aAt was assayed using the two-hybrid system. A gene encoding a truncated Cks1At protein was created by mutating the Tyr^{73} codon to a stop codon, thereby deleting the last 16 amino acids. Subsequently, this mutated gene was cloned in frame with the GAL4 activation domain, resulting in the pGADCKSQ plasmid. This vector was introduced into the HF7c yeast reporter strain, together with pGBTCDC2A or the empty pGBT9 vector. Only transformants harboring both pGADCKSQ and pGBTCDC2A grew in the absence of histidine (data not shown), demonstrating that the C-terminal part of Cks1At is not essential for the binding of Cdc2aAt in the yeast system.

To study the genomic organization of *cks1At*, *Arabidopsis* DNA was digested with four different enzymes. Fig. 3 shows that only one band for every digest is found after hybridization with the *cks1At*-coding region at low stringency, indicating the presence of only one *cks1At* gene per haploid genome of *Arabidopsis*.

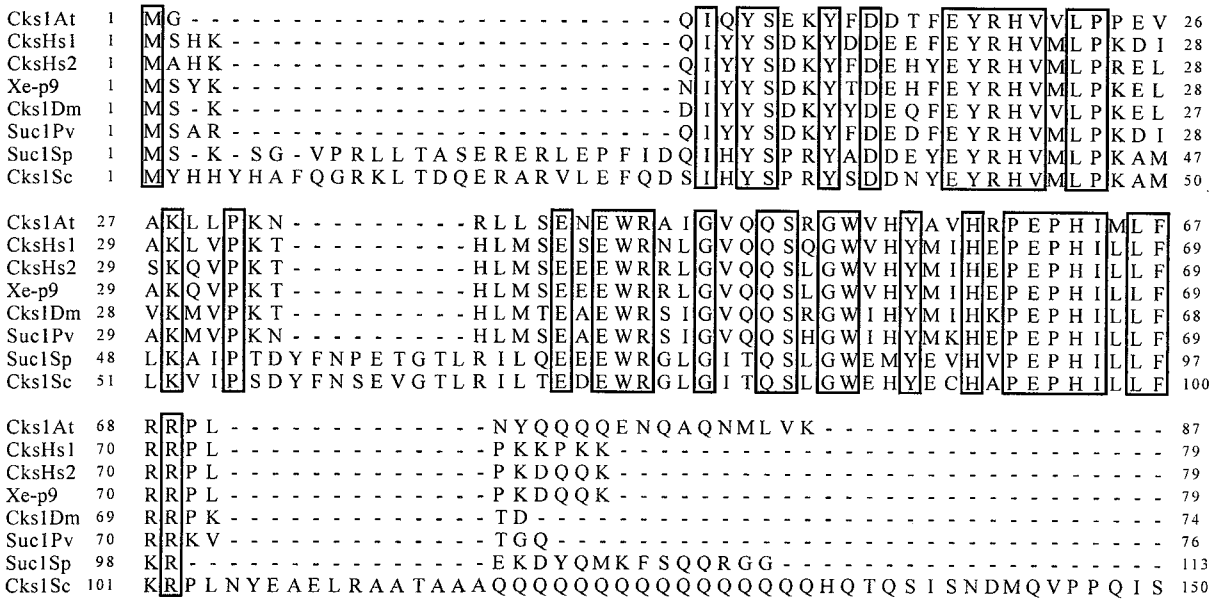

Fig. 2. Alignment of the Cks1At amino acid sequence with Suc1/Cks1 homologues of *Homo sapiens* CksHs1 and CksHs2 [6]; *Xenopus laevis* Xe-p9 [8], *Drosophila melanogaster* Cks1Dm [7], *Patella vulgata* Suc1Pv [32], *Schizosaccharomyces pombe* Suc1Sp [10], and *Saccharomyces cerevisiae* Cks1Sc [4]. Alignment was obtained using the Clustal W software (version 1.5; [33]. Amino acid residues conserved among all homologues are boxed.

