Cyclin-dependent kinase inhibitors of *Arabidopsis thaliana*

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Preface

This work was carried out at the Department of Plant Systems Biology, Flanders Interuniverstity Institute for Biotechnology (VIB), University of Ghent, Belgium. The “Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen” (IWT), University of Ghent, VIB and European Molecular Biology Organization (EMBO) are gratefully acknowledged for the fellowships granted.

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Aurine

Content

Abbreviations

ABA	Abscisic acid
APC/C	Anaphase-promoting complex/Cyclosome
BY-2	Bright Yellow-2 tobacco cell suspension culture
CAK	CDK activating kinase
CaMV	Cauliflower mosaic virus promoter
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CHX	Cycloheximide
Cip/Kip	CDK inhibitor protein
CKI	CDK kinase inhibitor
CKS/Suc	CDK kinase subunit/Subunit of CDK
Col	*Arabidopsis thaliana* Colombia ecotype
CTD	Carboxy-terminal domain
CYC	Cyclin
DAS	Days after sowing
DEL	DP-E2F like protein
DNA	Deoxyribonucleic acid
DP	Dimerization partner
E2	Ubiquitin-conjugating enzyme 2
E2F	Adenovirus E2 promoter binding factor
E3	Ubiquitin-ligase enzyme 3
G1/G2	Gap-phases 1/2
INK4	Inhibitor of CDK4
KRP	Kip-related protein
M	Mitosis
MM1	MM1 *Arabidopsis thaliana* cell suspension culture
MSA	M-phase specific activator
MTs	Microtubules
MYB	Myeloblastosis viral oncogene
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis
PPB	Preprophase band
RB/RBR	Retinoblastoma protein/related
RC	Replicative complex
PCR	Polymerase chain reaction

RFM	Ring finger motif protein
RNA	Ribonucleic Acid
RT	Reverse transcription
S	DNA synthesis
SCF	Skp1, Cullin, F-box complex
SDS	Sodium dodecyl sulfate
SKP	S-phase kinase associated protein
STM	Shootmeristemless promoter
TF	Transcription factor
WEE1	Wee1 kinase

Scope

Progression through the cell cycle is central to cell proliferation and fundamental in growth and development of all multicellular organisms, including higher plants. A central role in the regulation of the cell cycle is played by the cyclin-dependent kinases (CDKs) (chapter 1). CDK activity is controlled by a variety of mechanisms, including by docking of a family of mainly low molecular weight proteins called CDK inhibitors (CKIs), which negatively regulate CDK activity by tight association with CDK/cyclin complexes. The *Arabidopsis thaliana* genome contains seven *CKI* genes called *KRPs* (Kip related proteins), because of their homology with the mammalian CKIs of the Kip/Cip family (chapter 2). The goal of this research was to decipher the role of KRPs during the cell cycle and in plant growth and development.

Most part of this work (chapter 3, 4 and 5) focuses on a detailed analysis of KRP2.

Analysis of *Arabidopsis* plants overexpressing *KRP2* illustrated a role for KRP2 as activator of the mitosis-to-endocycle transition (chapter 3). Moreover, we showed that KRP2 protein abundance is regulated through CDK phosphorylation and proteasomal degradation and propose a mechanism in which CDKB1;1 controls the level of CDKA;1 activity through regulating KRP2 protein abundance.

In chapter 4, the effect of CDK phosphorylation on KRP2 function and stability was studied in more detail and proven to alter the CDK binding-specificity and to enhance the degradation of KRP2. We also show that CKS1 regulates KRP2 function, indirectly by competing with KRP2 for CDKA;1 interaction, and directly through binding and destabilization of KRP2.

Deletion mapping and mutagenesis of KRP2 identified its functional domains involved in protein stability and CDK and cyclin interaction and revealed that CDK inhibition requires tight association of KRP2 with both the CDK and cyclin subunit (chapter 5). Furthermore, analysis of KRP2 mutants with impaired CDK inhibitory activity suggested a possible dual role for KRP2 as CDK regulator.

Finally, in chapter 6, we present the partial molecular analysis of a KRP interacting KBR1 RING finger protein and suggest a possible function of RFP1 in the degradation of KRPs and other stress induced negative regulators of cell cycle and plant growth.

Chapter 1

Chapter page: Inflorescence of a wild-type *Arabidopsis thaliana* plant (Col-0).

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The plant cell cycle

Introduction

Cell division is one of the most conspicuous features of life, and thus the main events in the control of cell cycle regulation are highly conserved throughout evolution (Amon, 1998; Leatherwood, 1998). However, despite this overall evolutionary conservation, individual mechanisms of cell cycle control vary considerably between different species. Plants have unique features that give the control of cell division particular importance, including their post-embryonic organ initiation and growth throughout the entire plant lifespan from specialized regions called meristems, the absence of cell migration, and the responsiveness of growth rate and development to changes in environmental conditions. Plant cell division therefore plays a role both in the developmental processes that create plant architecture and in the modulation of plant growth rate in response to the changing environment (Cockcroft et al., 2000; Menges et al., 2002).

The central convergence point of eukaryotic cell cycle control, where intrinsic and extrinsic cues are integrated, is a group of heterodimeric Ser/Thr kinase complexes, consisting of a catalytic subunit, the cyclin-dependent kinase (CDK), and an activating cyclin subunit (Nigg, 1995). CDKs comprise several subtypes, based on the constituents of a catalytic and a regulatory subunit (Pines, 1994). CDK activity is highly regulated at specific stages of the cell cycle by a number of events including availability of cell cycle components (through transcriptional and translational regulation and proteolysis) (Peters, 1998), post-translational modifications, subcellular localization, and interaction with other regulatory proteins (Lees, 1995). The coordinated progression through the cell cycle occurs through the phosphorylation of a plethora of substrates by activated CDKs, resulting in the entry into a new round of DNA replication and the entry into mitosis, respectively. This chapter focuses on the different classes of plant CDKs and cyclins, and summarizes our current knowledge on cell cycle regulation in plants, compared to what is known in other eukaryotes.

Plant CDKs

Since the identification of the yeast cdc2 (S. pombe) and CDC28 (S. cerevisiae) (Lörincz and Reed, 1984; Nasmyth and Reed, 1980) a multitude of CDK related kinases have been isolated and characterized in different organisms, including plants (Mironov et al.,1999). While in yeast a single CDK is responsible for initiating all cell cycle transitions, different CDKs with specialized functions during the cell cycle exist in higher eukaryotes. The plant CDKs have been classified into six (A to F) subtypes (Joubès et al., 2000a). In the *Arabidopsis thaliana* genome, 12 CDKs have been identified, displaying a high degree of homology with yeast and animal CDKs (Vandepoele et al., 2002). The functions of the A- and B-type CDKs are the best documented.

CDKA

A-type CDKs are related to yeast cdc2/CDC28 and animal CDK1/CDK2. All members of this class contain the conserved PSTAIRE motif in the cyclin-binding domain and have been shown to functionally complement yeast cdc2/CDC28 mutations (Colasanti et al., 1991; Ferreira et al., 1991; Hirt et al., 1991, 1993; Fobert et al., 1996). *CDKA* mRNAs are expressed throughout the cell cycle, and their protein kinase activity becomes activated at both the G1/S and G2/M transitions (Martinez et al., 1992; Hemerly et al., 1993; Bögre et al., 1997; Magyar et al., 1997).

CDKA;1 transcripts are confined to tissues in which the cells have retained the capacity to divide and are induced upon cell division stimulating treatments, suggesting the involvement of CDKAs in cell proliferation and maintenance of cell division in non-proliferating tissues (Hemerly et al., 1993). However, CDKA activity within cells is not regulated at the level of its expression as constitutive overexpression of *Arath;CDKA;1* in either *Arabidopsis* or tobacco did not affect plant development (Hemerly et al., 1995). Rather its activity is regulated at the posttranscriptional level, and constitutive overexpression of a *CDKA;1* dominant-negative (*CDKA;1.DN*) mutant allele, which does not posses kinase activity, causes lethality in transgenic *Arabidopsis* plants and a severe inhibition of cell proliferation in transgenic tobacco plants (Hemerly et al., 1995). Interestingly, this reduction in cell number was accompanied by an increase in cellsize, but did not affect plant morphogenesis of *CDKA;1.DN* tobacco plants, illustrating the flexibility of plant development in relation to cell number.

