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Mutational analysis of two *Arabidopsis thaliana* cyclin-dependent kinases in fission yeast

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Abstract We have analyzed five mutant alleles of two cyclin-dependent kinases from *Arabidopsis thaliana*, *CDC2aAt* and *CDC2bAt*, in *Schizosaccharomyces pombe*. Two of the five mutant alleles produced similar phenotypes for both cyclin-dependent kinases. The other three mutants caused phenotypes dependent on the particular cyclin-dependent kinase. Of all the mutant alleles, only two were found to possess a detectable kinase activity. Our mutational analysis lends further support for CDC2aAt being the true orthologue of the yeast cdc2. CDC2bAt, even though quite divergent from *S. pombe* cdc2, still retains the ability to interact with at least some essential cell cycle regulators, suggesting some functional homology with the yeast protein. Additionally, we demonstrated that the three amino acid deletion in the DL50 mutants results in the loss of the ability to interact with the suc1/CKS1 proteins.

Key words: Cell cycle; Cyclin-dependent kinase; CDC2 kinase subunit; *Arabidopsis thaliana*; *Schizosaccharomyces pombe*

1. Introduction

The control of cell cycle progression in eukaryotic cells is mainly exerted at two transition points. One in late G1, before DNA synthesis, and one at the G2/M boundary. Cyclin-dependent kinases (CDKs) regulate the passage through these control points by the phosphorylation of key substrates. CDK activity requires binding of the CDKs with regulatory proteins, called cyclins, and the phosphorylation of a Thr residue located in the 'T loop'. Activated CDKs may be inhibited by the phosphorylation of two residues (Thr-14 and Tyr-15) located in the catalytic cleft and by the binding of the so-called cyclin-dependent kinase inhibitors. Most of these regulatory steps are conserved from yeast to human [1].

The two CDKs characterized so far in *Arabidopsis thaliana*, CDC2aAt and CDC2bAt [2,3], share 60% and 56% identity with the fission yeast cdc2 protein, respectively, and 56% identity between themselves. Both CDC2aAt and CDC2bAt have a unique motif in the cyclin-binding domain (PSTAIRE and PPTALRE, respectively), suggesting that each binds to different cyclins. *CDC2aAt* can partially complement (for just two rounds of cell division) the temperature sensitive mutant *cdc2-33* of *Schizosaccharomyces pombe*, whereas *CDC2bAt* cannot [4]. The only conclusion that may be drawn from these results is that *CDC2aAt*, but not *CDC2bAt*, is able to substitute for some unidentified function(s) of the multifunctional CDC2 kinase that is impaired by the *cdc2-33* mutation. The question remains about the identity of that (those) function(s) and about the proficiency of both *Arabidopsis* CDKs as far as other functions are concerned.

Their differential behavior suggests that *CDC2aAt* and *CDC2bAt* play a unique role in the regulation of the cell cycle rather than being redundant. However, the individual roles of *CDC2aAt* and *CDC2bAt* in the cell cycle control remain to be determined. The use of dominant mutants provides a way to address this problem. Previously, two mutations were introduced into *CDC2aAt*. One expected to inactivate the kinase (*CDC2aAt.DN*) and one that abolished the inhibitory phosphorylation of the Thr-14 and Tyr-15 residues (*CDC2aAt.AF*). Their effects on the cell cycle were analyzed both in fission yeast and in planta [5]. Here we attempted to extend this work by constructing three additional mutant alleles of *CDC2aAt*. The mutations were chosen by analogy with dominant negative mutations identified in the fission yeast *cdc2* gene [6]. In *Schizosaccharomyces pombe*, the mutants interfered with three distinct elements of the cell cycle control: (i) entry into mitosis (*cdc2.DL50*), (ii) progression through mitosis (*cdc2.DL41*) and (iii) coordination of the DNA synthesis phase with mitosis (*cdc2.DL36*). Although *CDC2bAt* fails to complement the *cdc2-33* mutation, this fact does not preclude that it may share some of the cell cycle functions with *cdc2*. Because the residues affected by the yeast mutations described above are conserved in *CDC2bAt*, we introduced the analogous mutations into *CDC2bAt* as well.

As a first step in the analysis of these new mutant *CDK* alleles, all were expressed in fission yeast and their effects upon cell cycle progression were compared with those described for the original fission yeast *cdc2* mutants. As experimental system, fission yeast offers a number of advantages for comparing different CDKs and their alleles, such as the availability of promoters allowing inducible expression to different levels and the possibility to analyse the effect of the mutant proteins in the absence of the endogenous cdc2 activ-

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Abbreviations: CDK, cyclin-dependent kinase; CKS, CDC2 kinase subunit; DAPI, 4′,6-diamidino-2-phenylindole

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ity. Moreover, the identification of cell cycle mutant phenotypes in fission yeast is a relatively straightforward process. A cell cycle arrest leads typically to cell elongation (cdc$^-$ phenotype), whereas an advancing of mitosis produces typical cells with a reduced cell size (wee phenotype) when compared to wild-type cells.