3.3. Cks1At can rescue a temperature-sensitive cdc2 mutant of fission yeast

Both *suc1* and *cks1* from yeast were initially identified as suppressors of temperature-sensitive alleles of *cdc2* and *CDC28*, respectively [2–4]. To determine the functionality of the Cks1At protein, we tested whether it was able to rescue the temperature-sensitive fission yeast *cdc2-L7* strain. For this purpose, the full-length *cks1At*-coding region was cloned in the pREP3, pREP41, and pREP81 vectors, resulting in pREP3CKS, pREP41CKS, and pREP81CKS, respectively. These three vectors contain the thiamine-repressible promoter *nmt1* and allow inducible expression of *cks1At* to different levels [9,20]. Strongest induction can be achieved using the pREP3CKS vector, intermediate with pREP41CKS, and lowest with pREP81CKS. All constructs were introduced into wild-type and *cdc2-L7* yeast.

Wild-type cells of fission yeast transformed with pREP81CKS grew normally under both inductive (without thiamine) and non-inductive (with thiamine) conditions (data not shown). In contrast, in the absence of thiamine, cell growth was completely or partially inhibited in cells transformed with pREP3CKS and pREP41CKS, respectively (Fig. 4A; data not shown). Microscopic analysis revealed the *cks1At*-overexpressing cells to have an elongated phenotype (Fig. 4E). No cell elongation was seen under non-inductive conditions (Fig. 4D), nor in cells harboring the empty pREP3 or pREP41 vector (Fig. 4B,C), demonstrating that the observed phenotype was linked with *cks1At* expression.

Fig. 5 presents the *cdc2-L7* transformants grown in the presence and absence of thiamine, both at the permissive (28°C) and restrictive (35°C) temperature. At the permissive temperature a behavior similar to that of the wild-type strain was observed: cell growth was inhibited in the absence of thiamine for cells transformed with pREP41CKS or pREP3CKS, but not for pREP81CKS transformants (data not shown). At the restrictive temperature only pREP81CKS- and pREP41CKS-transformed cells grew in the absence of thiamine (Fig. 5A; data not shown). No growth was observed in the presence of thiamine, showing that the rescue of the *cdc2-L7* strain was specifically associated with low to intermediate expression levels of *cks1At*. At the microscopic level, the rescued *cdc2-L7* cells showed a cellular morphology intermediate to that of cells grown either at the restrictive temperature or at the permissive temperature (Fig. 5B–E).

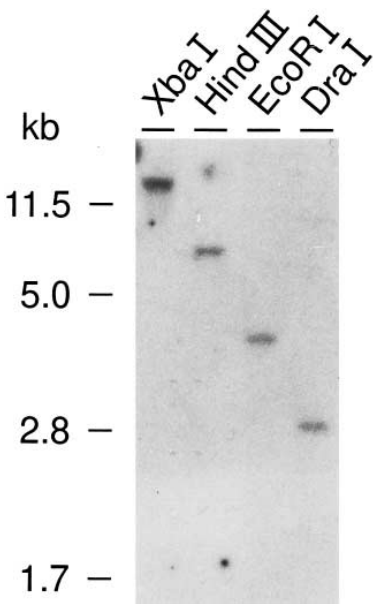

Fig. 3. DNA gel blot analysis of the *Arabidopsis cks1At* gene. *Arabidopsis* (ecotype Col-0) genomic DNA was digested with indicated restriction enzymes and hybridized with a probe derived from the *cks1At* cDNA at low stringency. Molecular length markers are given at the left in kilobases.

