ANALYSIS OF TWO PLANT PROTEIN COMPLEXES ASSOCIATED WITH TRANSCRIPTION AND CELL CYCLE PROGRESSION

PhD Thesis

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ABBREVIATIONS

aa	amino acid
APC	anaphase-promoting complex
CAK	CDK-activating kinase
ccs52, Ccs52	cell cycle switch 52 gene and protein, respectively
Cdc	cell division control
Cdh1	Cdc20 homologue 1
CDK	cyclin-dependent kinase
CKI	CDK-inhibitory subunit
CSM	Cdh1-specific motif
CTD	carboxy-terminal domain of RNA polymerase II
Da	dalton
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF	DRB-sensitivity inducing factor
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EST	expressed sequence tag
G1, G2	gap phases of cell cycle
GTF	general transcription factor
HA	hemagglutinin
M phase	mitosis
MPF	mitosis-promoting factor
mRNA	messenger RNA
NELF	negative elongation factor
nt	nucleotide
N-TEF	negative transcription elongation factor
PIC	preinitiation complex
Pol II	RNA polymerase II
Pol IIA	hypophosphorylated form of RNA polymerase II
Pol IIO	hyperphosphorylated from of RNA polymerase II
P-TEFb	positive transcription elongation factor b
Rb	retinoblastoma protein
S phase	DNA synthesis phase of cell cycle
SCF	Skp1/Cullin/F-box ubiquitin ligase complex
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
snRNP	small nuclear ribonucleoprotein particle
TFII	transcription factor of RNA polymerase II
TPR	tetratricopeptide repeat

CONTENTS

GENERAL INTRODUCTION

Plants and animals began their evolutionary history with a common unicellular ancestor. Then, after a long period of independent unicellular evolution, two different life strategies representing two lineages of multicellular development emerged that led to present-day plants and animals. Thus, kingdom-specific evolution started with a common set of genes that have progressively undergone independent diversification and today, besides serving conserved cellular mechanisms, they contribute also to lineage-specific functions (Meyerowitz, 1999).

The results presented in this thesis can be included in such large chapters as transcription and cell cycle regulation, where the basic molecular elements show clear conservation across kingdoms. The work began with the study of an alfalfa cyclin-dependent kinase that led us soon to the field of transcriptional regulation. In an independent study in *Arabidopsis*, the homologue of this kinase was identified as an interacting partner of one of the anaphase-promoting complex activator proteins. And although this point of contact was not confirmed by later experiments, this is where the work on plant anaphase-promoting complex and its activators originated leading finally to two independent « lineages » of results. The data of the two fields are presented and discussed separately in the dissertation.

As mentioned above, in the first part of the results experiments aiming at characterizing an alfalfa cyclin-dependent kinase are described. The family of cyclin-dependent kinases is well known from animals as fundamental members of the cell cycle machinery and, more recently, as basic regulators of transcription. Several members were identified also from plants based mainly on sequence homology with metazoan kinases, and those showing cell cycle regulation are thoroughly investigated. This study tries to complete the picture about plant cyclin-dependent kinases by describing a *Medicago* kinase-cyclin pair the function of which is unrelated to cell division and affects RNA polymerase II-mediated transcription.

The second part of results contributes to our current understanding on the plant anaphase-promoting complex and its activators. Controlled degradation of proteins via the ubiquitin – 26S proteasome pathway is an important regulatory mechanism in all eukaryotes. The anaphase-promoting complex-mediated ubiquitination of proteins and their subsequent degradation is essential for cell cycle progression, for mitotic exit in animals

and yeasts. Components of this machinery, such as the Ccs52 proteins, which are related to animal anaphase-promoting complex activators, have been identified in plants as well. In our work, we aimed at analyzing the anaphase-promoting complex in the model plant *Arabidopsis thaliana* and at investigating its interaction with Ccs52 proteins.

The results are presented according to the formal requirements of the doctoral schools of the University of Szeged (first part) and the University Paris XI (second part). Before presenting the results, the thesis gives an overview on the characteristics of cyclin-dependent kinases, thereafter discusses the process of messenger RNA transcription with a special interest on participating cyclin-dependent kinases. In the third part of the introduction, general aspects of cell cycle progression are briefly presented and followed by a more detailed description of the ubiquitin-mediated proteolysis and its role in cell cycle regulation. As basic aspects of both processes seem to be highly conserved in plants as well, the more thoroughly studied animal and yeast systems will be presented.

INTRODUCTION

I. Cyclin-dependent kinases: an overview

Cyclin-dependent kinases (CDKs) together with their regulatory subunits, the cyclins, are primarily known as key regulators of the eukaryotic cell cycle. Since the discovery of the first members of the family, the Cdc2/Cdc28 kinases from yeasts, a large number of CDKs have been described from various eukaryotes and often found to have function unrelated to cell division. The importance of CDKs in transcriptional regulation is well established nowadays. Other members are known to be implicated in as divergent processes as apoptosis or neural differentiation in vertebrates. Thus, CDKs represent a structurally related but functionally diverse family of protein kinases.

The knowledge about the structure of CDKs and the regulation of their activities relies mainly on studies of cell the cycle kinases (Morgan, 1995, 1997; Pines, 1995). Based on the data of yeast Cdc2/Cdc28 and vertebrate Cdk1 and Cdk2, CDKs are typically 35-40 kDa serine/threonine-specific kinases, whose catalytic activity is dependent on association with cyclins. Analysis of their primary structure reveals that CDKs possess conserved subdomains in addition to the structural characteristics shared by all protein kinases. Such is the helical motif close to the N-terminus of the protein which is involved in the binding of the adequate cyclin. The PSTAIRE sequence is the classical cyclin binding site that is conserved in yeasts, in the animal Cdk1 Cdk2 and plant CDKAs; but several variants are known and often used to classify CDKs. Another distinctive feature of CDKs is the so called T-loop which is involved in substrate binding, however, in monomeric, nonmodified CDKs it blocks the entry of the active site (De Bondt *et al.*, 1993).

To gain full activity, CDKs need to be complexed with cyclins. In contrast to the relatively high conservation of the catalytic CDK subunits, cyclins are very divergent proteins. Their similarity is restricted to the approx. 100-residue long cyclin box, a helix-rich structure which is responsible for CDK binding and activation (Nugent *et al.*, 1991). Cyclin binding flattens the T-loop so that the CDKs–substrate interface becomes accessible. Cyclins not only activate CDKs but define the temporal and spatial pattern of kinase activity and modulate the substrate choice as well (Loog and Morgan, 2005). Cyclins interacting with cell cycle kinases appear and disappear in a definite order during cell

division, which ensures the oscillation of CDK activities throughout the cell cycle. These cyclins are expressed in a stage-specific manner and contain sequence motifs that render them susceptible for degradation. Proteolytic elimination of cyclins via the ubiquitin-proteasome pathway is a fast and irreversible mode of CDK inactivation at defined time points of cell cycle (Chapter III).

Although cyclin binding is the main determinant of CDK function, additional mechanisms help to fine-tune the activity of the complex (Figure 1). Multiple phosphorylation-dephosphorylation events affect both positively and negatively the activity of CDKs. Most kinases require phosphorylation of a conserved threonine residue at position 160 (Thr160), whereas phosphorylation of threonine 14 and tyrosine 15 (Thr14 and Tyr15) inhibits CDK activity (numbering according to human Cdk2 sequence).

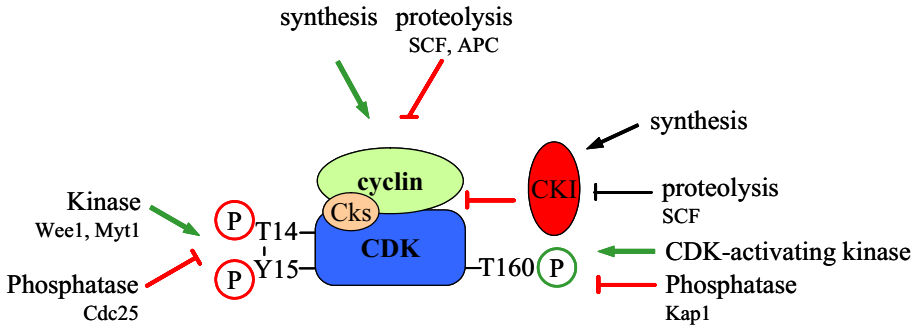

Figure 1. ***The activity of cyclin-dependent kinases is regulated by multiple mechanisms.*** The main mechanisms include ordered synthesis and proteolysis of regulatory subunits and phosphorylation/dephosphorylation of the CDK subunit. Names for enzymes known to modify cell cycle CDKs are given. Green arrows indicate activating, red ones inhibiting modifications. See Chapter III and the text for details.

Activating phosphorylation of Thr160 is carried out by a so called CDK-activating kinase (CAK) which is itself a CDK-cyclin pair in higher eukaryotes and is composed of Cdk7 and cyclin H subunits (Chapter II). Inhibitory phosphorylation of Thr14 and Tyr15 is particularly important in the timing of mitosis: Cdk1 is held inactive by phosphorylation in the phase preceding mitosis and its abrupt activation and the consequent onset of mitosis are triggered by dephosphorylation.

In addition to cyclins, other proteins are also known to associate with CDKs and influence their activity. Besides phosphorylation, CDK activity can be downregulated by binding to inhibitory subunits, termed CKIs (CDK-inhibitory subunits). These inhibitors are also of basic importance in timing of cell cycle events (Sherr and Roberts, 1999).
Moreover, CDKs known to be involved in cell cycle regulation associate with small proteins termed Cks (Cdc kinase subunit). Although the relevance of this interaction is still controversial, it was found to influence the affinity of CDKs towards the substrates and contribute to the proteolysis of cyclins and CKIs as well (Harper, 2001).

Finally, the subcellular localization of the cyclin-CDK heterodimer represents another level of regulation. It restricts the range of available substrates and the accessibility of the complex to regulators (Yang and Kornbluth, 1999).

The functional diversity of the cyclin-dependent kinase family can be recognized in every kingdom of eukaryotes. Principal structural and functional features of human CDKs and their budding yeast counterparts are summarized in Table 1.

CDK	Cyclin-binding motif	Cyclin partner	Function	*S. cerevisiae* homologue
Cdk1	PSTAIRE	A, B	cell cycle, mitosis	Cdc28
Cdk2	PSTAIRE	A, D, E	cell cycle, G1/S	Cdc28
Cdk3	PSTAIRE	C	entry into cell cycle, G0/G1	Cdc28
Cdk4	PISTVRE	D	cell cycle, G1	
Cdk5	PSSALRE	p35	neural differentiation	
Cdk6	PLSTIRE	D	cell cycle, G1	
Cdk7	NRTALRE	H	transcription, CAK	Kin28
Cdk8	SMSACRE	C	transcription	Srb10
Cdk9	PITALRE	T1, T2, K	transcription	Ctk1, Bur1
Cdk10	PISSLRE	?	cell cycle?, G2/M?	
Cdk11	PITSLRE	L	pre-mRNA splicing	

Table 1. ***Main characteristics of human cyclin-dependent kinases.*** Only CDKs designated according to the nomenclature of human kinases are listed, other CDKs or CDK-related kinases referred by their corresponding cyclin-binding sequences are not indicated.

In plants, six classes of CDKs are designated based on sequence homology which seems to reflect also functional similarities (Table 2) (Vandepoele *et al.*, 2002). The A-type CDKs are the functional homologues of the Cdc2-type kinases, while B-type CDKs are unique to plants in that their expression is regulated during the cell cycle. D and F classes include

kinases with CDK-activating kinase activity. C-type CDKs seem to act in the regulation of transcription, while the function of CDKEs remains to be defined. A-, B-, D- and F-type kinases are presented more thoroughly in Chapter III; C, D and E classes in Chapter II.

CDK class	Cyclin-binding motif	Cyclin partner	Function	Human homologue
CDKA	PSTAIRE	A, B, D	cell cycle, G1/S and G2/M	Cdk1
CDKB	PPT(A/T)LRE	B	cell cycle, G2/M	
CDKC	PITAIRE	T	transcription	Cdk9
CDKD	N(V/F/I)TALRE	C, H	CAK, transcription	Cdk7
CDKE	SPTAIRE	D?	transcription?	Cdk8?
CDKF	–	?	CAK-activating kinase	

Table 2. ***Overview of plant cyclin-dependent kinase classes.***

II. From DNA to messenger RNA – transcription by RNA polymerase II

In eukaryotes transcription of almost all protein-coding genes is carried out by RNA polymerase II (Pol II). Although Pol II is itself a multisubunit enzyme, the completion of a functional messenger RNA (mRNA) requires the coordinated activity of many other proteins. Protein complexes help to release the repression exerted by chromatin structure, to recognize core promoter elements, melt the double helix of DNA, keep elongating the nascent RNA and process it to mature form. Basal transcription is over-regulated at multiple levels rendering it responsive to changes in external and internal environment, to growth, development, proliferation and even death. In this introduction, only basal transcription is discussed with special interest on phosphorylation events and a transcriptional activator complex involved in this process, termed positive transcriptional elongation factor b (P-TEFb).

A. The transcription cycle

1. RNA polymerase II

RNA polymerase II shows high homology among eukaryotes. It is composed of 12 subunits, Rpb1 to Rpb12, forming a complex of >0.5 MDa. Moreover, the two largest

subunits, Rpb1 and Rpb2 are homologous in structure and function to the β and β' subunits of the *E. coli* RNA polymerase (Cramer *et al.*, 2000). Although these two subunits are the most conserved ones, at least ten yeast subunits can substitute for their mammalian counterparts (Cramer *et al.*, 2000; Hampsey, 1998). Other five subunits are common to all three eukaryotic RNA polymerases.

A unique feature of the largest subunit of RNA polymerase II is its carboxy-terminal domain (CTD) containing multiple repeats of the consensus heptapeptide Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7 (Figure 2) (Allison *et al.*, 1985; Corden *et al.*, 1985). This sequence is highly conserved among the different species, while the number of repeats seems to increase with genome complexity. CTD is essential for cell viability; deletion of more than the half of the repeats is lethal in yeast, whereas deletion of shorter sections impairs growth in mouse (Litingtung *et al.*, 1999; Nonet *et al.*, 1987).

YSPSSPG	YSPTSPA	YNPQSAK
YSPTSPG	YSPTSPA	YSPSIA
YSPTSPG	YSPTSPA	YSPSNAR
YSPTSPG	YSPTSPS	LSPASP
YSPTSPT	YSPTSPS	YSPTSPN
YSPSSPG	YSPTSPS	YSPTSPS
YSPTSPA	YSPTSPS	YSPTSPS
YSPTSPS	YSPTSPS	YSPSSPT
YSPTSPS	YSPTSPA	YSPSSP
YSPTSPS	YSPTSPG	YSSGASPD
YSPTSPS	YSPTSPS	YSPSAG
YSPTSPS	YSPTSPS	YSPTLPG
YSPTSPS	YGPTSPS	YSPSSTGQ
		YTPHEGDKKDKTGKKDASKDDKGNP

Figure 2. ***The C-terminal domain of RNA polymerase II of*** **Arabidopsis thaliana** (Nawrath *et al.*, 1990). Residues that differ from the consensus sequence YSPTSPS are highlighted by red.

The CTD from different species contains variable number of repeats that are less well conserved. Generally, the repeat sequence of the CTD increasingly deviates from the consensus sequence with proximity to the carboxy-terminus. The evolution of these non-consensus repeats and longer CTDs has been speculated to attribute specific functions to different regions of the CTD (Fong and Bentley, 2001).

2. Initiation of transcription

Transcription begins with the recruitment of Pol II to the promoter and the synthesis of the first phosphodiester bond of the nascent RNA. Since eukaryotic DNA is embedded in

chromatin, the first step in the regulation of transcription is the recruitment of Pol II to promoter regions. For this, the tight structure of chromatin must be loosened which is carried out by chromatin remodelling complexes and histone modifying enzymes (Belotserkovskaya and Berger, 1999; Vignali *et al.*, 2000).

Eukaryotic Pol II is unable to initiate transcription by itself. Core promoter elements are recognized by additional factors termed general transcription factors (GTFs). The Pol II-GTF complex can not respond to activators and inhibitors, for this, another complex termed Mediator needs to be recruited.

General transcription factors are required at all promoters used by Pol II, where they assemble together with the polymerase in the so called preinitiation complex (PIC). *In vitro* reconstitution experiments suggested that GTFs assemble in a defined order to form the PIC, but later studies identified subsets of GTFs pre-assembled with Pol II and proteins from the Mediator complex (Koleske and Young, 1995).

Based on the conventional model of ordered assembly, TFIID recognizes and binds core elements in the promoter, followed by TFIIB, TFIIF and Pol II, TFIIE, finally TFIIH. TFIIA can join the complex at any step after TFIID (Martinez, 2002; Orphanides *et al.*, 1996). The start site or initiator element of the transcript is embedded in a pyrimidine-rich region and preceded by the TATA box, which is located approx. 25-30 basepairs upstream. The TATA-binding protein (TBP), a subunit of TFIID, recognizes and binds the TATA element. Additional subunits of TFIID termed TAFs (TBP-associated factors) contribute to the specificity of promoter recognition and function in activated transcription. TFIIA has been shown to be essential for transcription from TATA-less, initiator element-containing promoters. The binding of TFIIB to regions flanking the 5' and 3' sides of the TATA box and to TBP stabilizes the DNA-TBP interaction (Hahn, 2004). Besides recruiting Pol II to the complex, TFIIF probably stimulates its dephosphorylation as well by associating with the CTD phosphatase Fcp1 (Chambers *et al.*, 1995). TFIIH, the only GTF possessing ATP-dependent catalytic activity is tethered to the assembled complex via TFIIE. TFIIH contains two helicases needed for DNA melting and the cyclin-dependent kinase-cyclin pair Cdk7-cyclin H possessing CTD kinase activity. Following the assembly of PIC, DNA is melted at the initiation site by the DNA helicase of TFIIH and the first phosphodiester bond of the nascent RNA is synthesized.

The model of ordered recruitment is based on *in vitro* data; various off-DNA complexes containing Pol II, TFIIF, TFIIE, TFIIH and components of the Mediator complex has been described *in vivo* both in yeast and mammals. The existence of such holoenzymes suggests that transcription might not be primarily regulated through the assembly of PIC from individual components, but rather by the rate with which activators recruit the holoenzyme to the promoter. Although some GTFs can bind to activators, these interactions are not sufficient for the activation of Pol II *in vitro*, the addition of Mediator is needed to render the reaction activator-dependent (Lewis and Reinberg, 2003).

The 20-subunit Mediator complex was originally identified in budding yeast by genetic screens for suppressors of truncations in the CTD. Biochemical experiments aiming at reconstituting activator-dependent transcription identified additional subunits. Based on biochemical, genetic and structural data, Mediator is thought to have a modular structure with sets of subunits more or less stably associated (Lewis and Reinberg, 2003). Srb4 and Srb6 are core components of the complex; their inactivation decreases transcription of most genes *in vivo* (Holstege *et al.*, 1998). The cyclin-dependent kinase-cyclin pair Srb10-Srb11 is part of a negative regulatory subcomplex.

Data are somehow contradictory regarding the time and mode of Mediator recruitment to the promoter; it seems to be widely dependent on the transcribed gene. Mediator has been found important for reinitiation of transcription as well. When Pol II starts elongation, a scaffold complex left on the promoter composed of TFIIA, TFIID, TFIIH, TFIIE and Mediator can be isolated, which might facilitate the rebuilding of PIC (Yudkovsky *et al.*, 2000).

3. Elongation of transcription

Elongation is not simply the monotonous addition of nucleotides to the growing RNA chain. Instead, it is a complex process where the activity of the elongating polymerase is highly regulated. In early steps, Pol II escapes the promoter and is dissociated from the majority of initiation factors. During elongation, Pol II is associated with a variety of elongation factors that can arrest, reactivate or abort the transcriptional complex. These elongation factors can be divided into several classes based on their function. There are factors to reactivate the arrested Pol II, while a plethora of complexes alter the overall rate of transcription by influencing the catalytical activity of the enzyme. The transition from

initiation to elongation is marked by the phosphorylation of the CTD, and even the pattern of phosphorylation is highly dynamic during elongation. It is thought to offer a platform for diverse enzymatic activities involved in remodelling of the chromatin structure and in co-transcriptional processing of the nascent RNA.

a) Elongation factors facilitate elongation through different mechanisms

After the synthesis of the first bond of the transcript, Pol II moves away from the promoter and begins elongation. During elongation, Pol II meets blocks imposed by inhibitory DNA structures or proteins bound to the DNA template. These blocks cause pausing of elongation complexes and in some cases may lead to stable arrest. Arrest results in backtracking of Pol II and subsequent translocation of the 3' end of the transcript from the catalytic centre. If not realigned, Pol II may release the transcript and terminate transcription. Pol II has intrinsic endonuclease activity that cleaves the transcript upstream of its 3' end creating a new, correctly positioned 3' terminus thus permitting its re-extension. The elongation factor TFIIS interacts directly with Pol II and stimulates its endonucleolytic activity possibly by inducing allosteric changes in the catalytic centre of the enzyme (Sims *et al.*, 2004).

The *in vivo* elongation rate on chromatin template of Pol II is 1500-2000 nucleotides per minute, whereas the purified enzyme even on naked DNA template is only able to incorporate 100-300 nt/min (Shilatifard *et al.*, 2003). Several factors has been isolated that interact directly with Pol II and boost its elongation rate. These include TFIIF, ELL and Elongin among others; all seem to increase elongation efficiency by preventing transient pausing of Pol II which occurs otherwise at all or most steps of nucleotide addition. These factors are thought to maintain the proper alignment of the 3' end of the nascent mRNA with the catalytic site of Pol II and thus prevent its backtracking and arrest (Shilatifard, 2004).

Purified Pol II is unable to transcribe *in vitro* from chromatin template due to the repressive nature of chromatin structure. Elongation factors like Elongator and FACT do not alter the catalytic properties of Pol II, but promote elongation by modifying nucleosome structure and so facilitating the passage of Pol II.

Elongator was originally identified as a six-subunit complex stably associated with hyperphosphorylated Pol II. One of the subunits, Elp3 has histone acetyl-transferase

activity and acetylates histone H3 and H4 *in vitro*. However, the role of Elongator in transcription is still controversial; recent studies found that the majority of the complex is cytoplasmic and could not demonstrate its transcription-dependent recruitment to Pol II (Pokholok *et al.*, 2002).

FACT (facilitates chromatin transcription) is a two-subunit complex capable of binding directly to histones H2A-H2B and probably dissociates nucleosomes formed by histone octamers. Besides facilitating transcription, yeast FACT is involved in other DNA-related processes like DNA replication and repair (Svejstrup, 2002).

b) Control of elongation by CTD-phosphorylation

The phosphorylation state of CTD varies during the transcription cycle and is essential for its regulation. Only the non- or hypophosphorylated form (IIA form) can be recruited to the pre-initiation complex, while for productive elongation CTD needs to be heavily phosphorylated (IIO form) (Cadena and Dahmus, 1987). This shift from IIA to IIO form is thought to dissociate GTFs and Mediator from Pol II and changes in the phosphorylation pattern of CTD during elongation may serve as platform for different factors regulating transcription and pre-mRNA processing (Figure 3). Although the heptade contains five phosphorylatable residues, Ser^2 and Ser^5 phosphorylation is predominant. It affects the elongation properties of Pol II and when mutated, interferes with viability in yeast (Hengartner *et al.*, 1998; West and Corden, 1995). Mainly Ser^5 phosphorylation is found in initiating Pol II, while Ser^2 phosphorylation increases towards the 3' end of the transcribed gene. This differential phosphorylation of CTD seems to govern RNA processing by recruiting enzymes for RNA capping, splicing and polyadenylation at different phases of transcription cycle (Cho *et al.*, 2001; Komarnitsky *et al.*, 2000; Schroeder *et al.*, 2000).

Although multiple kinases are known to phosphorylate CTD, genetic and biochemical data indicate that in budding yeast the Cdk7 homologue Kin28 and the Cdk9-related Ctk1 kinases are responsible for the bulk phosphorylation of Pol II on Ser^5 and Ser^2, respectively (Cho *et al.*, 2001; Komarnitsky *et al.*, 2000). The function of these kinases together with Srb10/Cdk8 is discussed in Chapter II.B.

The mitotic kinase Cdk1 inhibits transcription *in vitro*; it may be involved in the repression of transcription during mitosis by pre-initiation hyperphosphorylation of Pol II and phosphorylation of TFIID, Cdk7 and various other transcriptional activators (Gebara *et*

al., 1997; Oelgeschlager, 2002). The extracellular signal-related kinase 1/2 (ERK1/2) belonging to mitogen activated protein kinases (MAPK) also efficiently phosphorylates the CTD and generates a novel form, Pol IIm migrating between IIA and IIO on SDS-PAGE analysis and might respond to stress and mitogenic stimuli (Bonnet *et al.*, 1999; Kobor and Greenblatt, 2002). To date, c-abl is the only kinase known to phosphorylate tyrosine within the CTD, although biochemical data suggest the existence of additional kinases targeting the same residue (Oelgeschlager, 2002).

Changes in CTD phosphorylation during elongation and the finding that only hypophosphorylated CTD can enter a new round of transcription imply the activity of specific CTD phosphatases (Figure 3F). Contrarily to the large number of CTD kinases, only a few phosphatases have been described. The yeast FCP1 phosphatase (TFIIF-stimulated CTD phosphatase 1) counteracts the effect of Ctk1, whereas its mammalian homologue can remove phosphates from both Ser^2 and Ser^5 (Cho *et al.*, 2001; Lin *et al.*, 2002a). Another mammalian CTD phosphatase, SCP (small CTD phosphatase) seems to have preference towards Ser^5 (Yeo *et al.*, 2003).

4. Transcription and pre-mRNA processing

The synthesis of eukaryotic mRNA is not completed with its transcription, additional processes are required to produce stable, fully functional transcript. The highly interdependent events of mRNA capping, splicing and polyadenylation occur co-transcriptionally and seem to be orchestrated by the dynamic phosphorylation pattern of the C-terminal domain of Pol II (Figure 3).

The cap structure found at the 5' end of all eukaryotic mRNA is synthesized soon after transcription initiation, when the transcript is about 20-30 nucleotides long (Figure 3C) (Coppola *et al.*, 1983). Capping requires the consecutive activity of three enzymes: RNA 5' triphosphatase removes the γ-phosphate of the first nucleotide of the transcript, RNA guanylyltransferase transfers GMP to the resulting diphosphate end and RNA (guanine-7)-methyltransferase methylates the N7 position of the transferred GMP. All three enzymes bind only to phosphorylated CTD; moreover, guanylyltransferase is activated allosterically by Ser^5-phosphorylated CTD heptade (Bentley, 2002; Ho and Shuman, 1999).

Introns interrupting the coding regions of genes are removed from the primary transcript by a large complex called spliceosome. This macromolecular complex is composed of

small nuclear ribonucleoprotein particles (snRNPs) and members of the serine/arginine-rich protein family. Consensus elements at the ends of and within the intron define the site of the cleavage reaction. Although splicing can be reconstituted *in vitro* using pretranscribed RNA, several lines of evidence suggest that *in vivo* the process is connected with transcription. Spliceosome assembly is initiated by the binding of U1 snRNP to the 5' splice site. One of its components interacts directly with phospho-CTD and might direct the recruitment of other splicing signals on the nascent transcript (Figure 3E) (Morris and Greenleaf, 2000).

Polyadenylation of the 3' end of almost all eukaryotic mRNAs is required for correct termination of transcription. For the addition of polyadenyl (poly(A)) tail, pre-mRNA needs to be cleaved at its correct 3' end. This cleavage is directed by several multiprotein complexes and occurs 10-30 nucleotides downstream of the conserved AAUAAA sequence; finally, poly(A) polymerase extends poly(A) tail. (Proudfoot *et al.*, 2002). The role of CTD phosphorylation has been recently connected with polyadenylation. Phosphorylation of Ser^2 by the Ctk1 kinase in yeast and by its metazoan homologue Cdk9 is required for correct 3' end processing: it acts synergistically with the emerging poly(A) site on the nascent transcript to recruit polyadenylation factors (Figure 3D, E) (Ahn *et al.*, 2004; Ni *et al.*, 2004).

5. Termination of transcription

It is well established that functional polyadenylation signal is required for transcription termination. There are two models to explain the coupling of 3' end processing and termination. In one, passing through the polyadenylation signal causes a conformational change in the transcription complex which renders it competent for termination. According to the other model, the cleavage of the transcript and the rapid degradation of the resulting downstream products turn the polymerase prone to termination (Hirose and Manley, 2000). Recent data support a kind of combination of these models, where both 3' end processing and cleavage of the downstream transcript are required. First the non-coding part of the product is cleaved at multiple sites, followed by the cleavage at the poly(A) site which would mediate the release of the transcript from the polymerase. Transcriptional pause sites located downstream of the poly(A) site have been identified and are thought to slow down Pol II progression and thus facilitate poly(A) site recognition (Proudfoot *et al.*, 2002).

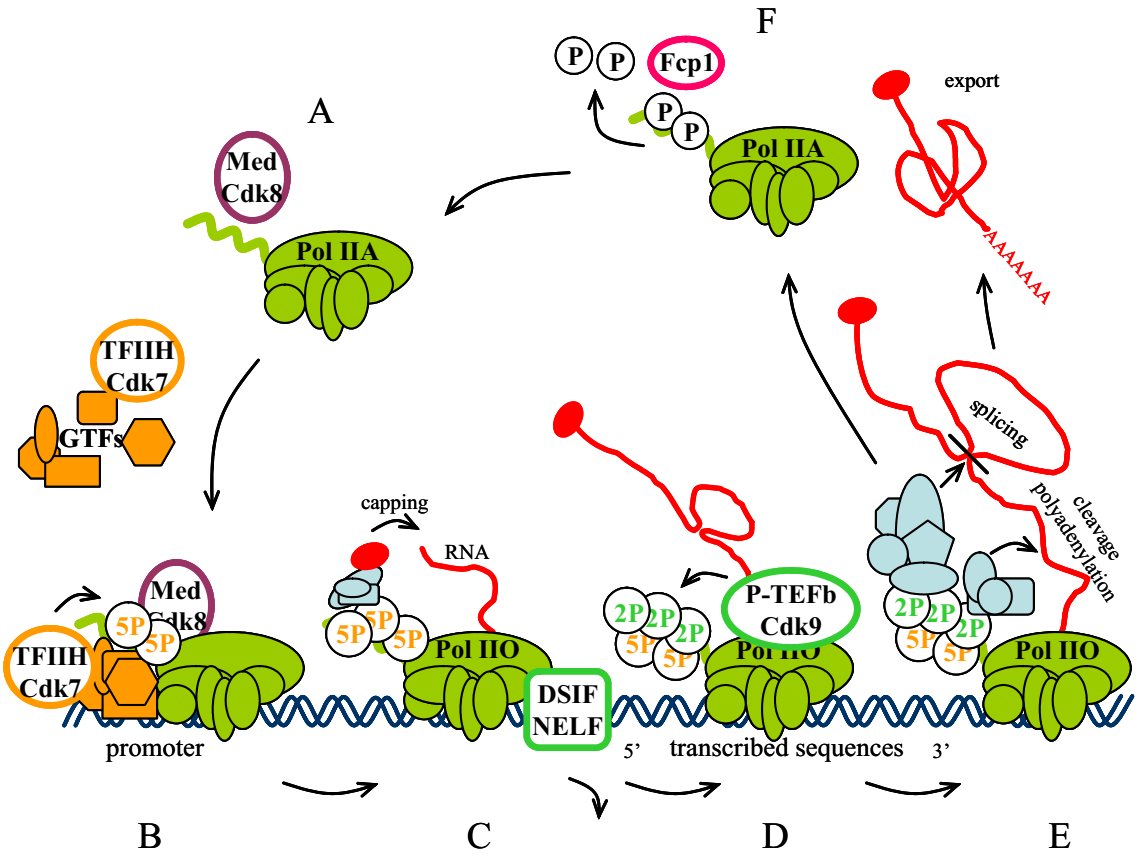

Figure 3. ***The transcription cycle: CTD phosphorylation and pre-mRNA processing.*** **(A)** Recruitment of RNA polymerase II. The nonphosphorylated Pol II core or the holoenzyme containing coactivators (such as Mediator (Med)) is recruited to the promoter together with general transcription factors (GTFs). Phosphorylation of CTD by Cdk8 prior to preinitiation complex assembly prevents the entry of Pol II into the complex. **(B)** In the preinitiation complex the TFIIH subunit Cdk7 phosphorylates Ser^5 (5P) within the CTD of Pol II. **(C)** At the initiation of transcription capping enzymes recruited by Ser^5-phosphorylated CTD cap the emerging pre-mRNA at its 5' end. **(D)** In elongation, the activity of the P-TEFb subunit Cdk9 generates phospho-Ser^2 (2P) on the CTD and removes the block of DSIF/NELF. **(E)** Phosphorylated CTD recruits splicing factors which remove introns and cleavage/polyadenylation factors that process the 3' end of the transcript. When transcription is terminated, Pol II leaves the DNA and the mature transcript is exported to the cytoplasm. **(F)** For the recycling of Pol II to another round of transcription the CTD is dephosphorylated by Fcp1 phosphatase. (After Palancade and Bensaude, 2003.)