We found that two of the three new mutant alleles of *CDC2aAt* (*CDC2aAt.DL41* and *CDC2bAt.DL50*) caused an alteration of the cell cycle almost indistinguishable from those observed for the corresponding yeast cdc2 mutants, whereas the third one (*CDC2aAt.DL36*) displayed only a partial similarity. We also show that expression of *CDC2bAt*, and four of the five mutant alleles thereof, blocked the cell cycle in G2, whereas the *CDC2bAt.DL50* allele caused a mitotic catastrophe phenotype identical to that of *cdc2.DL50* and *CDC2aAt.DL50* mutant genes.

2. Materials and methods

2.1. Site-directed mutagenesis

CDC2aAt and *CDC2bAt* cDNAs were cloned in pGem7Z-f$^-$ (Promega, Madison, WI, USA) and in pUC18, respectively. The site-directed mutagenesis was performed with the use of the ExSite PCR-based, site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The primers used to introduce the mutations were (the mutagenized residues being underlined): 5′-TTGTCAGGACATTTACTCAT-3′ and 5′-GGATACCGAATGCTCTG-3′ for *CDC2aAt.DL41*, 5′-TCTCTGGTACCGAGCAC-3′ and 5′-ATAACAACCTCATGAGTAAAT-3′ for *CDC2aAt.DL36*, 5′-TATAAATCTGCTTTCCCTAAA-3′ and 5′-AGAAGTTACCCCACGC-3′ for *CDC2aAt.DL50*, 5′-ATTCGGGAAAGTCTACAAA-3′ and 5′-GCTCCTTCTCCCGACCTT-3′ for *CDC2bAt.AF*, 5′-AATTTGGGTCTTGGTCGT-3′ and 5′-AGCAATCTTAAGAAGCTCTT-3′ for *CDC2bAt.DN*, 5′-TCTTAAGTCTTATACGCATGA-3′ and 5′-AGAACAGTAAAAGCACGAC-3′ for *CDC2bAt.DL41*, 5′-TTCTTTGGTATAGAGCTCCT-3′ and 5′-TAACAATCTCATGCGTATAA-3′ for *CDC2bAt.DL36* and 5′-TGGCATGTTTACCCTAA-3′ and 5′-TGTGGAAACACCCG-3′ for *CDC2bAt.DL50*. The fidelity of the mutagenesis was confirmed by sequencing.

2.2. Plasmids and recombinant DNA

CDC2aAt, *CDC2aAt.AF* and *CDC2aAt.DN* cDNAs were transcriptionally fused to the *nmt1* promoter in the fission yeast expression vector pREP3 [7]. The *CDC2aAt.DL41*, *CDC2aAt.DL36* and *CDC2aAt.DL50* cDNAs were cloned in pREP3 by replacing the *Nco*I-*Bam*HI fragment of the *CDC2aAt* cDNA with the respective fragments carrying the mutations. To clone *CDC2bAt* cDNA in pREP3, one of the constructs above was opened with *Nco*I, trimmed with mung bean exonuclease and subsequently cleaved with *Xma*I. The *CDC2bAt* cDNA was then ligated into the vector as *Hpa*I-*Xma*I fragment. All the other cDNAs carrying the *CDC2bAt* mutations were cloned into pREP3 replacing the *Sal*I-*Bam*HI fragment of *CDC2bAt* with the respective fragments carrying the mutations.

For cloning *CDC2aAt* alleles in pREP41 [8], a *Nco*I linker was ligated to the filled in *Nde*I site and the respective cDNAs were cloned as *Nco*I-*Xma*I fragments into the *Nco*I-*Xma*I site of pREP41. *CDC2bAt* cDNA was ligated as *Kpn*I (blunted)-*Bam*HI fragment into the *Nde*I (filled in)-*Bam*HI site of pREP41. All the *CDC2bAt* mutations were cloned, replacing the *Sal*I-*Bam*HI of the construct above with the respective fragments carrying the mutations.

2.3. Two hybrid assay

Vectors and strains used were provided with the Matchmaker two hybrid system kit (Clontech, Palo Alto, CA, USA). The preys and *CDC2aAt* bait were cloned as described [9]. The *CDC2aAt.DL50* bait was constructed by cloning the *CDC2aAt.DL50* cDNA as *Nco*I (filled)-*Xma*I fragment into the pGBT9 vector opened with *Eco*RI (filled)-*Xma*I. The baits and preys were co-transformed into the budding yeast HF7c strain (*MAT*$_a$ *ura3-52 his3-200 ade2-101 lys 2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 URA3::GAL4*$_{17MERS(3X)}$*-CYC1*$_{TATA}$*-LacZ*) and the association between the baits and the preys was assessed by the ability of the co-transformed strains to grow on histidine lacking medium.

2.4. Fission yeast strains and methods

The two temperature sensitive *cdc2* mutant strains used were *cdc2-33 leu1-32 h*$^-$ and *cdc2-L7 leu1-32 h*$^-$ [10]. Basic fission yeast techniques were performed as described [11].

2.5. Immuno blots and H1 kinase assays

Cell extracts were prepared [11] and histone H1 kinase activity in crude protein extracts was assayed [12]. Histone H1 kinase assays and immunoblotting of the CDK complexes, purified from crude extracts by p13^{suc1} affinity chromatography, were done according to [9]. Phosphorylated histone H1 was visualized and quantified through PhosphorImager scanning (Molecular Dynamics, Eugene, OR, USA).