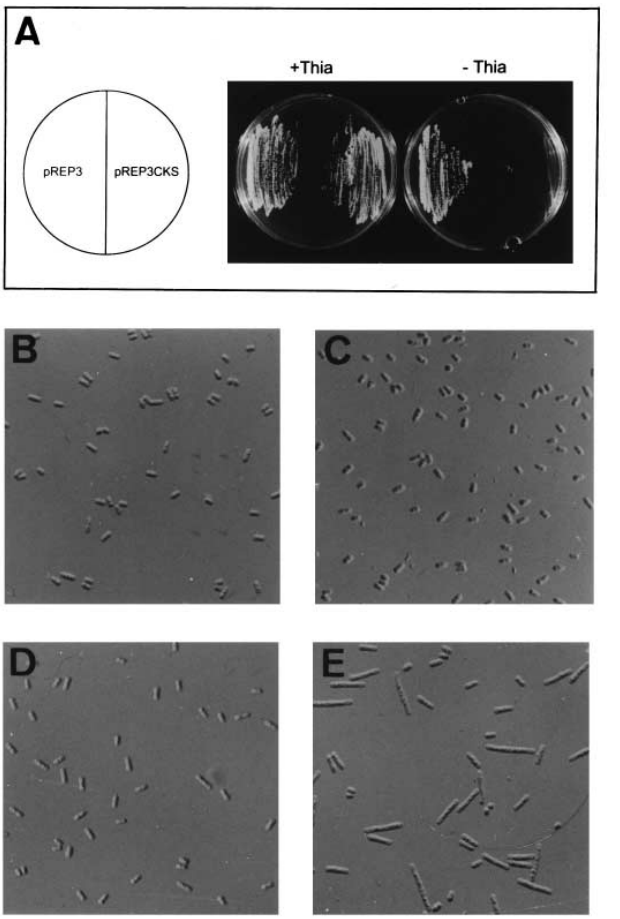

Fig. 4. Inhibition of cell division by *Cks1At* expression in fission yeast. A: Fission yeast *972 leu1-32 h⁻* cells transformed with the pREP3 control vector or with pREP3CKS. Transformants were streaked on plates with (+Thia) or without (−Thia) thiamine and grown for 3 days at 30°C. B,C: Transformants harboring the pREP3 vector grown at 30°C in the presence and in the absence of thiamine, respectively. D,E: Transformants harboring the pREP3CKS vector grown at 30°C in the presence and in the absence of thiamine, respectively.

3.4. Cks1At binds both Cdc2aAt and Cdc2bAt

Previously, it has been shown that not every CDK protein associates with Suc1/Cks1. Human Cdk4 and Cdk5 do not bind to CksHs1 or fission yeast Suc1 [24–26]. To test whether Cks1At binds Cdc2bAt in addition to Cdc2aAt, the *cdc2bAt* full-length coding region was cloned in fusion with the GAL4 DNA-binding domain. The resulting plasmid (pGBTCDC2B) was transformed in yeast together with the plasmid pGADCKS bearing the GAL4 activation domain fused to Cks1At. As controls, the pGBTCDC2B plasmid was cotransformed with the pGAD424 vector, which contained the

GAL4-activating domain only, and the pGADCKS plasmid was cotransformed with pGBT9. Transformants were plated on medium containing or lacking histidine. Only yeast containing both pGBTCDC2B and pGADCKS grew in the absence of histidine, indicating a stable interaction between Cks1At and Cdc2bAt (Fig. 1, lower panel). Also the pGADCKSQ plasmid, encoding the truncated form of Cks1At, enabled growth on histidine-lacking medium demonstrating that the C-terminal part of Cks1At is non-essential for the binding of Cdc2bAt, as was observed for Cdc2aAt (data not shown).

To obtain independent evidence for the interaction of Cks1At with the *Arabidopsis* CDKs, the Cks1At protein was overproduced in *E. coli*, purified to homogeneity, and coupled to Sepharose beads (see Section 2). Protein extracts from 2-day-old actively dividing cell suspensions were loaded on these Cks1At–Sepharose beads. As control, the same amount of protein was loaded on mock Sepharose beads. After incubation for 2 h at 4°C the beads were extensively rinsed with salt- and detergent-containing buffer to wash away unspecifically bound proteins. The bound proteins were resolved by SDS-PAGE. Immunoblotting was performed using antibodies specific for both Cdc2aAt (34 kDa) and Cdc2bAt (35 kDa). Both Cdc2aAt and Cdc2bAt were detected in the bound material obtained from Cks1At–Sepharose beads which had been incubated with total *Arabidopsis* extracts (Fig. 5A). No immunoreactive proteins were recovered from the extracts using the control Sepharose beads. These data clearly demonstrated that Cks1At bound both Cdc2aAt and Cdc2bAt. Assaying the Cks1At–Sepharose and control beads for kinase activity using histone H1 as substrate showed that only Cks1At beads were able to bind the activated form of the CDK complexes (Fig. 5B).