B. Cyclin-dependent kinases in transcriptional regulation

It is now clear that despite the relatively high number of CTD kinases including certain cyclin-dependent kinases, these proteins are not functionally redundant (Prelich, 2002).

Mutational and deletional analysis in yeast clearly support this idea, since (i) some, but not all kinases are essential for viability; (ii) mutations in different kinases cause distinct phenotypes; and (iii) these mutations affect to different extent the global gene expression pattern. Moreover, (iv) CTD kinases have distinct properties when assayed in biochemical experiments.

Most probably specific biochemical mechanisms underlie these differences, such as (i) different residues are targeted by these kinases within the CTD; (ii) the kinases differ in the timing of their activation; (iii) other substrates are also targeted; and (iv) the recruitment of the kinases might depend on the structural features of the promoter.

At least four CTD kinases, namely Cdk1, Cdk7, Cdk8 and Cdk9, belong to the cyclin-dependent kinase family of protein kinases. Cdk1 fulfils most probably cell cycle-dependent regulation of Pol II (inhibition of its activity during mitosis) and won't be discussed in this chapter (Oelgeschlager, 2002). In contrast, Cdk7, Cdk8 and Cdk9 are the major cellular kinases that contribute to the regulation of basal transcription. The activity of Cdk7 and Cdk9 promotes transcription, whereas Cdk8 seems to have negative effect on it (Figure 3).

1. Cdk7 and Cdk8 act on early steps of transcription

The general transcription factor TFIIH has several enzymatic activities including DNA-dependent ATPase, ATP-dependent DNA helicase and CTD kinase. The CTD kinase activity resides in Cdk7 which forms a trimeric subcomplex also called TFIIK with its cyclin partner, cyclin H and an assembly factor, the ring finger protein MAT1 (ménage à trois 1). Besides promoting the assembly of the CDK-cyclin complex, MAT1 renders Cdk7 independent of the activating phosphorylation on the conserved threonine residue (Devault *et al.*, 1995; Fisher *et al.*, 1995). The vertebrate subcomplex has also CDK-activating kinase activity towards the CDKs involved in cell cycle regulation (Kaldis, 1999). The budding yeast complex is somewhat different; the subcomplex is formed by the CDK-cyclin pair Kin28-Ccl1, whereas the homologue of MAT1, Tfb3 seems to be part of the TFIIH core (Hampsey, 1998). Kin28-Ccl1 is 'solely' a CTD kinase; another kinase, the monomeric Cak1/Civ1 is responsible for the regulatory phosphorylation of the cell cycle kinase Cdc28 (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996).

In vivo data in accordance with previous *in vitro* experiments indicate that the kinase responsible for the majority of Ser^5 phosphorylation is Cdk7/Kin28 (Ramanathan *et al.*, 2001; Trigon *et al.* 1998; Komarnitsky *et al.*, 2000). The kinase activity of Cdk7 is modulated by its recruitment to the preinitiation complex: it increases its efficiency towards the CTD and TFIIE promotes its preference for Ser^5 (Rossignol *et al.*, 1997; Yamamoto *et al.*, 2001). Cdk7/Kin28 activity is essential in transcription complexes nearby the promoter region: by generating phospho-Ser^5 it ensures the recruitment of capping enzymes (Figure 3B, C) (Kim *et al.*, 2002; Komarnitsy *et al.*, 2000).

The Srb10-Srb11 cyclin-dependent kinase–cyclin pair and its metazoan orthologue Cdk8-cyclin C are nonessential negative regulatory subunits of the Pol II-associated Mediator complex (Borggrefe *et al.*, 2002). Although similarly to Cdk7, Cdk8/Srb10 targets Ser^5 within the heptapeptide, the functions of the two kinases are not redundant (Rickert *et al.*, 1999). While Cdk7/Kin28 has a general promoting effect on transcription, Cdk8/Srb10 seems to affect only a small subset of genes (Carlson *et al.* 1997; Holstege *et al.*, 1998). Biochemical data reinforce the repressive effect of Cdk8/Srb10. Srb10-Srb11 prevents the recruitment of Pol II to the preinitiation complex by phosphorylating the CTD prior to PIC formation (Figure 3A) (Hengartner *et al.*, 1998). The inhibitory effect of Cdk8-cyclin C might be indirect as well: in addition to CTD, it phosphorylates also cyclin H from TFIIH which leads to the inactivation of Cdk7 (Akoulitchev *et al.*, 2000).

2. Cdk9 is the kinase subunit of positive transcription elongation factor b

a) P-TEFb facilitates productive transcript elongation

The nucleotide analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) has been found to cause premature transcription termination resulting in short, abortive transcripts (Chodosh *et al.*, 1989). Studies investigating the mechanism of DRB inhibition revealed that DRB does not affect Pol II itself, additional factors are required to mediate the inhibition. This led to the establishment of negative and positive transcription elongation factors (N-TEF and P-TEF) and to the model where early elongation is blocked by N-TEF and this block is released by the action of positively acting factors (Kephart *et al.*, 1992; Marshall and Price, 1992).

A single complex corresponding to P-TEF activity and showing DRB-sensitivity has been purified and termed positive transcription elongation factor b (P-TEFb) (Marshall and Price, 1995). The complex was found to possess kinase activity and phosphorylate the CTD of Pol II (Marshall *et al.*, 1996). Subsequent cloning of the subunits of P-TEFb revealed that it is composed of a cyclin-dependent kinase–cyclin pair (Peng *et al.*, 1998a; Zhu *et al.*, 1997). The CDK was identical with the previously described PITALRE kinase named after its cyclin-binding motif which was shown to phosphorylate itself and the retinoblastoma protein in a constitutive manner during the cell cycle (Grana *et al.*, 1994). PITALRE, renamed Cdk9 according to the nomenclature of mammalian CDKs, interacts with four different cyclins: cyclin T1, T2a, T2b and K (Fu *et al.*, 1999; Peng *et al.*, 1998a, 1998b). These cyclins are encoded by three genes, cyclin T2a and T2b being splice variants and differing only in their extreme carboxy-terminal part (Peng *et al.*, 1998b). The cyclin box is the most conserved region of these proteins; cyclin T1 and T2 are highly homologous, whereas cyclin K shares only about 30 % identity with cyclin Ts in this region (Table 4) (Price, 2000).

Extensive *in vitro* and, more recently, *in vivo* analysis of P-TEFb function reinforced its role in transcriptional regulation. It is not only required for efficient elongation but contributes also to the establishment of the phosphorylation pattern of CTD. Analysis of the biochemical properties of P-TEFb determined that it preferentially targets Ser^2 within the CTD, however, to a slight extent phospho-Ser^5 is also detectable (Figure 3D) (Kim *et al.*, 2002; Ramanathan *et al.*, 2001; Zhou *et al.*, 2000). *In vivo* data from systems depleted of P-TEFb function (by RNA interference in *C. elegans* or inhibition of Cdk9 by flavopiridol in *Drosophila*) reinforce Ser^2 phosphorylation (Ni *et al.*, 2004; Shim *et al.*, 2002). These results are further supported by data from budding yeast. In yeast, two kinases, Bur1 and Ctk1 are equally homologous to Cdk9 and seem to share metazoan P-TEFb function. Deletion of Ctk1 results in loss of Ser^2 phosphorylation, but does not obviously affect elongation (Ahn *et al.*, 2004; Cho *et al.*, 2001). In contrast, loss of Bur1 function causes defective elongation with apparently normal CTD phosphorylation. This suggests that additional substrates might also be targeted by the kinase analogously to P-TEFb, which was found to phosphorylate subunits of N-TEFs (Spt5 and NELF-E/RD, see below) (Fujinaga *et al.*, 2004; Keogh *et al.*, 2003; Kim and Sharp, 2001; Ping and Rana, 2001).

In accordance with the model of positive and negative elongation factors, two complexes of N-TEF activity have been identified acting in the DRB-dependent inhibition of elongation. DRB-sensitivity inducing factor (DSIF) and negative elongation factor (NELF) are recruited to the hypophosphorylated form of RNA Pol II and cooperatively inhibit early steps in elongation (Figure 3C).

DSIF is a two-subunit complex composed of p14/Spt4 and p160/Spt5. Genetic and biochemical data indicate that this heterodimer may have both positive and negative effects on transcription. Spt4 promotes elongation on chromatin templates, whereas Spt5 facilitates late elongation by preventing premature termination (Bourgeois *et al.*, 2002; Morillon *et al.*, 2003; Rondon *et al.*, 2004). Pol II-bound DSIF recruits NELF to the elongation complex and this association triggers transcriptional pausing (Renner *et al.*, 2001; Yamaguchi *et al.*, 2002). NELF consists of five subunits, NELF-A to NELF-E. The smallest subunit, NELF-E, also called RD, contains an RNA recognition motif which has been found essential for the repressive function of the complex (Yamaguchi *et al.*, 2002).

Based on these data it is now well established that P-TEFb/Cdk9, together with TFIIH/Cdk7, is particularly important for stimulating transcription and pre-mRNA maturation via the phosphorylation of CTD (Figure 3B, C, D). These two kinases act consecutively: Cdk7 generates phospho-Ser^5 close to the promoter, whereas the activity of Cdk9 in elongation complexes results in mainly Ser^2-phosphorylated CTD. The DSIF/NELF/P-TEFb checkpoint of early elongation is proposed to ensure correct capping of pre-mRNA and thus prevent the synthesis of non-functional transcripts (Sims *et al.*, 2004). In this model, DSIF is recruited shortly after transcription initiation. Whether it precedes or follows Ser^5 phosphorylation of CTD by TFIIH/Cdk7 remains to be clarified. NELF binds to the Pol II-DSIF complex and induces pausing of Pol II which probably involves the binding of the nascent 17–22-nt-long transcript by NELF-E/RD (Yamaguchi *et al.*, 2002). This pausing allows the binding of capping enzymes to the Ser^5-phosphorylated CTD which is further facilitated by Spt5 (Pei *et al.*, 2001). DSIF/NELF-mediated arrest is then relieved by the kinase activity of P-TEFb, and the transcription complex resumes elongation. Most probably phosphorylation of Ser^2 within the CTD by Cdk9 is not sufficient to release Pol II, targeting of Spt5 and NELF-E/RD is also required. Moreover, the presence of capping enzymes also facilitates reinitiation (Mandal *et al.*, 2004). Finally, the increasing proportion of phospho-Ser^2 towards the 3' end of the transcribed gene

generated by P-TEFb, together with the emerging poly(A) site leads to the binding of polyadenylation factors (chapter II.A.4).

b) Regulation of the activity of Cdk9

CDK regulatory mechanisms are highly conserved and involve phosphorylation of definite residues and binding to inhibiting and activating factors (Chapter I). Similarly to most other CDKs, the activity of Cdk9 and its complex is regulated at multiple levels.

1) Cyclins: substrate choice, stability and localization of P-TEFb

Cyclins are known not only to trigger but also to direct some features of CDK activity like substrate range and subcellular localization (Loog and Morgan, 2005; Morgan, 1997; Yang and Kornbluth, 1999). Similarly to other CDKs, the activation of Cdk9 requires the binding to one of its cyclin subunits, cyclin T1, one of the T2s or cyclin K (Fu *et al.*, 1999; Peng *et al.*, 1998a, 1998b). Despite the marked differences between T-type cyclins and cyclin K, the relevance and consequences of cyclin choice is still unclear. The vast majority of Cdk9 is complexed with T-type cyclins *in vivo* with cyclin T1 being the major partner present in approx. 80% of the complexes (Peng *et al.*, 1998b). T-type cyclins have a C-terminal histidine-rich motif, which has been found essential in recognizing the carboxyl-terminal domain of Pol II (Lin *et al.*, 2002b; Taube *et al.*, 2002). Cyclin K is quite divergent from the other cyclin partners of Cdk9. Although it lacks the CTD recognition motif, the protein forms an active complex with Cdk9 which can promote transcription (Fu *et al.*, 1999). Interestingly, cyclin K was originally identified by its ability to rescue G1 cyclin deletion in yeast. In addition to CTD, the immunecomplex of cyclin K displays potent CDK-activating kinase activity *in vitro*, however its kinase partner in this reaction was not identified (Edwards *et al.*, 1998).

Several lines of evidence indicate that the steady-state level of Cdk9 is controlled in mammalian cells by its association with cyclin T. When the kinase alone is ectopically overexpressed, both exogenous and endogenous Cdk9 will have high turnover rate in order to compensate for the elevated protein level, while overexpression of cyclin T1 stabilizes exogenous Cdk9 (Garriga *et al.*, 2003; O'Keeffe *et al.*, 2000). Over physiological levels, the degradation of the kinase is probably carried out by the ubiquitin–proteasome pathway, where Cdk9 would be targeted for ubiquitination by interaction of cyclin T1 with the

SCF^{Skp2} ubiquitin ligase complex (Garriga *et al.*, 2003; Kiernan *et al.*, 2001). If this mechanism is relevant *in vivo* at normal Cdk9 level remains to be clarified. Once bound to its cyclin partner, Cdk9 is no more prone to degradation; cyclin T1 is a stable protein and its interaction with Cdk9 stabilizes the kinase (Garriga *et al.*, 2003; O'Keeffe *et al.*, 2000). During the maturation of P-TEFb complexes, newly synthesized Cdk9 is protected and folded by chaperones: it will be transferred from Hsp70 to the kinase-specific Hsp90/Cdc37 chaperone complex and finally to cyclin T1 (O'Keeffe *et al.*, 2000).

The interaction of Cdk9 with cyclin T seems to define its subcellular localization as well. Cyclin T1 can be found in the nucleus in considerable overlap with speckle-like structures (Herrmann and Mancini, 2001). Nuclear speckles are microscopically visible structures that are highly enriched in factors involved in transcription and pre-mRNA splicing. Although their exact function is still not clear, they are proposed to serve as storage and assembly areas for these factors (Spector, 1996). Cyclin T1 has been suggested to enhance the nuclear localization of Cdk9 and to direct the kinase to speckle-like structures. The complex shows only partial co-localization with different phosphorylated forms of Pol II suggesting that only a fraction of P-TEFb is associated to the polymerase (Herrmann and Mancini, 2001; Napolitano *et al.*, 2002). To enter to the nucleus, Cdk9 needs to be phosphorylated at multiple serine and threonine residues at the C-terminal part, which is carried out by the Cdk9-cyclin T complex itself (Napolitano *et al.*, 2003).

2) Inactive and active pools of P-TEFb exist in dynamic equilibrium

The most relevant regulatory mechanism of mammalian P-TEFb activity seems to be its inhibition by sequestrating it into an inactive pool. P-TEFb is present in two complexes of distinct size in these cells. One is identical with the active P-TEFb core comprising Cdk9 and cyclin T1 or T2, whereas the other complex of larger size contains P-TEFb with significantly reduced ability to promote Pol II elongation (Nguyen *et al.*, 2001; Yang *et al.*, 2001). Two additional subunits can be identified in the inactive complex: surprisingly, an RNA species, the small nuclear RNA (snRNA) 7SK and a protein termed MAQ1 (ménage à quatre 1) or HEXIM1 (Michels *et al.*, 2003; Nguyen *et al.*, 2001; Yang *et al.*, 2001; Yik *et al.*, 2003). 7SK snRNA and HEXIM1 act together to inhibit the kinase activity of P-TEFb and form a new, unusual type of CDK inhibitors. Recent studies suggest that the 7SK/HEXIM1 dimer is assembled first, which might affect the folding of HEXIM1 and

promote its binding to the cyclin subunit of P-TEFb (Michels *et al.*, 2004; Yik *et al.*, 2003). The assembly of P-TEFb/HEXIM1/7SK complex requires the phosphorylation of Cdk9 at Thr187. This residue corresponds to the conserved phosphorylation site Thr160 in Cdk2 situated in the T-loop and targeted by CDK-activating kinase (Chen *et al.*, 2004; Morgan, 1997). However, neither the autophosphorylation activity of Cdk9 nor the CAK activity of Cdk7 was able to induce 7SK/HEXIM1 binding to P-TEFb, the kinase responsible for this phosphorylation remains to be identified (Chen *et al.*, 2004).

About 50% of total P-TEFb is in inactive state in human cells. This inhibition is reversible; upon treatments impairing transcription (e.g. actinomycin D, UV irradiation, DRB) inactive P-TEFb is rapidly converted into active form suggesting a dynamic equilibrium between the two forms and the existence of a critical threshold of kinase activity required for maintaining normal transcription (Nguyen *et al.*, 2001). This hypothesis is further supported by data from RNA interference experiments in human cells, where despite the decreased Cdk9 protein level, the kinase activity remains normal (Chiu *et al.*, 2004). How cells perceive Cdk9 level and kinase activity and how these and other signals like stress converge on dissociating finally 7SK/HEXIM1 from P-TEFb is not known yet. One possible mechanism could involve the kinase targeting Thr187 on Cdk9.

3) Control of gene expression by P-TEFb

The significance of P-TEFb activity is well established *in vitro*. Inhibition of Cdk9 activity *in vivo* by RNA interference or by the highly specific inhibitor flavopiridol reinforce that P-TEFb is required for the transcription of many class II genes (genes transcribed by Pol II) (Chao and Price, 2001; Shim *et al.*, 2002).

Numerous promoters and proteins involved in transcription have been found to associate with P-TEFb. The most studied example is certainly the recruitment of P-TEFb by the Tat transactivator protein of human immunodeficiency virus type 1 (HIV-1). Tat protein, encoded by the HIV-1 genome promotes efficient transcription of viral genes by enhancing the processivity of Pol II from the viral long terminal repeat region. Tat binds to a highly structured element on the 5' end of the nascent viral transcript and through its interaction with cyclin T1 recruits P-TEFb to the Pol II elongation machinery (Taube *et al.*, 1999). Several transcription factors, e.g. NF-κB, the androgen receptor and c-Myc among others, are also known to bind and direct P-TEFb to specific promoters (Garriga and Grana, 2004).

3. Plant cyclin-dependent kinases possessing CTD kinase activity

Our current knowledge about non-cell cycle plant kinases is very poor, although three of the six classes of plant CDKs group proteins that are related in sequence to metazoan CTD kinases (Table 2). D-, E- and C-type CDKs are homologous to Cdk7, Cdk8 and Cdk9 kinases, respectively. They all show CTD kinase activity *in vitro*, but the relevance of this phosphorylation in the regulation of basal transcription is not established so far.

D-type CDKs are known from to species; the R2 kinase described in rice as well as Cak2At and Cak4At from *Arabidopsis thaliana* target both cyclin-dependent kinases and the CTD of Pol II (Shimotohno *et al.*, 2003; Yamaguchi *et al.*, 1998). R2 interacts with cyclin H and interestingly, also with cyclin C, although the positive effect of the latter on CDK activity was not verified (Yamaguchi *et al.*, 2000). Unlike its metazoan homologue, the expression and CTD kinase activity of R2 varies during the cell cycle with peak in S phase (Fabian-Marwedel *et al.*, 2002).

Very little is known about the function of Cdk8- and Cdk9-related plant proteins. CDKE kinases are the closest homologues of Cdk8; however, their SPTAIRE cyclin binding motif is more reminiscent of the PSTAIRE sequence of Cdk1 or A-type plant CDKs than the SMSACRE motif of Cdk8. Intriguingly, AtCDKE is not binding to one of the two predicted C-type cyclins of *Arabidopsis*, but similarly to A-class CDKs interacts with D-type cyclins in yeast two-hybrid system. Mutation of AtCDKE impairs floral organ identity suggesting a function for the kinase in differentiation (Wang and Chen, 2004).

CDKC class proteins from alfalfa, *Arabidopsis* and tomato are only recently connected with transcription (Barroco *et al.*, 2003; Joubes *et al.*, 2001; Magyar *et al.*, 1997). The *Arabidopsis* CDKC;2 protein was found to bind cyclins related to human T and K cyclins. Its interaction with a ribonucleoprotein and the GT-1 transcription factor further suggests that C-type kinases may have role in the control of transcription (Barroco *et al.*, 2003).

III. The regulation of cell cycle in eukaryotes

A. Overview of cell cycle events

Cell division, i.e. the reproduction and division of the genome into the newly forming cells is the basis for growth, development and inheritance in all living organisms. It is a unidirectional succession of events divided into four phases; the two main events, the synthesis of the genome (S phase) and its partition into the daughter cells (mitosis, M phase) are preceded by gaps (G1 and G2, respectively) to ensure the proper completion of the previous phase and the preparation of the next one. Mitosis, the only microscopically visible stage is further divided into five steps: prophase, metaphase, anaphase, telophase and cytokinesis, whereas the rest of the cell cycle is often referred to as interphase. The formation of the chromosomes, which begins in prophase and is completed in metaphase, is accompanied by the breakdown of the nuclear envelope and the appearance of spindle microtubules radiating from the poles. At metaphase, chromosomes are aligned in the metaphase plate attached to kinetochore microtubules. The extension of spindle microtubules and the depolymerization of kinetochore microtubules result in the separation of sister chromatids and their movement towards the opposite spindle poles in anaphase. Separated chromatids decondense in telophase and will be surrounded by the newly forming nuclear envelope. Finally, mitosis is completed by the division of the cytoplasm, the cytokinesis. The plane of the newly forming cell wall in plants is defined by the preprophase band visible in early prophase, while the building of the cell wall is driven by the phragmoplast. This plant-specific cytokinetic organelle is composed of microtubules, actin filaments and vesicles delivering proteins and polysaccharides to the site of cell wall formation.

Cell cycle is rigorously surveyed; if any error is sensed the progression will be blocked and the next stage delayed to ensure the correction of errors and finishing of the previous stage. Several checkpoints exist and control (i) the external and internal environment (nutrients, hormones, DNA damage, cell size, etc.) at the G1/S transition, (ii) the error-free achievement of DNA replication in S phase, (iii) the correct attachment of the chromosomes to the kinetochores and so to the spindle apparatus at the metaphase/anaphase transition.

The molecular mechanisms underlying the complex events of cell cycle progression and control largely involve cyclin-dependent kinases in all organisms studied so far. In yeasts, a single kinase, Cdc28/Cdc2 is sufficient to drive the division cycle, while higher eukaryotes have several CDKs to promote cell cycle progression (Table 1, 2). The activity of CDKs is regulated at multiple levels in all eukaryotes: association with activating and/or inhibiting proteins and phosphorylation/dephosphorylation turn their catalytical activity on and off (Chapter I, Figure 1). Binding to the activating subunits, the cyclins, defines the substrate specificity and subcellular localization of CDKs (Loog and Morgan, 2005). Cyclin partners of cell cycle kinases are present only for a definite period during the cycle; G1 cyclins Cln1-3 in budding yeast and metazoan D- and E-type cyclins are abundant around G1/S transition, whereas cyclins A and B and the yeast Clb1-4 cyclins drive the activity of CDKs in mitosis (Morgan, 1997).

In animal cells mitogenic stimuli converge on D-type cyclins leading to their accumulation. D-type cyclins bind to Cdk4 and Cdk6 directing the complex to the nucleus, where it will be activated by the CDK-activating kinase. The Cdk7-cyclin H-MAT1 heterotrimer is the only CAK identified to date; it targets the conserved residue corresponding to threonine 160 of Cdk2 on the kinase (Chapter I). Cdk4/6-cyclin D phosphorylates and inactivates the transcriptional repressor retinoblastoma protein (Rb). Unphosphorylated Rb binds and sequesters E2F transcription factor responsible for S-phase specific gene expression. Moreover, Rb interacts with chromatin remodelling enzymes and thus inhibits probably the loosening of chromatin structure required for efficient transcription initiation. Rb phosphorylation leads to E2F release which in turn binds to DP transcription factor and this heterodimer activates the transcription of S-phase genes (Harbour and Dean, 2000). The Cdk4/6-cyclin E complex is required to overcome the restriction point at the G1/S transition and commit cells to divide. When any DNA damage is sensed, cell cycle is arrested prior to the synthesis of DNA by CDK inhibitors from the Cip/Kip and the INK4 family (Sherr and Roberts, 1999). Once the role of cyclins D and E is fulfilled and the integrity of DNA verified, cyclins and inhibitors are eliminated. The ubiquitin ligase complex SCF marks these proteins by the addition of ubiquitin and so targets them for degradation by the 26S proteasome (Chapter II.C.2) (Yew, 2001). Next, cyclin A appears in early S phase and activates Cdk2; the activity of this complex is necessary for correct S phase progression. In late S and G2 phase, mitotic A and B cyclins

accumulate in the cytoplasm. The onset of mitosis is delayed until DNA is completely and correctly replicated by repressing the activity of the M-phase promoting factor (MPF) Cdk1-cyclin B complex. In interphase, MPF is mainly cytoplasmic and held inactive by phosphorylation on the inhibitory sites Thr14 and Tyr15. The activity of Wee1 and Myt1 kinases targeting these residues exceeds that of Cdc25 phosphatase in G2 phase. At the onset of mitosis MPF is abruptly transported to the nucleus and in the same time dephosphorylated on Thr14 and Tyr15 due to Cdc25. The balance between the activity of Wee1 and Cdc25 is reversed by phosphorylation: they are targeted by Polo-like kinases (Plk) and while phosphorylation inhibits Wee1, it raises the activity of Cdc25 (Ohi and Gould, 1999). Completion of mitosis is mainly governed by ubiquitin-dependent proteolysis orchestrated by the ubiquitin ligase termed anaphase-promoting complex (APC). It drives the degradation of anaphase inhibitors and mitotic cyclins during M and early G1 phase (Chapter III.C.3).

B. The plant cell cycle

Considering the basic importance of cell division in every living organism, it is not surprising that its molecular mechanisms are highly conserved among eukaryotes. Similarly to the well-studied yeast and metazoan systems, plant cell cycle is governed by the successive activation of different cyclin-CDK complexes. A growing number of data indicate the conservation of CDK regulatory mechanisms as well. However, the very different cell-environment relations originating from the divergent life strategies of plants and animals are certainly reflected at molecular level as well. Studies in the different plant species and homology-based searches in the completed *Arabidopsis* genome identified candidates for most of the components of metazoan cell cycle machinery (Figure 4) (Dewitte and Murray, 2003; Vandepoele *et al.*, 2002; Wang *et al.*, 2004).

Two of the six designated classes of plant CDKs are the most studied groups being directly involved in cell cycle regulation (Table 2). A-type CDKs are the closest homologues of the Cdc28/Cdc2/Cdk1-type kinases and have the conserved PSTAIRE cyclin-binding motif. Accordingly, CDKA activity is high at both G1/S and G2/M transitions (Magyar *et al.*, 1997; Sorrel *et al.*, 2001). B-type CDKs with PPTALRE or PPTTLRE motifs are plant-specific kinases with the unique feature of cell cycle-dependent

expression pattern. CDKB proteins are present from late S until the end of mitosis with maximum activity in M (Magyar *et al.*, 1997; Porceddu *et al.*, 2001; Segers *et al.*, 1996).

Plant CDK-activating kinases represent two types (Table 2). The so-called vertebrate-type CAKs are grouped in CDKD class and as discussed in Chapter II.B.3, – consistent with their homology to Cdk7 – have both CDK activating and CTD kinase activity. The rice R2 kinase is preferentially expressed around S phase but the pattern of its CAK activity remains to be defined. Cak1At, (CDKF;1) the only known F-type kinase to date, is active as a monomer. Recently, it has been shown to phosphorylate and activate the CTD kinase activity of CDKD class kinases and thus act as a CAK-activating kinase (CAKAK) (Shimotohno *et al.*, 2004).

A, B and D classes of cyclins can be found in plants as well; however all three families are much more complex than that of animals: the analysis of the *Arabidopsis* genome identified 10 A-type, 11 B-type and 10 D-type cyclins (Wang *et al.*, 2004). A-type cyclin transcripts appear at different points of S-phase, persist in G2 and disappear during mitosis. However, the kinase activity associated to one of the three subclasses of A cyclins shows two peaks, one in mid-S and a second in G2-M (Roudier *et al.*, 2000). Transcript and protein level of B-type cyclins are restricted to G2 and M phases in plants as well and their degradation at the onset of anaphase is most probably carried out via the 26S proteasome (Genschik *et al.*, 1998). Common to animals and plants, D-type cyclins contain the Rb-interaction motif and, in most cases, a PEST sequence responsible for their degradation. Plant D-type cyclins might mediate effects of mitogenic stimuli: the expression of different members of D-type cyclins is regulated by sugars and various plant hormones (Dewitte and Murray, 2003).

Seven CDK inhibitors have been isolated from *Arabidopsis* with highest homology to the Cip/Kip class of inhibitors and named therefore Kip-related proteins (KRP). Several KRPs interact with CDKA and D-type cyclins, while their overexpression correlates with reduced kinase activity indicating that the inhibitor-mediated control of CDK activity is conserved in plants (De Veylder *et al.*, 2001; Wang *et al.*, 1998, 2000).

The identification of other regulatory proteins supports that the basic mechanisms of cell cycle regulation are conserved in plants as well (Figure 4). Members of the Rb-E2F pathway, homologues of Wee1 kinase and Cdc25 phosphatase were identified from different species, and a growing number of data indicate that ubiquitin-mediated proteolysis

is important in plant cell cycle progression as well (Dewitte and Murray, 2003; Genschik *et al.*, 1998; Landrieu *et al.*, 2004).

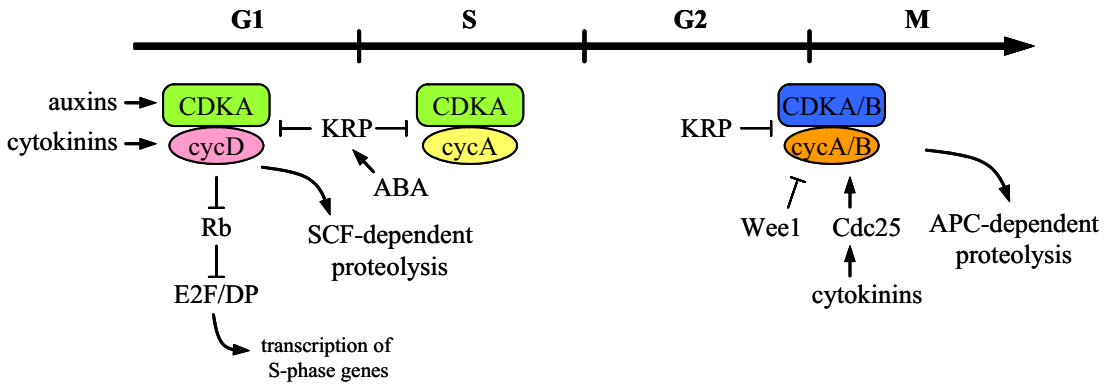

Figure 4. ***Model for cell cycle control in plants.*** Mitogenic signals (e.g. hormones) promote the activation of A-type CDKs via D-type cyclins. The catalytic activity of these complexes releases the E2F/DP transcription factor sequestered by Rb; S-phase specific genes are transcribed. D-type cyclins are degraded at the G1/S transition via SCF-dependent proteolysis. A-type cyclins appear in S phase and activate A-type CDKs in S and G2 phases. The G2/M transition is regulated by complexes of A- and B-type CDKs with A- and B-type cyclins. These complexes are further regulated by phosphorylation (Wee1) and dephosphorylation (Cdc25). At the exit of mitosis, cyclins are eliminated by APC-mediated proteolysis. CDK inhibitors (KRP) act most probably both at the G1/S and the G2/M transitions.

C. Ubiquitin-mediated protein degradation in cell cycle control

1. The ubiquitin system

Regulated proteolysis is of basic importance in many cellular processes. Proteins destined for degradation are modified by ubiquitin, a small 76 amino-acid-residue protein and then eliminated by the 26S proteasome. The covalent ligation of ubiquitin to target proteins requires the sequential activities of three enzymes. First, ubiquitin is bound via its C-terminus by a high energy thiol esther linkage to a cysteine residue in the ubiquitin-activating enzyme (E1), which leads to its activation. Next, this activated ubiquitin is transferred to the ubiquitin-conjugating enzyme (E2). Finally, ubiquitin ligase (E3) catalyses the binding of ubiquitin to a lysine residue in the target protein. Multiple rounds of these reactions lead to the formation of multiubiquitin chain on the substrate, which is recognized by the 26S proteasome and results in the degradation of the ubiquitin-marked

protein. While there is usually a single E1 enzyme, many variants of E2 and E3 enzymes are present providing specificity for the system (Hershko and Ciechanover, 1998).

Ubiquitin-mediated proteolysis is largely involved in the ordering of cell cycle transitions. By targeting inhibitors of the phase to come and activators of the completed phase for degradation it ensures progression of cell cycle in an abrupt and irreversible manner. Protein degradation culminates in the two main events of cell cycle, DNA synthesis and mitosis, mediated by the SCF and the anaphase-promoting complex (APC) ubiquitin ligases, respectively.