3. Results

A map of the mutations introduced into the two *A. thaliana* CDKs is presented in Fig. 1. The wild-type and mutant alleles were cloned under the control of the *nmt1* promoter and its attenuated derivative *nmt1-T4* in the pREP3 and pREP41 expression vectors, respectively [7,8]. The *nmt1* promoter is repressed when cells are grown in the presence of thiamine and can be induced by growing the cells in thiamine-free medium. For the analysis, the designed constructs were transformed into two temperature sensitive fission yeast strains, *cdc2-33* and *cdc2-L7*. The growth of the transformed strains was analyzed to assess the ability of the mutant alleles to

Table 1
Overview of overexpression analysis of the *A. thaliana* wild-type and mutant CDK alleles in fission yeast

Alleles	Complements *cdc2-33*	Phenotype in *cdc2-33* at permissive temperatures	H1 kinase activity in *cdc2-L7* at 36°C[a]	H1 kinase activity in *cdc2-33* at 25°C[b]
CDC2aAt	+	Wee	42%	Decrease
CDC2aAt.AF	+	Severe wee	96%	Increase
CDC2aAt.DN	−	Interphase arrest, oversized nuclei	–	Decrease
CDC2aAt.DL36	−	Interphase arrest	–	Decrease
CDC2aAt.DL41	−	Mitotic arrest	11%	Decrease
CDC2aAt.DL50	−	Mitotic catastrophe	261%	No change
CDC2bAt	−	Interphase arrest	–	Decrease
CDC2bAt.AF	−	Interphase arrest	–	Decrease
CDC2bAt.DN	−	Interphase arrest, oversized nuclei	–	Decrease
CDC2bAt.DL36	−	Interphase arrest	–	Decrease
CDC2bAt.DL41	−	Interphase arrest	–	Decrease
CDC2bAt.DL50	−	Mitotic catastrophe	–	Decrease

[a]The increase upon induction
[b]In comparison with non-induced cells

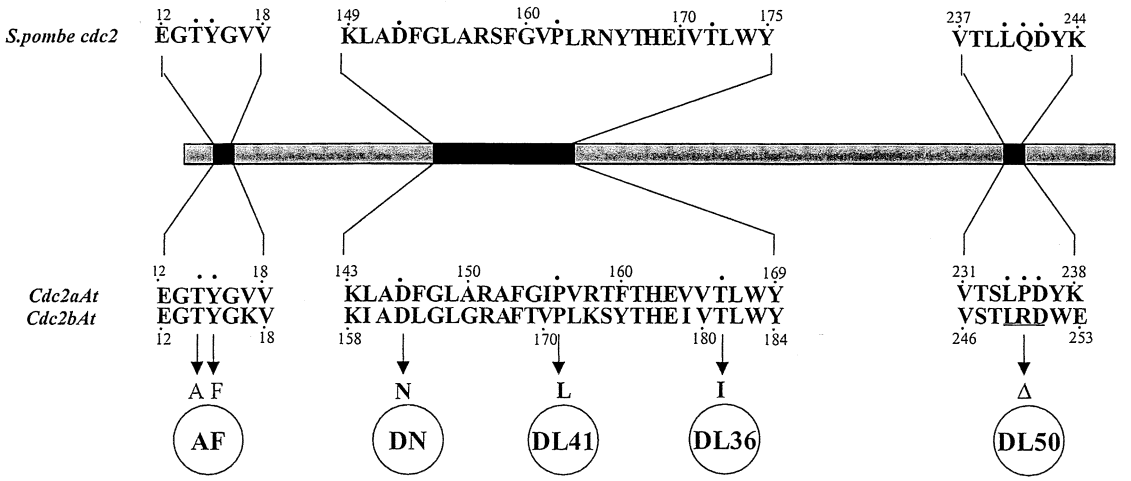

Fig. 1. Map of the characterized mutant alleles of *CDC2aAt* and *CDC2bAt*. The bar in the middle represents the complete coding region. The amino acid sequences of the mutated regions (in one letter code) are given below and above the bar for the plant CDKs and fission yeast cdc2, respectively. The dots above the sequence indicate the mutated amino acids with the arrows pointing to the corresponding changes.

restore or to interfere with cell division activity at the restrictive (36°C) or permissive (25°C) temperatures, respectively. Cells stained with a DNA-binding fluorochrome were subjected to further analysis by fluorescent microscopy and flow cytometry. Because the pREP3 vector gave a basal expression level in the non-induced conditions, most analyses were done with the pREP41 constructs.

3.1. *Effect of CDC2aAt and CDC2bAt mutant alleles on the cell cycle progression in yeast*

Expression of the different *A. thaliana CDK* alleles in the fission yeast *cdc2-33* strain grown under permissive conditions interfered in different ways with cell cycle progression (Table 1). Expression of the wild-type *CDC2aAt* gene led to the production of cells that divided at a reduced size (Fig. 2B) in comparison to cells transformed with the control vector (Fig. 2A), suggesting that mitosis is advanced in these cells. Flow cytometric analysis showed an accumulation of 1C cells (data not shown). Cells that overexpressed *CDC2bAt* were more elongated (Fig. 2J). Flow cytometric analysis indicated that most of the cells expressing *CDC2bAt* had a 2C DNA content, pointing to an arrest in the G2 phase of the cell cycle (data not shown).