In a similar way as described for the Cks1At–Sepharose beads, fission yeast Suc1 and human CksHs1 beads were loaded with protein extracts from 2-day-old cell suspensions.

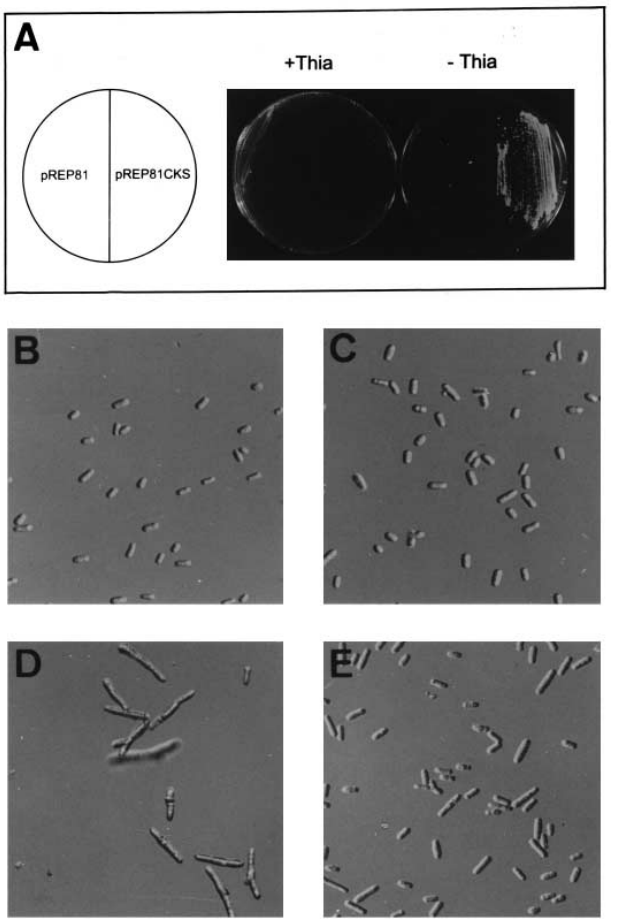

Fig. 5. Rescue of the temperature-sensitive *cdc2-L7* strain of fission yeast at the restrictive temperature by *cks1At* overexpression. A: Fission yeast *cdc2-L7 leu1-32 h*$^-$ cells transformed with the pREP81 control vector or with pREP81CKS. Transformants were streaked on plates with (+Thia) or without (−Thia) thiamine and grown for 5 days at 35°C. B,C: Transformants harboring the pREP81CKS vector grown at 28°C in the presence and the absence of thiamine, respectively. D,E: Transformants harboring the pREP81CKS vector grown at 35°C in the presence and the absence of thiamine, respectively.