CDKB

B-type CDKs constitute the second major group of CDKs involved in plant cell division. Unlike the *CDKA*, the expression of the B-type *CDK* genes is under strict cell cycle control as transcripts and proteins peak mainly from S phase till mitosis (Hirayama et al., 1991; Fobert et al.,1996; Segers et al.,1996; Magyar et al.,1997; Joubès et al., 2000a). In accordance, kinase activity of B-type CDKs is predominantly linked to mitosis (Sorrell et al., 2001; Porceddu et al.,2001). B-type CDKs are subdivided in two groups B1- and B2-type CDKs, distinguished by the PPTALRE or PPTTLRE amino acid motif in their respective cyclin-binding domain and are unable to functionally complement temperature-sensitive cdc2 yeast mutants (Imajuku et al., 1992; Fobert et al., 1996).

In etiolated seedlings, expression of the *Arabidopsis CDKB1;1* gene is correlated with the elongation rate of the hypocotyls. Inhibition of *CDKB1;1* gene expression by an antisense construct resulted in short hypocotyls and open cotyledon phenotypes when transgenic seedlings were grown in darkness, indicating a role for CDKB1;1 in hypocotyl cell elongation and cotyledon cell development (Yoshizumi et al., 1999). Moreover, cotyledons of antisense lines fail to green when transferred from darkness to light, which is attributed to the conversion of etioplasts to amyloplasts. Recently CDKB1;1 has been postulated to have a function in stomatal development, as it is highly expressed in guard cells and stomatal precursor cells of cotyledons (Boudolf et al., 2004a). In accordance, transgenic *Arabidopsis* plants with reduced B-type CDK activity show a decreased stomatal index due to an early block of stomatal precursor cell division. Many aberrant stomata cells were observed that were blocked in the G2 phase of the cell cycle. Although division of stomatal precursors was inhibited, cells still acquired stomatal identity, illustrating that stomatal cell differentiation is independent of cellular and nuclear division. In addition to their stomatal phenotype, *Arabidopsis* plants overexpressing a dominant negative allele of *CDKB1;1* were found to undergo premature endoreduplication, demonstrating a function of CDKB1;1 in the control of the timing of endocycle onset (Boudolf et al., 2004b).

CDKC

Several plant C-type CDKs have been isolated and characterized from different species (Feiler and Jacobs, 1991; Magyar et al., 1997; Burssens et al., 1998; Joubès et al., 2001; Barroco et al., 2003; Fulop et al., 2005). Members of the C-type CDK group are characterized by the presence of a PITAIRE motif in the cyclin binding domain. This motif is also present in the human cholinesterase-

related cell-division controller (CHED) kinases that are suspected to be involved with megakaryocyte differentiation in hematopoiesis (Lapidot-Lifson et al., 1992).
Although CDKC function is poorly documented, CDKCs were recently partially characterized and suggested to have a function in the control of the transcriptional machinery (Barroco et al., 2003; Fulop et al., 2005). T-type cyclins were identified as binding partner of the CDKCs. Plant CYCT shares homology with animal cyclins from the T and K group that bind CDK9, a CDK containing the divergent PITALRE motif that has a function in transcription elongation (De Falco and Giordano, 1998; Bregman et al., 2000), suggesting that CDKC proteins are functional homologues of CDK9. *Arath;CDKC* expression was not detected in actively dividing *Arabidopsis* tissues and transcripts were mainly confined to epidermal cells of differentiated flower tissues (Barroco et al., 2003). This is in accordance to what has been described for animal CDK9, that is present in elevated levels in terminally differentiated cells (Price, 2000), suggesting the implication of CDKCs in the molecular mechanisms triggering cell differentiation.

CDKE

E-type CDKs harbour the SPTAIRE motif and were identified in alfalfa and *Arabidopsis* (Joubès et al., 2000a; Vandepoele et al., 2002). CDKE expression in synchronized alfalfa cells displays a weak constitutive signal throughout the cell cycle (Magyar et al., 1997).
Recently, the *Arabidopsis* HUA ENHANCER3 (HEN3) gene was mapped and found to encode CDKE (Wang and Chen, 2004). HEN3 exhibits CTD kinase activity, like human CDK8, which regulates transcription through the phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II (Rickert et al., 1996). This means that CDKE could play a regulatory role in transcription. Moreover, E-type CDKs are proposed to have a function in cell differentiation since the HEN3 mutation affected the specification of stamen and carpel identity and the proper termination of stem cells in the floral meristem.

CDK-activating kinases CDKD and CDKF

CDK activation requires, beside cyclin interaction, the phosphorylation of a conserved Thr residue by CDK-activating kinases (CAKs). In plants CAKs were classified in two groups: the D- and the F-type CDKs (Vandepoele et al., 2002; reviewed by Umeda et al., 2005). Till now the D-type class of CDKs comprises one rice CAK (Orysa;CDKD;1, R2) and three *Arabidopsis* CAKs (Arath;CDKD;1-3).
CDKDs are related to the mammalian CDK7 kinases that regulate both cell cycle progression through the phosphorylation of other CDKs (CAK activity), and basal

transcription through phosphorylation of the CTD of the largest subunit of RNA polymerase II (CTD kinase activity) (Harper and Elledge, 1998). Rice CDKD;1 was shown to be able to complement a CAK-deficient mutant of budding yeast, to display both CAK and CTD kinase activity and to be positively regulated by cyclinH, as described for CDK7 kinases (Yamaguchi et al., 1998 and 2000). From the *Arabidopsis* CDKDs only CDKD;2 and CDKD;3 were shown to phosphorylate both CDK and CTD, and CDKD;3 was able to complement temperature sensitive CAK1 mutants of budding yeast (Shimotohno et al., 2003).
The *Arabidopsis* CDKF;1 displays similarity to CDK7 only in restricted domains and has CAK activity, but no CTD kinase activity, despite the observation that it can complement yeast CAK1-deficient mutants (Shimotohno et al., 2003). Recently, it was shown that CDKF;1 acts as a CAK-activating kinase (CAKAK) since it possesses cyclinH-independent kinase activity and is able to phosphorylate D-type CDKs, resulting in the activation of the CTD kinase activity of CDKD;2 *in vitro* and in root protoplasts (Shimotohno et al., 2004). Inducible co-suppression of *Arabidopsis* CDKF;1 using either sense or antisense constructs resulted in a gradual reduction of CDK activity and caused premature differentiation of initial cells in the root meristem, suggesting a function of CDKF;1 in differentiation (Umeda et al., 2000).

Plant cyclins

By definition, all CDKs share the feature that their enzymatic activation requires the binding of a regulatory cyclin subunit. Crystallographic studies of human CDK2 and its partner cyclinA revealed that CDKs display a bilobal structure (De Bondt et al., 1993; Jeffrey et al., 1995; Russo et al., 1996). The cofactor ATP binds deep within the cleft between the two CDK lobes, with its phosphates oriented outward. In protein kinases other than CDKs, the protein substrate interacts with the entrance of the active site cleft, but this region is obscured in monomeric CDKs by a large flexible loop called the T-loop. Moreover, key residues in the ATP-binding site are misoriented in the CDK monomer, further contributing to its inactivity. CDK activation through cyclin interaction therefore is accompanied with significant structural changes in the CDK active site. Major changes involve the repositioning of the T-loop that `releases´ the protein substrate-binding site, and the reconfiguration of the ATP binding site that corrects the positioning of the ATP phosphates for the phophotransfer reaction to the substrates (Jeffrey et al., 1995).

Multiple cyclins have been isolated in plants according to their sequence homology with well characterized cyclins in other eukaryotic systems as well as by complementation of yeasts deficient in the expression of specific cyclins (Sauter et al., 1995; Umeda et al., 1999b, Yamaguchi et al., 2000). They can broadly be classified into G1 and mitotic cyclins, according to their expression pattern during the cell cycle. Genome-wide analysis of the *Arabidopsis* cyclin gene family revealed a much greater complexity than that reported for cyclin genes in animals (Vandepoele et al., 2002). Plant cyclins are classified in three main groups (A-, B-, and D-type cyclins) according to their expression pattern and function during the cell cycle, and three other types (C-, H-, and T-cyclins) that are less well characterized (Renaudin et al., 1996; Yamaguchi et al., 2000; Barroco et al., 2003; Fulop et al., 2005).