2. The SCF complex and the G1/S progression

In yeasts and mammals protein degradation mediated by the SCF ubiquitin ligase (named after its subunits Skp1, cullin, F-box) is essential for the onset of DNA replication. SCF is a multisubunit complex that contains at least the E2 enzyme Ubc3/Cdc34 or Ubc5 and the subunits Skp1, cullin1/Cdc53, the RING finger protein Roc1/Rbx1/Hrt1 and an F-box protein. Besides the F-box motif which is required for binding to Skp1, F-box proteins contain other sequence elements that mediate protein-protein interactions (e.g. WD40 and leucine-rich repeats) and so determine the substrate specificity of the complex. The large number of proteins with F-box signature in each genome studied so far (over 19 in budding yeast, 50 in vertebrates and around 700 in *Arabidopsis*) suggests that the stability of a broad variety of proteins – and thus many cellular processes – might be regulated via the SCF complex (Gagne *et al.*, 2002; Kipreos and Pagano, 2000). The complex is thought to be constantly active but target only phosphorylated proteins (Bachmair *et al.*, 2001; Deshaies, 1999; Jackson *et al.*, 2000).

Ubiquitination of cell cycle-related proteins is carried out by the SCF^{Cdc4} and SCF^{Grr1} complexes in budding yeast and by SCF^{Skp2} in mammals (the index denotes the F-box protein present in the complex). In *S. cerevisiae*, S-phase Cdc28/Clb5-6 complexes are held inactive by the Sic1 CDK-inhibitor. The accumulation of Cdc28/Cln1-3 during G1 phase leads to the phosphorylation of Sic1 and thus directs it to SCF^{Cdc4}. Several other G1- and S-specific proteins are also targeted by this form of SCF: the Far1 mating pheromone-responsive CDK inhibitor, Cdc6 involved in the licensing of replication origins and the Cdc4 F-box protein itself. The increase in Cdc28/Cln1-3 activity leads to autophosphorylation on Cln1-2 which is recognized by the SCF^{Grr1} complex.

Similarly, mammalian S-phase progression is accompanied by extensive protein degradation mediated by SCF^{Skp2} or by other, yet unidentified SCF complexes. Ubiquitinated proteins include inhibitors of S phase onset like $p21^{Cip1}$ and $p27^{Kip1}$ CDK inhibitors of the Cip/Kip family; G1 cyclins A, D and E; Cdc6, an essential component of the pre-replication complex; and the S-phase specific transcription factor E2F-1 (Yew, 2001). Interestingly, Skp2 itself and so the activity of SCF^{Skp2} is regulated by degradation: its level is kept low in early G1 by the M-phase regulator ubiquitin ligase, the anaphase-promoting complex (APC^{Cdh1}) (Bashir *et al.*, 2004; Wei *et al.*, 2004).

3. Control of mitosis by the anaphase-promoting complex

The other cell cycle-related ubiquitin ligase, the anaphase promoting complex, was discovered as the ubiquitin ligase responsible for mitotic degradation of cyclin B and found necessary for sister chromatid separation (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). The complex of 20S size was originally thought to contain eight subunits, nowadays 13 subunits are known from yeasts and 11 from mammals (Appendix III) (Peters, 2002). Although the function of the individual subunits is poorly understood, they are highly conserved in all eukaryotes: based on sequence homology, *in silico* analysis of the *Arabidopsis* genome identified 11 putative APC components (Capron *et al.*, 2003a).

a) Composition of APC

Similarly to SCF, APC is a multisubunit RING-cullin based ubiquitin ligase (Figure 21). The catalytical core is formed by the RING-H2 protein Apc11 and the cullin-homologue Apc2; these two subunits are sufficient to recruit E2 activity and efficiently ubiquitinate target proteins although with reduced specificity towards the substrates. Apc11 binds the E2 enzyme Ubc4, while UbcH10 is recruited via the cullin domain of Apc2 (Gmachl *et al.*, 2000; Leverson *et al.*, 2000; Tang *et al.*, 2001). Several subunits (Cdc16, Cdc23, Cdc27 and Apc7 in mammals) contain multiple copies of a 34-amino-acid motif called tetratricopeptide repeat (TPR), which acts in mediating protein-protein interactions. These TPR proteins are supposed to form a scaffold where other subunits will anchor. Cdc27 and Apc7 interact with proteins containing isoleucine-arginine motif at their C-terminus, like co-activator proteins of both Cdc20- and Cdh1-type and the Doc-domain subunit Apc10 (Vodermaier *et al.*, 2003). Apc10 is suggested to improve the processivity of the

ubiquitination reaction by limiting substrate dissociation and it might contribute to substrate recognition and recruitment as well (Carrol and Morgan, 2002; Passmore *et al.*, 2003). Most of the other subunits of APC do not even share any homology with proteins of known function, their function such as the reason of the high complexity of APC compared to other E3 enzymes remains elusive.

To gain full activity, Cdc20/Fizzy and Cdh1/Hct1/Fizzy-related activator proteins bind to APC in a cell cycle-dependent manner and regulate its substrate specificity (Burton and Solomon, 2001; Hilioti *et al.*, 2001; Pfleger *et al.*, 2001; Schwab *et al.*, 2001). APC^{Cdc20} is present in meta- and anaphase, while APC^{Cdh1} functions in mitotic exit and G1 phases (Castro *et al.*, 2005; Harper *et al.*, 2002). APC^{Cdh1} activity is detected in postmitotic, terminally differentiated cells as well (Gieffers, *et al.*, 1999). Both activators contain WD40 repeats also found in several F-box proteins, which is, once again, reminiscent of the structure of the SCF complex.

b) Substrates

The activity of APC is needed for sister chromatid separation and through CDK inactivation for mitotic exit. When APC function is impaired, cells arrest in late metaphase before sister chromatids have been separated, while expression of non-degradable cyclins blocks mitosis in telophase. These data suggest that other substrate(s) than cyclins must exist that has/have to be eliminated for anaphase onset. This substrate is called securin. After DNA synthesis, sister chromatids are held together by the cohesin protein complex. Cleavage of cohesin by a cysteine protease, called separase sets anaphase on. The timing of separase activation is critical and it depends on the proteolysis of its inhibitor, the securin protein (Nasmyth, 2001). The initiation of anaphase is marked by decreasing CDK activity. Besides eliminating securin, APC^{Cdc20} ubiquitinates the bulk of S- and M-phase specific cyclins, like Clb5 and Clb3 in yeast. Activated APC^{Cdh1} continues the degradation of mitotic cyclins and the resulting low CDK activity triggers mitotic exit. APC^{Cdh1} activity is also present in early G1 phase and maintains ubiquitination of mitotic cyclins and several proteins involved in spindle function (Castro *et al.*, 2005). These and other substrates of APC are summarized in Table 3.

Several sequence motifs have been found necessary for the degradation of APC substrates. Destruction box or D box was identified in the N-terminus of mitotic cyclins

with the consensus sequence RxxLxxxxN (King *et al.*, 1996). The other motif frequently found in proteins targeted by APC is the KEN box named after its consensus and determined originally in Cdc20 (Pfleger and Kirschner, 2000). While APC^{Cdc20} seems to recognize exclusively D box containing proteins, APC^{Cdh1} interacts with proteins containing D, KEN or other motifs (Fang *et al.*, 1998). New motifs have been determined recently, the A box in the Aurora-A kinase and a GxEN sequence in Xkid directs the proteolysis of these proteins (Castro *et al.*, 2003; Littlepage and Ruderman, 2002). If these domains are directly involved in the activator-substrate interaction, remains a field of debate.

Function of substrate	Substrate	APC complex
Anaphase inhibitor	securin	APC^{Cdc20}
Protein kinases	Plk1	APC^{Cdh1}
	Hsl1	APC^{Cdc20}, APC^{Cdh1}
	Aurora A, B	APC^{Cdh1}
	Nek2A	APC^{Cdc20}
Protein phosphatase	Cdc25A	APC^{Cdh1}
Regulatory subunits of protein kinases	B-type cyclins	APC^{Cdc20}, APC^{Cdh1}
	A-type cyclins	APC^{Cdc20}, APC^{Cdh1}
	Dbf4	APC^{Cdc20}
APC cofactors	Cdc20	APC^{Cdh1}
	E2-C	APC^{Cdh1}
Motor, spindle and kinetochore proteins	Ase1	APC^{Cdh1}
	Kip1	APC^{Cdc20}
	Cin8	APC^{Cdh1}
	Xkid	APC^{Cdc20}, APC^{Cdh1}
Regulators of DNA replication	geminin	APC
	Cdc6	APC^{Cdh1}

Table 3. ***Substrates of the anaphase-promoting complex.*** After Peters, 2002.

c) Regulation of APC activity

The ubiquitin ligase activity of APC oscillates during the cell cycle. Its regulation involves multiple mechanisms: phosphorylation of several subunits in the core complex, phosphorylation and proteolysis of the activator proteins, and association of the APC with inhibitory proteins (Figure 5).

In the APC several subunits are phosphorylated including Apc1 and the TPR subunits Cdc16, Cdc23 and Cdc27 (Harper *et al.*, 2002). CDK phosphorylation sites were identified in the TPR-containing subunits and Cks-mediated Cdk1 phosphorylation of Cdc27 was found to stimulate the activity of the complex (Patra and Dunphy, 1998). Polo-like kinases (Plk) target Apc1, Cdc27 and Cdc16 probably on other residues than Cdk1 (Kotani *et al.*, 1998). Although the physiological relevance of these phosphorylation events is unclear, they might facilitate Cdc20 recruitment to the complex, whereas binding of Cdh1 to the APC does not seem to be affected by phosphorylation of the APC subunits.

The activator proteins Cdc20 and Cdh1 contain also several phosphorylation sites. While the significance of Cdc20 phosphorylation is uncertain, phosphorylation of Cdh1 by CDK-mitotic cyclin complexes affects basically its activity; when the protein is phosphorylated, it becomes unable to bind the core APC.

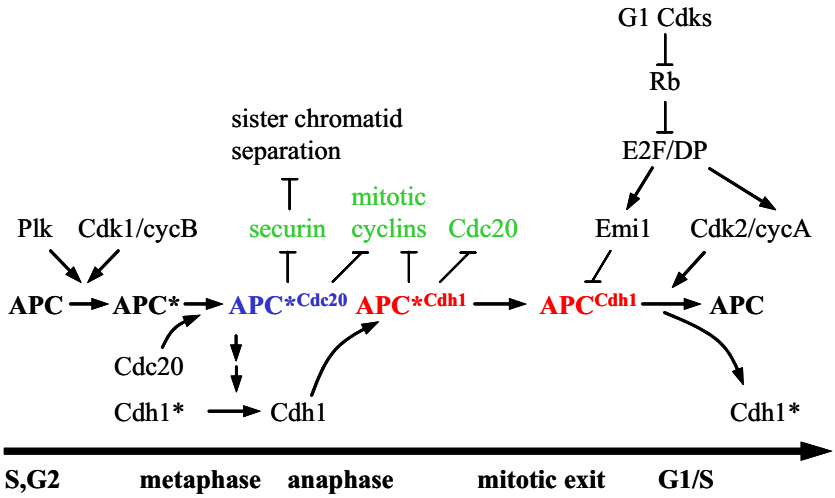

Figure 5. ***The anaphase promoting complex in the control of mitosis.*** See the text for details. Active APC is highlighted by blue and red, APC substrates in green. Asterisks mark phosphoproteins.

Cdc20 has a narrow expression profile, similar to that of mitotic cyclins peaking at the G2/M transition which is then followed by a rapid decrease. In contrast to Cdc20, the protein level of Cdh1 is constant in the cell cycle but its phosphorylation state varies. When

mitotic CDK activities are high, Cdh1 is inactive. Cdc20 provokes degradation of mitotic cyclins, thereby leading to activation of Cdh1, which then elicits degradation of Cdc20. (Harper *et al.*, 2002). In the next cycle, G1 and S cyclins activate CDKs which by targeting the Rb protein allow the S phase-specific transcription of Emi1, an inhibitor of Cdh1 and Cdc20. Emi1 interacts with and blocks the N-terminal part of the activators involved in substrate recognition. The increasing CDK activities in S phase facilitate APC^{Cdh1} inactivation by phosphorylation of Cdh1 (Rape and Kirschner, 2004; Sorensen *et al.*, 2001). Binding of Emi1 to Cdc20 in late S and early M phase prevents premature degradation of mitotic cyclins. Emi1 contains an F box motif and several Cdk1-cyclin B phosphorylation sites suggesting that SCF could be responsible for its mitotic degradation (Reimann *et al.*, 2001a, b).

PRELIMINARIES AND OBJECTIVES

Six members of the cyclin-dependent kinase family representing four classes of plant kinases have been isolated from *Medicago sativa* in the Laboratory of Plant Cell Division and Differentiation (Hirt *et al.*, 1993; Magyar *et al.*, 1997). MsCDKA;1 and MsCDKA;2 (formerly Cdc2MsA and B) containing the classical PSTAIRE sequence at their cyclin-binding site are the closest homologues of yeast Cdc28/Cdc2 kinases. Consistently, their kinase activities peak at both G1/S and G2/M cell cycle transitions. MsCDKB1;1 and MsCDKB2;1 (Cdc2MsD and F) belong to the two subclasses of the plant specific B-type CDKs. CDKB1 group is characterized by the PPTALRE motif as well as its transcript being present from late S-phase until mitosis, while the B2 class is marked by the PPTTLRE motif, and peaks slightly later, in G2 and mitosis (Magyar *et al.*, 1997; Mészáros *et al.*, 2000). The other two kinases are classified as E- (Cdc2MsE or MsCDKE;1) and C-type (Cdc2MsC or MsCDKC;1) CDKs that represent the less studied groups of plant CDKs. At the beginning of this work, the only information about the CDKC;1 kinase was that its transcript was detected at a constant level in all cell cycle phases (Magyar *et al.*, 1997).

The aim of our work was to get an insight into the possible function of the alfalfa CDKC;1 protein. The cDNA is predicted to encode a 509-amino acid-long protein with an approx. molecular weight of 57 kDa and a PITAIRE motif at its cyclin-binding site. Compared to A- and B-type CDKs, CDKC;1 possesses a long C-terminal extension.

Since CDKs act generally in complex with cyclins, the identification of interacting partners for the CDKC;1 kinase was necessary. Thereafter, the activity of the kinase complex was analyzed using CDKC;1-specific antibodies in alfalfa cell extracts and by epitope-tagged proteins expressed in *Arabidopsis* protoplasts. The subcellular localization of the kinase-cyclin heterodimer was also determined. Finally, the effect of the catalytical activity of the complex on RNA polymerase II-mediated transcription was studied in an *in vitro* transcription system.

Parallel to our work, in the laboratory of Éva Kondorosi, the *Arabidopsis* homologue of the alfalfa CDKC;1 kinase was identified as interacting partner of the Ccs52-type APC activator proteins. Our subsequent studies did not confirm the proteolytical degradation of

the kinase or the cyclin protein and the complex failed to phosphorylate the Ccs52 proteins (data not presented in the thesis). Despite the negative results, the original observation initiated my work on plant anaphase-promoting complexes and its activators.

The composition of the anaphase-promoting complex has been studied extensively in yeast and mammalian systems while the number of identified subunits is growing steadily (Chapter III.C). Together with other members of the ubiquitin – proteasome pathway, homologues of most APC subunits can be identified in the *Arabidopsis* genome as well (Appendix III) (Bachmair *et al.*, 2001; Capron *et al.*, 2003a). Most subunits of APC are encoded by single-copy genes; the only subunit with two related gene copies is Cdc27. However, other components of the APC-mediated ubiquitination, like E2 enzymes, activators and even the potential substrates, are represented by complex families in plants suggesting an intricate regulation of protein stability during the cell cycle.

Previous work from our laboratory identified the first plant orthologue of the Cdh1 APC activator from *M. sativa*, called *cell cycle switch* or *ccs52* (Cebolla *et al.*, 1999). Further detailed study in the model legume *M. truncatula* defined two classes of Ccs52 activators, Ccs52A and B, which displayed distinct functions and regulation (Tarayre *et al.*, 2004; Vinardell *et al.*, 2003). These two types of activators can also be distinguished in *Arabidopsis*; its genome contains three *ccs52* genes: *Atccs52A1, Atccs52A2* and *Atccs52B* (Appendix III).

In this work we aimed at characterizing the *Arabidopsis ccs52* genes and their encoded proteins. The association of AtCcs52 proteins to intact APC was tested in fission yeast and *in planta*. By binary interaction analyses using the individual APC subunits, Ccs52-type activators as well as Ubc19 and Ubc20 ubiquitin-conjugating enzymes, we aimed at outlining the basic molecular organization of the plant APC. To test the eventual substrate-selectivity, binding of the activator proteins to mitotic cyclins was also investigated. Finally, the cell cycle-dependent expression of the studied genes was analyzed in synchronized suspension cultured cells.

To facilitate orientation within the complex structure of APC, Appendix III summarizes the main structural and functional properties of its subunits, while a model for its molecular organization is given on Figure 21A.

MATERIALS AND METHODS

Note: Those techniques that are described in details in the enclosed manuscript "*Arabidopsis* Anaphase-Promoting Complexes: Multiple activators and the wide range of substrates might keep APC perpetually busy" (pp.68-85) are only referred by their respective page numbers.

cDNA isolation

The cDNA coding for MsCDKC;1 was originally isolated and described by Magyar *et al.* (1997).

cDNAs coding for APC subunits, E2 enzymes, activators and cyclins were amplified directly by PCR with Pfu enzyme (Promega) from an *Arabidopsis thaliana* ecotype Columbia cell suspension (kind gift of Csaba Koncz) or plantlet cDNA library. The oligonucleotides used in these reactions were: 5'agagatatggcgacagagt and 5'tcatctcagtgttgaataagt for *apc10*; 5'atggcttttgatggttgttg and 5'ttactctttgaactgccattc for a*pc11*; 5'atgagggaagaaattgag and 5'actgaccaattcctagcagag for c*dc16*; 5'atggcttctaaagagtgttgc and 5'ggaacagtacagctaaatagg for c*dc23*; 5'atgatggagaatctactggc and 5'ttagggttagtccacaag for *cdc27A*; 5'atggaagctatgcttgtgga and 5'tcacgggctctcatcgatctc for *cdc27B/hobbit*; 5'aaaaggatggcgacggttaat and 5'aagagatatgatccaatacagttga for *ubc19*; 5'atggccgccgtaaatggat and 5'agcttgcagaaatcatgcact for *ubc20*; 5'ggatccatggaagaagaagatcctacagc and 5'ggaaccaacattcaacacaaccgg for *Atccs52A1*; 5'ggatccatggaagaagatgaatcaac and 5'cctgatttcgagaatcatgtcaagac for *Atccs52A2*; 5'ggatccatggcatcgccacagagtacc and 5'gtttcaatctccgaccaaaccgtgc for *Atccs52B*; 5'catgtcgaacattcttcagaatcg and 5'-ttagctgttgttgaagaactc for *AtcycA1;1*; 5'aatgtcttcttcgtcgagaaatc and 5'tcaacaatatgtacaatacacg for *AtcycA1;2*; 5'taagatgatgacttctcgttcg and 5'cttcttcgtcttcttaaaggg for *AtcycB1;1*. The PCR fragments were introduced into EcoRV-digested pBluescript SKII+ (Invitrogen) for subunits and E2s or into pGEMeasy (Invitrogen) for acivators and cyclins. cDNAs coding for Apc2 and Cdc26 in pGEM-T were kindly provided by Pascal Genschik, AtCycB1;2 in pBluescript SKII+ by Arp Schnittger.

cDNAs from the cloning vectors were subcloned into specific plasmids by standard molecular cloning techniques (Sambrook *et al.*, 1989).

Yeast manipulations

Yeast two-hybrid interaction analysis

The yeast two-hybrid screens and directed interaction tests were performed using the GAL4-based Matchmaker Two-Hybrid System (Clontech) according to the manufacturer's protocol. Full-length cDNAs were introduced into pGAD424 and/or pGBT9 vectors. For the transformations, the PJ69-4a strain [*MATa, leu2-3, 112, ura3-52, trp1-901, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, lys2::GAL1-HIS3, met2::GAL7-lacZ*] was used (James *et al.*, 1996). Transformation mixtures were plated on yeast drop-out selection media lacking either leucine and tryptophan to estimate the transformation efficiency or lacking leucine, tryptophan and histidine for testing protein-protein interactions. Positive colonies from selective plates were recovered after 4-6 days and tested for growth on selective plates lacking leucine, tryptophan, histidine and adenine. The β-galactosidase activities were assayed as described by Gindullis *et al.* (1999).

Complementation assay

For the complementation assay with MtCyclin T, the yeast strain Y145 that carries a triple deletion of *cln1*, *cln2* and *cln3* genes was used (Edwards *et al.*, 1998). Yeasts were kept alive by an integrated *GAL::CLN3* gene induced when cells are plated on galactose-containing medium. Transformation of the yeast strain with cyclin T inserted into pVT-U103 (Vernet *et al.*, 1987), p416MET25 or p426MET25 (Mumberg *et al.*, 1994) was performed as above. The transformation mixture was plated on yeast drop-out selection media lacking uracil and containing 2% galactose. Colonies were replica-plated onto yeast drop-out selection media lacking uracil and containing 2% glucose and on media lacking uracil and methionine containing 2% glucose.

Heterologous expression in fission yeast

The expression of AtCcs52 proteins in *S. pombe* is decribed on page 77.

Protein techniques

Bacterial protein expression and antibody purification

An EcoRI-XmnI fragment encoding the C-terminal 21 kDa region of alfalfa CDKC;1 was cloned into pTrcHisA vector (Invitrogen) and expressed in *E. coli*. The hexahistidine fusion protein was purified under denaturing conditions as described in the manufacturer's protocol and used to immunize rabbits. For the affinity purification of the antiserum the cDNA coding for the whole protein was introduced into pET-35b vector (Novagen) and expressed in *E. coli*. After induction bacterial pellet was resuspended in 20 mM Tris-HCl pH 7.5, lysed by sonication and centrifugated. Because the recombinant protein formed inclusion bodies, this fraction was used for the antibody purification. The insoluble pellet was washed twice with the initial buffer, resuspended in 1% SDS and boiled to solubilize.

Purification of antibodies was based on the method described by LeGendre, 1990. Briefly, the solubilized pellet was separated by SDS-PAGE and blotted onto nitrocellulose membrane. The strip containing the antigenic protein was cut out after staining the membrane with Ponceau S. The membrane was saturated with 3% BSA in TBS supplemented with 0.05% Tween 20 (TBST) for two hours. Immunoglobulins were precipitated from the crude serum with 40% ammonium sulphate, redissolved and dialyzed in PBS. 500 µL of this serum was added to the membrane in a total volume of 2 mL TBST containing 1% BSA and 0.05% NaN_3 and incubated for four hours at room temperature. The blot was rinsed three times with TBST and once with 10 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.5. Bound antibodies were eluted in 2x500 µL elution buffer (0.1 M glycine, 0.5 M NaCl, 0.05% Tween 20, pH 2.6), 2 min each. The solutions containing eluted antibodies were immediately neutralized with 60 µL 1 M Tris pH 8. BSA was added to a final concentration of 1 mg/mL to the pooled eluate, which was dialyzed against TBS plus 0.01% Tween 20. Finally, purified antibodies were frozen in liquid N_2 and stored at -80°C.

For the generation of wild type and phosphorylation site-mutated GST fusion proteins containing two copies of the Pol II C-terminal heptapeptide repeat, the following oligonucleotides were annealed, then digested with BamHI-EcoRI (restriction sites are underlined) and inserted into pGEX-4T-2 expression vector (Pharmacia):

wt upper: 5'cgggatcctactccccgacctccccgtcctactccccgac-ctccccggaattccg,

wt lower: 5'cggaattccggggaggtcggggagtaggacggggaggtcggggagtaggatcccg,

S2A upper: 5'cgggatcctacgccccgacctccccgtcctacgccccgacctccccggaattccg,

S2A lower: 5'cg- gaattccggggaggtcggggcgtaggacggggaggtcggggcgtaggatcccg,

S5A upper: 5'cgggatcctactcccc- gaccgccccgtcctactccccgaccgccccggaattccg,

S5A lower: 5'cggaattccggggcggtcggggagtaggacggggcggtcggggagtaggatcccg.

Recombinant proteins were expressed in *E. coli* strain BL21 and purified as recommended by the manufacturer. To summarize, 50 mL of culture at an OD_{600} of 0.6 was induced with 0.2 mM IPTG for 4h at 37°C. Cells were pelleted by centrifugation, resuspended in PBS pH 7.4, lysed by the addition of lysozyme (100 µg/mL for 20 min) followed by sonication. To facilitate the solubilization of the expressed proteins, 1% Triton X-100 was added to the sonicated lysate and incubated on ice for 30 min. The lysate was cleared by centrifugation, glutathione-Sepharose was added to the supernatant and incubated on ice for 1h. Beads were washed three times with ten volumes of PBS then GST-fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris pH 8.0.

Protein extraction from plant cells

Protein extracts from both *Medicago sativa* and *Arabidopsis thaliana* cells were prepared in the same buffer containing 25 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 60 mM β-glycerophosphate, 1 mM DTT, 0.1 % NP40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF and protease inhibitors (Complete, Roche).

Immunoprecipitation

Immunopreipitation of epitope-tagged proteins from lysates of transfected *Arabidopsis* protoplasts is decribed on page 78.

Immunoblot analysis

For the western blot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Amersham) as described by Sambrook *et al.* (1989). The membrane was saturated for 2h with 5% non-fat dried milk in TBST.

Then, the blot was incubated for 2 hours with the primary antibody diluted as recommended by the manufacturers; the polyclonal anti-CDKC;1 antibody was used in 1:1000 dilution. After three washes with TBST, horseradish peroxidase-conjugated secondary antibodies diluted according to the manufacturer's instructions were added to the membrane and incubated for 1h. Following three washes with TBST, blots were revealed by enhanced chemiluminescence detection (Amersham).

Kinase assay

For the kinase assays CDKC;1 protein was immunoprecipitated from 150 µg total protein extract from *M. sativa* A2 suspension cells with 50 µL affinity-purified polyclonal anti-CDKC;1 antibody and immobilized on protein A agarose. For assaying the kinase activity of epitope-tagged CDKC;1, proteins were immunoprecipitated from lysates of *Arabidopsis* protoplasts as described there. Immuncomplexes were washed three times in TBST and once with kinase buffer. Kinase assays were performed in buffer containing 25 mM Tris pH 7.8, 15 mM $MgCl_2$, 1 mM DTT, 0.2 mg/mL substrate and 2.5 µCi [γ-^{32}P]ATP for 30 min at room temperature. To stop the reactions SDS loading buffer was added and samples were boiled. Reactions were separated by SDS-PAGE and the dried gels were autoradiographed to reveal phosphorylation.

Subcellular fractionation of alfalfa cells

For the separation of the fractions, protoplasts were prepared by incubating alfalfa A2 suspension cells in 2% cellulose R10, 0.5% driselase, 0.5% macerozyme, 1% pectinase, 0.1% pectolyase, 0.18 M mannitol, 0.18 M sorbitol, 3.5 mM $CaCl_2$, 0.4 M NaH_2PO_4, 0.5% MES, pH 5.6. Protoplasts were filtered through a 50-micron mesh, washed three times with GH solution (0.38 M glucose, 1.36 mM $CaCl_2$) and incubated in the same solution for 10 min on ice. Then 0.1% Triton X-100 was added, nuclei were released by pipetting and collected after 5 min with centrifugation at 1000 g. The supernatant was used as cytoplasmic fraction. Pelleted nuclei were washed twice with GH solution and lysed in 25 mM Tris pH 7.6, 15 mM $MgCl_2$, 275 mM NaCl, 60 mM β-glycerophosphate, 1 mM DTT, 0.1% NP40, 20 mM NaF and protease inhibitors (Complete, Roche).

The nuclear extract was used for the glycerol gradient fractionation. 10-35% linear glycerol gradient was prepared in 16 mL volume in the buffer used for the lysis of nuclei but containing 200 mM NaCl and 20 mM β-glycerophosphate. Nuclear extract containing 1 mg of total protein was layered on the top and centrifuged with 115 000 g for 16 hours at 4°C. Fractions of 0.75 mL were collected, 20 µL from each was used for immunoblot analysis.

Plant manipulations

Plant materials

Medicago sativa ssp. varia genotype A2 cell suspension culture is maintained by weekly subculturing in MS medium (Sigma) supplemented with 1 mg/L 2,4-D and 0.2 mg/L kinetin.

Arabidopsis thaliana cell ecotype Columbia suspension was used for the protoplast transformations. The culture is maintained by weekly subculturing in MS medium supplemented with 3% w/v sucrose, 240 µg/L 2,4-D and 14 µg/L kinetin.

For the synchrony, *Arabidopsis thaliana* ecotype Landsberg erecta cell suspension cultures were maintained as described on page 78.

Synchronization of cell cycle

Synchronization of A2 cell suspension by hydroxyurea treatment was performed as described by Ayaydin *et al.*, 2000.

The reversible G1/S blockage of *Arabidopsis thaliana* (Landsberg erecta ecotype) cells is described on page 78.

Transfection of *Arabidopsis* protoplasts

cDNAs tested for interaction were cloned into pRT104-derived vectors modified to incorporate three tandem copies of the c-myc and HA epitopes as N-terminal fusions (Topfer *et al.*, 1987). Protoplasts from *Arabidopsis* cell suspension were prepared and transfected as follows. Cells were collected from 40 mL of four-day-old suspension culture by centrifugation. Cell walls were digested for 5 hours in MS-GM (MS supplemented with

0.17 M glucose and 0.17 M mannitol) containing 0.25% cellulase R10 and 0.05% macerozyme R10 (Yakult Honsha Co. Ltd.). Protoplasts were washed with MS-GM and collected in MS containing 0.28 M saccharose. The transformation mixture contained 10^6 cells in 50 µL, 10 µg of each plasmid DNA and 150 µL PEG solution (25% PEG 6000, 0.45 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 9). After 20 min, 1 mL of 0.1 M $Ca(NO_3)_2$ was added. Cells were then centrifuged, resuspended in 500 µL MS-GM and incubated for 16 hours. Protein extracts were prepared in the previously described buffer by freezing the protoplasts in liquid N_2 and allowing them to thaw on ice. Lysates cleared by centrifugation were used in subsequent immunoprecipitation assays.

RNA isolation and RT-PCR analysis

Total RNA from plant samples was isolated according to Menges and Murray (2002). To summarize, to approx. 150 mg of plant material ground in liquid N_2 600 µL extraction buffer (50 mM TrisCl pH 9, 10 mM EDTA pH 9, 2% SDS, 100 mM LiCl, 10 µg/mL proteinase K) was added, vortexed for 15 sec to shear DNA and incubated for 15 min at 65°C. Cell debris were pelleted by centrifugation for 5 min and the supernatant extracted with equal volume of phenol pH 9 first, then with phenol/chloroform (1:1) and finally with chloroform/isoamilalcohol (24:1). RNA was precipitated from the liquid phase by adding 1 volume of 4 M LiCl for 16 hours at 4°C. Precipitated RNA was pelleted by 20 min of centrifugation and washed with ice-cold 2 M LiCl. Redissolved RNA was re-precipitated by adding 1/10 volume of Na acetate pH 5.2 and 2.5 volume of ethanol, centrifuged and washed with 70% ethanol.

For the first strand synthesis, samples were treated with DNase I (Promega) for 30 min at 37°C and 5 µg of total RNA was used as template for reverse transcription. The cDNA synthesis was carried out in a final volume of 20 µL using 500 ng of oligo(dT) primer and PowerScript Reverse Transcriptase (Clontech) according to the manufacturer's recommendations. The cDNA was diluted to 100 µL with 10 mM Tris pH 8, 0.1 mM EDTA and 1 µL was used per PCR reaction with the following steps: 94°C 2 min, [94°C 30 sec, 55°C 40 sec, 72°C 45 sec] for various numbers of cycles. The primers are listed on pages 78-79.

PCR products were separated on agarose gel, blotted by capillary transfer onto nylon membrane (Biotrans, ICN), hybridized in Church buffer (0.5 M Na-phosphate pH 7.2, 7%

SDS, 2 mM EDTA) at 65°C and washed as described by Sambrook *et al.* (1989). The respective PCR fragments labelled with [α-^{32}P]dCTP by random priming (Megaprime DNA labelling system, Amersham) were used as specific probes for hybridization. Signals were analyzed by PhosphorImager (Molecular Dynamics).