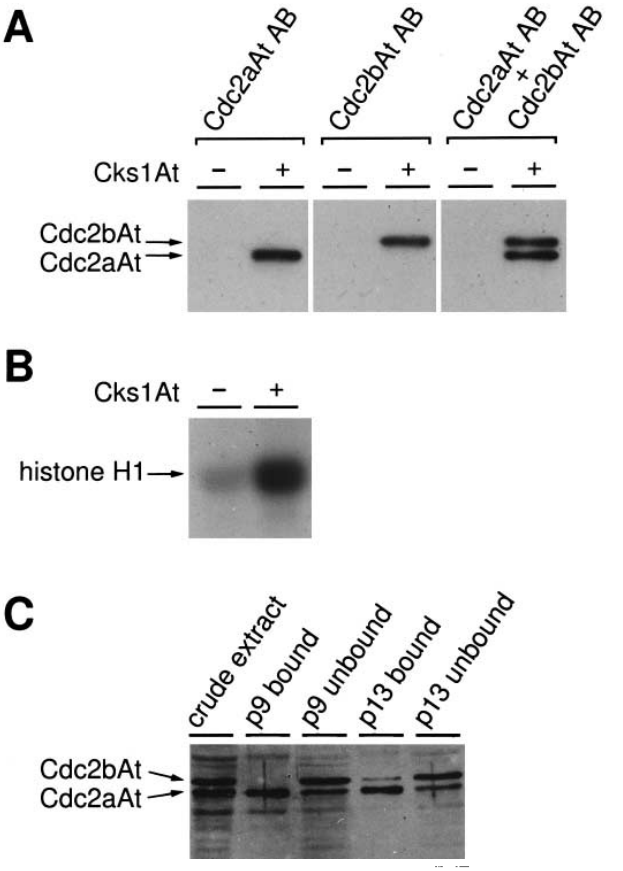

Fig. 6. In vitro binding of Cks1At to Cdc2aAt and Cdc2bAt. A: Protein extracts from 2-day-old *Arabidopsis* (ecotype Col-0) cell suspensions loaded on mock (−) or Cks1At–Sepharose (+) beads. After 2 h of incubation the beads were extensively washed with salt- and detergent-containing buffer. Binding of Cdc2aAt and Cdc2bAt to Cks1At was monitored by immunoblotting (see Section 2), using a Cdc2aAt-specific antibody (Cdc2aAt AB), a Cdc2bAt-specific antibody (Cdc2bAt AB), or both simultaneously (Cdc2aAt AB+Cdc2bAt AB). B: Cks1At–Sepharose bound Cdk activity measured using histone H1 as a substrate. C: Protein extracts loaded on CksHs1 (p9) and fission yeast Suc1 (p13) Sepharose beads. After 2 h of binding, the supernatants were collected and the beads were extensively washed with salt- and detergent-containing buffer. CksHs1 and Suc1 bound (p9 bound and p13 bound, respectively) and unbound (p9 unbound and p13 unbound) fractions were analyzed for the presence of Cdc2aAt and Cdc2bAt by immunoblotting using a mixture of Cdc2aAt- and Cdc2bAt-specific antibodies. As control, a crude protein extract was loaded (left).

After 2 h, the supernatants were removed and the beads were washed several times. Both the affinity bound and unbound fractions were separated by SDS-PAGE. Immunoblotting was performed using a mixture of Cdc2aAt- and Cdc2bAt-specific antibodies. As shown in Fig. 6C, Suc1–Sepharose (p13) bound Cdc2aAt but only weakly Cdc2bAt. CksHs1 beads (p9) associated with Cdc2aAt but not at all with Cdc2bAt, which remained in the unbound fraction.

4. Discussion

Here, we reported the characterization of the *Arabidopsis suc1/cks1* homologue, designated *cks1At*. The *cks1At* gene encodes a protein of 10.5 kDa. Previously, by immunoblotting with a fission yeast Suc1-specific antibody a cross-reacting band of 13 kDa in wheat and pea extracts has been detected [27]. On an SDS-PAGE gel the Cks1At protein migrates in between the fission yeast Suc1 (13.5 kDa) and human CksHs1 (9.6 kDa) proteins (data not shown). The difference between the molecular mass of the Cks1At protein and the cross-reacting band in pea and wheat extracts could be due to the difference in length of the Suc1/Cks1 proteins of different plant species. Alternatively, the fission yeast Suc1 antibody possibly recognizes an unrelated protein, sharing a Suc1 epitope. Such an unrelated CDK-binding protein of 15 kDa was identified during an attempt to purify the starfish Suc1/Cks1 homologue using a fission yeast Suc1 antibody [26].