Plants contain a large number of mitotic cyclins, no fewer than 21 in *Arabidopsis*, belonging to three A-type (CYCA1, CYCA2 and CYCA3) and three B-type (CYCB1, B2 and B3) subclasses (Renaudin et al., 1996; Vandepoele et al., 2002; Wang et al., 2004).
In synchronized tobacco BY2 cells, different A-type cyclins are expressed sequentially at different time-points during the cell cycle. The expression of two CYCA3s was upregulated at the G1/S transition, whereas a CYCA1 was induced at mid-S phase (Reichheld et al., 1996). The alfalfa A2-type cyclin Medsa;CYCA2;2 is expressed in all phases of the cell cycle, but its associated kinase activity peaks both in S-phase and during the G2/M transition (Roudier et al., 2000). These molecular data, together with different cellular compartmental localizations suggest that A-type cyclins may have different functions in plants (Roudier et al., 2000; Mews et al., 1997; Criqui et al., 2001; Yu et al., 2003). Antisense expression of Medsa;CYCA2;2 in alfalfa halted regeneration of somatic embryos, suggesting a role for CYCA2;2 in the formation or activity of apical meristems (Roudier et al., 2003). *Arabidopsis* plants overexpressing Nicta;CYCA3;2 show a dramatically modified morphology and a reduced cell differentiation and endoreduplication, implying a positive association with cell proliferation (Yu et al., 2003).
B-type cyclins are expressed within a narrow time window from late G2- to mid M-phase, and the ectopic expression of both Arath;CYCB1;1 and Oryza;CYCB2;2 accelerates root growth without altering root morphology (Doerner et al., 1996; Lee et al., 2003). Ectopic expression of Medsa;CYCB2;2 in developing tobacco plants interferes with differentiation events and specifically blocks root regeneration (Weingartner et al., 2003).

In addition to mitotic cyclins, plants also contain a large number of D-type cyclins. CYCDs have been described as G1-specific cyclins on the basis of their capacity to rescue yeast mutants lacking endogenous G1 cyclins (Soni et al., 1995).The expression of several D-type cyclins is regulated by nutrient availability and hormones (reviewed by Dewitte et al., 2003). In synchronized *Arabidopsis* cell cultures, most CYCDs increase during cell cycle re-entry, but during the subsequent cycles their transcripts remain relatively constant (Menges et al., 2003). They are proposed to be sensors of growth conditions and presumed to regulate the G1/S transition by activating the Rb/E2F-DP pathway (Gutierrez et al., 2002; Shen, 2002; Trimarchi and Lees, 2002). However, evidence points to an additional function of plant D-type cyclins at the G2-to-M transition (Sorrell et al., 1999; Schnittger et al., 2002a; Kono et al., 2003; Koroleva et al., 2004). Transgenic tobacco plants overexpressing Arath;CYCD2;1 show an increased cell division rate and a shortened G1 cell cycle phase (Cockcroft et al., 2000). Ectopic expression of CYCD3;1 in *Arabidopsis* results in hyperplasia in leaves and dramatically affects plant morphogenesis, demonstrating that CYCD3;1 promotes the proliferative phase of mitotic cycles and inhibits differentiation (Riou-Khamlichi et al., 1999; DeWitte et al., 2003).
Genes encoding H-type cyclins have been identified in *Arabidopsis*, rice and poplar and are believed to be the regulatory subunit of CAKs (Yamaguchi et al., 2000; Vandepoele et al., 2002). In rice and poplar high CYCH transcript levels are associated with dividing cells. Rice cyclinH interacts specifically with Orysa;CDKD;1, increases its kinase activity and enhances the rescue by CDKD;1 of a CAK mutant in budding yeast (Yamaguchi et al., 2000).
C-, and T-type cyclins are till now not experimentally characterized in plants. They are proposed to be involved in the control of the transcription machinery (Barroco et al., 2003; Fulop et al., 2005).

The coordinated progression of the cell cycle depends on the timely activation of CDKs that is, in part, regulated by the cell-cycle dependent oscillation of cyclins. Regulation of cyclin abundance occurs through repetitive rounds of highly controlled cyclin gene transcription and cyclin proteolysis.
Little information about transcriptional regulation of plant cyclins is available. Plant B-type cyclin promoters contain a common cis-acting element, called the M-specific activator (MSA) element, which is necessary and sufficient to direct G2/M-specific gene expression (Ito et al., 1998; Tréhin et al., 1999; Ito et al., 2001). Three different MYB-like proteins (Nicta;MYBA1, A2 and B) were shown to specifically interact with the MSA element (Ito et al., 2001). Nicta;MYBA1 and

Nicta;MYBA2 are expressed specifically at the G2/M transition, when B-type cyclins are transcribed, while Nicta;MYBB is expressed constitutively. Moreover, A-type MYBs are activators of MSA-containing promoters, whereas Nicta;MYBB is a repressor. Interestingly, a MYB protein also activates cyclinB transcription in Drosophila, indicating some similarity between the mechanisms of cyclin transcription regulation in plant and animal cells (Okada et al., 2002).

Cyclin degradation occurs mainly through ubiquitin-dependent proteolysis. Mitotic cyclins are degraded during or at the end of mitosis by the proteasome, after ubiquitination by the highly conserved anaphase-promoting complex/cyclosome (APC/C) (King et al., 1996; Zachariae et al., 1996). G1 cyclins are highly unstable owing to the action of non-APC/C ubiquitinating Skp1-Cdc53/Cullin-F-box protein (SCF) complexes (Willems et al., 1996; Patton et al., 1998). APC/C complex substrates are characterized by the presence of a short peptide motif, called the D-box, which is necessary for their degradation during mitosis. The fact that most plant mitotic cyclin sequences contain a D-box and that the APC/C subunits are conserved in plants suggest that plant mitotic cyclins may be APC/C targets (Vandepoele et al., 2002; Capron et al., 2003b). Indeed, the existence of a D-box pathway in plants was proven (Genschik et al., 1998). Moreover, both an A2-type cyclin from Medicago and an A3-type cyclin from tobacco seemed to be degraded in early M phase (Roudier et al., 2000; Criqui et al., 2001). Furthermore, it is shown that a proteasome inhibitor induces tobacco cyclinB1 accumulation, suggesting degradation by the ubiquitin-dependent pathway (Criqui et al., 2000). The functional relevance of this cyclin destruction is exemplified by the observation that overexpression of a nondegradable B1-cyclin, lacking the D-box, causes severe growth retardation and abnormal development, with a higher percentage of cells exhibiting duplicated ploidy levels than in the controls (Weingartner et al., 2004). The proteolysis of a B2-type cyclin in early mitosis has also been reported, but surprisingly seemed proteasome-independent (Weingartner et al., 2003). Little is known of the stability or mechanisms of turnover of G1 cyclins in plants. Recently, Arath;CYCD3;1, but not CYCD2;1, has been shown to be a highly unstable protein whose proteolysis is mediated by the ubiquitin-dependent proteasome (Planchais et al., 2004). Interestingly, in plants silenced for Arath;Rbx1, a component of the SCF complex, CYCD3;1 protein was stabilized (Lechner et al., 2002). However, the identity of the F-box component of the SCF complex involved in CYCD3;1 recognition and targeted proteolysis remains elusive.

CDK/cyclin regulation

Although cyclin binding is the primary determinant of CDK functioning, layers of additional regulatory subunits and protein kinases restrict CDK/cyclin activity to ensure the correct temporal progression of events leading to the faithful duplication of cells (Figure 1).

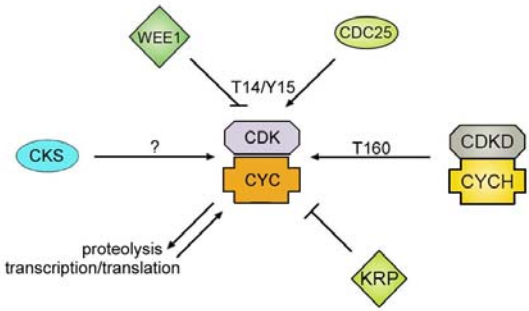

Figure 1. Regulation of CDK activity. For details, see text. ⊥ represents inhibitory events, → activating events. Both cyclins and some KRPs are regulated by synthesis and ubiquitin-mediated proteolysis.