Dephosphorylation of Tyr-15 in the fission yeast cdc2 leads to entry into mitosis through activation of the kinase [13]. In higher eukaryotes, an additional control is imposed through phosphorylation of Thr-14 [14,15]. Expression in fission yeast of *CDC2aAt.AF*, a mutant *CDC2aAt* in which the Thr-14 and Tyr-15 residues are mutated into the two non-phosphorylatable residues Ala-14 and Phe-15, has already been described at the morphological level [5]. A mixed population of cells was observed with abnormal phenotypes resembling those described for the fission yeast cdc2.F15 mutant [13]. We introduced the AF mutation in *CDC2bAt* and expressed this mutant allele in yeast. No morphological difference with cells expressing *CDC2bAt* was seen. Cells appeared elongated as a consequence of continued growth in the absence of cell division (data not shown).

The DN mutation, analyzed previously in a number of PSTAIRE kinases including the fission yeast cdc2, inactivates the kinase causing a cell cycle arrest [16]. As shown before [5], expression of *CDC2aAt.DN* in fission yeast leads to a tight cell cycle block in the interphase. We found additionally that the majority of the cells contained oversized nuclei (Fig. 2C) and that, at later time points, a subpopulation of cells developed grossly swollen nuclei that distended the yeast cell wall (Fig. 2D). An identical phenotype was also observed upon expression of *CDC2bAt.DN* (Fig. 2K).

The DL41 mutation was originally identified in a fission yeast cdc2 mutant which, when expressed, blocked the cells in mitosis. Cells showed condensed chromatin, elongated spindles and aberrantly formed septa. The condensed chromatin was often resolved in three bodies which were considered to be the three chromosomes of fission yeast [6]. Similarly, expression of *CDC2aAt.DL41* induced an accumulation of cells with condensed chromatin and misplaced or partially formed septa. The condensed chromatin was often resolved in three bodies variously located within the cells (Fig. 2E). In agreement with the observed phenotype, the cells were found by flow cytometric analysis to contain a 2C DNA content (data not shown). At a higher expression level, *CDC2aAt.DL41* led to the formation of a population of elongated cells with centrally located chromatin and misplaced or multiple septa (Fig. 2F). Thus, the expression of *CDC2aAt.DL41* seems to block cells at different points depending on the expression level. In contrast, expression of *CDC2bAt.DL41* induced a cell cycle arrest similar to that observed for the expression of the wild-type *CDC2bAt* gene.

Expression of *cdc2.DL36* in fission yeast arrested the cell division and caused an increase in the DNA content from 2C up to 16C, resulting in highly elongated cells with very large nuclei [16]. The phenotype obtained when *CDC2aAt.DL36* was expressed in the yeast cells was partially different (Fig. 2G). The cells were strongly elongated, pointing to a cell cycle arrest. Although DAPI staining revealed a clear increase in the size of the nuclei, flow cytometric analysis showed the presence of only 2C cells (not shown). The phenotype of *CDC2bAt.DL36* was quite similar to that seen upon expression of *CDC2bAt* (data not shown).