In contrast to all other non-yeast Suc1/Cks1 homologues, Cks1At contains a longer polyglutamine-rich C-terminal domain. Polyglutamine segments are found in a broad variety of proteins and are thought to be involved in protein–protein interactions [28–30]. We have shown that the last 16 amino acids of Cks1At, including the polyglutamine stretch, are dispensable for the binding of both Cdc2aAt and Cdc2bAt. Therefore, this part of the Cks1At protein might be involved in the interaction with other proteins or may stabilize the CDK–Cks1At interaction.

It has been shown that both human *cksHs1* and *cksHs2* can substitute the *cks1* gene of *S. cerevisiae* [9]. Expression of *cksHs1* or *cksHs2* from a GAL1 promoter enabled a Cks1-deficient yeast strain to propagate. We took a different approach to test the functionality of *cks1At*. The fission yeast *suc1* gene was originally identified as a suppressor of certain temperature-sensitive *cdc2* mutants [2]. One of these alleles, *cdc2-L7*, contains a Pro-to-Ser substitution at amino acid position 208 [31]. This residue is located immediately upstream of the GDSEID motif which, as revealed by crystallographic studies, is involved in the binding of Suc1/Cks1 [12]. The $p34^{cdc2-L7}$ protein was demonstrated to have a strongly reduced affinity for Suc1 [5]. We have shown that low to moderate expression levels of *cks1At* allowed the *cdc2-L7* strain to divide at the restrictive temperature, suggesting that Cks1At can substitute for the fission yeast Suc1. Additionally, *cks1At* is able to complement a fission yeast suc1 deleted strain (data not shown).

In contrast, high expression levels of Cks1At inhibited cell division in both the mutant and wild-type strain. Similarly, a Suc1/Cks1 excess caused a G_2 arrest or delay of G_2/M transition in yeast and *Xenopus* egg extracts, respectively [3,8,10]. It is postulated that Suc1/Cks1 proteins regulate the interaction of CDKs with positive and negative regulators. An excess of the Cks1At protein might therefore bind and sequester these regulators, resulting in an inappropriate activation or inactivation of Cdk activity, causing a cell cycle arrest.

Cks1At was shown to bind both Cdc2aAt and Cdc2bAt in vivo and in vitro, using a two-hybrid approach or Cks1At–Sepharose affinity selection, respectively. This suggests that both *Arabidopsis* CDKs interact functionally with the possible regulatory Cks1At protein. Whereas the fission yeast Suc1 and human CksHs1 proteins show high amino-acid identity with Cks1At, they bind Cdc2bAt only weakly or not at all. Cdc2aAt and Cdc2bAt are distinguished by several features, including the presence of different cyclin-binding motifs. Cdc2aAt contains the PSTAIRE motif, commonly found in CDK homologues from other organisms. Cdc2bAt contains a PPTALRE motif which has also been identified in other plant CDKs, but has not been found in any other organism. Possibly, Cdc2bAt has a role in regulating some plant-specific features of the cell cycle such as the appearance of a cytoskeletal structure during preprophase, called the preprophase band. Seen from an evolutionary point of view, *cdc2bAt* might have evolved from *cdc2aAt*. During its evolution, the Cdc2bAt might have lost its ability to bind to any non-plant Suc1/Cks1 homologue, but still retained its capacity to bind Cks1At. Another interesting difference between the two *Arabidopsis* CDKs is that *cdc2aAt* can complement yeast temperature-sensitive $p34^{cdc2/CDC28}$ mutants, whereas *cdc2bAt* cannot [14]. The incapacity of Cdc2bAt to associate strongly with the fission yeast Suc1 protein offers a probable explanation of its inability to complement $p34^{cdc2}$ mutants.