In addition to cyclin binding, full activation of the CDK/cyclin complex requires the phosphorylation of a conserved Thr residue in the T-loop of the CDK (Thr160 in human CDK2) by a CDK activating kinase (CAK) (Russo et al., 1996; Brown et al., 1999). In plants several CAK kinases, that complement S. cerevisiae CAK mutations and that phosphorylate CDKs, have been described (Shimotohno et al., 2003).

Although inactivation of CDK/cyclin complexes can be achieved by ubiquitin-mediated degradation of cyclins (Peters, 2002), a distinct mode of reversible inactivation can be triggered through phosphorylating crucial residues, equivalent to Thr14 and Tyr15 of human CDK2, located within the CDK ATP-binding loop (Nurse, 1997). This phosphorylation is catalyzed by the WEE1 kinase and is counteracted by the dual-specificity phosphatase CDC25. Plants possess a WEE1 kinase which is involved in the inhibitory phosphorylation of CDKs (Sun et al., 1999; Sorrell et al., 2002). Recently, the primitive unicellular algae, Ostreococcus tauri has been found to contain a bona fide CDC25, that is able to activate purified CDK/cyclin complexes and to suppress a cdc25-22 mutant in S.

pombe (Khadaroo et al., 2004). However, in both *Arabidopsis* and rice, no genes with high homology to yeast or animal CDC25 genes have been identified (Buell, 2002; Vandepoele et al., 2002). Nevertheless, evidence suggests that higher plants also have a phosphatase that can activate CDK/cyclin complexes. Inactive CDK complexes purified from tobacco cell suspensions arrested in G2 phase by cytokinin starvation can be activated upon incubation with the yeast CDC25 protein (Zhang et al., 1996). Recently, a small protein with dual-specificity CDC25-like phosphatase activity has been identified in *Arabidopsis*. This CDC25-like protein consists of a sole catalytic domain, has tyrosine phosphatase activity and is able to stimulate *Arabidopsis* CDK activity (Landrieu et al., 2004). The *in vivo* role of this CDC25-like protein, however, remains to be determined.

Further mechanisms of modulating CDK activity exist through the action of inhibitors and scaffolding proteins.
CDK inhibitors inhibit CDK activity by tight association with CDK complexes (Nakayama and Nakayama, 1998). All known plant CDK inhibitors share a 31-amino-acid domain with $p27^{Kip1}$, a member of the mammalian Kip/Cip family of protein inhibitors. Based on this similarity, plant CDK inhibitors were designated Kip-related proteins (KRPs, De Veylder et al., 2001a). KRPs interact with CDKA and D-type cyclins, but not with CDKBs (Wang et al., 1998; Lui et al., 2000; De Veylder et al., 2001a; Jasinski et al., 2002; Zhou et al.,2002). They were shown to inhibit CDK activity *in vitro* (Wang et al., 1997; Lui et al., 2000; Jasinski et al., 2002; Verkest et al., 2005; Coelho et al., 2005), whereas their overexpression in plants results in a decrease in kinase activity *in vivo* (Wang et al., 2000; De Veylder et al., 2001a; Zhou et al., 2002). Plant KRPs function in regulating the cell cycle during development and in response to environmental signals (chapter 2).
The CKS (CDK subunit) proteins represent another class of conserved proteins that interact with CDKs and regulate their activity. *Arabidopsis* contains two homologues of the yeast $p13^{Suc1}$ (De Veylder et al., 1997; Vandepoele et al., 2002). Although CKS proteins are widely used as an affinity matrix for the purification of CDKs their function remains mostly elusive. Crystallographic studies of the human CKS/CDK2 complex suggest that these proteins function as a docking factor for both positive and negative regulators of CDKs (Bourne et al., 1996). In situ expression analysis of Arath;CKS1 revealed that CKS1 transcripts are present in both mitotically dividing and endoreduplicating cells (Jacqmard et al., 1999), suggesting that it is required for both modes of the cell cycle. Overexpression of Arath;CKS1 in *Arabidopsis* causes a prolongation of cell-cycle duration and strongly reduced meristem size, implying a role in the inhibition of the mitotic cell cycle (De Veylder et al., 2001b).

Mitotic cell cycle

Several variations in the cell cycle occur in nature, but the most common, and best studied, form is the mitotic cell cycle comprising four different stages. In this cycle DNA replication (S phase) is directly followed by the segregation of the duplicated genetic material between two daughter cells, a process called mitosis (M phase). Both the S and M phase are proceeded by a preparative phase (G1 and G2 phase). During the G1 phase, cells monitor their size and environment, whereas one of the tasks performed during the G2 phase is to ensure that DNA duplication has been completed. Although many core cell cycle genes have been cloned, the mechanisms that control the orderly progression through the cell cycle in plants have only recently started to be understood (reviewed by De Veylder et al., 2003).

G1-S transition and DNA replication

The mechanism that regulates progression through G1 and transition to S-phase, thereby initiating genome duplication, appears to be conserved between mammals and plants and is controlled by the retinoblastoma/E2F pathway. Mitogenic stimulation induces accumulation of D-type cyclins that bind specific CDKs and initiate the phosphorylation of the retinoblastoma (RB) protein, a key regulator of the start of DNA replication (Weinberg, 1995). In its non-phosphorylated form RB binds E2F/DP transcription factors which activate DNA-synthesis genes, but its phosphorylation counteracts the inhibitory function of RB, and releases E2F/DP.

RB-related genes (RBR) were first discovered in maize and later in other plant species (Grafi et al., 1996; Vandepoele et al., 2002). RBR functions as a negative regulator of cell proliferation because Arath;RBR1 mutants fail to arrest mitosis of haploid nuclei (Ebel et al., 2004). Similarly, inhibition of Nicta;RBR1 using virus-induced gene silencing induced prolonged cell proliferation coupled with delayed cell differentiation in the leaves and stems of tobacco plants (Park et al., 2005). The RBR protein is phosphorylated by CDKA/CYCD complexes in a cell-cycle-dependent manner, reaching its maximum at the G1/S transition and remaining high until mid/late S phase (Boniotti et al., 2001; Nakagami et al., 2002). Although not experimentally proven, it is believed that in analogy with mammalian systems, this phosphorylation inactivates RBR and relieves repression of E2F/DP transcription factors. CYCD3 cyclins seem to play an important function in this process, because overexpression of *Arabidopsis* CYCD3;1 or tobacco CYCD3;3 stimulates cells to exit the G1 phase (Nakagami et al., 2002; Dewitte et al., 2003).

Conversely, KRPs are suggested to prevent CYCD3/CDK phosphorylation of RBR, as overexpression of KRPs in *Arabidopsis* completely complements the phenotype induced by CYCD3;1 overproduction (Jasinski et al., 2002; Zhou et al., 2003). In addition, overexpression of Arath;KRP1 in trichomes is able to restore the phenotype induced by CYCD3;1 overexpression (Schnittger et al., 2003). In parallel, additional mechanisms control CYCD3/CDK activity. Full kinase activity and nuclear localization of CYCD3 was shown to require its phosphorylation by an unknown upstream working kinase (Nakagami et al., 2002). Moreover, it is suggested that CAK activity could be responsible for activation of CYCD/CDK complexes, since overexpression of rice CDKD accelerates S-phase entry (Fabian-Marwedel et al., 2002).
E2F/DPs regulate the expression of genes involved in DNA synthesis and replication. The *Arabidopsis* genome comprises three E2Fs, two DPs and three monomeric DP-E2F-like (DEL) genes (Vandepoele et al., 2002). Both E2Fa and E2Fb are transcriptional activators, whereas E2Fc and DELs are believed to be repressors of E2F/DP target genes (Kosuhi and Ohashi, 2002; Mariconti et al., 2002). Transient overexpression of E2Fa and DPa induces non-dividing mesophyll cells to re-enter S phase (Rossignol et al., 2002), whereas their constitutive overexpression induces ectopic cell divisions because of a delay in cell differentiation (De Veylder et al., 2002; Kosugi and Ohashi, 2003). Co-overexpression of E2Fb and DPa in tobacco BY-2 cells leads to a shortening of cell cycle duration due to the promotion of both the G1-S and G2-M transitions (Magyar et al., 2005). The repressive function of E2Fc was proven by the negative effect of the overproduction of a stable form of E2Fc on cell division. Furthermore, this overexpression was correlated with decreased expression of the known E2F/DP target gene CDC6 (del Pozo et al., 2002). Interestingly, E2Fc was shown to be a CYCD/CDKA substrate, whose phosphorylation results in ubiquitin-mediated proteasomal degradation of E2Fc. In a competition assay, DEL expression antagonizes E2F/DP function, suggesting their role as transcriptional repressors (Kosugi et al., 2002). In agreement, DEL1 was proven to suppress transcription of DNA replication genes (Vlieghe et al., 2005). The function of DEL3 on the other hand is less clear, since DEL3 doesn't affect the endocycle or the expression of genes involved in S-phase. Instead it affects a subset of genes involved in plant cell wall biogenesis, and is believed to regulate hypocotyl cell growth and expansion (Ramirez-Para et al., 2004).