The mutant cdc2.DL50 protein contains a deletion of three

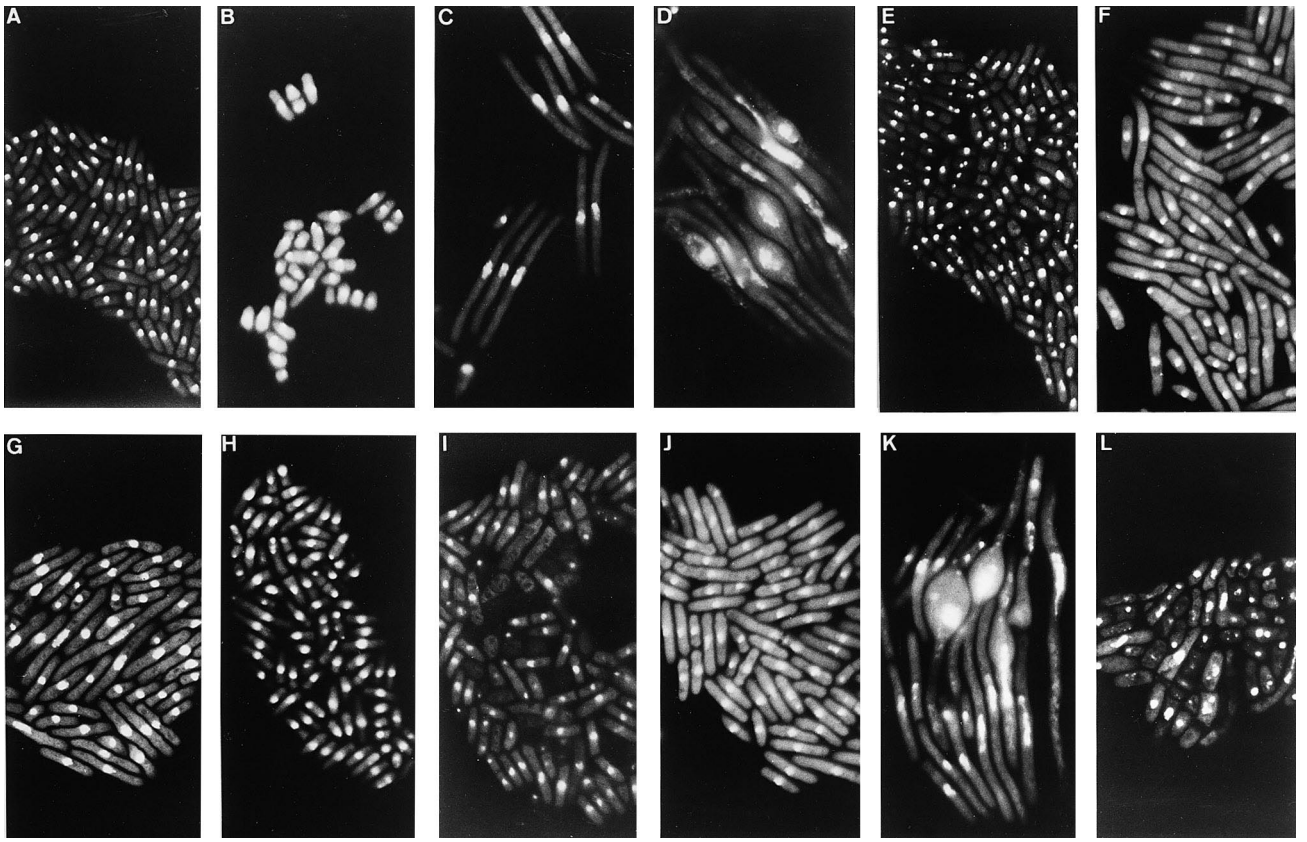

Fig. 2. A–L: Cytological analysis of the yeast cells expressing the mutant alleles of *CDC2aAt* and *CDC2bAt* at 25°C. REP41 and REP3 vectors were used to express the wild-type and mutant alleles of *CDC2aAt* and *CDC2bAt*. After the beginning of the induction of expression (0 time point), the samples were taken at different time points up to 38 h. The nuclei were stained with DAPI and the cells were analyzed microscopically as described in Section 2. Cells were maintained in exponential growth during the course of the experiment. (A) REP41 (38 h); (B) REP41/*CDC2aAt* (24 h); (C) REP41/*CDC2aAt.DN* (24 h). (D) REP41/*CDC2aAt.DN* (38 h); (E) REP41/*CDC2aAt.DL41* (24 h); (F) REP3/*CDC2aAt.DL41* (38 h); (G) REP3/*CDC2aAt.DL36* (24 h); (H) REP41/*CDC2aAt.DL50* (24 h); (I) REP3/*CDC2aAt.DL50* (24 h); (J) REP3/*CDC2bAt* (24 h); (K) REP41/*CDC2bAt.DN* (38 h); (L) REP41/*CDC2bAt.DL50* (38 h).

residues [6]. Deletion of this region in cdc2 leads to a mitotic catastrophe. The cells enter mitosis prematurely when the S phase is not yet completed with a rapid loss of viability as a consequence [6]. Expression of *CDC2aAt.DL50* induced also a premature advancement into mitosis as can be deduced from the small cell size after 24 h of induction (Fig. 2H). At a higher expression level the phenotype looked even more severe. A large proportion of cells were either anucleate, had nuclei cut by septa or showed fragmented chromatin (Fig. 2I). Flow cytometric analysis showed the appearance of diffused peaks at both sides of the typical 2C peak, likely to represent a mixed population of cells with a 1C DNA content unable to pass the cell size check point as a consequence of the reduced cell size, and cells with a DNA content ranging between 1C and 2C or 2C and 4C as a result of abnormal chromatin segregation (data not shown). Surprisingly, a very similar phenotype was observed when *CDC2bAt.DL50* was expressed in the yeast cells (Fig. 2L).

3.2. Complementation of cdc2-33 with different alleles of CDC2aAt and CDC2bAt

The wild-type proteins and the mutant isoforms were tested for their ability to rescue the growth defect imparted by the *cdc2-33* mutation at the restrictive temperature of 36°C. Upon induction of the attenuated *nmt1-T4* promoter at the permissive temperature, cells were plated on inducing medium and incubated at the restrictive temperature for 4 days. Only *CDC2aAt* and *CDC2aAt.AF* were able to restore the growth of the fission yeast *cdc2-33* cells to an extent sufficient to form colonies at the restrictive temperature (Table 1).

3.3. Determination of the specific kinase activity associated with the A. thaliana CDKs

The specific kinase activity associated with the mutant *A. thaliana* kinases was determined in protein extracts prepared from the fission yeast *cdc2-L7* strain transformed with the corresponding expression cassettes. This strain was reported to have a negligible CDK activity at 37°C [12], so the kinase activity measured at this temperature is mainly determined by the *A. thaliana* CDKs. All of the transformed strains were grown in medium without thiamine for 16 h at the permissive temperature of 25°C to induce the *nmt1* promoter. Control cultures were grown in the same conditions but in the presence of thiamine. After protein extraction, the CDK activity was determined at 37°C in crude protein extracts with histone H1 as a substrate [12].