Immunolocalization

For immunolocalization, *Arabidopsis* protoplasts were co-transfected with HA-tagged cyclin T and myc-tagged CDKC;1. After 16h, protoplasts were fixed in 3% paraformaldehyde in microtubule stabilization buffer (MSB) containing PBS pH 6.8, 10 mM $MgSO_4$, 10 mM EGTA, 0.17 M glucose and 0.17 M mannitol for 50 min. After five washes with MSB, protoplasts were spread on slides and dried to immobilize. Cells were permeabilized with MSB containing 0.5 % Triton X-100 for 20 min and blocked with 5% normal donkey serum in MSB for 30 min. Slides were then incubated overnight at 4°C with anti-HA monoclonal antibody (clone 16B12, Covance) and polyclonal anti-myc (Molecular Probes) at a 1:200 dilution. Cells were washed with MSB and incubated with the secondary Alexa 488- and TRITC-conjugated antibodies (Jackson Immunochemicals) at 1:1000 and 1:150 dilutions, respectively for 1h at room temperature. Cells were washed, counterstained with 1 μg/mL DAPI for 10 min and mounted using Citifluor (Citifluor Ltd.). Confocal laser scanning microscopy was performed using a Leica TCS SP2 microscope (Leica Microsystems).

In vitro *transcription reactions*

Transcription assays were performed with the HeLaScribe nuclear extract (Promega) according to the protocol provided by the manufacturer with the following modifications. As template, supercoiled pML(C_2AT) plasmid DNA carrying the adenovirus major late promoter in front of a synthetic 400bp-long G-less cassette was used (Sawadogo and Roeder, 1985). Reaction mixtures in a final volume of 25 μL contained 1x transcription buffer, 4 mM $MgCl_2$, 100 ng of template DNA and 8U (50 μg) of HeLa nuclear extract were assembled on ice then incubated for 15 min at room temperature. Reactions were initiated by adding ATP and CTP to 400 μM and UTP containing 5 μCi of [α-^{32}P] UTP (3000 Ci/mmol, Amersham) to 15 μM final concentrations, incubated for 45 min at 30°C then terminated with 175 μL of stop solution. After phenol/chloroform and chloroform

extractions RNAs were ethanol precipitated, dried, redissolved in formamide loading buffer and resolved on denaturing 5% polyacrylamide 0.5x TBE gels (Sambrook *et al.*, 1989). Radioactive transcription products were detected by autoradiography. When biochemical complementation was tested, Cdk9 complexes were depleted from the HeLa nuclear extract by three consecutive rounds of immunoprecipitation, each for one hour with 2.5 µg of polyclonal anti-Cdk9 antibody (sc-484, Santa Cruz Biotechnology) immobilized on Dynabeads Protein G paramagnetic particles (Dynal). At the same time alfalfa CDKC;1 and cyclin T proteins as well as CDKC;1-cyclin T complexes were immunoprecipitated either with anti-myc or with anti-HA antibodies from lysates of *Arabidopsis* protoplasts expressing the epitope-tagged proteins. Aliquots of the depleted nuclear extract were then supplemented with the different immunocomplexes captured on Protein G Dynabeads and transcriptional activity was analysed as described above.

RESULTS AND DISCUSSION

PART I. CHARACTERIZATION OF A C-TYPE CYCLIN-DEPENDENT KINASE FROM ALFALFA

Cyclin T is a specific interactor of the CDKC;1 kinase

The catalytical activity of a given CDK is basically defined by its cyclin partner. Cyclins modulate the temporal and often the spatial pattern of kinase activity and contribute to the substrate recognition of the complex (Morgan, 1997; Loog and Morgan, 2005). Therefore, it is crucial to know the cyclin partner(s) of a given CDK for its characterization.

To identify cyclins and possibly other proteins associating with the alfalfa CDKC;1 protein, a yeast two-hybrid screen using the kinase as bait was performed in a *Medicago truncatula* cDNA library.

Due to its small, diploid genome and favourable generation period, M. truncatula is the model organism for legume research. In addition, it has a dynamically growing EST database and shares very high sequence identity with the tetraploid *M. sativa* (Bell *et al.*, 2001). In this case, the translated sequence of the *M. truncatula* EST BE205235 was found to be completely identical with the MsCDKC;1 protein and to cover a region of 203 amino acids at its N-terminus including also the cyclin-binding PITAIRE motif.

The cDNA library used in the screen was made of young, emerging nodules and characterized by sequencing a limited number of ESTs. This analysis showed that in addition to nodulin genes that are specifically expressed during nodule development, housekeeping genes (e.g. genes playing role in cell wall synthesis, membrane transport, transcription, translation, etc.) and cell cycle genes are also present in the library (Györgyey *et al.*, 2000).

Screening of approx. $9x10^5$ independent transformants yielded 78 positive interactors, all representing cDNA clones of various lengths coding for the same protein. The longest cDNA was of 1390 bp harbouring a complete open reading frame of 1119 bp that codes for a 372-amino acid protein with a relative molecular mass of 42.6 kDa (Figure 6). Three groups of shorter cDNAs were isolated: all were truncated at the 5' end, while the 3' end contained in each case the stop codon. These subclones were 297, 417 and 426 bp shorter than the longest cDNA but all interacted with similar intensity to the CDKC;1 protein.

```
                        M  S  F  V  R  N  F  Q  A  E  D  C  N      13
ttcgctgcttcctcaggaattATGTCTTTTGTTCGGAATTTTCAAGCTGAAGACTGTAAT     60

L  N  G  G  Y  R  S  T  Y  D  G  N  N  T  D  R  K  R  S  R      33
TTAAACGGTGGTTATCGGTCCACCTATGATGGAAACAACACTGATAGGAAAAGAAGCCGA    120

N  H  Y  N  Q  R  N  Y  N  N  Y  Y  D  N  H  N  Q  A  N  F      53
AACCATTATAATCAGAGGAATTATAACAACTACTATGACAATCACAATCAGGCCAACTTT    180

G  Y  Y  G  G  N  F  N  Q  C  N  A  D  Y  A  N  Y  A  N  V      73
GGCTACTATGGTGGCAACTTCAACCAATGCAATGCTGACTATGCTAATTATGCCAATGTT    240

A  S  S  S  L  K  K  R  K  Y  S  A  P  V  R  G  E  S  Q  K      93
GCCTCATCATCTCTGAAGAAGAGAAAATATTCAGCTCCTGTTCGTGGAGAAAGCCAGAAG    300

F  T  L  P  A  T  V  Y  D  S  I  P  S  S  R  N  F  Q  A  Y     113
TTCACTCTTCCAGCCACAGTCTATGACAGTATCCCTTCATCCCGCAATTTTCAAGCTTAT    360

P  A  R  S  I  A  Y  N  S  T  S  A  S  L  K  P  D  F  S  I     133
CCTGCAAGATCCATTGCTTATAACTCTACCTCAGCTAGCCTGAAGCCCGATTTCTCTATA    420

F  D  D  D  K  P  I  F  M  S  R  D  D  I  D  R  N  S  P  S     153
TTTGATGATGATAAGCCTATCTTTATGTCAAGGGATGATATTGATAGAAACTCTCCATCA    480

R  K  D  G  I  D  V  L  H  E  T  H  L  R  Y  S  Y  C  A  F     173
AGAAAAGATGGCATTGATGTGCTTCATGAAACACACTTGCGGTATTCTTATTGTGCCTTC    540

L  Q  N  L  G  T  R  L  E  M  P  Q  T  T  I  G  T  S  M  V     193
CTTCAGAATCTCGGAACAAGGCTTGAGATGCCTCAAACTACCATTGGGACATCCATGGTT    600

L  C  H  R  F  F  V  R  R  S  H  A  C  H  D  R  F  L  I  A     213
CTGTGCCACCGTTTTTTTGTTCGGCGATCGCATGCTTGCCATGACAGATTTTTGATAGCT    660

T  A  A  L  F  L  A  G  K  S  E  E  S  P  C  P  L  N  S  V     233
ACTGCTGCTCTTTTTCTTGCTGGGAAGTCTGAAGAATCTCCATGCCCTTTGAATAGCGTA    720

L  R  T  S  S  E  L  L  H  K  Q  D  F  A  F  L  S  Y  W  F     253
TTGAGAACATCCAGTGAACTCTTACACAAACAAGATTTTGCTTTCTTATCCTACTGGTTT    780

P  V  D  W  F  E  Q  Y  R  E  R  V  L  E  A  E  Q  L  I  L     273
CCTGTTGATTGGTTTGAACAGTATCGTGAACGGGTGCTTGAAGCTGAACAGTTGATACTC    840

T  T  L  N  F  E  L  G  V  Q  H  P  Y  A  P  L  T  S  V  L     293
ACTACTCTAAATTTTGAACTCGGTGTACAGCATCCATACGCACCTCTTACATCTGTTCTG    900

N  K  L  G  L  S  K  T  V  L  V  N  M  A  L  N  L  V  S  E     313
AACAAATTAGGTCTTTCAAAGACCGTTTTGGTGAATATGGCGTTAAACTTGGTCAGTGAA    960

G  L  R  S  S  L  W  L  Q  F  K  P  H  Q  I  A  A  G  A  A     333
GGGCTTCGTAGCTCTCTTTGGCTTCAATTTAAACCTCATCAAATTGCTGCTGGAGCTGCT   1020

Y  L  A  A  K  F  L  N  M  D  L  A  A  Y  K  N  I  W  Q  E     353
TACCTTGCTGCCAAGTTTTTGAACATGGACCTTGCTGCCTATAAGAATATCTGGCAAGAG   1080

F  Q  A  T  P  S  V  L  Q  D  V  S  Q  Q  L  M  E  L  F  *     372
TTTCAGGCAACCCCATCTGTTCTTCAAGATGTCTCTCAGCAGTTAATGGAGCTCTTTTAG   1140

aagatagaagaaatgctgtataccatgatttcagtatagagtagattttaaatacttaac   1200

atacttgagaagtagaattactacaggtcatcagtgttgtttctggtgtttggatcctac   1260

ccgtgcatgcacataaaagagtttgaaacttggaatatgcgattatttggtttggatgtt   1320

aattttgttgtttgtctaggcgtcattggaaataagataattaatttttttaaaaaaaaa   1380

aaaaaaaaaa  1390
```

Figure 6. ***cDNA and protein sequence of*** **Medicago truncatula** ***cyclin T.*** Double underline marks the beginning of original subclones isolated by the yeast two-hybrid screen. Cyclin box is highlighted in bold; amino acid residues predicted to form α-helices outside the cyclin box are underlined. The sequence is deposited in GenBank under the accession number AY338488.

Analysis of the predicted protein sequence identified a cyclin box domain between amino acids 137 and 281. This domain contains the CDK-binding site and is present in all cyclins identified so far (Nugent *et al.*, 1991). Consistently, all the truncated clones isolated in the screen contained the whole-length cyclin box indicating that this domain is required for the interaction with the CDKC;1 protein.

Sequence similarity searches classified the newly identified cyclin among *Arabidopsis* cyclin T and T-like proteins and metazoan T and K-type cyclins (alignment in Appendix II, Table 4).

	MtCycT	AtCycT	AtCycT-like1	AtCycT-like2	HsCycT1	HsCycT2	HsCycK
MtCycT	–	45	49	48	36	30	29
AtCycT	69	–	59	57	37	31	22
AtCycT-like1	69	73	–	93	42	38	35
AtCycT-like2	68	75	95	–	41	37	32
HsCycT1	53	60	60	60	–	75	39
HsCycT2	52	59	61	60	86	–	34
HsCycK	52	45	56	56	57	58	–

Table 4. Similarity (blue) **and identity** (red) **among the cyclin box of T-type cyclins** (given in percentage).

T cyclins of animals are quite divergent in their sequence except for the cyclin box located in the N-terminal part and a histidine-rich stretch in the carboxy-terminal part of the proteins. This His-rich region binds to and probably facilitates the recognition of the carboxy-terminal domain of RNA polymerase II, whereas the recruitment of kinase-cyclin complex to the promoter is probably carried out via promoter-specific activator proteins. Human cyclin T1 contains a PEST signature at its extreme C-terminus, but it is still uncertain whether it directs the complex for ubiquitination (Chapter III). Similarly to metazoan cyclin Ts, the highest homology among the recently identified three T-type cyclins from *Arabidopsis* and the new *Medicago* cyclin is detected in the cyclin box region (Table 4) (Barroco *et al.*, 2003). Plant T-type cyclins do not show any additional recognizable motifs, but analysis of their predicted secondary structure shows four helical domains after the five-helix bundle of the cyclin box. This feature seems to be conserved in animal T and K-type cyclins (Figure 7), and some degree of sequence homology can be detected in this region as well suggesting a structural similarity for this family of cyclins. It

is reminiscent of the second five-helix bundle, also called cyclin_C domain, found in many cyclins. The cyclin core comprising the cyclin box (or cyclin_N domain) and the cyclin_C domain displays a conserved folding. The kinase-cyclin interface is basically provided by helices of the cyclin box, whereas other helical structures (such as cyclin_C domain or helices outside the cyclin core) may contribute to this interaction or to the binding of substrates and/or regulators (Goda *et al.*, 2001; Jeffrey *et al.*, 1995; Morgan, 1997). That no cyclin subclone lacking this four-helix bundle was identified in the screen suggests that, in addition to the cyclin box, this domain might also be involved in the interaction with CDKC;1.

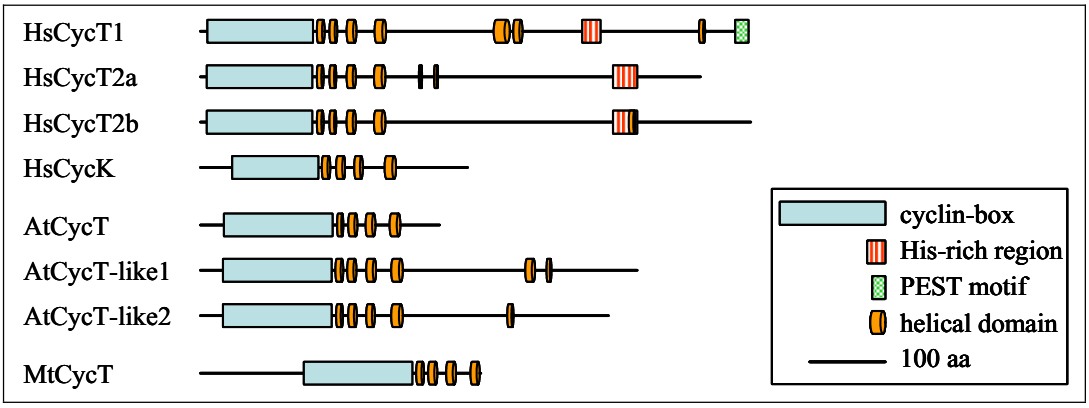

Figure 7. ***Structure of human and plant T-type cyclins.***

Sequence homology alone turned out to be insufficient to classify the newly identified cyclin, as it is almost equally homologous to members of both K and T classes (Appendix II, Table 4). To clarify this question, we took advantage of the ability of human cyclin K to rescue the lethality of G1 cyclin deletion in yeast (Edwards *et al.*, 1998). The *S. cerevisiae* strain Y145 lacks *cln1*, *cln2* and *cln3* genes and is kept alive by an integrated *GAL::CLN3* gene on galactose-containing medium. When plated on medium containing glucose instead of galactose, the inserted *CLN3* gene is not expressed and yeast cells fail to grow. We tested whether the cDNA coding for the *Medicago* cyclin could rescue this conditional lethality when introduced into this strain. These complementation experiments gave negative results independently of the choice of expression plasmid. This suggested that the identified *Medicago* cyclin belongs to the cyclin T rather than to the cyclin K family and thus was designated as MtCycT;1.

The specificity of the interaction of CDKC;1 with the cyclin protein was tested using different *Medicago* cyclins and cyclin-dependent kinases (Figure 8). In these pairwise yeast two-hybrid assays, CDKC;1 and cyclin T bound strongly and exclusively to each other, but not to other cyclins or CDKs.

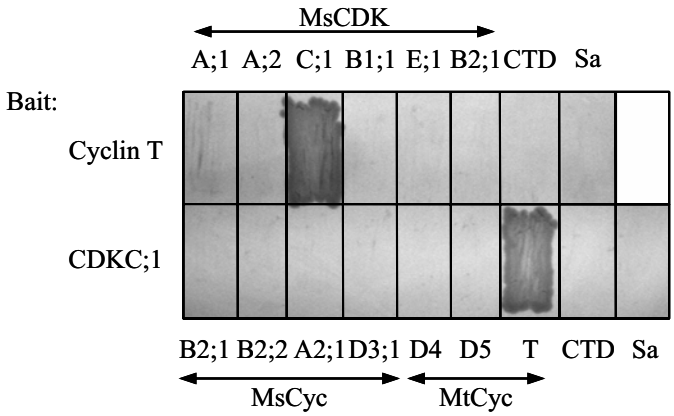

Figure 8. ***The interaction between cyclin T and CDKC;1 is specific.*** In pairwise yeast two-hybrid assays cyclin T binds to CDKC;1 but not to other classes of CDKs (upper row). Similarly, CDKC;1 fails to interact with other types of cyclins than cyclin T (lower row). Control of the bait construct for self-activation is shown on the right (Sa). Co-transformed yeast cells were plated on synthetic drop-out medium lacking tryptophan, leucine, histidine and adenine.

To confirm the interaction between CDKC;1 and cyclin T *in vivo*, the proteins were expressed transiently in *Arabidopsis* cell suspension protoplasts and tested for binding in immunoprecipitation assay. The lack of a reproducible transfection method for alfalfa protoplasts compelled us to opt for this heterologous assay system. This choice was validated by later biochemical experiments where proteins expressed in this way behaved identically to the endogenous CDKC;1 immunocomplex. For transient expression, cDNAs coding for CDKC;1 and cyclin T were introduced in frame with three myc- or hemagglutinin (HA)-epitopes and expressed under the control of the cauliflower mosaic virus 35S promoter either alone or together. The proteins were then immunoprecipitated from the *Arabidopsis* protein extracts with anti-HA antibodies and the presence of the myc-tagged partner was verified by anti-myc immunoblot. These experiments clearly demonstrated the complex formation between CDKC;1 and cyclin T proteins *in vivo* (Figure 9).

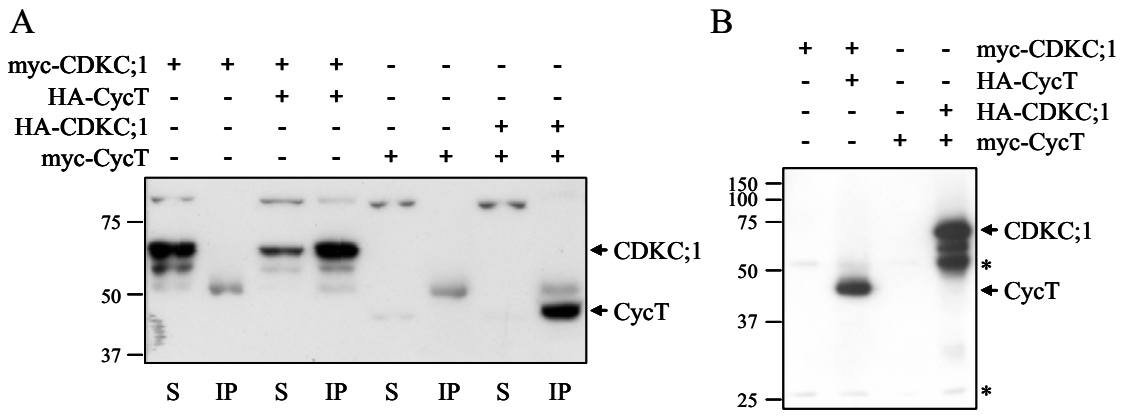

Figure 9. ***CDKC;1 and cyclin T interact*** **in vivo.** HA- and myc-tagged proteins were expressed in *Arabidopsis* protoplasts and immunoprecipitated from lysates using anti-HA antibody (3F10 clone). **(A)** myc-tagged proteins were revealed by anti-myc immunoblot in the supernatants of the reactions (S) and immunoprecipitated complexes (IP). **(B)** The efficiency of immunoprecipitation is verified by anti-HA (12CA5 clone) immunoblot. Asterisks mark the positions of heavy and light chains of IgG.

Characterization of the kinase activity of CDKC;1

Plant cyclin-dependent kinases characterized so far all participate in the regulation of cell cycle (Dewitte and Murray, 2003). However, from recent experimental data it is becoming evident that some plant CDKs similarly to animal ones, participate in other cellular processes as well.

The CDKC;1 protein is quite divergent in its sequence from the other alfalfa kinases. Its cyclin-binding motif differs from that of the A- and B-type CDKs and is most related to metazoan Cdk9 kinases. In addition to the highly homologous kinase domains, all known plant C-type kinases contain a long, divergent C-terminal extension with as yet undefined function (alignment in Appendix I).

For the characterization of this *Medicago* kinase-cyclin pair, specific antibodies were prepared against the CDKC;1 protein. To rule out possible cross-reaction with other CDKs, only the region coding for the non-conserved C-terminal part was cloned as translational fusion with six histidine residues into a bacterial expression vector. The purified protein was used to immunize rabbits and the obtained serum was tested for specific recognition on

in vitro translated [^{35}S]methionine-labelled CDKC;1 protein (Figure 10A). For affinity-purification, the whole-length protein was expressed in a different bacterial system. The purified polyclonal antibody detected a single band on alfalfa protein extracts with a size of approx. 56 kDa matching well to the predicted 56.4 kDa size of the CDKC;1 protein (Figure 10B).

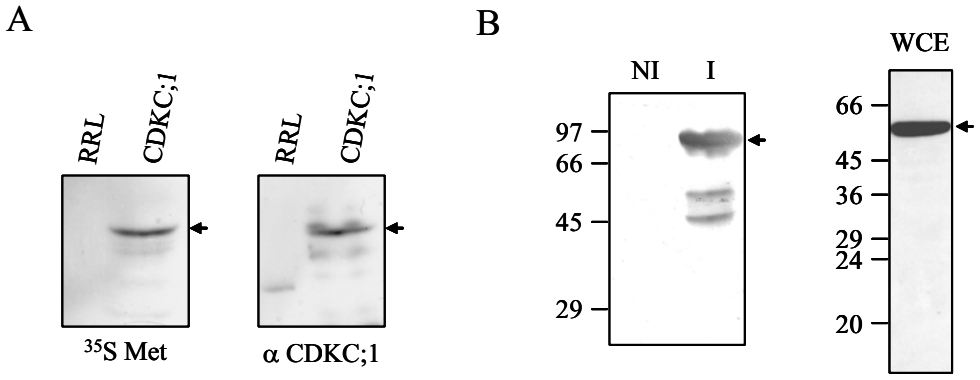

Figure 10. ***The polyclonal anti-MsCDKC;1 antibody recognizes the MsCDKC;1 protein specifically.*** **(A)** The crude, non purified serum recognizes *in vitro* translated [^{35}S]methionine-labelled MsCDKC;1. The protein was expressed in rabbit reticulocyte lysate, separated by SDS-PAGE and blotted onto PVDF membrane. The membrane was autoradiographed (left panel) and then immunoblotted with anti-CDKC;1 serum (right panel). RRL (rabbit reticulocyte lysate) denotes the control reaction, where no DNA encoding CDKC;1 was present. **(B)** The affinity purified antibody detects CDKC;1 fused to cellulose-binding domain when assayed on lysate of bacteria induced to express the protein (I) but not in non-induced culture (NI) (left panel). It gives a single band on alfalfa whole cell protein extract (WCE) when assayed in immunoblot experiments. Molecular mass standards are given in kDa. Arrowheads mark the signal corresponding to the CDKC;1 protein.

To assess the activity of the kinase, CDKC;1-containing complexes were immunoprecipitated from lysates of alfalfa suspension cells and tested in kinase assays with different proteins known to be good *in vitro* substrates for cyclin-dependent kinases. Histone H1 is the most widely used substrate, its (S/T)PK(K/R) motif is a target for most CDKs. Myelin basic protein is a typical MAP kinase target but is also phosphorylated by Cdc2-type kinases. The C-terminal domain of RNA polymerase II is targeted by metazoan Cdk7/8/9 kinases *in vitro* and *in vivo* as well. The retinoblastoma protein is an *in vivo* substrate for many CDKs that are active at the G1/S transition.

In our experiments, the immunocomplex of CDKC;1 failed to phosphorylate the typical CDK substrates histone H1 and β-casein (Figure 11). In contrast, a very potent kinase

activity could be detected towards the synthetic peptide comprising six copies of the repetitive heptapeptide of the CTD of RNA polymerase II. Myelin basic protein and the human retinoblastoma protein were also phosphorylated, although to a lesser extent. Autophosporylation of the kinase was not detected in these and any subsequent *in vitro* kinase assays.

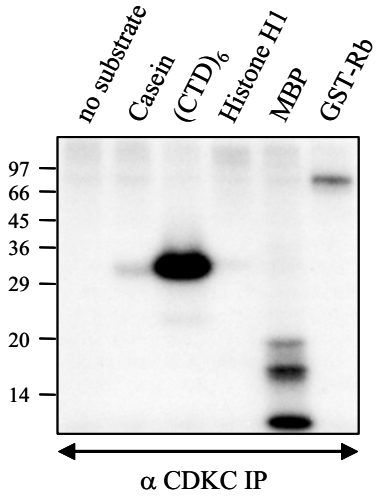

Figure 11. In vitro *substrate specificity of the CDKC;1 kinase.* CDKC;1 complexes were immunoprecipitated from protein extracts of alfalfa suspension cells and assayed for kinase activity with the indicated substrates. Proteins were separated by SDS-PAGE and revealed by autoradiography. Molecular mass standards are given in kDa.

	MsCDKA;1/A;2	MsCDKB1;1	MsCDKB2;1	MsCDKC;1
Histone H1	+++	+	++	–
MBP	+++	–	–	++
CTD	+++	–	–	+++
Retinoblastoma	+++	+	–	+

Table 5. In vitro *substrate specificity of alfalfa cyclin-dependent kinases* (after Mészáros, T., PhD thesis, 1999; and this work).

These substrate specificity data clearly distinguish the CDKC;1 kinase from the already described *Medicago* and other plant CDK kinases (Table 5) and suggest a distinct phosphorylation site preference. This substrate range resembles that of human Cdk9 except for the lack of autophosphorylation. Autophosphorylation of Cdk9 at several C-terminal residues has been shown to affect the subcellular localization of the kinase in human cells (Chapter II).

The regulation of CDK activity is generally posttranslational; i.e. constant mRNA and protein levels during the cell cycle are associated with fluctuating catalytical activity. Plant

A-type kinases follow this pattern, whereas B-type kinases have phase-specific expression as well. Since it was shown previously that the mRNA of CDKC;1 is invariably present in all phases of the cell cycle (Magyar *et al.*, 1997), we tested whether its protein level or the associated kinase activity would show any cell cycle dependent fluctuation. For this, alfalfa suspension cultured cells were blocked by hydroxyurea treatment to the G1/S transition. After the removal of the blocking agent, cell cycle progression was followed by flow cytometric analysis and samples were taken by 2-hour intervals.

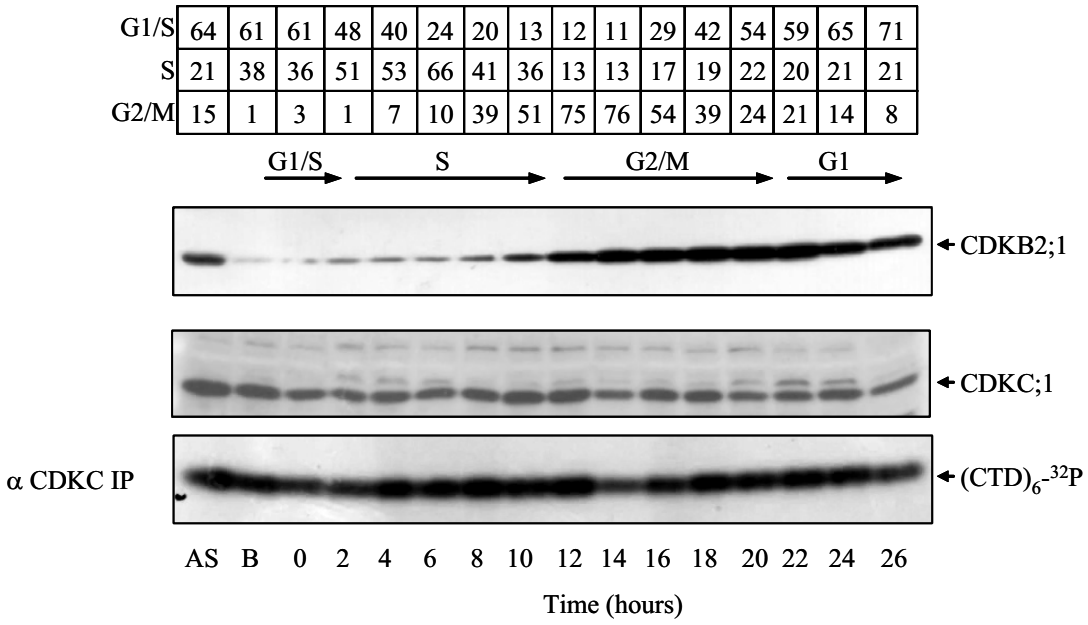

Figure 12. ***Both the protein level and the kinase activity of MsCDKC;1 are constant in different cell cycle phases.*** The distribution of cells among different cell cycle phases in percentage was assessed by measuring the nuclear DNA content by flow cytometry. MsCDKB2;1 and CDKC;1 protein levels were detected by immunoblotting with the respective antibodies from synchronized samples containing equal amount of proteins (first two panels). For the kinase assay, CTD peptide was added to CDKC;1 immunocomplexes, reaction mixtures were resolved by SDS-PAGE and visualized by autoradiography (lower panel). Cells were harvested for analysis at the indicated times where 0h corresponds to the release of the block. AS, asynchronous culture; B, hydoxyurea block.

To characterize the synchronicity, MsCDKB2;1 protein levels were determined by immunoblot in the different samples. This B-type alfalfa kinase is known to be present only in G2/M phases (Magyar *et al.*, 1997). In contrast, the level of the CDKC;1 protein remained fairly unchanged throughout the progression of the cell cycle. Similarly, no peak could be detected when the kinase activity of the CDKC;1 complex was analyzed by

immunoprecipitation-CTD kinase assay (Figure 12). This result further supports the idea, that the *Medicago* CDKC;1 kinase is not or not directly involved in the control of cell cycle. This result is in accordance with data on the human Cdk7/8/9 kinases which show substantially constant CTD kinase activities throughout the cell cycle (Oelgeschlager, 2002).

Cyclin function is primarily controlled by changes in protein level due to controlled transcription/expression and degradation (Morgan, 1995). Similarly to CDKC;1, the transcript of cyclin T was present in all cell cycle phases (data not shown). Unfortunately, its protein level could not be verified in the samples of synchronized cells because of the lack of specific antibodies. However, the finding that the kinase activity is constant during the cell cycle suggests a similar pattern for the level of cyclin T as well, since cyclins are required for the full activity of CDKs. To demonstrate that the kinase activity observed in the latter and other experiments is really due to the promoting effect of cyclin T binding we had recourse to epitope-tagged proteins expressed in *Arabidopsis* protoplasts. CDKC;1 and cyclin T proteins were expressed alone and together in protoplasts, immunoprecipitated with anti-epitope antibodies from the protein lysates and assayed by *in vitro* kinase assays (Figure 13).

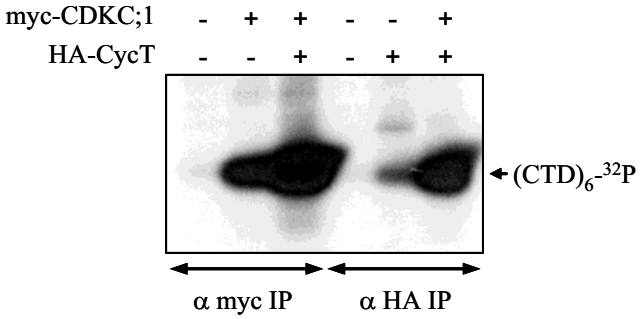

Figure 13. ***Cyclin T binding to CDKC;1 promotes its kinase activity.*** Tagged proteins were expressed in *Arabidopsis* and immunoprecipitated with anti-tag antibodies as indicated on the bottom of the figure. Immunocomplexes were assayed for CTD kinase activity, and subjected to SDS-PAGE. The dried gel was autoradiographed.

When both proteins were present, a strong CTD kinase activity was detected independently of which of the proteins was used for the immunoprecipitation. In the absence of *Medicago* CDKC;1, cyclin T alone had only a slight kinase activity probably

due to cross-reaction with endogenous *Arabidopsis* C-type kinases. CDKC;1 alone displays somewhat higher kinase activity towards the CTD which can be explained in two ways: (i) the crossreactivity between MsCDKC;1 and *Arabidopsis* T-type cyclins is stronger than in the inverse case; (ii) alternatively, although the extent of crossreactivity is comparable, the *Medicago* kinase is more potent than its *Arabidopsis* homologues. In *Arabidopsis*, two CDKC and three cyclin T proteins have been described, and a recent analysis identified two additional T-type cyclins (Barroco *et al.*, 2003; Wang *et al.*, 2004). Despite the possible cross-reaction, the increase in the CTD kinase activity detected in the presence of both *Medicago* proteins is convincing. This reinforces that the binding of cyclin T promotes the activity of the kinase and thus CDKC;1 and cyclin T form a functional complex.