E2F/DPs regulate target gene expression through interaction with E2F-sites in their promoters. A database search, in which the *Arabidopsis* genome was screened for genes harboring the E2F binding promoter element, identified

numerous genes involved in DNA synthesis and replication (Ramirez-Parra et al., 2003), and micro-array analyses on E2Fa/DPa overexpressing plants has identified some of these as bona fide targets (Vlieghe et al., 2003; Vandepoele et al.,2005). Several of these are pre-replicative complex (RC) genes (e.g. CDC6, Cdt1, ORC1, MCMs) that regulate initiation of DNA replication. Initiation of DNA replication requires licensing of sequences called replication origins. Licensing consists of the sequential recruitment of pre-RC multiprotein structures to DNA replication origins (Takeda and Dutta, 2005). When DNA synthesis begins, pre-RC components are inactivated, preventing the origins of replication from re-replicating the genome until M-phase is completed. Despite the high conservation of pre-RC components in eukaryotes, the regulatory network controlling their function is extremely diverse in different model systems and very little is known of its complexity in plants (Bryant et al., 2001). It is generally accepted that the proper and timely control of DNA replication is regulated at several levels, including transcription, phosphorylation, subcellular localization, or proteolysis. CDK activity plays a crucial role both in triggering DNA initiation of pre-RCs at G1/S and in inhibiting pre-RC formation during S and G2 phase. In *Arabidopsis* ectopic expression of CDC6 and Cdt1 induces in a cell-type specific manner either enhanced cell proliferation or enhanced DNA endoreduplication, illustrating their function in S-phase (Castellano et al., 2001 and 2004). Both E2F targets are regulated by proteasome-mediated degradation, which at least in the case of Cdt1 occurs through CDK phosphorylation.

Although plants clearly share all of the elements needed for G1/S entry with other eukaryotes, they lack the typical class of E-type cyclins, which are known to be essential regulators of DNA replication (Duronio et al., 1996). Presumably, some of the A- or D-type cyclins assume the role of the CYCEs. Indeed, the lack of a consensus RBR binding motif in some D-type cyclins suggests that these might have gained other novel functions during evolution. Moreover, the distinct transcriptional accumulation of Arath;CYCD5;1 during G1 and CYCD4;1 in late G1/S suggests a possible role as functional equivalents of cyclinE (Menges et al., 2005). Similarly, some A-type cyclins display transcriptional accumulation in S-phase. Accordingly, Nicta;CYCA3;2 was suggested to perform an analogous role to that of animal cyclinE in the control of plant cell division and differentiation, based on similarities in function such as the requirement of CYCA3;2 for reentry into cell division and its function in enhancing the expression of S-phase-specific histone genes (Yu et al., 2003).

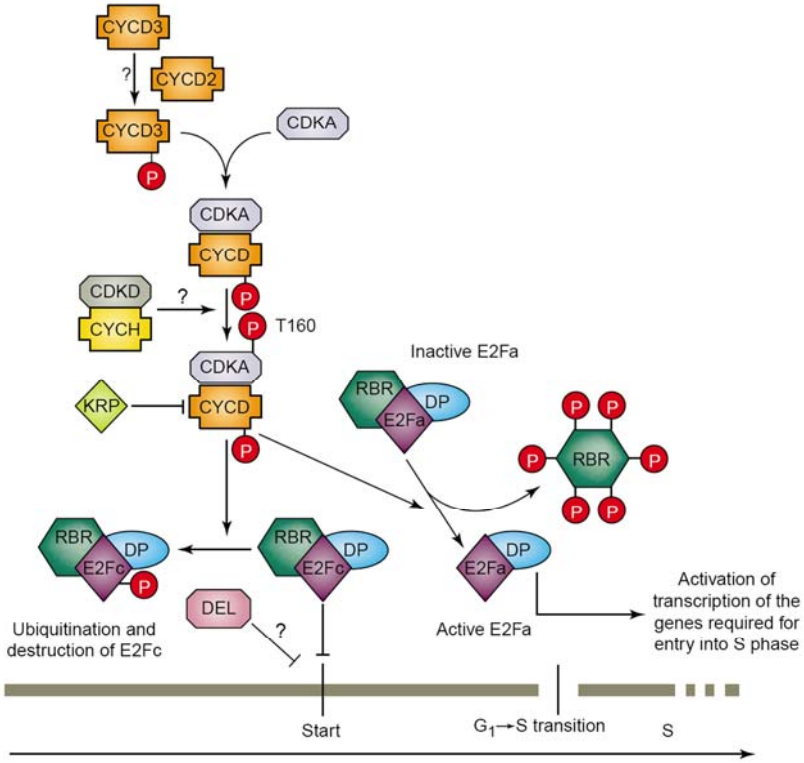

Figure 2. Schematic overview of the regulation of the G1-S transition in plants. Pathways that are still not fully demonstrated are marked by question marks.

G2-M transition and mitosis

The regulation of entry in mitosis in plants is still poorly understood. In mammals and insects, the G2/M transition is specifically regulated by CDKs that associate with A- and B-type cyclins. In plants, kinase activity assays suggest that two types of CDKs (A and B) control the G2/M transition (Magyar et al., 1997; Porceddu et al., 2001). In planta overexpression of a dominant negative kinase mutant indicated that A-type CDKs are involved in both the G1/S and G2/M transition (Hemerly et al.,1995), and down-regulation of the activity of a B-type CDK in transgenic tobacco plants delayed G2/M transition (Porceddu et al., 1999). Based

on their transcriptional levels, both plant A- and B-type cyclins are responsible for mitotic events. Ectopic expression of alfalfa CYCB2;2 and *Arabidopsis* CYCB1;2 cyclins indeed stimulates G2/M transition (Schnittger et al., 2002b; Weingartner et al., 2003). However, the activity of the CDK complexes is regulated by additional mechanisms. In yeast and mammalia, CDK/cyclin complexes are maintained in an inactive state during the S and G2 phases by WEE1 kinases, and become activated at the G2/M boundary by the dual-specificity CDC25 phosphatase. Overexpression of plant WEE1 genes in S. pombe inhibits cell division (Sorrell et al., 2002). Interestingly, purified WEE1 can inhibit mitotic CDK activity *in vitro*. Although no CDC25 homolog has been identified in plants, accumulating data suggest the existence of a CDC25-like regulation of CDKs in plants (see above). In accordance, overexpression of the S. pombe *CDC25* gene in tobacco plants resulted into increased lateral root primordium formation and into a new threshold size for cell division in the primordia (Bell et al., 1993; McKibbin et al., 1998). Whereas, microinduction techniques in tobacco plants clearly demonstrated that *CDC25* expression in young leaf primordia leads to changes in cell division patterns (Wyrzykowska et al., 2002).