CDC2aAt, *CDC2aAt.AF* and *CDC2aAt.DL50* showed a readily detectable kinase activity as manifested by an increase of the signals upon induction (approximately 1.4-, 2- and 3.6-fold, respectively) (Table 1). Only a marginal increase was observed in case of *CDC2aAt.DL41* expression (11%). None of the CDC2bAt proteins, including wild-type, showed any

detectable kinase activity upon overproduction in the *cdc2-L7* strain (Table 1).

3.4. Determination of the total histone H1 kinase activity in cdc2-33 background

To correlate the above described phenotypes with changes in the total CDK activity, we analyzed the histone H1 kinase activity in the strains used for the cytological studies. Protein extracts were prepared from the yeast *cdc2-33* strains transformed with the different pREP41 constructs grown at the permissive temperature under inducing and non-inducing conditions. Protein extracts were prepared from cells harvested one generation time before the first changes in cell division were observed (16 h after the removal of thiamine). The assays were performed at the permissive temperature (25°C) with CDK complexes partially purified by $p13^{suc1}$-affinity chromatography [17].

For the *CDC2aAt* alleles, an increase of histone H1 kinase activity was only observed in case of expression of *CDC2aAt.AF* (Table 1). Expression of *CDC2aAt.DN* and *CDC2aAt.DL36* strongly reduced the total CDK activity. *CDC2aAt* and *CDC2aAt.DL41*, when overexpressed in yeast, also compromized the total CDK activity, though less than *CDC2aAt.DN*. Unexpectedly, expression of *CDC2aAt.DL50* in the *cdc2-33* background seemed to have almost no influence on the total CDK activity, in spite of the high specific kinase activity associated with this allele (see above). The expression of the *CDC2bAt* alleles in the *cdc2-33* strain induced a reduction in the total CDK activity (data not shown).

3.5. Impact of the DL50 mutation on the interaction of CDC2aAt with the fission yeast suc1 protein and its A. thaliana homologue CKS1At

The apparent discrepancy between the high kinase activity associated with *CDC2aAt.DL50* on the one hand and the absence of any increase in the total CDK activity upon its expression in yeast cells on the other hand, prompted us to further investigate the nature of the DL50 mutation. The amino acids deleted in DL50 are located within the region involved in the interaction of human CDK2 with the CKS1Hs protein [18]. CKS1Hs is a member of a conserved family of small proteins (suc1/CKS1) binding tightly to many but not all CDKs. The current view is that they may function as docking factors mediating the interaction of CDKs with phosphoproteins [19]. The suc1/CKS1 proteins are routinely used to partially purify CDK containing complexes from crude extracts because of their high affinity for CDKs. We speculated that by using $p13^{suc1}$ beads to measure the kinase activity, the complexes containing CDC2aAt.DL50 could have been lost. To verify this hypothesis, we tested in vitro the ability of CDC2aAt.DL50 to bind $p13^{suc1}$. Fig. 3 shows the result of an immunoblot with an anti-CDC2aAt antibody of $p13^{suc1}$-purified complexes: no CDC2aAt.DL50 protein was recovered by $p13^{suc1}$ affinity chromatography in contrast to CDC2aAt, which was efficiently retained on the $p13^{suc1}$ beads. Moreover, to investigate whether we could extend these results to the interaction of CDC2aAt.DL50 with the *A. thaliana* suc1/CKS1 homologue CKS1At [9], we performed an in vivo two hybrid assay using CDC2aAt.DL50 as bait and CKS1At as prey. The strain producing both the bait and the prey is able to grow on the selective medium only if the two proteins interact. In contrast to the wild-type CDC2aAt protein, the CDC2aAt.DL50 was unable to associate with the CKS1At protein in the two hybrid system (Fig. 4: compare sectors 1 and 4). However, CDC2aAt.DL50 could still associate with two other proteins known to interact with CDC2aAt, namely CYCD1;1 and the putative substrate TH65 (Fig. 4: sectors 2, 5, and 3, 6 respectively, [20]). These results demonstrate that the deletion of three amino acids in CDC2aAt.DL50 specifically abolishes the binding of CKS1At.

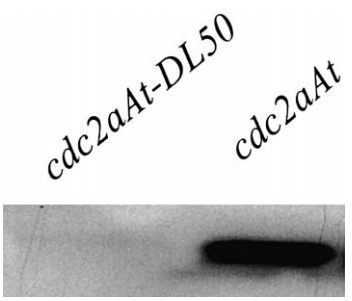

Fig. 3. In vitro interaction of CDC2aAt and CDC2aAt.DL50 with suc1. Proteins were prepared from the strains expressing either *CDC2aAt.DL50* or *CDC2aAt* and were used for $p13^{suc1}$ agarose affinity chromatography. The proteins retained on the beads were analyzed by immunoblotting with a CDC2aAt-specific antibody.