Subcellular localization of CDKC;1 and cyclin T

The subcellular localization of CDK-cyclin complexes is generally under strict control. Besides designating the range of substrates it renders the complex accessible for regulatory proteins (Yang and Kornbluth, 1997).

As first approach to study the subcellular localization of the CDKC;1-cyclin T complex, nuclear and cytoplasmic fractions were prepared from alfalfa suspension cells. Immunoblots with anti-CDKC;1 antibody revealed the vast majority of the protein in the nuclear lysate, whereas practically no signal was detected in the cytoplasmic fraction (Figure 14A). The CTD kinase activity of the complex showed the same pattern further supporting that the activity detected in our kinase assays was really associated with CDKC;1. As expected, the potential target RNA polymerase II was also highly enriched in the nuclear fraction.

The localizations of both proteins were determined by immunostaining on *Arabidopsis* protoplasts expressing transiently the epitope-tagged versions of CDKC;1 and cyclin T (Figure 14B). Consistently with the result of subcellular fractionation, CDKC;1 was found exclusively in the nucleus, evenly dispersed in the nucleoplasm and completely excluded from the nucleolus. The same pattern was found independently of the presence of cyclin T. Identical distribution could be observed for cyclin T in single and co-transformed cells as well, although in this case a weak cytoplasmic signal was also visible, which might mean that a small fraction of cyclin T was localized to the cytoplasm. However, it is more likely

that this signal was due to non-specific labelling as similar staining could be observed in the control samples lacking the tagged protein.

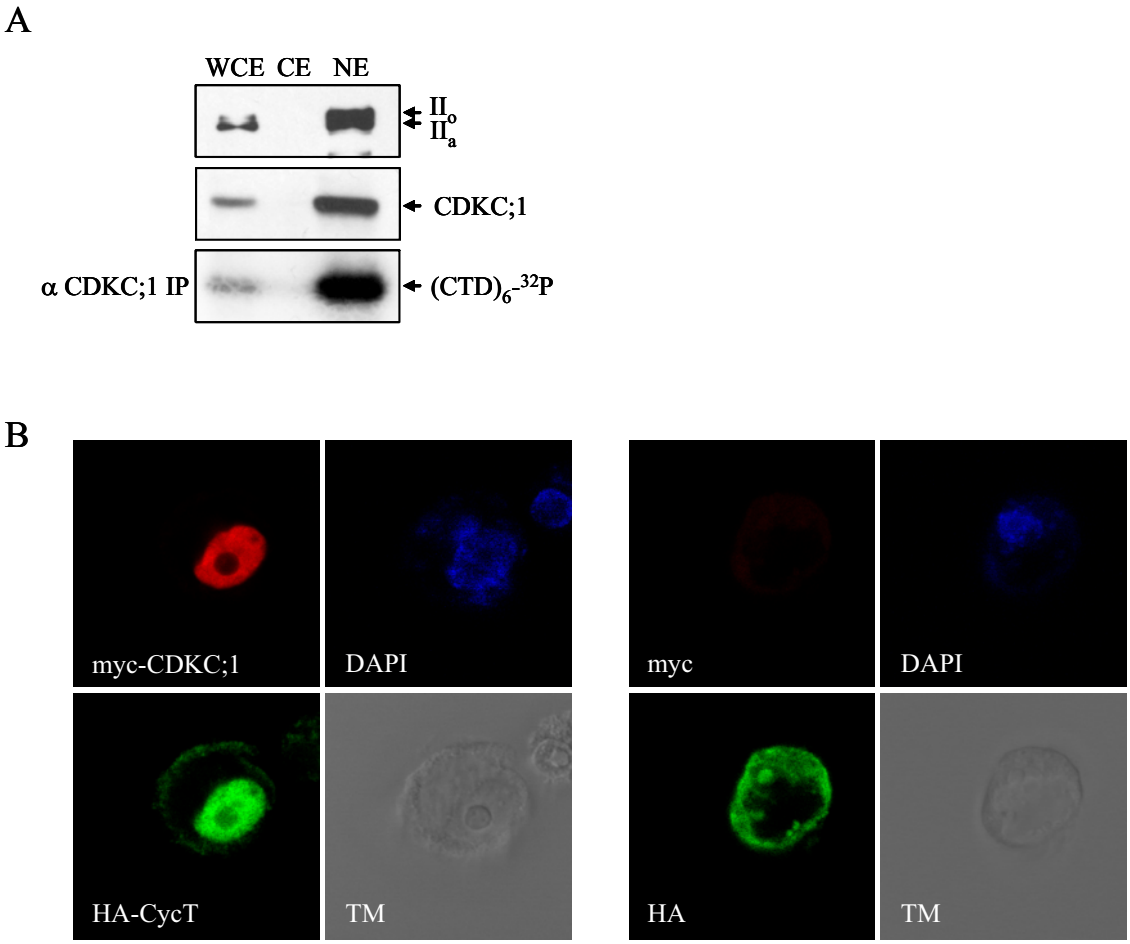

Figure 14. ***The CDKC;1-cyclin T complex is nuclear.*** **(A)** Protein extracts from whole cells (WCE), cytosolic (CE) and nuclear (NE) fractions were prepared from alfalfa suspension cells and tested for the presence of CDKC;1 and its associated CTD kinase activity as described previously. Hypo- and hyperphosphorylated forms of RNA polymerase II (indicated by II_a and II_o, respectively) were detected using the 8WG16 anti-Pol II antibody. **(B)** *Arabidopsis* protoplasts were co-transformed with plasmids expressing either myc-CDKC;1 and HA-cyclin T (left panel) or with plasmids expressing only the epitopes (right panel). Primary anti-tag antibodies, anti-myc and anti-HA, were labelled with TRITC- or Alexa 488-conjugated secondary antibodies, respectively. Nuclei were counterstained with DAPI; TM is for transmission.

In human cells, cyclin T1 is invariably localized to the nucleus to speckle-like structures, while Cdk9 is predominantly nuclear but a small proportion is present in the cytoplasm as well. The nuclear localization of Cdk9 is promoted by cyclin T and the autophosphorylation

of the kinase on residues close to its C-terminus is also required (Chapter II). In our kinase assays, we failed to detect any autophosphorylation of CDKC;1 and consistent with this, sequence homology of plant and human kinases does not extend to the carboxy-terminal region (Appendix I). Interestingly, algorithms predicting subcellular localization for proteins (http://psort.ims.u-tokyo.ac.jp) place all known plant C-type kinases with high certainty to the nucleus, as they detect in each protein a domain containing mainly basic residues. This motif, found around the 360 aa position, is highly conserved amongst the plant proteins but is completely absent from metazoan kinases (Appendix I). Although positively charged residues are abundant in nuclear localization signals in plants, animals and yeast as well, no real consensus for nuclear targeting can be designated, the experimental determination of such sequences is still the best method of identification (Cokol *et al.*, 2000; Moriguchi *et al.*, 2005). Keeping this in mind, the hypothesis that plants define the subcellular localization of the CDKC;1-cyclin T complex differently from animal Cdk9-cyclin T is appealing. The lack of autophosphorylation of CDKC;1 and the fact that both CDKC;1 and cyclin T displayed nuclear localization when expressed separately suggests that the nuclear localization might be an intrinsic property of both proteins or at least not directly influenced by each other.

In contrast to the human Cdk9-cyclin T complex, neither CDKC;1 nor cyclin T showed accumulation at discrete foci within the nucleus; although nuclear speckles, that function most probably in the storage and assembly of splicing components, can be detected in plants as well (Kalyna and Barta, 2004). The even distribution of the *Medicago* CDKC;1-cyclin T complex resembles more the pattern of two other human CTD kinases, Cdk7 and Cdk8 (Herrmann and Mancini, 2001). However, several factors might disturb the proper subnuclear localization of CDKC;1-cyclin T in our experimental system: (i) the proteins are significantly overexpressed relative to the endogenous level, thus physiological anchoring sites might be saturated; (ii) the *Medicago* complex can not or not sufficiently bind to corresponding *Arabidopsis* proteins due to heterologous expression; or (iii) the N-terminal tagging of the proteins might interfere with the subnuclear targeting of the complex. The distribution of endogenous CDKC;1 within the nucleus could be revealed by immunolocalization studies on alfalfa cells using the polyclonal anti-CDKC;1 antibody.

Besides nuclear localization, which is a prerequisite for kinases targeting RNA polymerase II, another important characteristic of CTD kinases is whether they are stably

associated with the Pol II transcription complex at any time of transcription. From the most studied human kinases it is known, that while complexes of Cdk7 and Cdk8 are found in association with preinitiation complexes, the binding of Cdk9 to Pol II is not detectable (Chapter II). Direct association of CDKC;1 or cyclin T to CTD is not supported by yeast two-hybrid data (Figure 8); however, the kinase-cyclin complex could still be tethered to Pol II via other proteins present in the holoenzyme.

To determine whether the *Medicago* CDKC;1 complex could be tightly bound to Pol II, nuclear extract of alfalfa cells was size-fractionated by glycerol gradient centrifugation.

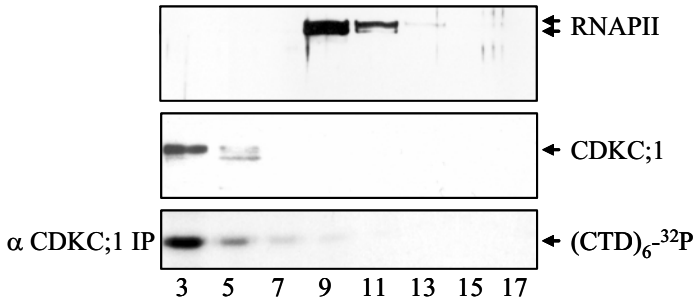

Figure 15. ***The CDKC;1 complex is not stably associated with RNA polymerase II.*** RNA polymerase II and CDKC;1 were detected in different glycerol gradient fractions by immunoblot (fraction numbers indicated at the bottom). CTD kinase activity of the CDKC;1 immunocomplex was revealed by autoradiography.

This method allows gentle isolation of large macromolecular complexes and thus suits to study associated proteins. Immunoblot analysis of the fractions revealed the presence of the bulk of CDKC;1 protein and its associated kinase activity in a different complex of smaller size than that of Pol II which argues against the idea of stable interaction between the C-type kinase and the Pol II holoenzyme (Figure 15). The result is further supported by immunoprecipitation data, where no interaction was detected between Pol II and CDKC;1 (data not shown). This implies that recruitment of the CDKC;1 complex to Pol II might be highly dynamic similarly to the animal Cdk9-cyclin T complexes (Herrmann and Mancini, 2001, Lis *et al.*, 2000).

Function of the CDKC;1-cyclin T complex in basal transcription

The properties of the CDKC;1 kinase, as revealed by all the previous experiments, are very distinct from other plant CDKs and more related to that of metazoan Cdk9 proteins.

Besides sequence homology, the *in vitro* substrate range of the two kinase complexes is similar. Moreover, the protein level and catalytical activity of both types of kinases remains unchanged during cell cycle transitions. The functional resemblance is further supported by the nuclear localization of both plant and animal complexes. All these findings together support the hypothesis that the CDKC;1-cyclin T complex might be the plant counterpart of metazoan positive transcription elongation factor b.

In order to test whether the *Medicago* CDKC;1 complex has similar functions as the Cdk9-containing P-TEFb, its effect on basal transcription was studied.

The first step towards the characterization of a potential CTD kinase is to identify its target specificity within the repetitive heptapeptide. In studies aiming at characterizing CTD kinases, two different methods are used to determine the site preference of the enzymes. Kinase activity can be assayed (i) using synthetic peptides consisting of a few repeats where phosphorylatable residues are replaced by non-phosphorylatable alanine one by one or (ii) mapping site preference with phosphoepitope-specific antibodies on intact Pol II or isolated CTD of variable length. Although there are five potential phosphorylation sites within the peptide and four of them can be potentially targeted by the Ser/Thr-specific CDKs, only phosphorylation of serines in positions 2 and 5 has been correlated with transcription elongation (Chapter II).

To assess the target site preference of the CDKC;1 kinase, a set of GST-fusion peptides were constructed containing either the wild type or Ser-mutated versions of the CTD repeat. In kinase assays, the wild type peptide but not GST alone was efficiently labelled, supporting that the reaction is specific towards the peptide. Mutation of Ser^2 to alanine barely altered the efficiency of the reaction, whereas replacement of Ser^5 with nonphosphorylatable alanine completely abolished phosphorylation. This suggests, that at least *in vitro* and on short CTD peptides CDKC;1-cyclin T targets Ser^5 within the repetitive heptapeptide.

However, these *in vitro* data have to be considered with precaution as they might differ from data obtained by other approaches and especially from what is observed *in vivo*. This discrepancy can be resolved by considering the complexity of CTD where each phosphorylation site is unique and may be influenced by the phosphorylation state of neighbouring residues or the presence of degenerated repeats. Metazoan Cdk7/8/9 kinases

all target Ser[5] *in vitro* on short peptides, whereas *in vivo* Cdk9 generates mostly phospho-Ser[2] (Palancade and Bensaude, 2003; Ramanathan *et al.*, 2001).

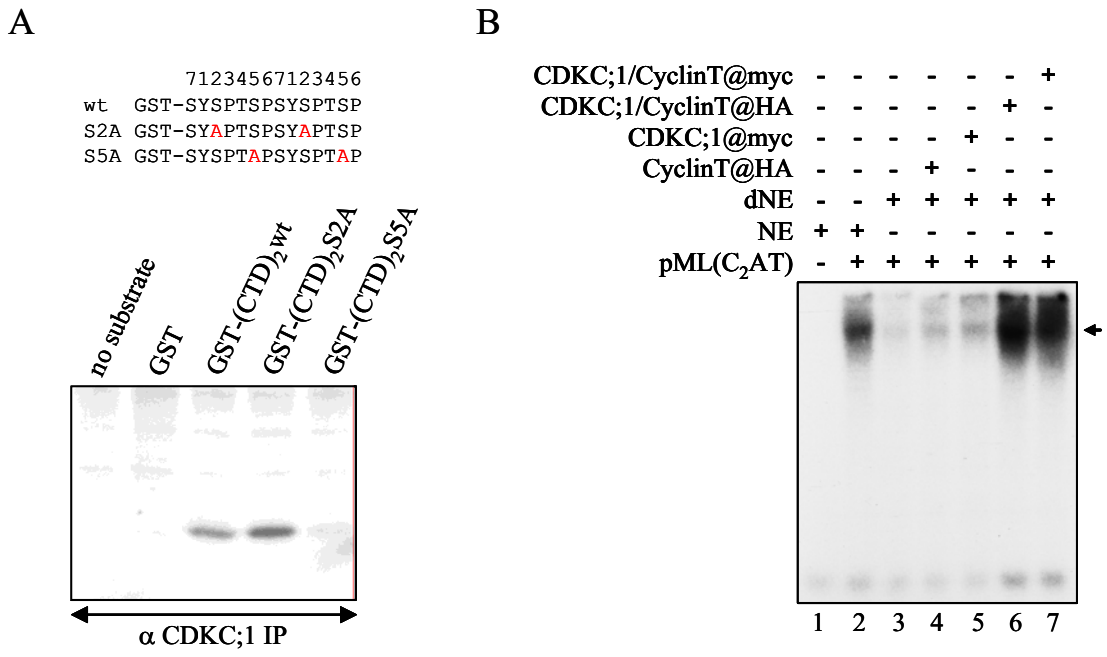

Figure 16. ***The CDKC;1-cyclin T complex targets Ser[5] within the heptapeptide and functions in basal transcription.*** **(A)** For the kinase reaction CDKC;1 was immunoprecipitated from alfalfa protein extract and assayed with the indicated wild-type and Ser-mutated CTD repeats fused to GST. Phosphorylation was detected as previously. **(B)** Aliquots of Cdk9-depleted HeLa nuclear extract (dNE) were supplemented with *Medicago* HA-cyclin T and myc-CDKC;1 proteins or myc-CDKC;1-HA-cyclin T complexes that were immunoprecipitated from transfected *Arabidopsis* protoplasts. Transcription reactions were performed in the presence of [α-^{32}P]UTP and pML(C_2AT) plasmid DNA as template. Reaction products were analyzed on a 5% denaturing gel and visualized by autoradiography. Arrowhead marks the position of the promoter-specific radiolabelled transcript.

The effect of the CDKC;1-cyclin T complex on RNA polymerase II-mediated transcription was studied in an *in vitro* transcription system. These systems allow to study the individual steps of transcription and the identification of participating factors. The method is well established in vertebrate and yeast cells but reported only for a few plant species (Schwechheimer *et al.*, 1998; Sugiura, 1997). In plants, both the presence of a rigid cell wall which renders difficult the mild lysis of cells and high protease and nuclease activities hamper the preparation of transcriptionally active extracts. In addition, secondary metabolites accumulated in vacuoles might also inhibit transcription. Since all our attempts

to work out an *in vitro* transcription system using alfalfa cell extracts failed, we decided to use a heterologous system based on nuclear extracts of human cells. This system permitted to study the effect of *Medicago* CDKC;1-cyclin T on transcription and to test whether this complex could represent the plant counterpart of metazoan P-TEFb as it was suggested by its biochemical properties. As transcription template, a plasmid DNA containing the adenovirus major late promoter in fusion with a G-less cassette was applied (Sawadogo and Roeder, 1985). This cassette is an approx. 400-bp region that lacks G residues on the sense strand. If transcription is carried out in the absence of GTP, RNA synthesis terminates at the first G in the plasmid sequence, which – as the length of the G-free sequence is known – facilitates the detection of the promoter-specific transcript.

In order to render the transcriptional activity of the extract P-TEFb-dependent, endogenous Cdk9 was depleted from the HeLa extract by repeated rounds of immunoprecipitation with a Cdk9-specific antibody. It has been shown that such an immunodepletion doesn't reduce the concentration of other factors needed for transcription (Peng *et al.*, 1998a; Zhu *et al.*, 1997). As compared to the untreated nuclear extract, depletion of Cdk9 almost completely abolished transcriptional activity and reduced the amount of the radiolabelled transcript to a barely detectable level (Figure 16B, lanes 2 and 3).

When the transcription reaction mixture was supplemented either with HA-cyclin T or myc-CDKC;1 proteins that were immunoprecipitated with tag-specific antibodies from lysates of transfected *Arabidopsis* protoplasts, a slight increase in the rate of transcription could be observed (lanes 4 and 5). As discussed previously, this is probably due to complex formation, a small fraction of the expressed *Medicago* subunits associated with endogenous *Arabidopsis* proteins. Addition of immunoprecipitated *Medicago* CDKC;1-cyclin T complexes however not only restored the transcriptional activity of the depleted extract, but resulted in the activation of transcription (lanes 6 and 7).

All the previous results and that the *Medicago* CDKC;1-cyclin T complex is capable of complementing the missing P-TEFb function suggest that the complex is a functional orthologue of the human Cdk9-cyclin T pair.

SUMMARY AND CONCLUSIONS

Although it is now widely accepted that cyclin-dependent kinases represent a functionally heterologous family, the field of non-cell cycle kinases is still somehow neglected in plants. In this work we give the characterization of the alfalfa CDKC;1 protein that is the first detailed functional study of a plant C-type cyclin-dependent kinase. The major experimental results presented in this part of the thesis can be summarized as follows:

1. We identified a T-type cyclin as a specific partner of CDKC;1. The interaction occurs *in vitro* and *in vivo* as well; moreover, cyclin binding promotes the catalytical activity of CDKC;1.
2. The *in vitro* substrates of the CDKC;1 immunocomplex include the repetitive heptapeptide of the carboxy-terminal domain of RNA polymerase II, myelin basic protein and the retinoblastoma protein, but not the typical CDK substrates histone H1 and β-casein.
3. Both the protein level and the kinase activity of CDKC;1 remain unchanged during the cell cycle.
4. Both members of the complex, CDKC;1 as well as cyclin T are localized exclusively to the nucleus in a different complex than that of RNA polymerase II.
5. The kinase activity of CDKC;1-cyclin T targets the Ser^5 residue within the repetitive heptapeptide of CTD when assayed on short peptide substrates in *in vitro* conditions.
6. The *Medicago* CDKC;1-cyclin T complex can complement missing Cdk9 function in promoter-specific transcription assays using nuclear extracts of human cells.

Most of these basic functional characteristics of the *Medicago* CDKC;1-cyclin T complex overlap with that of metazoan P-TEFb. This, together with the high conservation of the structure of RNA polymerase II including also the specific carboxy-terminal domain

(Figure 2) and of the general transcription factors in plants as well (Nawrath *et al.*, 1990; Pan *et al.*, 2000) strongly supports the idea that the *Medicago* CDKC;1-cyclin T complex acts in the regulation of RNA polymerase II-mediated transcription and represents the plant orthologue of metazoan positive transcription elongation factor b.

Although the fundamental catalytical characteristics of the metazoan and plant complexes are similar, several data indicate that we can certainly expect specific features in their regulation and function as well.

The activity of the metazoan kinase is basically defined by its association with one of its cyclin partners. Cyclins promote the stability of Cdk9, define its localization and they might direct different functions of the complex.

The genomes of the model plants *Arabidopsis* and rice code for several T-type cyclins (Wang *et al.*, 2004). Despite this, yeast two-hybrid screen performed with the *Arabidopsis* CDKC;2 protein identified only one cyclin T (At1g27630) as interactor but not the other T-type cyclins (Barroco *et al.*, 2003). Similarly, although we do not know whether the *Medicago* genome codes for additional T-type cyclins, the screen for interactors of the alfalfa CDKC;1 protein yielded only clones of a single cyclin T. The closest structural homologue of the alfalfa cyclin is the identified cyclin partner of AtCDKC;2 indicating that this subtype might represent the predominant form of plant CDKC-cyclin complexes.

In mammalian T-type cyclins a histidine-rich motif interacting with the CTD directs the activity of Cdk9 to its substrate, thus these complexes are able to phosphorylate CTD even when they are not recruited directly to the CTD. Consistent with this, several transcription factors were found to bind and probably direct Cdk9-cyclin T to promoters. In contrast, cyclin K that lacks this motif can activate transcription in these *in vitro* experiments only when tethered to the proximity of CTD. RNA binding proteins might direct the complex *in vivo* as the nascent RNA strand moves along the CTD (Lin *et al.*, 2002b); however, the exact mechanism remains elusive. Similarly to cyclin K, plant T-type cyclins lack this histidine-rich motif. And although other sequences involved in the interaction of cyclin T2 and CTD were identified recently (Kurosu *et al.*, 2004), our yeast two-hybrid and immunoprecipitation data do not support the idea of a direct, stable interaction between the *Medicago* proteins and the CTD. This would suggest a cyclin K-like recruitment, but the fact that a transcription factor was also identified among the interactors of the *Arabidopsis*

CDKC;2 kinase could mean that the mechanism of recruitment might be similar to mammalian T-type cyclins.

Both the mammalian and the plant kinase complexes are nuclear, however their targeting likely involves different mechanisms. In contrast to mammalian cells where binding to cyclin T together with autophosphorylation activity is required for the targeting of Cdk9 to the nucleus, no autophosphorylation of the CDKC;1 kinase was detected. In addition, CDKC;1 was exclusively localized to the nucleus when expressed alone in *Arabidopsis* protoplasts. Thus, nuclear localization might be an intrinsic property of this protein as a nuclear localization signal is predicted at all known plant C-type kinases in a region non-homologous to metazoan kinases. Although speckled pattern, as it was found for Cdk9-cyclin T, is more consistent with a role in pre-mRNA processing, the fact that AtCDKC;2 interacts with a ribonucleoprotein (Barroco *et al.*, 2003) suggests that plant CDKC-cyclin T complexes could contribute to pre-RNA maturation.

The inhibition of P-TEFb activity in mammalian cells is exerted via the 7SK snRNA/HEXIM1 complex. Orthologues of these subunits can be identified in higher metazoan, but no 7SK RNA has been reported from plants (Gursoy *et al.*, 2000) and homology-based searches using the conserved domains of HEXIM1 do not yield significant matches from plant sequences. Consistently, we found a single complex of CDKC;1 by glycerol gradient fractionation, and RNase A treatment of isolated CDKC;1 immunocomplexes did not resulted in increased kinase activity.

Metazoan Cdk9 is a multifunctional kinase; besides acting in basal transcription it is involved in differentiation as well. The activity of the complex is essential in the differentiation of muscle cells by activating MyoD-dependent transcription which could also involve the phosphorylation of the retinoblastoma protein (Simone *et al.*, 2002). The *Medicago* complex is also capable of phosphorylating the Rb protein. Furthermore, *Arabidopsis* and tomato CDKC transcripts are present in differentiating generative tissues and developing fruit that might reinforce a possible role for the kinase in differentiation (Barroco *et al.*, 2003; Joubes *et al.*, 2001). To complete the picture about the possible functional diversity of plant CDKCs, it has to be mentioned that in addition to their homology to the PITALRE kinase Cdk9, they show homology also to metazoan PITAIRE kinases. This latter group is far from being characterized. These proteins bear long N- and

C-terminal extensions and could be involved in hematopoietic differentiation and pre-mRNA processing (Ko *et al.*, 2001; Lapidot-Liftson *et al.*, 1992; Marques *et al.*, 2000).

By describing the basic properties of the *Medicago* CDKC;1-cyclin T complex, we give the first indication on the function of plant C-type CDKs; at the same time our studies raise a large number of questions. In the coming years, it will be certainly worth answering them and discovering this unexplored field of plant science.

PART II. ANAPHASE-PROMOTING COMPLEXES IN *ARABIDOPSIS*

Arabidopsis Anaphase-Promoting Complexes: Multiple activators and the wide range of substrates might keep APC perpetually busy

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Key words: APC, Cdh1, mitotic cyclins, cyclin-dependent kinases, E3 ubiquitin ligase, E2 ubiquitin conjugating enzymes

ABSTRACT

The anaphase-promoting complex (APC), a multisubunit E3 ubiquitin ligase, is an essential regulator of the cell cycle from metaphase until S-phase in yeast and metazoans. APC mediates degradation of numerous cell cycle-related proteins, including mitotic cyclins and its activation and substrate-specificity are determined by two adaptor proteins, Cdc20 and Cdh1. Plants have multiple APC activators and the Cdh1-type proteins, in addition, are represented by two subclasses, known as Ccs52A and Ccs52B. The *Arabidopsis* genome contains five *cdc20* genes as well as *ccs52A1, ccs52A2* and *ccs52B*. In *Schizosaccharomyces pombe*, expression of the three *Atccs52* genes elicited distinct phenotypes supporting non-redundant function of the AtCcs52 proteins. Consistent with these activities, the AtCcs52 proteins were able to bind both to the yeast and the *Arabidopsis* APCs. In synchronized *Arabidopsis* cell cultures the *cdc20* transcripts were present from early G2 until the M-phase exit, *ccs52B* from G2/M to M while *ccs52A1* and *ccs52A2* were from late M until early G2, suggesting consecutive action of these APC activators in the plant cell cycle. The AtCcs52 proteins interacted with different subsets of mitotic cyclins, in accordance with their expression profiles, either in free- or CDK-bound forms. Expression of most APC subunits was constitutive, whereas *cdc27a* and *cdc27b*, corresponding to two forms of *apc3*, and *ubc19* and *ubc20* encoding E2-C type ubiquitin-conjugating enzymes displayed differences in their cell cycle regulation. These data indicate the existence of numerous $APC^{Cdc20/Ccs52/CDC27}$ forms in *Arabidopsis*, which in conjunction with different E2 enzymes might have distinct or complementary functions at distinct stages of the cell cycle.

INTRODUCTION

The ubiquitin-26S proteasome pathway is a key regulatory mechanism in eukaryotes, which ensures that specific protein functions are turned off at the right time, in the right place, and in a unidirectional fashion. Protein ubiquitination is a multistep enzymatic process that involves at least three enzyme activities (Hershko and Ciechanover, 1998). Ubiquitin-activating enzyme (E1) forms a high-energy bond with ubiquitin, which is then transesterified to an ubiquitin-conjugating enzyme (E2). E3 ubiquitin ligases play a prominent role in selection and specific timing of polyubiquitination of the target proteins. The E3 enzymes can transfer the ubiquitin either directly to the substrates or indirectly by simply bringing the E2 enzyme in close proximity of the substrates. The anaphase-promoting complex is one such E3 enzyme, which has a crucial role in the cell cycle from metaphase until S-phase, orchestrating mitosis as well as formation of the prereplicative complexes.

The APC is a multiprotein complex, which has similar structure in all eukaryotes. In vertebrates 11 subunits (APC1-11) have been described, while the yeast APC is composed of minimum 13 subunits (reviewed by Castro *et al.*, 2005). The majority of the core subunits are stably associated throughout the cell cycle (Peters *et al.*, 1996); however, it is largely unknown how these subunits assemble to a functional E3. The minimal ubiquitin ligase module of the APC comprises APC2, a distant member of the cullin family and the RING-finger protein APC11. These two subunits interact with each other and with E2 ubiquitin conjugating enzymes, and result in ubiquitination of proteins *in vitro* but without substrate specificity (Gmachl *et al.*, 2000; Leverson *et al.*, 2000; Tang *et al.*, 2001). Stage-specific activation of the APC as well as selection and binding of the substrates are defined by two WD40 proteins, Cdc20 and Cdh1 (known as Ccs52 in plants). Binding of these APC

activators to the core APC is cell cycle regulated, and mediated by two consensus APC binding motifs; the C-box (DR(F/Y)IPxR) at the N-terminus (Schwab *et al.*, 2001) and the C-terminal IR tail that interacts with the tetratricopeptide repeat subunits, APC3 and APC7 in the human APC (Vodermaier *et al.*, 2003).

In somatic animal cells and in yeast, the cell cycle regulation of Cdc20 and Cdh1 is different. Cdc20 levels are controlled by transcriptional and proteolytic mechanisms that result in Cdc20 synthesis during S and G2 phase and in its degradation at the end of mitosis (Fang *et al.*, 1998; Kramer *et al.*, 1998; Prinz *et al.*, 1998; Shirayama *et al.*, 1998; Weinstein, 1997). In contrast, the levels of the Cdh1 RNA and protein are constant throughout the yeast cell cycle (Prinz *et al*., 1998). In mammals, the cdh1(fzr) message fluctuates during the cell cycle and peaks in mitosis (Inbal *et al.*, 1999). Moreover, the Cdh1 protein mediates its own degradation in G1 phase in a D-box dependent manner (Listovsky *et al.*, 2004).

Both the Cdc20 and the Cdh1 proteins contain several cyclin-dependent kinase (CDK) phosphorylation sites. Mitotic cyclin-CDK complexes have antagonistic roles in regulating APC^{Cdc20} and APC^{Cdh1}. The phosphorylation of APC subunits is required to allow APC activation by Cdc20 (Kotani *et al.*, 1998; Kraft *et al.*, 2003), whereas the phosphorylation of Cdh1 prevents its association with the APC (Jaspersen *et al*., 1999; Kramer *et al*., 2000; Zachariae *et al*., 1998) and leads to its translocation from the nucleus to the cytoplasm (Jaquenoud *et al*., 2002; Zhou *et al*., 2003). Cdc20 and Cdh1 act consecutively in the cell cycle. APC^{Cdc20} is activated as cells approach metaphase, and this activity is enforced by high CDK activity. As APC^{Cdc20} degrades mitotic cyclins and drops the activity of mitotic CDKs, APC^{Cdh1} becomes activated during exit from mitosis, which provokes degradation of Cdc20 (reviewed in Harper *et al.*, 2002; Zachariae and Nasmyth, 1999). Activity of APC^{Cdh1} persists until the G1/S transition.

Mitotic cyclins contain a degradation signal (RxxLxxxxN) called destruction box (D-box) (Glotzer *et al.*, 1991), that is recognized both by APC^{Cdc20} and APC^{Cdh1} and leads to polyubiquitination and degradation of mitotic cyclins. As the D-box sequences are highly variable, it is possible that the APC activators may selectively interact with specific sets of mitotic cyclins. Binding of cyclin A to the mammalian Cdh1 was dependent on the presence of the RVL sequence within the last WD40 domain (Sorensen *et al*., 2001). This motif is conserved in the different organisms and similarly to human it likely functions as a cyclin-binding motif in eukaryotes (Loog and Morgan, 2005). APC can function with multiple E2 family members. Degradation of mitotic cyclins involves two subclasses of E2s (E2-C/UBC-X and UBC4) (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996). However, it remains a question how these E2s are selectively recruited to and recognized by the APC.