Our knowledge of mitosis in higher plants is still in its infancy. Mitosis is viewed as the final phase of the cell cycle during which the mother cell splits itself into two daughter cells. During this process, not only are the replicated chromosomes split by the mitotic spindle (karyokinesis) but the plasma membrane, organelles and cytoplasm must also be divided (cytokinesis). Plant cells divide by building a new cell wall between daughter nuclei (called a cell plate while under construction). Cell plate positioning involves two major steps. First the establishment of the division site early in M phase by a structure called the preprophase band (PPB), and then the guidance of the cell plate during cytokinesis by the phragmoplast. Both the phragmoplast and the PPB are structures composed of microtubules (MTs) and unique to plant cells (Smith, 2001). Although the architectural changes of the MTs during the course of the cell cycle have been extensively studied in plants, it is not known how MT dynamics are regulated. Studies in animal cells have implicated CDKs and mitotic cyclins in the control of MT dynamics, and several evidences suggest similar functions of plant CDKs and cyclins.
Microinjection of purified active mitotic CDK complexes in Tradescantia cells accelerates PPB depolymerization and induces a premature breakdown of the nuclear envelope (Hush et al., 1996). A role for CDKA in MT stability and movement is prompted by its specific localization (Colasanti et al., 1993; Bogre et al., 1997; Mews et al., 1997; Stals et al., 1997; Weingartner et al., 2001). CDKA is located at the nucleus and cytoplasm during interphase but associates with parts

of the PPB, spindle, and phragmoplast during mitosis and cytokinesis. Subcellular localization experiments have shown that B-type CDKs and certain A- and B-type cyclins also associate with MT structures, such as the PPB, the mitotic spindle and the phragmoplast, suggesting their possible involvement in MT array regulation throughout mitosis (Mews et al., 1997; Ayaydin et al., 2000; Lee et al., 2003). Additionally, A- and B-type CDKs and B-type cyclins have been reported to bind to chromosomes, signifying their possible participation in chromosome condensation (Stals et al., 1997; Mews et al., 1997; Criqui et al., 2001; Lee et al., 2003). How plant CDK/cyclin complexes are involved in chromosome condensation is unknown, but it has been shown that the microinjection of mitotic CDK complexes significantly accelerated this process in plant cells (Hush et al., 1996).

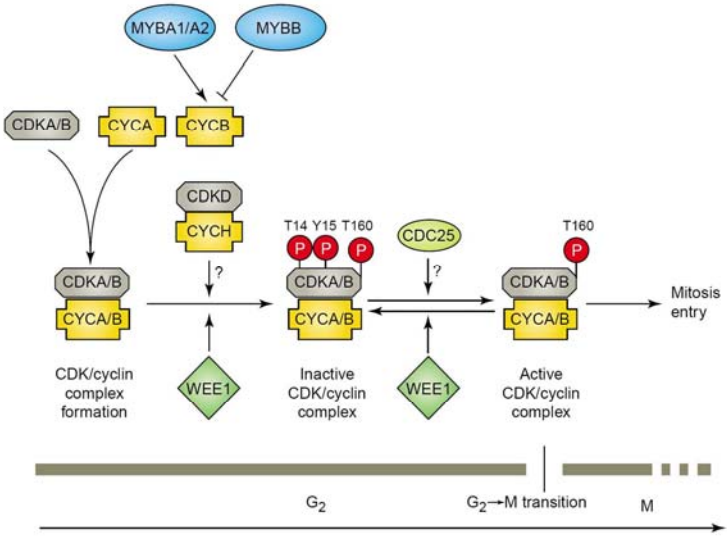

Figure 3. Schematic overview of the mechanistic regulation of the G2-M transition in plants. Question marks indicate pathways that are still not fully demonstrated experimentally.

In animal cells, the CDK complex is inactivated at the metaphase/anaphase transition by the degradation of mitotic cyclins by the APC/C protein degradation machinery (Genschik et al., 1998). As such, the localization on the phragmoplast

and the prolonged presence through anaphase and telophase of some plant mitotic cyclins is intriguing and the necessity to destroy mitotic cyclins during plant mitosis is debated (Mironov et al., 1999; Capron et al., 2003b). However, inhibition of the proteasome in prophase blocks plant cells in metaphase, suggesting that at least some D-box containing proteins need to be degraded for progression through mitosis (Genschik et al., 1998). In accordance, mutations in several APC/C component, such as APC2 (Capron et al., 2003a), CDC16/APC6 (Kwee et al., 2003) or APC27 (Blilou et al., 2002) lead to the accumulation of CYCB1 and impaired mitosis. Interestingly, the *Arabidopsis* cullin protein CUL1, a component of the SCF protein-ligase, was found to be associated with the mitotic spindle (Farras et al., 2001; Shen et al., 2002). Although it is known that SCF primarily controls the G1/S transition in fungi and animals (Tyers and Jorgensen, 2000), this observation suggests that plant SCF-dependent degradation may also have a function during mitosis.

Endoreduplication cell cycle

Many plant and animal cells have a different cell cycle mode in which cells undergo iterative DNA replications without any mitosis and cytokinesis. Although endoreduplication is a largely widespread phenomenon in higher plants, its physiological role and molecular control in plants is poorly understood. Endoploidy is often observed in differentiating plant cells and a requirement for correct organ development (Kondorosi et al., 2000). The endoreduplication cell cycle is a variant of the mitotic cell cycle. It lacks M-phase regulators but shares with the classical cell cycle part of the key regulators, especially those controlling the G1/S transition (Grafi, 1998). The mechanisms necessary for the transition of the mitotic cell cycle to the endocycle are believed to involve nothing more than loss of M-phase CDK activity and oscillations in the activity of S-phase CDKs (Edgar and Orr-Weaver, 2001; Larkins et al., 2001). This is achieved through modulations in CDK activity to prevent mitosis and to allow re-licensing of origins of replication. Initiation of DNA replication is licensed to occur only once during each cell cycle and is regulated by pre-RC complex genes like CDC6, Cdt1, ORC and MCMs, which are E2F targets. Ectopic expression of either CDC6 (Castellano et al., 2001) or Cdt1 (Castellano et al., 2004) is sufficient to trigger extra endocycles. Similarly, E2Fa-DPa overexpression causes extra rounds of DNA replication (De Veylder et al., 2002). Consistent with this, suppression of the E2F inhibitory RBR function in tobacco resulted in a similar phenotype (Park et al., 2005). Interestingly,

the activation of DNA replication in all these transgenic lines results in a cell-type specific, mixed phenotype, with cells that are triggered to proliferate besides the cells that are stimulated to endoreduplicate. De Veylder et al. (2002) proposed that this phenotype depends on the presence of a mitosis-inducing factor (MIF) in the cell-types undergoing extra cell divisions. Recently, DEL1 was shown to inhibit the endoreduplication phenotype but not the ectopic cell divisions induced by E2Fa-DPa (Vlieghe et al., 2005), suggesting a function of DEL1 in preserving the mitotic state of proliferating cells.

Endoreduplication requires exit of the mitotic cell cycle. In maize endosperm and during tomato fruit and *Arabidopsis* leaf development endoreduplication is correlated with the inhibition of M-phase associated CDK activity (Grafi and Larkins, 1995; Joubés et al., 1999; Verkest et al., 2005). However, the identity of the CDK complexes and the mechanisms that account for the decrease in CDK activity remain poorly understood.
Two recent reports shed light on the identity of implicated CDKs. Leiva-Neto et al. (2004) demonstrated that CDKA activity plays an active role in the endocycle, since the specific overexpression of a dominant negative allele of the maize A-type CDK could inhibit endoreduplication in endosperm cells. The mitotic-to-endocycle transition is prevented by the activity of CDKB1;1 (Boudolf et al., 2004b). In an E2Fa-DPa-overproducing background, the overexpression of a dominant negative allele of Arath;*CDKB1;1* resulted in both the enhancement of the endoreduplication phenotype and the repression of the ectopic cell divisions, suggesting that CDKB1;1 is part of the MIF.

Several mechanisms for the control of CDK activity are likely to occur during endoreduplication. Dealing with the phosphorylation status of the kinase complex, it has been suggested that the WEE1 inhibitory kinase may play a role in endoreduplicating cells of maize endosperm (Sun et al., 1999) and of tomato fruit (Gonzalez et al., 2005). WEE1 transcripts are most abundant in mitotically active organs and in endoreduplicating tissues in maize endosperm and during tomato fruit development. This suggests that the observed inhibition of mitotic CDK activity by WEE1 (Sun et al., 1999) could be one of the mechanisms that inhibits the G2/M transition and activates the endocycle. Another molecular mechanism for the mitosis-to-endocycle transition is the CCS52 protein, an activator of the anaphase-promoting complex (APC/C) whose expression coincides with the onset of endoreduplication (Cebolla et al., 1999). CCS52 is thought to be involved in mitotic cyclin degradation, resulting in inhibition of M-phase CDK activity. Ectopic expression of CYCB in trichomes converts the endocycle program that is associated with their normal

development into a mitotic program, resulting in multicellular trichomes (Schnittger et al., 2002b). Likewise, *Arabidopsis siamese* mutants misexpress CYCB1 and, consistent with this, have multicellular trichomes (Walker et al., 2000). CDK inhibitors have been proven to be important regulators of the endoreduplication cycle in several organisms (chapter2). In plants, the involvement of CDK inhibitors in endoreduplication was suggested through the observation of accumulation of an unidentified inhibitor protein coincident with the onset of endoreduplication in maize endosperm (Grafi and Larkins, 1995). Recently, it has been demonstrated that KRPs participate in inhibiting M-phase associated CDK activity, and as such trigger the endocycle onset (Verkest et al., 2005; Weinl et al., 2005). Furthermore, an unidentified role for CDK inhibitors is suggested during endoreduplication since the *Arabidopsis* KRP1 and KRP2 and the maize CKIs are expressed in both mitotically dividing and endoreduplicating tissues (Ormenese et al., 2004; Coelho et al., 2005).