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References

[1] Morgan, D.O. (1995) Nature 374, 131–134.
[2] Hayles, J., Beach, D., Durkacz, B. and Nurse, P. (1986) Mol Gen Genet 202, 291–293.
[3] Hayles, J., Aves, S. and Nurse, P. (1986) EMBO J. 5, 3373–3379.
[4] Hadwiger, J.A., Wittenberg, C., Mendenhall, M.D. and Reed, S.I. (1989) Mol. Cell. Biol. 9, 2034–2041.
[5] Brizuela, L., Draetta, G. and Beach, D. (1987) EMBO J. 6, 3507–3514.
[6] Richardson, H.E., Stueland, C.S., Thomas, J., Russell, P. and Reed, S.I. (1990) Genes Dev. 4, 1332–1344.
[7] Finley Jr., R.L. and Brent, R. (1994) Proc. Natl. Acad. Sci. USA 91, 12980–12984.
[8] Patra, D. and Dunphy, W.G. (1996) Genes Dev. 10, 1503–1515.
[9] Basi, G., Schmid, E. and Maundrell, K. (1993) Gene 123, 131–136.
[10] Hindley, J., Phear, G., Stein, M. and Beach, D. (1987) Mol. Cell. Biol. 7, 504–511.
[11] Dunphy, W.G. and Newport, J.W. (1989) Cell 58, 181–191.

[12] Bourne, Y., Watson, M.H., Hickey, M.J., Holmes, W., Rocque, W., Reed, S.I. and Tainer, J.A. (1996) Cell 84, 863–874.
[13] Ferreira, P.C.G., Hemerly, A.S., Villarroel, R., Van Montagu, M. and Inzé, D. (1991) Plant Cell 3, 531–540.
[14] Hirayama, T., Imajuku, Y., Anai, T., Matsui, M. and Oka, A. (1991) Gene 105, 159–165.
[15] Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Nucleic Acids Res. 20, 1425.
[16] Breedon, L. and Nasmyth, K. (1985) Cold Spring Harbor Symp Quant Biol 50, 643–650.
[17] Hoffman, C.S. and Winston, F. (1987) Gene 57, 267–272.
[18] Pruitt, R.E. and Meyerowitz, E.M. (1986) J. Mol. Biol. 187, 169–183.
[19] Hemerly, A., de Almeida Engler, J., Bergounioux, C., Van Montagu, M., Engler, G., Inzé, D. and Ferreira, P. (1995) EMBO J. 14, 3925–3936.
[20] Maundrell, K. (1990) J. Biol. Chem. 265, 10857–10864.
[21] Moreno, S., Klar, A. and Nurse, P. (1991) Methods Enzymol. 194, 795–826.
[22] Chien, C.-T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) Proc. Natl. Acad. Sci. USA 88, 9578–9582.
[23] Fields, S. and Sternglanz, R. (1994) Trends Genet. 10, 286–292.
[24] Lew, J., Beaudette, K., Litwin, C.M.E. and Wang, J.H. (1992) J Biol Chem 267, 13383–13390.
[25] Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.-Y., Hanks, S.K., Roussel, M.F. and Sherr, C.J. (1992) Cell 71, 323–334.
[26] Azzi, L., Meijer, L., Ostvold, A.-C., Lew, J. and Wang, J.H. (1994) J. Biol. Chem. 269, 13279–13288.
[27] John, P.C.L., Sek, F.J. and Hayles, J. (1991) Protoplasma 161, 70–74.
[28] Hoey, T., Weinzierl, R.O.J., Gill, G., Chen, J.-L., Dynlacht, B.D. and Tjian, R. (1993) Cell 72, 247–260.
[29] Persengiev, S.P., Saffer, J.D. and Kilpatrick, D.L. (1995) Proc. Natl. Acad. Sci. USA 92, 9107–9111.
[30] Bao, J., Sharp, A.H., Wagster, M.V., Becher, M., Schilling, G., Ross, C.A., Dawson, V.L. and Dawson, T.M. (1996) Proc. Natl. Acad. Sci. USA 93, 5037–5042.
[31] Carr, A.M., MacNeill, S.A., Hayles, J. and Nurse, P. (1989) Mol Gen Genet 218, 41–49.
[32] Colas, P., Serras, F. and van Loon, A.E. (1993) J. Dev. Biol. 37, 589–594.
[33] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.