In plants more than 5% of the genome encode components of the ubiquitin-proteasome pathway (Vierstra, 2003). In *Arabidopsis*, there is a single E1, but thirty-seven E2s and hundreds of E3s have been predicted (Bachmair *et al.*, 2001; Schwechheimer and Villalobos, 2004). Counterparts of all the known vertebrate APC subunits could be identified also in the *Arabidopsis* genome (Capron *et al.*, 2003a). Single-copy genes encode all subunits, except APC3/Cdc27. *Atcdc27A* (At3g16320) and *Atcdc27B/hobbit* (At2g20000) encode two functionally distinct APC3/Cdc27 proteins leading to the formation of APC^{Cdc27A} and APC^{Cdc27B} in *Arabidopsis*. Cdc27A appears to be a closer homolog of the human APC3 than Cdc27B/Hobbit (Blilou *et al.*, 2002).

Analysis of the Cdc20s is lacking in plants. Cdh1-type activators, named as cell cycle switch Ccs52 proteins, have been identified in *Medicago* species, *M. sativa* and the model legume *M. truncatula* (Cebolla *et al.*, 1999; Tarayre et al., 2004). Ms/MtCcs52A is an

ortholog of the fission yeast Cdh1 protein known as Srw1/Ste9. Induced over-expression of *ccs52A* in fission yeast elicited mitotic cyclin degradation, resulting in growth inhibition, cell elongation and repeated endoreduplication cycles (duplication of the genome without mitosis). Functional analysis of the Mt/MsCcs52A proteins led to the discovery of another conserved motif (YxxLL(K/R)xxLFC) in the Cdh1-type proteins, named as Cdh1-specific motif (CSM) that is also essential for APC interaction (Tarayre *et al.*, 2004). Analysis of the potential phosphorylation sites revealed the critical importance of phosphorylation sites in front of the C-box in the interaction of the Mt/MsCcs52A proteins with the core APC. Later, a homologue was discovered in *M. truncatula*, MtCcs52B, that exhibited strikingly different cell cycle and developmental regulation when compared to MtCcs52A and represented a functionally distinct subclass of the Ccs52 proteins (Tarayre *et al.*, 2004; Vinardell *et al.*, 2003). These two subclasses of the Ccs52 proteins appear to be conserved in plants.

The *Arabidopsis* genome contains three *ccs52* genes: *Atccs52A1, Atccs52A2* and *Atccs52B*, and five *cdc20* genes. Moreover, ten A-type and eleven B-type cyclins are predicted in the *Arabidopsis* genome (Vandepoele *et al.*, 2002; Wang et *al.*, 2004). As D-box dependent degradation of mitotic cyclins has been demonstrated in plants (Genschik *et al.*, 1998), all these mitotic cyclins are potential targets of the APC. The involvement of Mt/MsCcs52A proteins has been demonstrated and similar function can be expected for the AtCcs52A proteins. In contrast, there is no evidence for the function of Ccs52B in cyclin degradation. It thus remains a question how the *Arabidopsis* mitotic cyclins are recruited to the APC? Are all APC activators able to bind mitotic cyclins and if so, are there certain specificities in these interactions? Moreover, as mitotic cyclin-CDK complexes can phosphorylate the AtCcs52 proteins, it is also not known how the Ccs52 proteins interact with the free and CDK-bound cyclins.

In this work we aimed at answering these questions. We report on the characterization of the *Arabidopsis ccs52* genes, cell cycle regulation of the APC activators, APC components, E2-Cs and mitotic cyclins. We demonstrate binding of the AtCcs52 proteins to the APC and show that the AtCcs52 proteins have distinct functions and display specificity toward mitotic cyclins. These results provide the first indication on high complexity and selective activities of different APC forms in *Arabidopsis*.

RESULTS

The AtCcs52A1, AtCcs52A2 and AtCcs52B proteins have similar structure but elicit different phenotypes in *Schizosaccharomyces pombe*

Atccs52A1 (At4g22910) and *Atccs52A2* (At4g11920) are located on chromosome 4 and appear to be the results of a recent duplication of a 7.5 Mb chromosome region. *Atccs52B* (At5g13840) is present on chromosome 5. Interestingly, the five *cdc20/fzy* genes are also carried by chromosomes 4 and 5. At4g33260 and At4g33270 are present in tandem on chromosome 4 while chromosome 5 carries At5g26900, At5g27570 and At5g27080. The *Atccs52A, Atccs52A1* and *Atccs52B* genes are composed of 9, 8 and 7 exons, respectively (Figure 1A). In each gene, the first exon codes for the N-terminal region, upstream of the WD40 repeats. AtCcs52A1 and AtCcs52A2 share higher homology with each other and MtCcs52A than with AtCcs52B (Figure 1B). Likewise, AtCcs52B was more similar to MtCcs52B than to AtCcs52A1 and AtCcs52A2 (Figure 1B).

The C-box, CSM and the IR motifs and the mitotic cyclin binding motif (RVL) are conserved in each AtCcs52 protein. Moreover, putative serine and threonine CDK

phosphorylation sites can be predicted in all of them. Among them, five potential sites are conserved, including the critical phosphorylation sites upstream of the C-box. In spite of certain similarities, the proteins also differ from one other. For example, the AtCcs52A2 and AtCcs52B proteins are basic; pI is 9.07 and 9.28, respectively, whereas AtCcs52A1 (pI=7.52) is more neutral. A PEST motif is predicted in AtCcs52A1 (score 8.47), similarly to Mt/MsCcs52A, while this is absent in AtCcs52A2 or AtCcs52B. In the *Arabidopsis* and *M. truncatula* Ccs52B proteins, the serine is replaced by cystein in the C-box. The CSM, except for the conserved core sequence, is different in all AtCcs52 proteins.

Based on structural properties, it was impossible to define which *Arabidopsis* Ccs52A is the functional homolog of Mt/MsCcs52A. Nor could it be excluded that AtCcs52A1 and AtCcs52A2 play redundant functions or share functions of the legume Ccs52A proteins. In our previous work, we demonstrated functional differences between MtCcs52A and MtCcs52B in *S. pombe* (Tarayre *et al.*, 2004). Using this heterologous system again, we studied the effect of the *Arabidopsis ccs52* genes on yeast growth (Figure 2A) and morphology (Figure 2B). Expression of the *ccs52* genes was controlled by the *nmt1* promoter, which is active in the absence and inactive in the presence of thiamine. Induced expression of *nmt1::Atccs52A1* and *nmt1::Atccs52A2* inhibited the growth of *S. pombe*, as *nmt1::Mtccs52A* did. In contrast to *Mtccs52B,* which had no visible effect on yeast, *Atccs52B* expression also prevented growth, though to somewhat weaker extent than *Atccs52A1* or *Atccs52A2*.

In addition to proliferation arrest, expression of the *Atccs52* genes provoked also drastic morphological changes that were characteristic for each gene (Figure 2B). Expression of *Atccs52A2*, similarly to that of *Mtccs52A*, resulted in elongation of yeast cells and enlargement of nuclei (Tarayre *et al.*, 2004). *Atccs52A1* provoked even more severe phenotypes; the cells were huge, frequently branched at their extremities or displayed out-growths and ballooning. In these cells, the nuclei were giant and signs of apoptotic events were also observed. Over-expression of *Atccs52B* did not provoke any of these morphological changes; both the size and shape of the cells were comparable to the control ones. However, in certain cells, the nucleus displayed polar localisation instead of the usual central position. Moreover, aberrant migration of chromosomes was also detected. These symptoms may indicate mitotic catastrophes provoked by over-expression of AtCcs52B, and explain the inability of yeast cells to divide.

AtCcs52A1, AtCcs52A2 and AtCcs52B proteins bind both to the yeast and the *Arabidopsis* APCs

The yeast phenotypes induced by over-expression of the *Arabidopsis ccs52* genes indicated that the AtCcs52 proteins may activate or interfere with the yeast APC. Binding of the *Arabidopsis* Ccs52 proteins to the *S. pombe* APC was tested by *in vitro* binding assays (Figure 3A). In these assays, MtCcs52A was used as a positive and the green fluorescent protein (GFP) as a negative control. The Ccs52 proteins were labelled with three hemagglutinin (HA) tags at their N-terminus and transiently overproduced from the CaMV 35S promoter in *Arabidopsis* protoplasts. The protein extracts prepared from the protoplasts were mixed with protein extract of *S. pombe* strain S813 expressing the APC subunit Lid1 with 9 Myc epitopes. The HA-tagged proteins were pulled down with anti-HA antibody and the presence of the APC was detected on immunoblots with anti-Myc antibodies (Figure 3A). In the case of the HA-GFP, the Lid1 protein was not detectable while Lid1 was present in the immunoprecipitates of all Ccs52 proteins. These results

demonstrated specific binding of the AtCcs52A1, AtCcs52A2, AtCcs52B and MtCcs52A proteins to the yeast APC.

To test the interaction of the AtCcs52 proteins with the *Arabidopsis* APC *in planta*, transgenic *Arabidopsis* plants were generated, expressing the HA-tagged version of the AtCcs52A1, AtCcs52A2 or the AtCcs52B proteins under the control of the 35S promoter. After selection of the HA-Ccs52 over-expressing lines out of 51 primary transgenic plants by Western analysis, leaf protein extracts were used for immunoprecipitation of the APC with polyclonal anti-Cdc27A/APC3 antibodies (Capron *et al.*, 2003b). In the anti-Cdc27A immunocomplexes, the presence of each HA-tagged AtCcs52A1, AtCcs52A2 and AtCcs52B could be demonstrated with anti-HA immunoblots (Figure 3B), which showed *in planta* the binding of the AtCcs52 proteins to the *Arabidopsis* APC.

Specific interaction pattern of AtCcs52 proteins with different A- and B-type cyclins

Mitotic cyclins are known APC substrates in animals and yeasts, which can be recruited by both Cdc20 and Cdh1. Previously we showed that expression of *Msccs52A* provoked mitotic cyclin degradation in fission yeast, which predicted similar function in plants. The *Arabidopsis* genome contains 21 mitotic cyclins, 10 A- and 11 B-types, each subdivided in 3 subclasses (Vandepoele *et al.*, 2002; Wang *et al.*, 2004), which might be common or specific partners of the APC activators. To study the interaction and the eventual substrate-specificity of the *Arabidopsis* Ccs52 proteins toward the mitotic cyclins, we have taken different approaches. To indentify the interacting partners of the AtCcs52 proteins we performed yeast two-hybrid screens with the AtCcs52 proteins as baits using an *Arabidopsis* library made of all organs and different developmental stages of *Arabidopsis*. One out of 200 interacting partners was an A-type cyclin, CycA3;4 that exhibited strong interaction with AtCcs52A1 and AtCcs52B but only a very weak one with AtCcs52A2 (Figure 4A).

As cyclins are generally expressed at low levels and might be underrepresented in the library, we studied the binding of selected cyclins, AtCycA1;1, AtCycA1;2, AtCycB1;1, AtCycB1;2 and AtCycA3;4 to the AtCcs52 proteins in the *Arabidopsis* protoplast system. The AtCcs52s as well as GFP, as negative control, were marked with the HA-tag and the cyclins with the myc-epitope: both were expressed from the 35S promoter. After co-expression of cyclins and AtCcs52s/GFP in different pairwise combinations, the HA-AtCcs52 proteins or HA-GFP were pulled down from lysates of transfected protoplasts with the anti-HA antibody and the presence of cyclins in the immunocomplexes was detected with anti-myc antibody on Western blots (Figure 4B). In this assay, AtCcs52A1 and AtCcs52A2 showed interaction with all the five tested cyclins, though, consistent with the yeast two hybrid result, binding of AtCycA3;4 was weaker to AtCcs52A2 than to AtCcs52A1 or to AtCcs52B. AtCcs52B interacted also with AtCycA1;1, AtCycA3;4, AtCycB1;1 and AtCycB1;2 but not with AtCycA1;2. These plant and yeast data indicate that both the AtCcs52A and AtCcs52B proteins are able to bind mitotic cyclins but they may interact with different subsets of mitotic cyclins, representing partially overlapping and partially distinct specificities.

The AtCcs52 proteins bind both free and CDK-bound mitotic cyclins

Mitotic cyclins are both APC substrates and potential APC regulators via phosphorylation of the Cdh1/Ccs52 proteins by mitotic CDKs. This suggested that the Ccs52 proteins might interact with free mitotic cyclins as well as mitotic cyclin-CDK complexes. To test this possibility, the HA-AtCcs52 proteins and myc-CycA3;4 were over-

expressed in *Arabidopsis* protoplasts and the presence of CDKA;1, carrying the PSTAIRE motif was tested in the anti-HA immunocomplexes with anti-PSTAIR antibody on Western blots (Figure 4C). Though the amounts of CDKA;1 was significantly lower than that of the myc-CycA3;4, it was clearly present in the immunocomplex of each AtCcs52 protein. As the AtCcs52 proteins did not show interaction with CDKs in the yeast two hybrid and the *Arabidopsis* protoplast systems, the presence of CDKA;1 in the HA-AtCcs52 immunocomplexes demonstrates that AtCcs52 proteins bind both the free and CDK-bound mitotic cyclins.

APC activities and substrate specificity might be controlled by different APC$^{Ccs52/Cdc20}$ forms in conjunction with specific E2 enzymes

In yeast and human, *cdc20* and *cdh1* are differentially regulated during the cell cycle. In addition, *Mt/Msccs52A* and *Mt/Msccs52B*, encoding two subclasses of the Cdh1 type activators in *M. truncatula* and *M. sativa* showed also distinct expression patterns. In *Arabidopsis*, the presence of numerous APC activators and E2 enzymes as well as duplication of APC3/Cdc27 indicated that different APC forms may act at different stages of the cell cycle or control degradation of given substrate sets. As coordinated regulation of genes may reflect coordinated functions, *Arabidopsis* cell suspension cultures were synchronized with aphidicolin at early S phase, and expression of the *Arabidopsis ccs52* and *cdc20* genes, as well as that of APC substrates, APC subunits and E2 enzymes was studied during cell cycle progression by RT-PCR (Figure 5).

As in *Medicago*, expression profiles of the *ccs52A* and *ccs52B* genes were different; *ccs52B* expression was restricted to G2/M and M phase, while *ccs52A1* and *ccs52A2* were expressed from late M until late S-early G2 phases with identical pattern, except that transcript level of *ccs52A2* increased slightly earlier (20h) in M phase than that of *ccs52A1* (24h). Global expression of the *cdc20* genes was revealed by the use of a conserved primer pair present in all the five genes. Though *cdc20* transcripts were detectable in all cell cycle phases, an increase in transcript accumulation occurred from early G2 (6h) until M-phase exit (24h).

Transcription profiles of the tested cyclin B genes; *AtcycB1;1* and *AtcycB1;2* were very similar to their animal counterparts: *AtcycB1;2* expression started at 13h, peaked at 16h and decayed at 24h, just as in the case of *Atccs52B.* Expression of *AtcycB1;1* and the M-phase marker *AtcdkB2;2* (Menges and Murray, 2002) was similar to that of *AtcycB1;2* and *Atccs52B*, however, transcripts of these genes were also present in G1 and S phases.

The A-type cyclins showed more divergence in their expression pattern. *AtcycA1;1* transcript accumulated from late S (6-8h), peaking in G2 and M and remaining high even in early G1 phase. In contrast to *AtcycA1;1* and animal A-type cyclins, *AtcycA1;2* did not show any mitotic expression. The *AtcycA1;2* transcripts appeared in G1 and were present in S-phase samples, with a very pronounced peak at the G1/S boundary in the 0h sample just after the removal of the blocking agent, which recalls the expression profile of the mammalian E-type cyclins (as proposed for *Nicta;cycA3;2* as well by Yu *et al.*, 2003). The *AtcycA3;4* transcripts were present in a large window, with increased amounts in the S and G2 phases and reduced levels in the M phase.

Among the genes coding for APC subunits, expression of *apc10*, *apc11*, *cdc16*, *cdc23*, *cdc27A* and *B*, has been studied. None of these genes, as shown for *apc10* (Figure 5B), displayed clear cell cycle-dependent expression. Difference could only be detected in the case of *cdc27A* and *cdc27B*/*hobbit*; *cdc27A* displayed slightly elevated transcript levels in S-G2 whereas *cdc27B*/*hobbit* was elevated in S phase.

The E2-C class of ubiquitin-conjugating enzymes has been shown to be involved in mitotic cyclin degradation and anaphase progression (Aristarkhov *et al.*, 1996; Townsley *et al.*, 1997; Yu *et al.*, 1996). Here we studied expression of two members of the *Arabidopsis* E2-Cs, *ubc19* and *ubc20* (Criqui *et al.*, 2002), which exhibited radically different patterns. The expression of *ubc19* was dominant in G1-S, whereas that of *ubc20* in G2-M, suggesting implication these two very closely related genes in different phases of the cell cycle.

DISCUSSION

The activities of the *Atccs52A1, Atccs52A2* and *Atccs52B* genes

Due to the duplication of the *ccs52A* gene, *Arabidopsis* contains three Cdh1-type APC activators; *Atccs52A1, Atccs52A2* and *Atccs52B*. Analysis of the *Atccs52* gene functions in *S. pombe* revealed that all three *ccs52* genes are active, blocking proliferation, but with distinctive phenotypic effects. Over-expression of *Atccs52A2* provoked the same yeast phenotype as that of the endogenous *srw1* or the *Medicago ccs52A* gene, manifested in the inhibition of cell proliferation and at the same time in elongation of yeast cells and enlargement of nuclei. These results indicate that *Atccs52A2* might be the closest homologue of the Cdh1-type activators from other organisms. Over-expression of *Atccs52A1* provoked similar but even more pronounced phenotypes; cell growth in addition to elongation also produced lateral branching and was coupled to extreme enlargement of nuclei. In our previous works we showed that the increase of nuclear and cell volumes correlated with the ploidy levels in *Medicago* and required *ccs52A* expression to trigger endoreduplication (Cebolla *et al.*, 1999, Vinardell *et al.*, 2003). These yeast data support that both AtCcs52A1 and AtCcs52A2 are able to modulate cell size and nuclear DNA content. The *Atccs52A1*-triggered outgrowths of yeast cells show a surprising similarity to ploidy-dependent branching of the unicellular trichomes in *Arabidopsis*, which suggests a possible involvement of AtCcs52A1 in trichome development.

Binding of the AtCcs52 proteins to the APC

In this work we showed *in vivo* interaction of the AtCcs52A1, AtCcs52A2 and AtCcs52B proteins with the *Arabidopsis* APC *in planta*. In our assays, the APC was pulled down with anti-Cdc27A antibodies, which may indicate that the AtCcs52 proteins, similarly to HsCdh1, interact with the Cdc27/APC3 subunits. However, testing pair-wise interactions of the AtCcs52 proteins with AtCdc27A or Cdc27B in the yeast two-hybrid system showed no interactions (data not shown). This suggests that the AtCcs52 proteins rather bind to other TPR subunits or other APC components, or their binding requires the assembly of APC or at least APC subcomplexes.

The AtCcs52A1 and AtCcs52A2 proteins, similarly to MtCcs52A, were also able to bind to the fission yeast APC. However, AtCcs52B and MtCcs52B behaved differently one from another. AtCcs52B was active in yeast and interacted with the *S. pombe* APC, while MtCcs52B was apparently inactive. The structure of the AtCcs52B and MtCcs52B proteins is highly similar. The C-bow is identical, the IR-tail is present and the At/MtCSMs differ only in a single amino acid (S/N). The WD-repeat region is highly similar and construction of chimeric MtCcs52A/MtCcs52B proteins demonstrated that inability of the MtCcs52B protein binding to the yeast APC was due to the N-terminal part of MtCcs52B (Tarayre *et al.*, 2004). It remains to be tested whether this is caused by the single amino acid change in CSM or other differences in the N-terminal sequence.

Substrate specificity of the AtCs52A1, AtCcs52A2 and AtCcs52B proteins

Mitotic cyclins are known APC substrates. Mitotic cyclin degradation elicited by MsCcs52A has been demonstrated in fission yeast (Cebolla *et al.*, 1999) but it was unknown whether both subclasses of the Ccs52 proteins or only the Ccs52A-type proteins interact with mitotic cyclins. Moreover, the high number and different subclasses of A- and B-type cyclins in *Arabidopsis* suggested that the APC activators might exhibit differences in their interactions toward mitotic cyclins. Using five selected cyclins we showed that both the AtCcs52A and the AtCcs52B proteins are able to bind mitotic cyclins. Interactions of AtCcs52 proteins and mitotic cyclins displayed certain specificity, which correlated with cell cycle regulation of the APC activator and mitotic cyclin genes. In our assays, however, both the *Atccs52* genes and mitotic cyclins were expressed from the constitutive 35S promoter; therefore, specificity of the interactions should also rely on structural properties of cyclins. The D-box sequences themselves are insufficient to explain the specificity. Most likely the highly divergent N-terminal sequences also play roles in the AtCcs52-cyclin A/B interactions.

Cell cycle regulation of the *Atccs52A1, Atccs52A2* and *Atccs52B* genes

The expression patterns during the cell cycle were consecutively *Atcdc20*; *Atccs52B*; and the *Atccs52A2* / *ccs52A1*. *Atcdc20* was active from early G2 until M-phase exit, *ccs52B* in G2/M and M phase, while *ccs52A2* and *ccs52A1* were displayed from late M until early G2 phases. Thus, the expression profiles of the APC activators overlap the entire cell cycle. Does this reflect constitutive APC activity?

In yeast and mammals, the APC is inactive from S phase until metaphase. The level and activity of the Cdc20 and Cdh1 proteins are regulated at multiple levels, including transcriptional regulation, mRNA stability, phosphorylation, interaction with inhibitory proteins and their APC^{Cdh1}-mediated degradation (reviewed in Castro *et al.*, 2005).

Degradation of the *Medicago* cyclin MsCycA2 occurs in prometaphase (Roudier *et al.*, 2000), thus if mitotic cyclins are only degraded by the APC, it should be active before metaphase. Compared to animals and yeasts, *Arabidopsis* has a multitude of cyclins. The majority of the 21 mitotic cyclins follows the classical expression profile of A- and B-type mitotic cyclins, but certain cyclins are expressed throughout the cell cycle or present, as we showed in this work for *Atcyc1;2*, only in S-phase (Menges and Murray, 2002; Menges *et al.*, 2002; Menges *et al.*, 2003; reviewed in Dewitte and Murray; 2003). Therefore it is not impossible that in plants, constitutive activity of different APC activator-core APC forms, is required for degradation of D-box containg cyclins throughout the cell cycle.

One can argue, however, that the transcript levels of the *Atcdc20* and *Atccs52* genes alone are not sufficient to draw conclusions on the APC activity. Indeed, the APC activators might be regulated posttranscriptionally. However, homologues of the inhibitory proteins (like Emi1 and Mad2L2) could not be identified in the *Arabidopsis* genome. This may mean that other structurally less related inhibitors exist in *Arabidopsis* or this regulatory mechanism is not conserved in plants.

D-box dependent degradation of the AtCcs52 proteins is rather unlikely. AtCcs52A1 has no D-box sequence. An **R**KI**L** sequence is present between positions 150-153 in AtCcs52A2 and **R**NI**L** (277-280) in AtCcs52B. In the mammalian Cdh1, two N-terminal RxxL destruction boxes (7-10, 28-31) were necessary for degradation of Cdh1 (Listovsky et al., 2004). In the AtCcs52A2 and AtCcs52B proteins, the second RxxL copy is missing and the existing RxxL sequence is not at the N-terminus and likely does not function as

degradation signal. On the other hand, a PEST sequence is present in AtCcs52A1 that provokes instability of the protein.

The MtCcs52A-APC interaction is negatively regulated by phosphorylation (Tarayre *et al.*, 2004). Similarly, the activity of the AtCcs52 proteins could also be regulated by phosphorylation. We have shown that the AtCcs52 proteins interact both with free and CDK-bound mitotic cyclins. It remains to be tested whether interaction of the AtCcs52 proteins with mitotic CDKs reflects CDK phosphorylation-dependent regulation of the AtCcs52 proteins or recruitment of mitotic cyclins from CDK complexes and thereby regulating CDK activities.

The tested *Arabidopsis* mitotic cyclins, as well as *ubc19* and *ubc20* encoding E2 enzymes are expressed in different phases of the cell cycle. There are also differences in the expression pattern of the APC sunubits*, cdc27A* and *cdc27B*. These data indicate that two APC^{Cdc27A} and APC^{Cdc27B} forms coupled with either of the eight APC activators and Ubc19 or Ubc20 may be required for degradation of specific cyclins and other cell cycle related proteins at given stages of the cell cycle. This complexity of APC might be needed for finely tuned regulation of the plant cell cycle.

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MATERIALS AND METHODS

Constructs, plasmids

For transient expression in *Arabidopsis thaliana* protoplasts, three HA or three myc epitopes followed by the multicloning site of pBluescript SKII+ (Invitrogen) were introduced into pRT104 plant expression vector (Topfer *et al.*, 1987), generating pRT-3HA and pRT-3myc transient expression vectors. *Atccs52A1*, *Atccs52A2*, *Atccs52B*, *AtcycA1;1* ; *AtcycA1;2* ; *AtcycB1;1* and *AtcycB1;2* were cloned into pGEMeasy vector (Invitrogen) by Pfu amplification from a plantlet cDNA library. After sequencing, *Atccs52A1*, *Atccs52A2* and *Atccs52B* cDNA were cloned into pRT-3HA, whereas *AtcycA1;1* ; *AtcycA1;2* ; *AtcycB1;2* and *AtcycB1;1* (kindly provided by Arp Schnittger) were introduced into pRT-3myc.

Yeast techniques

Yeast two-hybrid screens were performed using Clontech's Matchmaker Kit. Briefly, the yeast two-hybrid strainY187 (MATα) carrying the bait plasmid was mated with strain AH109 (MATa) carrying the premade *Arabidopsis* cDNA library (kind gift from Hans Sommer, Cologne). Bait-prey interactions were selected on synthetic drop-out media (SD) lacking Leu, Trp, His and Ade after a 7-day incubation at 20°C. The selected prey inserts were partially sequenced by direct sequencing of PCR amplicons derived from whole yeast cells (Ling *et al.*, 1995). Directed yeast two-hybrid interactions were conducted by mating the Y187 strain carrying bait plasmid with the AH109 strain carrying the pray plasmid. Diploid cells were selected on SD-Leu-Trp and subsequently tested for protein interactions by monitoring growth on solid SD-Leu-Trp-His-Ade media.

For the overexpression of AtCcs52 proteins in *S. pombe*, cDNAs coding for the proteins were introduced into pREP1 plasmid. Cells from the SP-Q01 strain (Stratagene) were transformed and cultured as described by Tarayre *et al.* (2004).

Transient expression in protoplasts and immunoprecipitation

Arabidopsis thaliana cell ecotype Columbia suspension was used for the protoplast transformations. The culture is maintained by weekly subculturing in MS medium (Sigma) supplemented with 3% w/v sucrose, 240 µg/L 2,4-D and 14 µg/L kinetin.

Protoplasts were transfected using the polyethylene glycol-mediated method (Kiegerl *et al.*, 2000). Proteins were extracted after 16h of incubation in buffer containing 25 mM Tris HCl pH 7.7, 10 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 15 mM β-glycerophosphate, 0.1 % Tween 20, 10 % glycerol, 1 mM DTT, 1 mM NaF, 0.5 mM $NaVO_3$, 0.5 mM PMSF and protease inhibitors (Complete, Roche). From 500 µg of total protein extract of transfected *Arabidopsis* protoplasts, HA-tagged proteins were immunoprecipitated using 500 ng of 3F10 anti-HA monoclonal antibody (Roche) and 50 µL of Protein G paramagnetic microbeads (Miltenyi Biotec) by overnight incubation at 4°C. Beads were then loaded onto columns and washed three times with extraction buffer and once with 20 mM Tris pH 7.5. Bound proteins were eluted with 50 µL of SDS loading buffer. Co-immunoprecipitation of the myc-tagged protein was revealed by SDS-PAGE followed by western blot using the 9E10 anti-myc monoclonal antibody (Roche) and enhanced chemiluminescence detection (Amersham).

Synchronization of cell cycle in suspension cultured *Arabidopsis* cells

For the synchronization of cell cycle, *Arabidopsis thaliana* ecotype Landsberg erecta cell suspension cultures were maintained by weekly subculturing in MS medium pH 5.7 supplemented with 3% w/v sucrose, 0.5 mg/L NAA and 0.05 mg/L kinetin.

For the reversible G1/S blockage of *Arabidopsis thaliana* (Landsberg erecta ecotype) cells, the cell material from an 8-day-old culture was collected by centrifuging 5 min at 1500 rpm and the medium was discarded. Cells were resuspended in fresh MS medium and after 8 hours of culture aphidicolin was added to a final concentration of 5 µg/mL (stocks were prepared at a concentration of 5 mg/mL diluted in DMSO). The blockage was maintained for 18 hours. Cells were washed with fresh medium lacking hormones and sucrose during approx. 2 hours changing the medium four times: aphidicolin was removed by centrifuging the culture 5 min 1000 rpm and cells were washed for 5 min, followed by three further washes of 20 min. Finally, the cell pellet was resuspended in fresh, complete MS and cultured under normal conditions. Samples were taken at the following time points: before adding (AS) and before removing (B) the blocking agent, after the washes (0h), and at 2, 4, 6, 8, 10, 13, 16, 20 and 24 hours. The cell cycle progression was followed by flow-cytometry analysis (ELITE ESP, Beckman-Coulter).

RNA extraction and RT-PCR analysis

Total RNA was extracted as described by Menges and Murray (2002). For the first strand synthesis, samples were treated with DNase I (Promega) and 5 µg of total RNA was used as template for reverse transcription. The cDNA synthesis was carried out in a final volume of 20 µL using 500 ng of oligo(dT) primer and PowerScript Reverse Transcriptase (Clontech) according to the manufacturer's recommendations. The cDNA was diluted to 100 µL with 10 mM Tris pH 8, 0.1 mM EDTA and 1 µL was used for PCR reaction. The primers were as follows: 5'-ggtggtattgacaagcgtg and 5'-tcacgggtctgaccatcc for *EF1α*; 5'-ttaaacccgctgtttccgcc and 5'-ccaacagaccacatatccactcc for *cdkB2;2*; 5'-gaccaactcaagctggctg and 5'-ggaaccaacattcaacacaaccgg for *Atccs52A1*; 5'-cctgatttcgagaatcatgtcaagac and 5'-

ctgtgaacacgccgcagcagtg for *Atccs52A2*; 5'-aaatagaatgcaggttttgcgtg and 5'-taaacagcatcgacactgggagc for *Atccs52B*; 5'-gctcttgcgtggtgccttttc and 5'-agaactcttgatgtatgacca *Atcdc20*s; 5'-agagatatggcgacagagt and 5'-tcatctcagtgttgaataagt for *apc10*; 5'- ccgctccccaagtactacaa and 5'- ttagggttagtccacaaga for *cdc27A*; 5'-gcatcacttcagaatggcttt and 5'- tcacgggctctcatcgatctc for *cdc27B*; 5'-aaaaggatggcgacggttaat and 5'-aagagatatgatccaatacagttga for *ubc19*; 5'-atggccgccgtaaatggat and 5'-agcttgcagaaatcatgcact for *ubc20*; 5'-actccatcgctttatattgc and 5'-gtgattcctacaagagtcctc for *AtcycA1;1*; 5'-accgaattgctccaaagc and 5'-ttgcaaccacatcagatgag for *AtcycA1;2*; 5'-tcttttgtctgcttcagctgtattt and 5'-ttacgccattcctctaatggtaata for *Atcyc3;4*; 5'-ccacagcgaagcgttcgtcc and 5'-tgatattgcagaccatgc for *AtcycB1;1*; 5'-tccaatcagcagcagcactc and 5'-ggagaatatggttcacttcc for *AtcycB1;2*.

PCR products were separated on agarose gel, blotted by capillary transfer onto nylon membrane (Biotrans, ICN) and hybridized with the respective PCR fragments labelled with [α-^{32}P]dCTP by random priming (Megaprime DNA labelling system, Amersham). Signals were analyzed by PhosphorImager (Molecular Dynamics).