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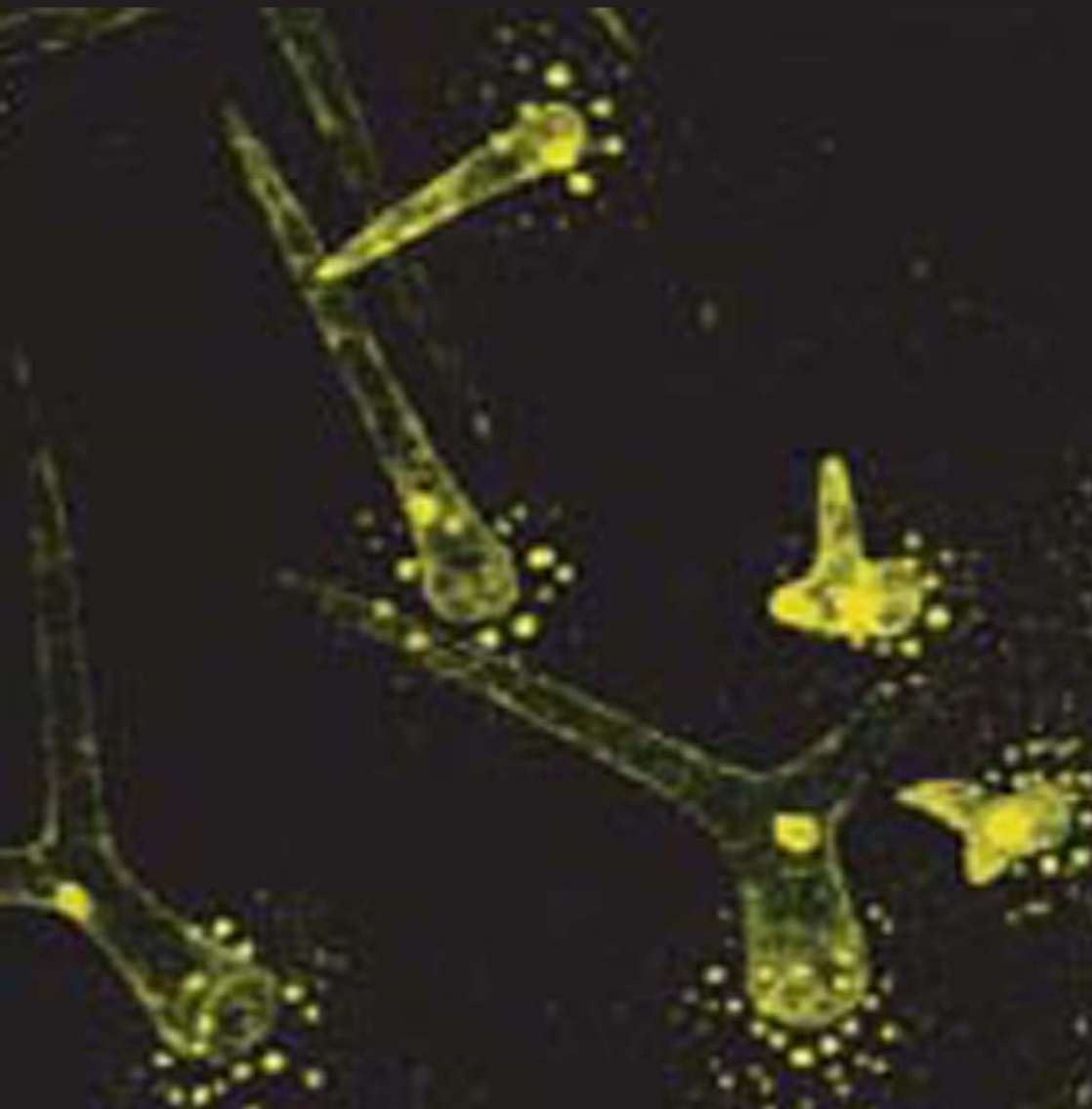
Chapter 2



Chapter page: Confocal laser scanning micrograph showing the localization of protein fusions of Arath;KRP1 with YFP expressed from the *GLABRA 2* promoter.

Table of contents

Switching the cell cycle: Kip-related proteins in plant cell cycle control

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During the development of multicellular organisms, many different cell types are created. These display characteristic cell cycle programs that can radically change during the organism's lifetime (Jakoby and Schnittger, 2004) (Figure 1). Usually, in young and less differentiated tissues, a proliferative cell cycle mode occurs. In this mitotic cell cycle program, DNA replication (also known as the synthesis or S phase) is followed by the segregation of the duplicated genetic material to two daughter cells in mitosis (M phase). Both the S and M phases are usually preceded by a preparative gap phase (G1 and G2 phase, respectively) during which cells scan whether all conditions are favorable to continue a new round of DNA replication or mitosis. As cells differentiate the rate of cell proliferation decreases. However, before complete withdrawal from the cell cycle and concomitant with differentiation, many plant cells switch from a mitotic cell cycle to an endoreduplication cycle (also called endocycle or endoreplication). During this endocycle the DNA is replicated without subsequent mitosis leading to polyploid cells. Eventually, cells exit the cell cycle completely.
Cell cycle progression is controlled by an evolutionary conserved molecular mechanism. A central role is played by kinase complexes, which in their minimal configuration consist of a Ser/Thr kinase (the cyclin-dependent kinase; CDK) and a regulating cyclin subunit. CDKs phosphorylate a plethora of substrates, as such triggering the transition from one cell cycle phase into the next one. The sequential and transient activation of different CDK-cyclin complexes dictates the unidirectional progression through the cell cycle.

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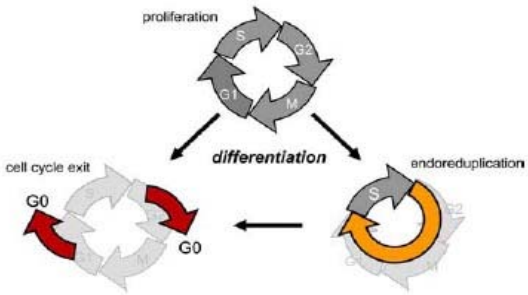


Figure 1. Cell cycle modes during development. During the growth of a multicellular organism, different cell cycle programs are executed. Typically, in young and less differentiated tissues, a proliferative cell cycle mode occurs by which more cells are generated. As cells differentiate, the rate of cell proliferation decreases and eventually, cells exit the cell cycle. However, before complete withdrawal from the cell cycle, and along with differentiation, many plant and animal cells switch from a mitotic cell cycle to an endoreduplication cycle, in which the nuclear DNA becomes replicated without subsequent nuclear and cellular division, leading to polyploid cells.

Because of its importance in growth and development, the CDK-cyclin activity must be strictly controlled. In addition, mechanisms must be operational that ensure a correct exit of the cell cycle in response to anti-mitogenic stimuli. In yeast and mammals, one of the major regulators of CDK activity are CDK inhibitory molecules (CKIs) that bind and inhibit or sequester CDKs. Recently, putative orthologs of CKI proteins have been identified in plants as well. In this update, we review the current knowledge on the biochemical properties of plant CKIs and discuss their physiological relevance during plant growth and development.