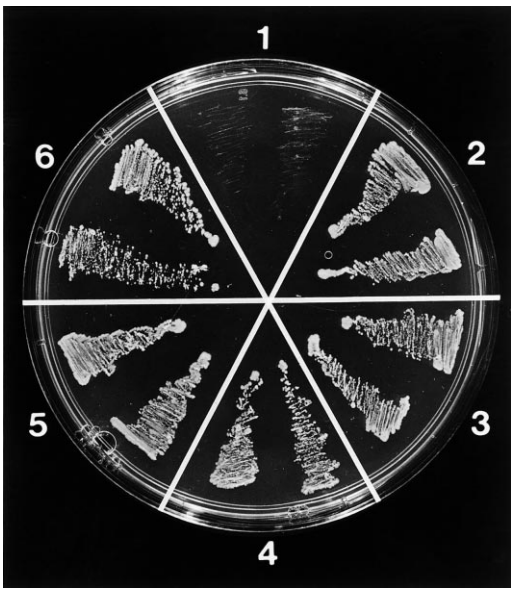

Fig. 4. In vivo interaction of CDC2aAt and CDC2aAt.DL50 with CKS1At. The yeast two hybrid system was used to compare the interaction of CDC2aAt.DL50 (sectors 1 through 3) and CDC2aAt (sectors 4 through 6) with CKS1At (sectors 1 and 4), and with two other *A. thaliana* proteins known to bind CDC2aAt, CYCD1;1 (sectors 2 and 5) and the TH65 protein (sectors 3 and 6).

4. Discussion

Here we have compared five mutant alleles of the two *A. thaliana* CDKs, *CDC2aAt* and *CDC2bAt*, in fission yeast. The results obtained can be summarized as follows: (i) only *CDC2aAt* and *CDC2aAt.AF* were able to complement the temperature sensitive mutant *cdc2-33* yeast strain. (ii) The DL50 mutation in both *CDC2aAt* and *CDC2bAt* gave rise to similar mitotic catastrophe phenotypes, though with apparently different mechanisms. Moreover, the mutation identifies

an important site for the interaction with the suc1/CKS1 docking factors. (iii) Two mutations, *CDC2aAt.DN* and *CDC2aAt.DL36*, abolished the kinase activity of CDC2aAt and the expression of the corresponding mutant proteins arrested yeast cells in G2. (iv) Expression of *CDC2aAt.DL41* interfered with the progression through mitosis. (v) All *CDC2bAt* mutant alleles, except for *CDC2bAt.DL50*, caused a cell cycle arrest in G2, accompanied by an increase in the size of the nuclei in case of the *CDC2bAt.DN* mutant.

Two mutant forms of CDC2aAt, CDC2aAt.DN (substitution of Asn-146 for Asp-146) and CDC2aAt.DL36 (substitution of Ile-166 for Thr-166) were found to be catalytically inactive in yeast cell extracts. The Asp-146 residue belongs to the triad of catalytic residues universally conserved in all eukaryotic protein kinases (Lys-33, Glu-51 and Asp-145 in the human CDK2). All three residues provide vital contacts for correct ATP orientation and Mg^{2+} coordination [21]. The Thr-166 residue is located amidst the residues, which were deduced from the three dimensional structure of the fully activated CDK2, to accommodate the substrate [22].

Both *CDC2aAt.DN* and *CDC2aAt.DL36* are therefore expected to behave as dominant negative mutants reducing the total CDK activity by sequestering important regulators of the yeast cdc2 protein. Indeed, when expressed in yeast, both *CDC2aAt.DN* and *CDC2aAt.DL36* caused a tight cell cycle arrest, similarly to the yeast *cdc2.DN* and *cdc2.DL36* genes [16]. Whereas for both yeast mutants the cell cycle arrest was followed by endoreduplication, our results provide an indication for endoreduplication (the appearance of grossly enlarged nuclei upon prolonged expression) only in case of *CDC2aAt.DN* expression. Expression of *CDC2aAt.DN* in tobacco caused a reduction in H1 kinase activity, accompanied by a decrease in the number of cells and an increase of the cell sizes [5]. Thus, the phenotype described in planta is consistent with that observed for *cdc2.DN* and *CDC2aAt.DN* expression in yeast, with the exception of lack of endoreduplication.

Because *CDC2bAt* is catalytically inactive when expressed in *S. pombe*, we did not expect a priori any specific phenotypes associated with the expression of *CDC2bAt.DN* and *CDC2bAt.DL36*. Therefore, it was surprising to find that CDC2bAt.DN conferred a much more severe cdc^- phenotype to *S. pombe* cells compared to the wild-type protein. The observed difference in the behavior of CDC2bAt and CDC2bAt.DN could be accounted for by an increased stability of the complexes formed by CDC2b.DN with limiting cell-cell regulators.

In contrast, expression of wild-type *CDC2aAt*, *CDC2aAt.AF* and *CDC2aAt.DL50* forced cells to divide at a smaller size, probably because of a precocious entry into mitosis, with the severity of the phenotype ranging from a moderate wee phenotype for *CDC2aAt* to a characteristically mitotic catastrophe for *CDC2aAt.DL50*.

Similarly to *CDC2aAt*, a wee phenotype was also observed when the fission yeast *cdc2* gene was expressed in yeast [23]. Although kinase activity measurements in the *cdc2-L7* strain showed that *CDC2aAt* is catalytically active in yeast cells, its expression in the *cdc2-33* background resulted in a reduction of the total CDK activity. These apparently contradictory results can be reconciled by assuming that the CDC2aAt kinase interacts inefficiently with yeast cell cycle regulators, leading to the formation of complexes with a specific kinase activity lower than that of the endogenous cdc2 complexes.