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FIGURES AND FIGURE LEGENDS

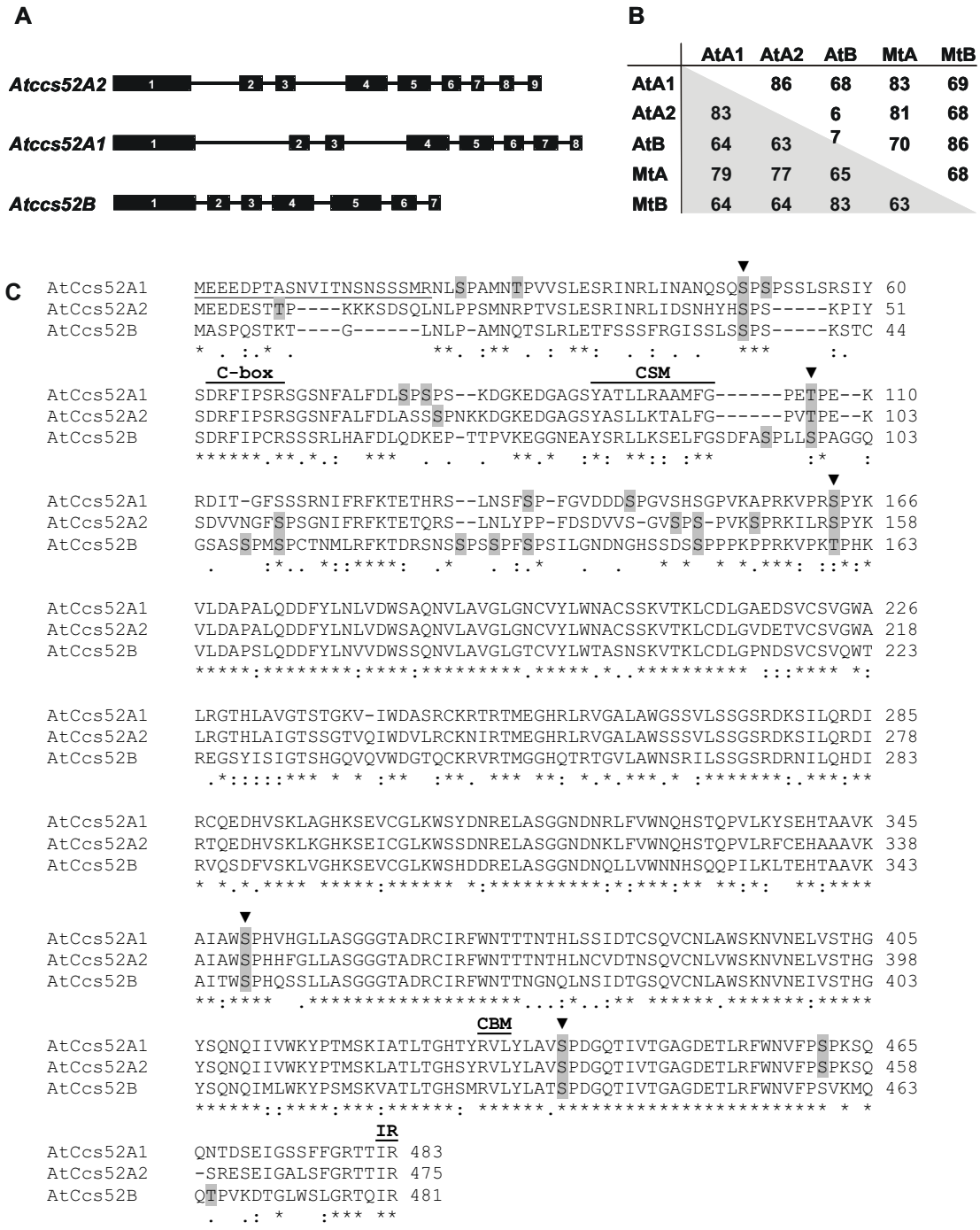

Figure 1. Organization of the *Atccs52* genes and relatedness of the AtCcs52 and MtCcs52 proteins. (A) Exon-intron organization of the *Atccs52* genes. (B) Identity (gray background) and similarity of the *Arabidopsis* and *M. truncatula* Ccs52 proteins in %. AtA1, AtCcs52A1; AtA2, AtCcs52A2; AtB, AtCcs52B; MtA, MtCcs52A; MtB, MtCcs52B. (C) The conserved APC interacting motifs (C-box, CSM, the C-terminal IR residues), the RVL cyclin binding site (CBS) and potential CDK phosphorylation sites (gray background) in the AtCcs52 proteins. Arrowheads mark conserved phosphorylation sites. Putative PEST motif at the N-terminus of AtCcs52A1 is underlined.

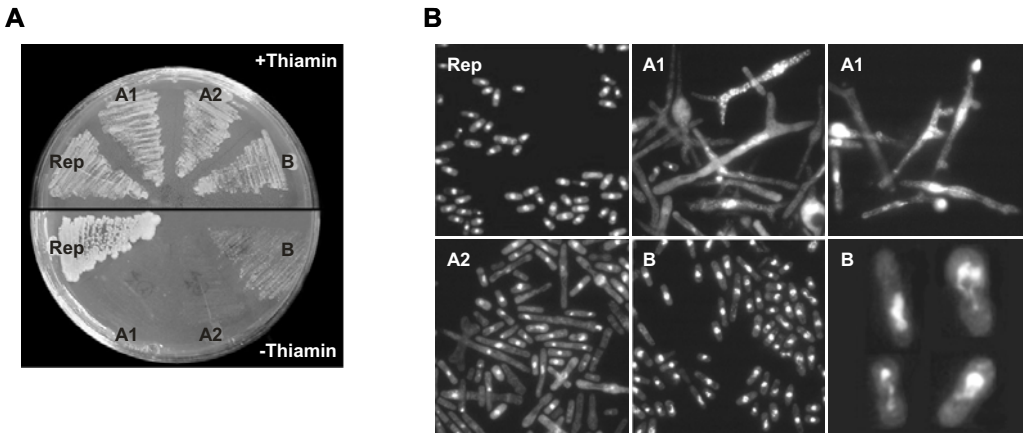

Figure 2. Induced expression of the *Atccs52* genes in fission yeast arrests growth (A) and affects cell size, cell shape and nuclear structure. (B) A1, *Atccs52A1*; A2, *Atccs52A2*; B, *Atccs52B*; Rep, empty vector.

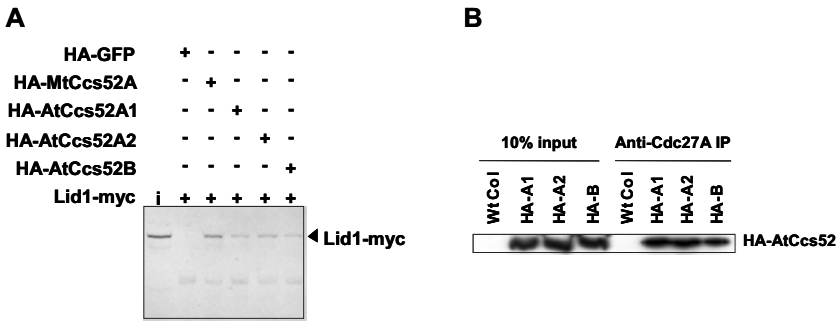

Figure 3. Binding of the Ccs52 proteins to the fission yeast and *Arabidopsis* APCs.
(A) *In vitro* binding of the HA-tagged Ccs52 proteins to the yeast APC labelled with myc epitopes on the Lid1 subunit (Berry *et al.*, 1999).
(B) Detection of the HA-tagged Ccs52 proteins in leaf extracts of transgenic *Arabidopsis* plants (input) and in the anti-Cdc27A immunoprecipitates (IP) of leaf extracts with anti-HA Western blots.

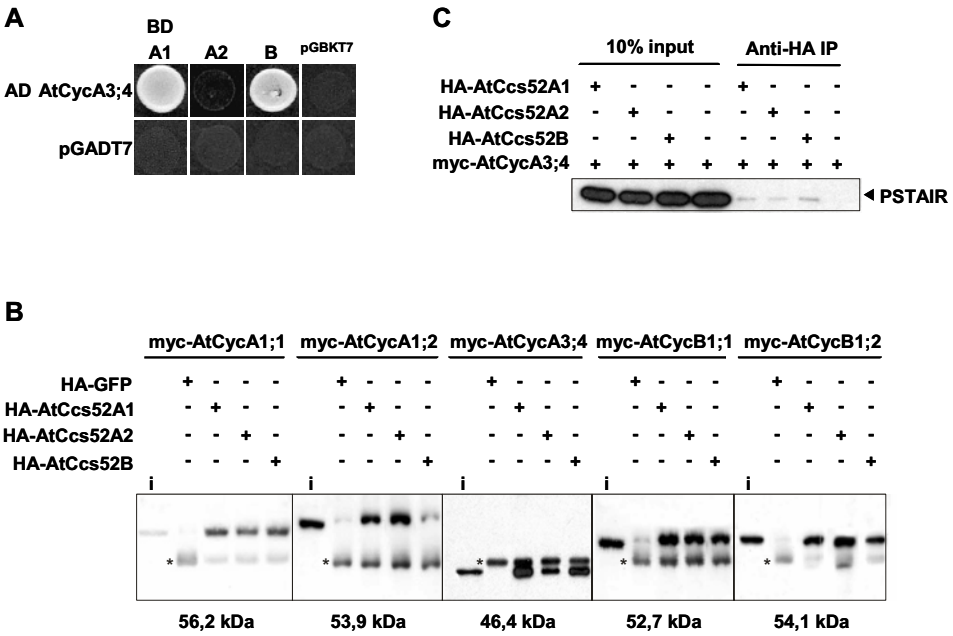

Figure 4. Interaction of AtCcs52 proteins with *Arabidopsis* A- and B-type mitotic cyclins, mitotic cyclin-CDK complexes and CDKs .
(A) Selective interaction of AtCycA3;4 with AtCcs52A1 and AtCcs52B in yeast two-hybrid system. (B) Interaction of HA-tagged Ccs52 proteins with myc-tagged A- and B-type mitotic cyclins in the *Arabidopsis* protoplast system. (C) Immunoprecipitates of protein extracts made of *Arabidopsis* protoplasts over-expressing HA-Ccs52s and myc-AtCycA3;4 contain CDKA;1 detected by anti-PSTAIR antibody.

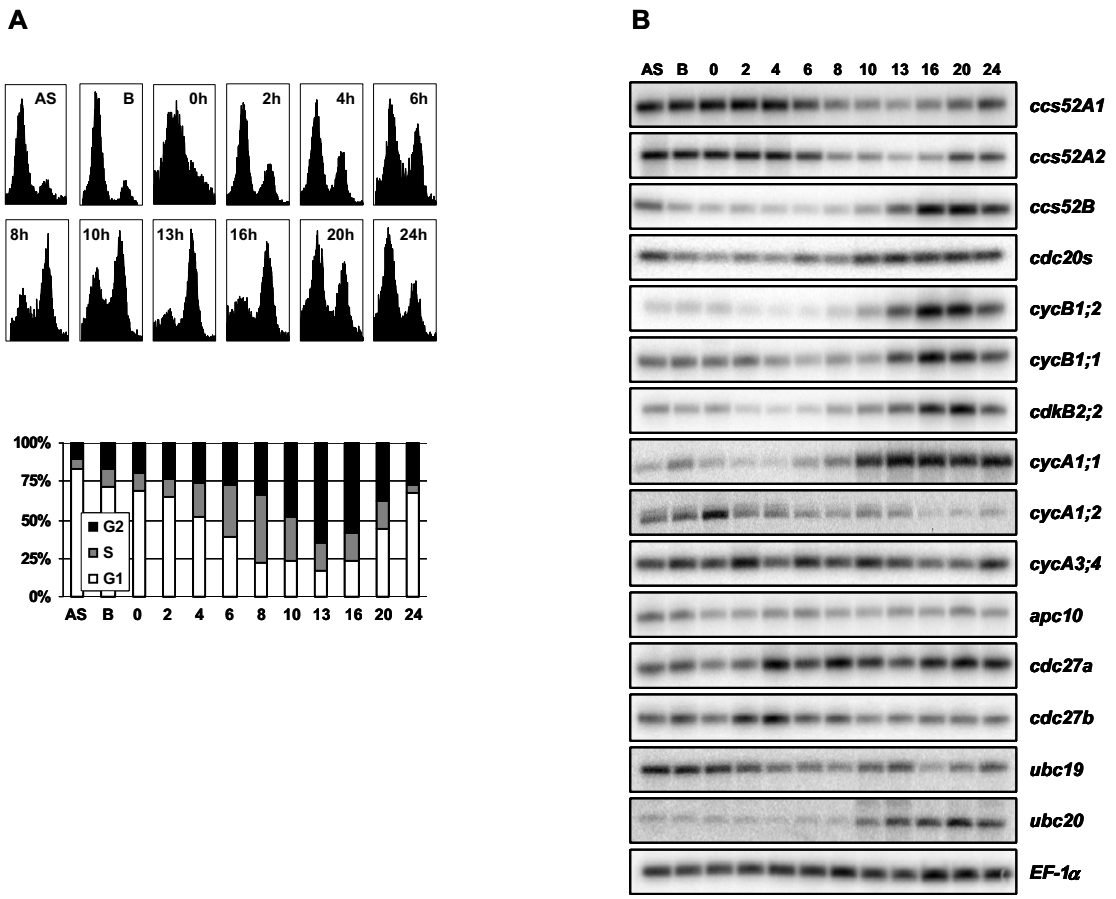

Figure 5. Differential regulation of APC activators, mitotic cyclins, APC subunits and E2-Cs during the cell cycle. (A) Distribution of nuclei in different phases of the cell cycle in asynchronously growing (AS) and synchronized *Arabidopsis* cell cultures. DNA content of DAPI-stained nuclei was measured by flow cytometry. B, aphidicolin-treated cells before wash; 0-24, hours after the release from the aphidicolin block. (B) Expression patterns of APC activators (*ccs52A1*, *ccs52A2*, *ccs52B*, *cdc20*s), APC substrates (*cycB1;1*, *cycB1;2*, *cycA1;1*, *cycA1;2*, *cycA3;4*), APC subunits (*apc10*, *cdc27A*, *cdc27B*), E2-Cs (*ubc19*, *ubc20*) and the mitotic *cdkB2;2* by RT-PCR analysis. Each gene, except the *cdc20*s, was amplified with gene-specific primers. Amplification of the constitutive control *EF-1α* served for quantification of the RNA in the different samples.

Interaction studies on the **Arabidopsis** *APC and Ccs52 activators*

The ability of all three AtCcs52 proteins to bind to the APC was demonstrated in fission yeast and *in planta* as well. However, we could not identify individual subunits that might be involved in the recruitment of these activator proteins. For this, we tried first to map out protein-protein interactions within the *Arabidopsis* APC.

To identify direct interactions among the APC subunits and the Ccs52-type activator proteins, as first approach we preformed Gal4-based pairwise yeast two-hybrid binding tests. This method seemed to be convenient as it has been used successfully in outlining interaction maps for such large complexes as the regulatory particle of the 26S proteasome and the COP9 signalosome in budding yeast (Fu *et al.*, 2001). In *Arabidopsis*, studying binary interactions among putative subunits by this approach helped to reveal the composition of diverse SCF complexes (Risseeuw *et al.*, 2003).

Full-length cDNAs coding for APC subunits and activators were isolated by PCR-based cloning from an *Arabidopsis* cell suspension (Columbia ecotype) cDNA library and verified by sequencing. The proteins tested for interaction (Appendix III) included the three Ccs52-type proteins, four subunits containing tetratricopeptide repeats (Cdc16, Cdc23, Cdc27A and Cdc27B), the cullin homology domain protein Apc2, the Doc domain protein Apc10, the RING finger subunit Apc11, and Cdc26. All cDNAs were cloned in translational fusion with both GAL4 DNA-binding (BD) and activation domain (AD) and yeasts were co-transformed with all possible paired combinations of these constructs. Interactions were detected by monitoring growth on medium selecting for the expression of both the *HIS3* and *ADE2* reporter genes.

Surprisingly, only few interactions were detected (Table 6). Although the trimeric module of TPR-containing subunits Cdc16, Cdc23 and Cdc27 is thought to serve as a scaffold for the assembly of APC, only binding of BD-Cdc16 to AD-Cdc23 was observed in our assays and no growth of yeasts transformed with the inverse configuration (BD-Cdc23/AD-Cdc16) was visible. The BD-fused version of Cdc16 was observed to bind with Cdc26. The two Cdc27 variants failed to show any positive reaction although both proteins were efficiently expressed as verified by anti-GAL4 BD and AD immunoblots. Moreover, N-terminal tagging did not influence their activities when expressed in *Arabidopsis* protoplasts; thus, it is possible that these subunits form multiple weak interactions within

the complex which are not detected in this assay due to the relatively high stringency of the selection. Other positive reactions were observed among BD-Cdc23/AD-Apc11 and BD-Apc10/AD-Apc11. This relative poverty of strong binary interactions between the subunits was also apparent from the results of yeast two-hybrid screens performed with Cdc16, Cdc27A or Cdc27B proteins as baits since no cDNAs coding for putative APC subunits were identified among the isolated binding partners.

	Apc2C	Apc2	Apc10	Apc11	Cdc16	Cdc23	Cdc26	Cdc27A/B
Apc2C			+/–	+/+		+/+		
Apc2		+		+/+		+/+		
Apc10	–/+	+/–		–/+				
Apc11	+/+	+/+	+/–			+/–		
Cdc16		+/–					+/–	
Cdc23	+/+	+/+		–/+		+		
Cdc26		+/–			–/+			
Cdc27A		+/–						
Cdc27B		+/–						

Table 6. ***Interactions among putative*** **Arabidopsis** ***APC subunits detected in yeast two-hybrid tests.*** Minus and plus signs separated by dash correspond to the following configuration: BD-protein in the upper row combined with AD-fused protein in left column/AD-fused protein in upper row combined with BD-fused protein in left column.

Two interactions involving Apc2 gave positive reactions in both combinations (Capron *et al.*, 2003b). The AD-Apc2 construct displayed a clear interaction with Apc11 and Cdc23. Although the BD-Apc2 construct exhibited strong transactivation of the selective reporter genes, it was still possible to confirm these interactions by measuring β-galactosidase activities of transformed yeast cells (Figure 17A). Apc2 is a large subunit containing a carboxy-terminal cullin homology domain and by analogy with cullin1/Cdc53 in the SCF complex (Zheng *et al.*, 2002), it might help to assemble components of the complex. In the human APC, the cullin homology domain of human Apc2 was shown to bind the RING-H2 protein Apc11 and, when supplemented with E2 activity, the heterodimer efficiently ubiquitinated APC substrates (Gmachl *et al.*, 2000; Tang *et al.*, 2001). Thus, Apc2-Apc11 is considered to be the catalytical centre of human APC. Considering the basic importance of the Apc2-Apc11 heterodimer within the APC, the binding of the two *Arabidopsis* proteins was verified *in vivo* as well. For this, HA-tagged Apc11 and myc-tagged Apc2

were co-expressed in *Arabidopsis* protoplasts as described previously. Next, co-immunoprecipitation of the proteins was demonstrated by anti-HA immunoprecipitation followed by an anti-myc immunoblot (Figure 17B).

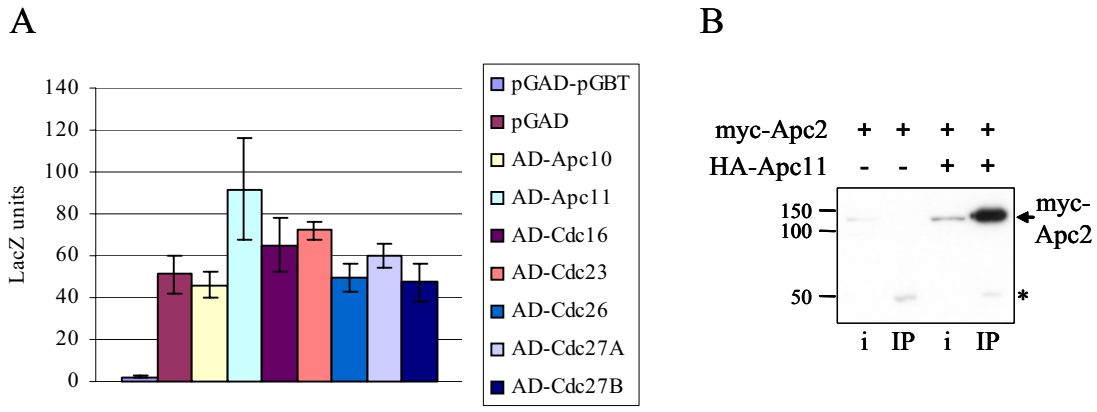

Figure 17. Arabidopsis *Apc2 interacts with Apc11* (Capron *et al.*, 2003b). **(A)** β-galactosidase activities of yeast cells transformed with BD-Apc2 and different APC subunits fused with AD. Yeast cells co-transformed with the empty vectors pGAD and pGBT together with colonies harbouring pGAD and BD-Apc2 were used as controls. **(B)** Apc2 co-immunoprecipitates with Apc11. i, input (one-tenth of the total input); IP, eluate of immunoprecipitation. Asterisk marks the heavy chain of IgG.

Additional data underlining the importance of the Apc2 subunit within the plant APC came from a different experiment, where proteins interacting with the two E2 enzymes Ubc19 and Ubc20 were investigated by a yeast two-hybrid screen of an *Arabidopsis* cDNA library prepared from suspension cultured cells. Among the approx. $1x10^6$ transformants 2 and 3 clones were identified, respectively, on medium selecting for the expression of both the *HIS3* and the *ADE2* reporter genes. All of these colonies contained the same cDNA corresponding to a partial clone of Apc2. The identified cDNA codes for the C-terminal part of the protein starting at amino acid residue 503, spanning the cullin homology domain and ending with a proper stop codon. When this partial clone (Apc2C) was tested in pairwise binding tests, Apc11 and Cdc23 showed positive reactions in both configurations confirming our previous results. In addition, AD-Apc2C interacted with BD-Apc10 (Table 6). Interestingly, the full-length Apc2 did not show any interaction with Ubc19 or Ubc20 in this assay, and a similar result was obtained previously for Apc10 as well (Figure 18). Difference between the binding properties of human Apc2 and the C-terminal cullin fragment of human Apc2 was reported by Tang *et al.* (2001). In their *in vitro* system,

full-length Apc2 failed to interact with Apc11, whereas the C-terminal fragment of Apc2 was sufficient to bind Apc11 and UbcH10 and efficiently supported ubiquitination reactions.

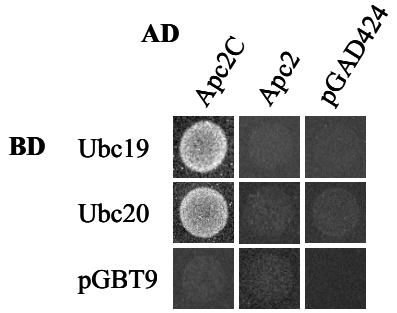

Figure 18. ***Ubc19 and Ubc20 interact with the C-terminal cullin homology domain of Apc2 in yeast two-hybrid system.*** Yeasts were co-transformed with the indicated constructs and plated on synthetic dropout medium lacking histidine and adenine.

The binding of Apc10 to the cullin homology domain of Apc2 seemed to be interesting, as in the previous assay both proteins interacted with Apc11 (Table 7) indicating that Apc10 could be in the proximity of the catalytical core. In budding yeast, Apc10/Doc1 is proposed to improve the catalytic activity of the complex by reducing the dissociation of substrates and to contribute to substrate recognition as well (Carroll and Morgan, 2002; Passmore *et al.*, 2003). Considering this, we further investigated the interactions of Apc10 with diverse APC subunits in co-immunoprecipitation experiments.

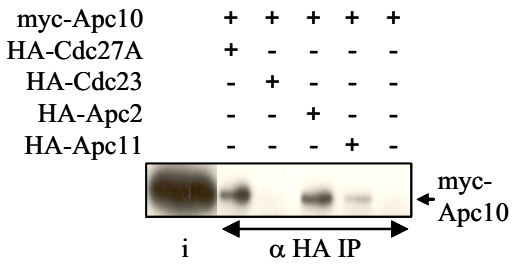

Figure 19. ***Apc10 binds to Apc2, Apc11 and Cdc27A.*** The indicated HA-tagged subunits were immuno-precipitated from *Arabiodpsis* protoplast lysates. The presence of myc-Apc10 in the immunocomplexes was revealed by anti-myc immunoblot. i, one-tenth of total myc-Apc10 input.

Consistent with previous yeast two-hybrid data, myc-Apc10 was detectable in the immunocomplexes of HA-Apc2, HA-Apc11 but not in that of HA-Cdc23. It was also possible to identify a new interaction: myc-Apc10 bound to HA-Cdc27A (Figure 19). Regarding the signal ratio of the input and the recovered proteins, all these interactions seemed to be quite weak. This might be an indication that the detected interaction is not direct: we recovered the whole APC, and myc-Apc10 was detected because it was incorporated to some extent into the endogenous complex. However, tagged proteins are

most probably present in large quantities relative to endogenous subunits, thus it is more likely that binary interaction occurs between them. Moreover, the lack of binding between Apc10 and Cdc23 further argues against detectable incorporation of exogenous proteins into endogenous APC. We suppose that in contrast to the strong, binary binding between Apc2 and Apc11, Apc10 is tethered to the complex by several weak interactions formed by the subunits we had identified (and possibly others as well).

The results of these interaction assays indicate that the Apc2-Apc11 heterodimer, where the catalytic activity of the human and yeast APC resides, is conserved in plants as well. This idea is further reinforced by the fact that E2 enzymes Ubc19/20 bind to the cullin domain of Apc2. Apc10 is close to this core, consistent with its proposed role in substrate ubiquitination. The TPR subunits are also connected to the catalytical centre as indicated by the Apc2/Cdc23, Cdc23/Cdc16 and Apc10/Cdc27A interactions. And although these interactions have to be confirmed by other methods as well (which is a part of ongoing research in our laboratory), the putative organization model of the plant APC matches well with the generalized model of the complex that integrates data from yeast and animal systems (Figure 21A, B in chapter 'Summary and conclusions').

In our next pairwise interaction experiments we aimed at characterizing the interactions that might participate in the association of AtCcs52 proteins to the APC.

For this, activator proteins together with individual *Arabidopsis* APC subunits were tested in binary binding assays. Again, interactions were tried first by the yeast two-hybrid approach. This, and subsequent yeast two-hybrid screens with the activator proteins as baits yielded no positive interactions among AtCcs52 proteins and APC subunits. Recent work of S. Tarayre on the *Medicago* Ccs52 proteins clearly demonstrated that several sequence motifs are important for the binding between the activators and the APC, including the N-terminal C box, the CSM (Cdh1-specific motif) and the C-terminal IR motif (Tarayre *et al.*, 2004). In human cells, the TPR proteins Apc3/Cdc27 and Apc7 were found to bind IR peptides; however, deletion of the IR motif and the C box diminished but did not completely abolish association of Cdh1 to the complex (Vodermaier *et al.*, 2003). Thus, it is highly probable that several subunits within the complex cooperatively mediate the recruitment of activators to the APC. We tried to detect such interactions in a homologous system using epitope-tagged proteins expressed in *Arabidopsis* protoplasts and concentrated first on one of the activators, AtCcs52A2.

It was clear from our previous experiments, that the catalytical core comprising Apc2 and Apc11 is conserved in plants. Moreover, this heterodimer was found to tether E2 activity necessary for the ubiquitination of substrates carried to the complex by the activators. Thus, we tested by co-immunoprecipitation whether one of these subunits could be involved in binding of AtCcs52 proteins. The presence of myc-AtCcs52A2 could be revealed in the immunocomplexes of HA-Apc2 (Figure 20A) but not in that of HA-Apc11 (data not shown). The amount of myc-AtCcs52A2 recovered via HA-Apc2 was approx. equal to 10 % of its total input, which might indicate the non-stochiometric nature of the interaction or, as discussed previously, suggest that several subunits participate in the recruitment of the activators.

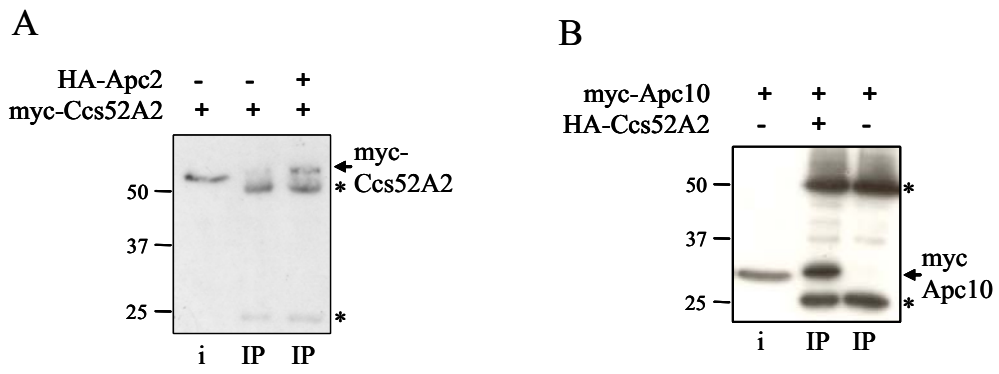

Figure 20. ***AtCcs52 proteins bind to the anaphase-promoting complex.*** **(A)** A fraction of Apc2 co-immunoprecipitates with AtCcs52A2. myc-AtCcs52A2 and HA-Apc2 were expressed in *Arabidopsis* protoplasts and used in anti-HA immunoprecipitation followed by an anti-myc immunoblot. **(B)** AtCcs52A2 binds efficiently to Apc10.
i, input (one-tenth of total input); IP, eluate of immunoprecipitation. Asterisks mark the heavy and light chains of IgG.

The identification of an additional interaction supports this latter possibility. We could demonstrate the binding between HA-AtCcs52A2 and myc-Apc10. Apc10 was previously identified to bind to Apc2, Apc11 and Cdc27A, thus it could be placed in the close proximity of the catalytic centre of the APC. Recent studies in other systems reinforce the role of Apc10 in substrate binding, however, the underlying mechanism is not yet clear. In budding yeast, loss of the Doc1/Apc10 function does not affect the binding of activators to the APC (Carroll and Morgan, 2002), whereas human APC devoid of Apc10 can not interact with Cdh1 (Vodermaier at al., 2003). Yeast data favour direct binding between

Doc1/Apc10 and the destruction box of the substrate and suggest a model, where the function of activators is to load the substrates onto APC – most probably on the Doc1/Apc10 subunit (Carroll *et al.*, 2005). The observed binding of Apc10 with the AtCcs52A2 protein suggests a role for Apc10 in activator recruitment; however, as AtCcs52A2 binds also to Apc2, it is more probable, that multiple low-affinity interactions contribute to their recruitment.

SUMMARY AND CONCLUSIONS

The composition, function and substrate-selective activation of the anaphase-promoting complex is widely studied in yeasts and animals and despite the great effort, our understanding is still far from being complete. APCs have not been isolated from plants so far; however, almost all subunits have homologues in *Arabidopsis*. Several components of the APC-mediated ubiquitination are represented by complex families in plants. In this work we aimed at characterizing the *ccs52* genes and the encoded APC activator proteins of *Arabidopsis*, including their expression during the cell cycle, their interactions with the APC as well as with mitotic cyclins. The major experimental results presented in this part of the thesis can be summarized as follows:

1. Binary interaction analyses show that the basic molecular organization of the APC is conserved in plants.
2. AtCcs52 proteins representing plant Cdh1-type APC activators bind to the fission yeast and Cdc27A-containing *Arabidopsis* anaphase-promoting complexes. The direct binding of the AtCcs52A2 activator to plant Apc2 and Apc10 subunits has been demonstrated.
3. Induced over-expression of AtCcs52A1, AtCcs52A2 and AtCcs52B proteins elicit distinct phenotypes in fission yeast, and the three activator proteins differentially bind plant A- and B-type cyclins indicating that they might have partially distinct and/or complementary functions.
4. In *Arabidopsis* cell suspension culture synchronized to G1/S phase by aphidicolin treatment, expression of most APC subunits was constitutive, whereas *cdc27a* and *cdc27b*, the three *Atccs52* genes, *ubc19* and *ubc20* encoding E2 enzymes displayed differences in their cell cycle regulation. These data indicate the existence of numerous $APC^{Cdc20/Ccs52/Cdc27}$ forms in *Arabidopsis*, which in conjunction with different E2 enzymes might have distinct or complementary functions at distinct stages of the cell cycle.

Compared to other known ubiquitin ligases, the APC is unusually complex with at least 11 subunits in vertebrates and 13 in yeast. Mapping of protein-protein interactions within the APC of these organisms has begun but the rationale of this high complexity is still elusive as well as the role of many subunits (Figure 21A) (reviewed in Castro *et al.*, 2005; Passmore, 2004). It is hypothesized that this complexity might allow intricate temporal and spatial regulation of APC activity that resides, similarly to the SCF complex, in the cullin-RING finger heterodimer. TPR-containing proteins might offer an assembly scaffold for other subunits and contribute to activator recruitment. Regulated binding of activators to the APC controls the timing of APC activation as well as the substrate specificity. The core APC itself seems to contribute to substrate binding via the Doc-domain subunit Apc10.

Our studies indicate, that the basic organization of APC is conserved in plants as well. It was possible to identify a strong interaction between the two components of the catalytical core, Apc2-Apc11, and connect them to the proposed processivity factor, Apc10 as well as the E2 enzymes Ubc19 and Ubc20. Moreover, we found that all three AtCcs52 are able to bind to the APC, and this interaction probably involves the Apc2 and Apc10 subunits. The proposed model of plant APC organization based on the detected interactions is depicted in Figure 21B.