The Thr-14 and Tyr-15 residues are located in the L2 loop forming the ATP-binding interface in the amino-terminal lobe of active CDKs [21]. Their phosphorylation is expected to interfere with the correct positioning of ATP through steric hindrance and electrostatic repulsion. Inhibitory phosphorylation of Tyr-15 (or Tyr-15 and Thr-14) has been shown to be important for the timing of mitosis in many, but not all, eukaryotes [24]. The involvement of the inhibitory phosphorylation in the cell cycle control in plants has been a matter of controversy [5,25]. Here we present an additional evidence for the relevance of Tyr-15 phosphorylation for the regulation of the kinase activity of plant CDKs. Yeast cells overproducing CDC2aAt.AF, in which both Thr-14 and Tyr-15 were substituted for two non-phosphorylatable residues, showed an increase in the total histone H1 kinase activity. These results provide further support for the negative regulation of CDC2aAt activity by the phosphorylation of the Tyr-15 residue, as it has been demonstrated for PSTAIRE kinases from tobacco [25].

Of the three amino acids deleted in the cdc2.DL50, only Asp-242 is conserved in functional cdc2 homologues of other eukaryotes. Surprisingly, the expression of both *CDC2aAt.DL50* and *CDC2bAt.DL50* induced a similar phenotype: cells entered mitosis inappropriately at a reduced size and eventually lost genome integrity. Such a phenotype is very similar to the mitotic catastrophe described for the yeast cdc2.DL50 mutant, which was shown to be caused by a premature onset of mitosis in cells that have not completed DNA replication. Therefore both *A. thaliana* DL50 mutants probably also cause a mitotic catastrophe by triggering entry into mitosis directly from the S phase. However, the mechanisms involved might be different: whereas CDC2aAt.DL50 retains a high level of kinase activity, no activity can be found associated with CDC2bAt.DL50. Thus, the mitotic catastrophe phenotype conferred by CDC2bAt.DL50 cannot be explained by a simple gain of function mechanism but rather involves interference with the normal cell cycle controls of the endogenous cdc2. On the contrary, the high specific kinase activity associated with CDC2aAt.DL50 suggests that it can be directly responsible for the premature activation of mitosis.

We have also shown that the DL50 mutation identifies a region of primary importance for the interaction with the suc1/CKS1 proteins. The crystal structure of the human CDK2 in complex with CKS1Hs indeed showed that the residues deleted in the DL50 mutants are located in a region forming the interaction interface for CKS1Hs [18]. Although the molecular details of the interaction are likely to vary depending on the particular complex, the overall structure of the interface should be well conserved, given the ability of CDKs and suc1/CKS1 proteins from different species to form stable complexes. One of the possibilities is that suc1/CKS1 modulates indirectly the Tyr-15 phosphorylation through the action of another protein. This would be consistent with the current view that the suc1/CKS1 protein mediates the interaction between CDKs and phosphoproteins [18]. Bourne et al. [18] also noticed the possibility for CKS1Hs to interfere with the activating Thr phosphorylation in the T loop. In both cases, the elimination of association with suc1 could contribute to premature activation of CDC2aAt.DL50. Alternatively, the DL50 mutation could act with a mechanism entirely independent of suc1 identifying, for instance, a site of interaction with a CDK inhibitor.

The Pro-162 residue of the yeast cdc2 protein, substituted with Leu-162 in the DL41 mutants, is found in all functional homologues of cdc2 in other eukaryotes, but is not universally conserved among other CDK proteins. Expression of *cdc2.DL41* in fission yeast induced an anaphase arrest by specifically preventing sister chromatids disjunction. Whereas expression of *CDC2bAt.DL41* in fission yeast blocked the cells in G2, *CDC2aAt.DL41* expression caused an effect similar to that described for *cdc2.DL41*. Moreover, the finding that the mutation of Pro-156 to Leu-156 in CDC2aAt negatively influences the activity of the enzyme further suggests analogy with cdc2.DL41. Taken together, these observations indicate that the *A. thaliana* PSTAIRE kinase retains at least some of the functions necessary for progression through mitosis in yeast, suggesting that it may be involved in mitosis in plants as well.

Our mutational analysis lends further support for CDC2aAt being the true orthologue of the yeast cdc2 as all the mutations tested in the present study induced a phenotype similar to those described for the homologous mutations in cdc2. Taken together, these data suggest that the functions shared by fission yeast cdc2 and CDC2aAt include regulation of the G1/S and G2/M transition points, G2 phase progression and sister chromatid separation.

CDC2bAt, even though quite divergent from fission yeast cdc2, still retains the ability to interact with at least some essential cell cycle regulators suggesting certain functional homology with the yeast protein. This can be deduced from the G2 arrest imposed by the expression of *CDC2bAt* and the accompanying decrease in the histone H1 kinase activity. On the other hand, we failed to detect any activity associated with CDC2bAt in fission yeast. A plausible interpretation of these observations could be that CDC2bAt interacts with components of the yeast cell cycle mechanism essential for the G2/M transition, most probably cyclins, but the complexes formed are inactive. The suggested interaction of CDC2bAt with G2/M transition regulators is in agreement with its expression pattern in plants, where CDC2bAt activity peaks at the G2/M transition point (G. Segers, unpublished data). We are currently analyzing the effects of overexpression of the *CDC2aAt* and *CDC2bAt* mutant genes in transgenic *A. thaliana* and tobacco plants.

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