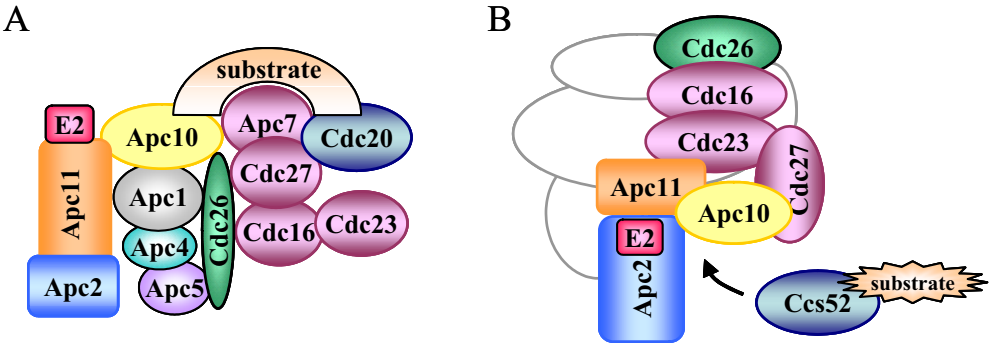

Figure 21. ***Outline of the molecular organization of anaphase-promoting complexes.*** **(A)** Generalized model built by integrating data from studies on the yeast and animal APC (after Castro *et al.*, 2005). **(B)** Proposed outline of the plant APC based on the putative interactions described in this thesis.

In *Drosophila melanogaster*, the APC may exist as several related complexes that are activated independently at different times and at different places and might perform at least

partially non-overlapping functions (Huang and Raff, 2002). The existence of two related copies coding for Cdc27 homologues in *A. thaliana* could lead to the formation of two functionally distinct APCs and suggests high complexity of APC forms in plants. Analysis of the transcript levels of Cdc27 homologues revealed that while *cdc27A* is expressed ubiquitously at high level in the entire embryo and in postembryonic meristems, *cdc27B/hobbit* transcripts show a low and punctuated pattern, indicating that Cdc27B might serve phase-specific functions (Blilou *et al.*, 2002). Our data support this idea as the two *cdc27* variants displayed different expression pattern during the cell cycle. The large array of APC-related genes involving E2 enzymes and activators that all show cell cycle-dependent expression further suggest the complexity of APC functions in plants. These patterns reveal the possibility that distinct APC^{Cdc20} and APC^{Ccs52} forms in conjunction with specific E2 enzymes succeed each other during cell cycle progression allowing a very intricate regulation of the G2/M transition. Moreover, these data support the idea that APC activity might be present in other phases as well. Besides differences in their timing, these complexes may display distinct substrate specificities as indicated by the differential affinities of AtCcs52 proteins towards A- and B-type cyclins. In addition, the different APC forms might function outside the cell cycle, similarly to the different APC^{Cdh1} complexes described from chick (Wan and Kirschner, 2001). The identification of several proteins involved in transcription, hormone biosynthesis and signalling as interactors of the AtCcs52 activators and the differential expression pattern of these genes in various plant organs seem to support this idea (unpublished data of Zsolt Kelemen and Sylvie Tarayre).

In summary, our data indicate that although the basic organization of APC is conserved in plants, several related complexes might exist. The Ccs52A, Ccs52B, and numerous Cdc20 proteins in plants interacting with APC forms containing one of the Cdc27 subunits together with diverse E2 enzymes can thus provide a finely tuned regulation and substrate specificity of the plant APCs during cell cycle and developmental programs.

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ABSTRACT

Regulation of basic cellular functions is conserved among plants and animals reflecting the common evolutionary origins of the two kingdoms. However, during the long independent evolution of these two lineages, the original sets of genes have progressively undergone independent diversification and today, besides serving conserved cellular mechanisms, they contribute to kingdom-specific functions as well. The results presented in this thesis can be included in such large chapters as transcription and cell cycle regulation, where the basic molecular elements show clear conservation across kingdoms.

In the ***first part*** of the thesis, experiments aiming at characterizing an alfalfa cyclin-dependent kinase (CDK) are described. The family of cyclin-dependent kinases is well known in animals as fundamental members of the cell cycle machinery and, more recently, as basic regulators of transcription. CDKs are serine-threonine specific kinases that are activated by a cyclin regulatory subunit. In yeasts, a single kinase, Cdc28/Cdc2 associated with phase-specific cyclins is sufficient to drive the cell division cycle, while higher eukaryotes have several CDKs to promote cell cycle progression. In yeasts and animals, certain CDKs are implicated in the control of transcription by altering the phosphorylation pattern of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II.

Similarly to animals, plants have multiple CDKs classified into six groups from A to F. A-, B-, D- and F-type kinases are involved in cell cycle regulation, while CDKC and E classes have been poorly investigated as of yet. C-type CDKs are constitutively expressed during the cell cycle and their cyclin-binding motif is divergent from the classical PSTAIRE sequence. This class groups the closest plant homologues of metazoan Cdk9 proteins known to be implicated in the regulation of transcription. The present work aimed at characterizing the alfalfa CDKC;1 protein.

First, we screened *Medicago* proteins for CDKC;1 interaction by the yeast two-hybrid system and identified a cyclin similar to human and plant T-type cyclins. The two proteins showed a high interaction specificity since *Medicago* A-, B- and D-type cyclins as well as A-, B- and E-class CDKs were unable to form complexes with the CDKC;1 and cyclin T proteins, respectively.

Specific antibodies were produced against the CDKC;1 kinase in order to characterize it biochemically. The CDKC;1 complex immunoprecipitated from alfalfa cells was found to possess protein kinase activity that phosphorylated proteins such as the myelin basic

protein, the C-terminal domain of RNA polymerase II and the retinoblastoma protein *in vitro*. Unlike complexes of the cell cycle kinases CDKA and CDKB, the CDKC;1 complex failed to phosphorylate the typical CDK substrate histone H1. Co-expression of epitope-tagged CDKC;1 and cyclin T in *Arabidopsis* protoplasts resulted in the formation of a CDK-cyclin complex that displayed a high CTD kinase activity indicating functional interaction *in vivo*.

In a cellular fractionation experiment, CDKC;1 was found in the nuclear protein fraction and its associated CTD kinase activity was detected therein. Nuclear localization of the complex was further confirmed by immunostaining of *Arabidopsis* protoplasts expressing the epitope-tagged CDKC;1 and cyclin T proteins. However, size-fractionation of alfalfa nuclear proteins showed that CDKC;1 was not included in the RNA polymerase II holoenzyme.

In alfalfa cell culture synchronized to G1/S phase by hydroxyurea treatment, both the protein level of CDKC;1 and its CTD kinase activity were constant throughout the cell cycle arguing against a direct involvement of the kinase in cell cycle control.

To assess the role of the CDKC;1 complex in RNA polymerase II-mediated transcription, first its site specificity within the heptapeptide repeat of CTD was determined. The mutation of Ser at position 2 in the repeat did not prevent substrate phosphorylation. In contrast, when Ser at position 5 was replaced by nonphosphorylatable Ala, phosphorylation by CDKC;1 was completely abolished indicating that the kinase complex targets Ser^5 within the CTD *in vitro*.

Finally, in promoter-specific *in vitro* transcription assays the CDKC;1-cyclin T kinase complex was able to replace human Cdk9 function in depleted HeLa nuclear extract and efficiently promoted transcription.

As the *Medicago* CDKC;1-cyclin T complex mirrors the most important features of metazoan Cdk9-cyclin T complexes, we conclude that CDKC;1-cyclin T is a plant orthologue of metazoan positive transcription elongation factor b, which consists of Cdk9-cyclin T.

The ***second part*** of the results contributes to our current understanding on the plant anaphase-promoting complex and its activators. Controlled degradation of regulatory proteins via the ubiquitin-proteasome pathway is an important mechanism in cell cycle regulation. A large multiprotein complex, the anaphase-promoting complex (APC) is responsible for the destruction of mitotic cyclins and other regulatory proteins by the exit from mitosis. Stage-specific activation and substrate selection of the APC are defined by

the binding of either of two activator proteins, Cdc20 or Cdh1. Plants have two classes of Cdh1-type activator proteins, Ccs52A and Ccs52B, that display different cell cycle control and developmental regulation in *Medicago*. The *Arabidopsis* genome contains three *ccs52* genes: *Atccs52A1*, *Atccs52A2* and *Atccs52B*. Moreover, the cyclin family is highly complex in plants, thus control of cyclin stability during the cell cycle might be intricately regulated. In this work we aimed at characterizing the *Arabidopsis ccs52* genes, cell cycle regulation of APC activators, APC components, E2 enzymes and mitotic cyclins.

In order to define whether the three *Arabidopsis* proteins have distinct or redundant functions, their corresponding genes were over-expressed from an inducible promoter in fission yeast. In addition to proliferation arrest, expression of the *Atccs52* genes provoked also drastic morphological changes that were characteristic for each gene. The binding of the *Arabidopsis* activators to the fission yeast APC was demonstrated by co-immunoprecipitation. The distinct phenotypes elicited by the expression of *Atccs52* genes in fission yeast indicated that all the AtCcs52 proteins may activate the yeast APC but have different functions.

The recruitment of AtCcs52 proteins to the plant APC was demonstrated *in planta* by immunoprecipitating the endogenous complex with anti-Cdc27A antibodies from transgenic plants expressing the tagged versions of the AtCcs52 proteins. Pairwise yeast two-hybrid analyses and co-immunoprecipitation experiments using *Arabidopsis* APC subunits indicated that the basic molecular organization of the complex is conserved in plants as well. It was possible to identify the Apc2-Apc11 heterodimer, the minimal ubiquitin ligase module of the human APC, and the binding of E2 enzymes Ubc19/20 to the cullin domain of Apc2. Further co-immunoprecipitation experiments demonstrated the direct binding of AtCcs52 activators to the Apc2 and Apc10 subunits.

To assess the eventual substrate-specificity of AtCcs52 proteins, their interaction with several mitotic cyclins, two A- and three B-type, was investigated. In these *in vitro* binding assays, the activators displayed distinct interaction patterns with the cyclins indicating that they might interact with partially different subsets of substrates.

In *Arabidopsis* cell suspension culture synchronized to G1/S phase by aphidicolin treatment, expression of most APC subunits proved to be constitutive, whereas *cdc27a* and *cdc27b*, the three *Atccs52* genes, *ubc19* and *ubc20* encoding E2 enzymes displayed differences in their cell cycle regulation. These data indicate the existence of numerous $APC^{Cdc20/Ccs52/Cdc27}$ forms in *Arabidopsis*, which, in conjunction with different E2 enzymes might have distinct or complementary functions at distinct stages of the cell cycle.

ÖSSZEFOGLALÁS

A sejtek fenntartásához szükséges alapfolyamatok nagyfokú konzerváltságot mutatnak állati és növényi szervezetekben egyaránt összhangban e két eukarióta fejlődési vonal közös evolúciós eredetével. A törzsfejlődés későbbi szakaszában azután az eredeti közös génkészlet fokozatosan megváltozott és a mai élőlényekben, az eredeti funkciók mellett egyéb, a fejlődési vonalra jellemző feladatok kiszolgálására is alkalmassá vált. A dolgozatban bemutatott eredmények olyan alapvető folyamatok részei, mint a sejtosztódás és a transzkripció, így nem meglepő, hogy a tanulmányozott növényi fehérjék igen hasonló tulajdonságokat mutatnak, mint állati homológjaik.

Az értekezés ***első rész***ében egy lucerna ciklinfüggő kináz (CDK) jellemzésére végzett kísérleteink kerülnek bemutatásra. A ciklinfüggő kinázok elsősorban mint a sejtosztódási ciklus kulcslépéseit szabályzó fehérjék ismertek; kevésbé tudott, hogy állati és élesztő sejtekben a transzkripció szabályzásásban is igen jelentős szerepük van. A CDK család tagjai szerin-treonin kinázok, amelyek ativitásukat és szubsztrátspecifitásukat ciklin reguláló alegységükkel való kapcsolódás után nyerik el. Élesztőkben egyetlen kináz, a Cdc28/Cdc2 fehérjék különböző ciklinekkel alkotott komplexei aktívak a sejtciklus mindkét átmenetében, míg a magasabbrendű eukarióta szervezetekben több kináz is részt vesz a folyamatban. Élesztőkben és állatokban több ciklinfüggő kináz aktivitása is alapvető fontosságú a fehérjekódoló gének átírásának szabályzásában; ezek a CDK-k az RNS polimeráz II karboxi-terminális doménjénét (CTD) foszforilálják.

Az állatokhoz hasonlóan a növényi szervezetek is több ciklinfüggő kináz fehérjével rendelkeznek. Ezek hat osztályba sorolhatók, CDKA-tól CDKF-ig. Az A, B, D és F típusú kinázok a sejtosztódási ciklus szabályzásában vesznek részt, míg a C és E típusú kinázok funkciója egészen az utóbbi időkig ismeretlen volt. A C típusú CDK-k mRNS-e a sejtciklus minden fázisában megtalálható és a ciklinkötésért felelős motívumuk eltér a klasszikus PSTAIRE szekvenciától. Ezek a fehérjék jelentik az állati Cdk9 kinázok legközelebbi homológjait, amelyekről ismert, hogy a transzkripció szabályzásában vesznek részt. A munkám elején a lucerna CDKC;1 kinázának jellemzését tűztük ki célul.

Ehhez először élesztő kéthibrid rendszerben vizsgáltuk a kinázzal kölcsönható fehérjéket. Így sikült azonosítanunk egy ciklin fehérjét, amely nagy hasonlóságot mutatott humán és növényi T típusú ciklinekhez. A két fehérje kölcsönhatása specifikusnak bizonyult, mivel további, irányított kéthibrid vizsgálatokban sem a kináz nem mutatott

kölcsönhatást egyéb, A, B és D típusú lucerna ciklinekkel, sem az azonosított ciklin A, B és E típusú CDK-kal.

A CDKC;1 kináz komplexének biokémiai jellemzéséhez specifikus ellananyagot termeltettünk a fehérje ellen. A lucerna sejtek fehérjekivonatából immunoprecipitált CDKC;1 komplex *in vitro* kinázaktivitással rendelkezett és hatékonyan foszforilálta a mielin bázikus fehérjét, az RNS polimeráz II C-terminális doménjét valamint a retinoblasztóma fehérjét, ellenben nem foszforilálta a tipikus CDK szubsztrát hiszton H1 fehérjét. Az *Arabidopsis* protoplasztokban kifejeztetett epitóp-jelölt fehérjék erős CTD kinázaktivitást mutató CDK-ciklin komplexet képeztek, alátámasztva ezzel a két fehérje *in vivo* funkcionális kölcsönhatását.

Lucerna sejtekből készített sejtmagi és citoplazmatikus frakciókban a CDKC;1 fehérje valamint a komplex CTD kinázaktivitása nukleáris lokalizációt mutatott, ami az epitóp-jelölt fehérjéket termelő *Arabidopsis* protoplasztokon immunolokalizációval is igazolható volt. Méret szerint elválasztott sejtmagi fehérjéken végzett immunoblotokkal bemutattuk, hogy a CDKC;1 kináz nem integráns része az RNS polimeráz II holoenzimnek.

Hidroxiureával G1/S fázisra szinkronizált lucerna sejtekben sem a CDKC;1 fehérje szintje, sem komplexének CTD kinázaktivtása nem változott a sejtciklus előrehaladtával, ami arra enged következtetni, hogy a fehérje nem vesz közvetlenül részt a sejosztódás szabályzásában.

A CDKC;1 fehérje transzkripcióban betöltött szerepét vizsgálva először a CTD ismétlődő heptapeptidjében a komplex által foszforilált aminosavat határoztuk meg. A kettes pozícióban található szerin alaninra cserélése nem befolyásolta a foszforiláció mértékét, ellenben az ötös pozícióban található szerin cseréje nem foszforilálható alaninra teljesen meggátolta a reakciót. Ezek alapján megállapítható, hogy a CDKC;1 kináz komplexe a Ser^5 aminosavat foszforilálja *in vitro*.

A kinázkomplex transzkripcióra gyakorolt hatását promóter-specifikus *in vitro* transzkripcióval vizsgáltuk humán sejtekből származó fehérjekivonatban. Ezekben a reakciókban a CDKC;1-cyclin T komplex képes volt a hiányzó Cdk9 funkció pótlására és pozitívan befolyásolta a transzkripciót.

A lucerna CDKC;1-cyclin T legfontosabb tulajdonságai megegyeznek az állati Cdk9-cyclin T komplexekével és hasonlóan ezekhez, a komplex elősegíti az RNS polimeráz II általi transzkripciót. Ezek alapján feltételezzük, hogy a CDKC;1-cyclin T komplex az állati pozitív transzripciós elongaciós faktor b (P-TEFb) növényi ortológja.

A dolgozat ***második rész***ében tárgyalt eredmények a növényi cikloszómáról és annak aktivátor fehérjéiről rendelkezésünkre álló képet árnyalják. A szabályzó fehérjék ubiquitin-közvetítette bontása igen fontos sejtciklus-szabályzó mechanizmus. A cikloszóma (anaphase-promoting complex, APC) aktivitása a mitotikus ciklinek és egyéb kontrollfehérjék lebontásán keresztül elengedhetetlen a mitózisból való kilépéshez. A komplex aktivitását és szubsztrátspecifitását két szabályzófehérje, Cdc20 és Cdh1, sejtciklusfüggő kapcsolódása szabja meg. Növényekben a Cdh1 típusú aktivátoroknak két osztálya különíthető el, a Ccs52A és Ccs52B fehérjék, amelyek különböző sejtciklus és fejlődési mintázatot mutatnak lucernában. Az *Arabidopsis* genomban három *ccs52* gén azonosítható: *Atccs52A1*, *Atccs52A2* és *Atccs52B*. Mivel a ciklinek családja is igen összetett növényekben, feltételezhető, hogy ezek stabilitása sokrétűen szabályzódik a növényi sejtciklus során. A értekezésben bemutatott munkánkkal az *Arabidopsis ccs52* gének sejtciklusfüggő expresszióját, a kódolt fehérjék APC-vel és lehetséges szubsztrátokkal való kölcsönhatásait kívántuk jellemezni.

Annak eldöntésére, hogy a három APC aktivátor fehérje redundáns vagy egyedi funkiókkal rendelkezik, a fehérjéket kódoló géneket hasadó élesztőben fejeztettük ki. Mindhárom aktivátor génje meggátolta a sejtek osztódását és mindegyik alapvető, génenként eltérő morfológiai változásokat indukált az élesztő sejtekben. Ko-immunoprecipitációs kísérletekkel valamennyi *Arabidopsis* fehérje kapcsolódása az élesztő APC-hez igazolható volt. A különböző fenotípusok élesztőben arra engednek következtetni, hogy az *Arabidopsis* Ccs52 fehérjék mind aktiválják az APC-t, de legalább részben eltérő funkcióval rendelkeznek.

Az AtCcs52 fehérjék kölcsönhatása az *Arabidopsis* APC-vel szintén igazolható volt; ehhez az endogén komplexet anti-Cdc27A ellenanyaggal immunoprecipitáltuk az aktivátor fehérjék epitóp-jelölt változatát kifejező transzgénikus növényekből készített fehérjekivonatból és az AtCcs52 fehérjéket az epitópjuk alapján detektáltuk immunoblottal. Élesztő kéthibrid vizsgálatok és ko-immunoprecipitálások sorozatával igazoltuk, hogy az APC komplex molekuláris szerkezete a növényekben is konzervált. Azonosítottuk az Apc2-Apc11 heterodimert, amely a humán komplex katalítikus aktivitásáért felelős, valamint megállapítottuk, hogy az Ubc19/20 E2 enzimek kötődnek az Apc2 cullin-homológ doménjéhez élesztő kéthibrid rendszerben. Ko-immunoprecipitációval igazoltuk az aktivátorok direkt kölcsönhatását az Apc2 és az Apc10 alegységekkel.

Az AtCcs52 fehérjék szubsztrátspecifitását A és B típusú mitotikus ciklinekkel vizsgáltuk. *In vitro* kötési kísérletekben a különböző aktivátorok egyedi kölcsönhatási

mintázatot mutattak, ami alapján feltételezhető, hogy csak részben átfedő szubsztrátkörrel rendelkeznek.

Afidikolinnal a G1/S átmenetre szinkronizált *Arabidopsis* sejtekben vizsgálva az egyes APC alegységek kifejeződése konstans volt, egyedül a *cdc27A* és *cdc27B* gének mutattak némi sejtciklusfüggő mintázatot. Ezzel szemben mindhárom aktivátor génje, valamint az Ubc19/20 E2 enzimeket kódoló gének kifejeződése erősen változott a sejtciklus különböző fázisaiban. Ezen adatok alapján több $APC^{Cdc20/Ccs52/Cdc27}$ formát tételezünk fel a növényi sejtekben, amelyek a különböző E2 enzimekkel kölcsönhatásban specifikus és/vagy egymást kiegészítő funkciókat látnak el a sejtciklus több fázisában.

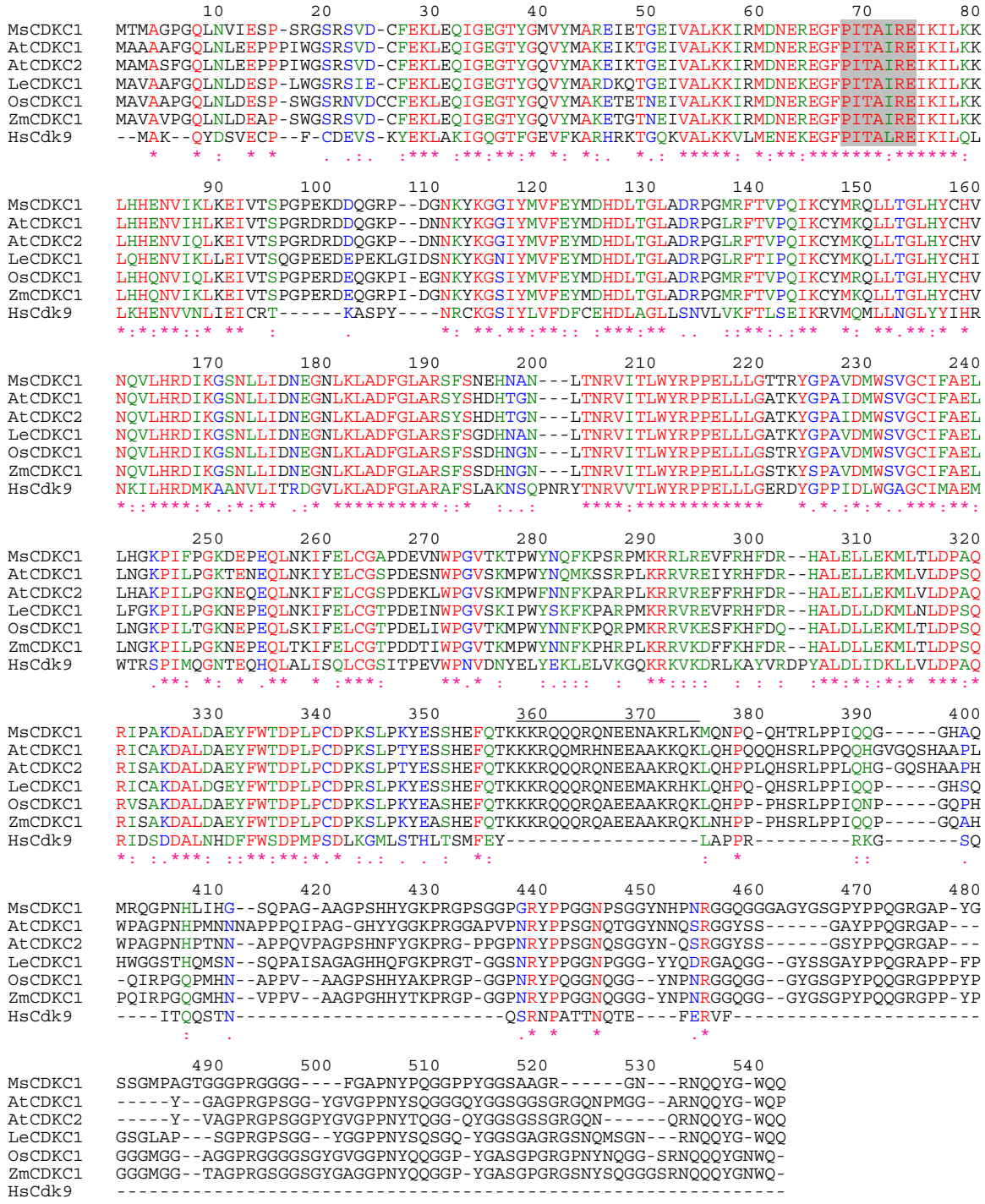
RÉSUMÉ

Les kinases cycline-dépendantes (CDK) sont des Ser/Thr kinases qui, en interagissant avec les cyclines, contrôlent principalement les transitions du cycle cellulaire. D'autres CDKs participent à la régulation de la transcription par phosphorylation du domaine C-terminal de l'ARN polymérase II. Chez les plantes, six familles des CDKs ont été décrites. Contrairement aux CDKs participant à la régulation du cycle cellulaire, la famille des CDKC est peu caractérisée. L'objectif de notre travail consistait en la caractérisation de la protéine CDKC;1 chez la luzerne.

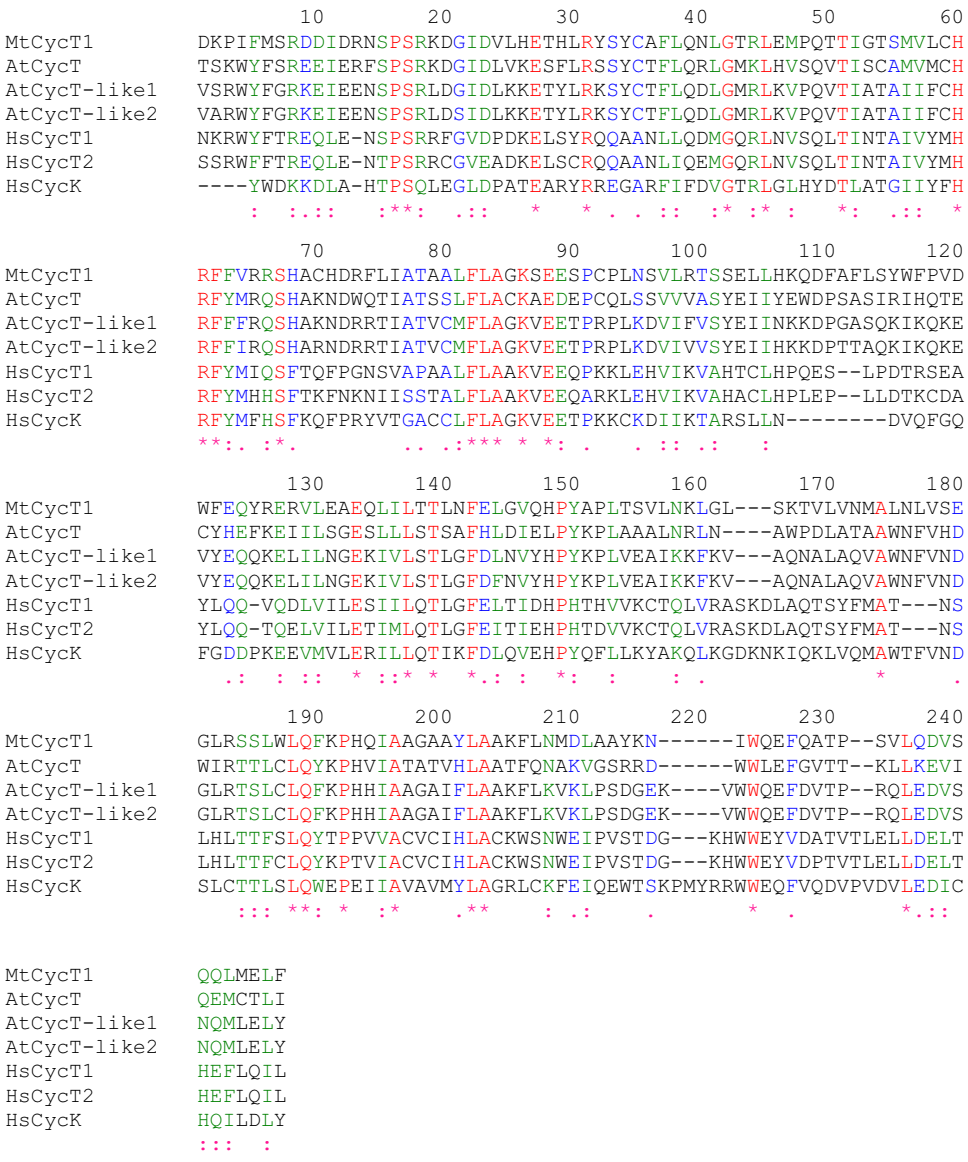
Le partenaire activateur de CDKC;1, une cycline T, a été identifiée par le système double-hybride chez la levure. L'activité catalytique de CDKC;1 analysée par différentes approches biochimiques, comme sa spécificité de substrat et sa localisation subcellulaire, indiquaient pour ce complexe un rôle dans la régulation de transcription. L'activité promotrice du complexe CDKC;1-cycline T est démontrée par des essais de transcription *in vitro*. Nos résultats indiquent que ce complexe serait l'orthologue du facteur d'élongation P-TEFb connu chez les animaux.

La seconde partie de la thèse aborde le sujet de la protéolyse ubiquitine-dépendante qui est un mécanisme important dans la régulation du cycle cellulaire. L'APC est une ubiquitine-ligase essentielle pour la mitose, dont l'activité est contrôlée par l'une de ses deux activateurs, Cdc20 et Cdh1/Ccs52, lui confèrant sa spécificité de substrat. Les protéines de type Cdh1/Ccs52 chez les plantes appartiennent à deux sous-familles: Ccs52A et B qui se distinguent aussi par leur expression différentielle au cours du cycle cellulaire et du développement.

Notre travail a consisté à caractériser les trois membres de la famille des gènes *ccs52* (*A1*, *A2* et *B*) chez *Arabidopsis thaliana*. L'organisation moléculaire de l'APC est étudiée chez les plantes et l'interaction entre les activateurs et l'APC est démontrée *in planta*. Des expériences d'interaction entre les protéines Ccs52 et des cyclines mitotiques ainsi que des études d'expression permettent d'envisager plusieurs formes $APC^{Cdc20/Ccs52/Cdc27}$ chez *A. thaliana*, ceux-ci ayant des fonctions différentes et complémentaires dans les différentes phases du cycle cellulaire.



Appendix I. ***Alignment of plant CDKC and human Cdk9 proteins.*** Cyclin-binding motifs are highlighted by grey shading. The position of the predicted nuclear localization signal in plant CDKCs is marked by black line. Red (*), identity; green (:), highly similar; blue (.), similar. Ms, *Medicago sativa*; At, *Arabidopsis thaliana;* Le, *Lycopersicon esculentum*; Os, *Oryza sativa*; Zm, *Zea mays*; Hs, *Homo sapiens*.



Appendix II. ***Alignment of the cyclin box and 4-helix bundle region of plant and human T-type cyclins.*** Numbering begins at the cyclin box domain for each protein.

	S. cerevisiae	*H. sapiens*	*A. thaliana*	**Protein motifs**	**Function**
APC subunits	Apc1	Apc1	At5g05560	Rpn1/2 protea-some repeats	scaffold?
	Apc2	Apc2	At2g04660*	cullin domain	catalytic core, recruits UbcH10
	Cdc27	Apc3/Cdc27	At3g16320 (Cdc27A)* At2g20000 (Hobbit, Cdc27B)*	TPR repeats	binds activators and Apc10
	Apc4	Apc4	At4g21530		
	Apc5	Apc5	[At1g06600, At1g06590]		
	Cdc16	Apc6/Cdc16	At1g78770 (Nomega)*	TPR repeats	protein-protein interactions?
		Apc7	At2g39090	TPR repeats	binds activators and Apc10
	Cdc23	Apc8/Cdc23	At3g48150*	TPR repeats	protein-protein interactions?
	Apc9				stabilization of APC structure?
	Doc1/Apc10	Apc10	At2g18290*	Doc domain	processivity factor
	Apc11	Apc11	At3g05870*	RING-H2 domain	catalytic core, recruits Ubc4
	Cdc26	Cdc26	not annotated*		stabilization of APC structure?
	Apc13				stabilization of APC structure?
	Mnd2				stabilization of APC structure?
Activators	Cdc20	p55Cdc	At4g33260 At4g33270 At5g27570 At5g26900 At5g27080	WD40	activation of APC substrate recruitment
	Cdh1/Hct1	Cdh1/Fzr	At4g22910 (AtCcs52A1)* At4g11920 (AtCcs52A2)* At5g13840 (AtCcs52B)*	WD40	activation of APC substrate recruitment
E2 enzymes	Ubc11p	UbcH10	At1g50490 (Ubc20)* At3g20060 (Ubc19)*		E2 activity
	Ubc4p	Ubc4	10 genes, Bachmair *et al.*, 2001		E2 activity

Appendix III. ***Composition of the APC in different species.*** (After Capron *et al.*, 2003; Castro *et al.*, 2005; Harper *et al.*, 2002). *Arabidopsis* proteins used in this study are marked by asterisk.