The plant cell cycle: a basic introduction

Similar to animals, progression through the cell cycle in plants is regulated by the conserved class of Ser/Thr kinases, the CDKs (De Veylder et al., 2003; Inzé, 2005). In *Arabidopsis thaliana*, at least two classes of CDKs are involved in cell cycle regulation: the A-type CDKs that are represented by only one gene in the model species *Arabidopsis* (designated Arath;*CDKA;1*) and the B-type CDK family that comprises four members, grouped into the B1 and B2 subclasses (Arath;*CDKB1;1*,

Arath;*CDKB1;2*, Arath;*CDKB2;1*, and Arath;*CDKB2;2*; Vandepoele et al., 2002). A-type CDKs display kinase activity from late G1 phase until the end of mitosis, suggesting a role for this particular CDK at both the G1-to-S and G2-to-M transition points (Magyar et al., 1997; Porceddu et al., 2001; Sorrell et al., 2001). A central role for CDKA;1 in controlling cell number has been demonstrated using transgenic tobacco (*Nicotiana tabacum*) plants with reduced A-type CDK activity (Hemerly et al., 1995). The requirement for Arath;CKDA;1 at least for entry into mitosis has been demonstrated as well by *cdka;1 null* mutants that fail to progress through the second mitosis during male gametophytic development (M. Nowack and A. Schnittger, unpublished data). The group of B-type CDKs displays a peak of activity at G2-to-M phase transition only (Magyar et al., 1997; Porceddu et al., 2001; Sorrell et al., 2001), suggesting that they play a role at the onset of or progression through mitosis. Correspondingly, cells of plants with reduced B-type CDK activity arrest in the G2 phase of the cell cycle (Porceddu et al., 2001; Boudolf et al., 2004a)

Although titration of CDK activity by the expression of dominant negative versions of both A- and B-type CDKs resulted in cell cycle defects, no extra cell divisions were stimulated by the overexpression of wild-type Arath;*CDKA;1*, Arath;*CDKB1;1*, and Arath;*CDKB1;2* alleles in plants (Hemerly et al., 1995, 2000; Boudolf et al., 2004a; Schnittger et al, 2003). This observation is consistent with the view that a cofactor is required for full CDK activity, i.e. a cyclin. Cyclins are regulated both transcriptionally and posttranslationally, mainly by the controlled protein degradation. By regulating the abundance of specific cyclins, the CDK activity is precisely tuned and targeted to substrates in a spatial and temporal manner.

The plant cyclin gene family is very complex. For instance, the *Arabidopsis* genome encodes at least 49 different cyclins (Vandepoele et al., 2002; Wang et al., 2004) that are classified into seven different subclasses (types A, B, C, D, H, P, and T). To date, only a few members of the A-type, B-type, and D-type cyclins have been analyzed. With a few exceptions, the expression patterns and activity profiles mimic those of their mammalian counterparts. A-type cyclins are important from S-to-M phase, while B-type cyclins primarily control the G2-to-M transition. D-type cyclins, whose expression is mainly correlated with the proliferative status of cells, are presumed to drive cells through the G1-to-S checkpoint in a mitogen-dependent manner (De Veylder et al., 2003; Inzé, 2005). In contrast to animals, some evidence points to an additional function of plant D-type cyclins at the G2-to-M transition (Schnittger et al., 2002; Kono et al., 2003; Koroleva et al., 2004).

Plant CDK inhibitors: the Kip-related proteins

Besides by binding of cyclins, CDK activity is regulated by docking of mainly small proteins, generally known as CKIs, which have been found to induce cell cycle arrest or to delay cell cycle progression in response to intracellular or extracellular signals. CKIs have been identified in many different organisms and, although all of them display CKI activity, they control a broad spectrum of often species-specific physiological processes. For example, in budding yeast (*Saccharomyces cerevisiae*), three CKIs have been described, Pho81, Far1, and Sic1 (Mendenhall et al., 1998). Pho81 inhibits a CDK-cyclin complex that controls gene expression under low phosphate conditions; Far1 binds and inactivates G1 CDK complexes to mediate pheromone-depedendent cell cycle blockage; and Sic1 plays a role in the timing of S-phase onset. In fission yeast (*Schizosaccharomyces pombe*), the CKI Rum1, is structurally and functionally related to Sic1, inhibits mitotic CDKs, and plays a central role in the regulation of the G1 phase.
In mammals, based on shared structural features and biochemical functions, CKIs have been divided into two major classes, the INK4 and the Kip/Cip class (Sherr and Roberts, 1999). Members of the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) are structurally similar to the Pho81 inhibitor of budding yeast and are characterized by the presence of multiple ankyrin-type repeats for CDK binding. They bind and inhibit a small subset of CDKs (CDK4 and CDK6) that are primarily responsible for passage through G1. INK4 protein binding to monomeric CDKs or CDK-cyclin complexes causes allosteric changes that impair cyclin binding or lead to the dissociation of the CDK-cyclin complex, respectively. In contrast, inhibitors of the Kip/Cip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) bind and inhibit a broader range of CDKs and function in dimeric as well as heterotrimeric complexes with CDKs and cyclins; all share a conserved inhibitory domain at their N-terminus. Kip/Cip binding does not dissociate the CDK-cyclin complex, but distorts the catalytic ATP-binding center of the CDK subunit.
The first plant CKIs have been detected in yeast two-hybrid screens performed to identify CDKA;1-associating proteins (Wang et al., 1997; Lui et al., 2000; De Veylder et al., 2001; Jasinksi et al., 2002b). Additional plant CKIs have been discovered in silico through genome data mining (De Veylder et al., 2001; Coelho et al., 2005). Overall, the plant CKIs have only low sequence identity to each other and the non-plant CKIs. Interspecies sequence homology is restricted to a short amino-acid region shared between the plant CKIs and the mammalian Kip/Cip inhibitors. Because of this homology, the name Kip-related proteins (KRPs) was suggested for the seven CKIs found in the *Arabidopsis* genome (De Veylder, et al.,

2001); but some family members are also known under different names such as ICK1 (KRP1) and ICK2 (KRP2) (Wang et al., 1997; Lui et al., 2000). No *Arabidopsis* homologs to the INK4 or yeast inhibitors have been identified so far (Vandepoele et al., 2002).

Biochemical properties

Despite their low sequence similarity with the mammalian CKIs, plant *KRP* genes encode functional CKIs, as demonstrated by their ability to inhibit CDK activity. *In vitro*, CKI activity was proven by adding recombinant KRP to partially purified CDK complexes (Wang et al., 1997; Lui et al., 2000; Jasinski et al, 2002b; Coelho et al., 2005). *In vivo* CKI activity was demonstrated by overexpressing diverse *KRP* genes in *Arabidopsis* (Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002a; Zhou et al., 2002). The reduced CDK activity observed upon *KRP* overexpression correlates with a decrease in cell division rate, resulting in leaves whose cell number is dramatically low. This decrease in cell number is accompanied by a change in leaf morphology (Figure 2).

Whereas KRPs have clearly been demonstrated to operate as inhibitors of CDK activity, the identity of the targeted CDK complexes remains unknown. Not all CDKs are KRP-sensitive, as even applying a high dose of recombinant KRP protein to purified CDK complexes only results into a partial inhibition of total CDK activity (Wang et al., 1997). In accordance with this observation, yeast two-hybrid interaction analysis demonstrated that the KRPs bind A-type, but not B-type, CDKs (Lui et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002b; Zhou et al., 2002). Recently a biochemical proof has been supplied: in *Arabidopsis* plants overexpressing Arath*;KRP2*, only the A-type CDK complexes are targeted for inhibition (Verkest et al., 2005). Consistently, the phenotype of plants misexpressing Arath*;KRP1* could be rescued by co-misexpression of Arath*;CDKA;1*, but not Arath*;CDKB1;2* (Schnittger et al., 2003).

Besides the CDK subunit, KRP binding is also directed by the cyclin subunit. Yeast two-hybrid assays have revealed interactions of *Arabidopsis* and tobacco KRPs with D-type cyclins, suggesting that KRPs are potential regulators of CDK-cyclinD complexes (Wang et al., 1998; Lui et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002b; Zhou et al., 2002). Furthermore, *in vivo* binding specificity between plant CKIs and different D-type cyclins has been proven by the observation that the aberrant cell and leaf phenotypes seen upon *KRP* overexpression can be complemented by co-overexpression of D-type cyclins (Jasinski et al., 2002a; Schnittger et al., 2003; Zhou et al., 2003b). Recently, it was demonstrated that not

only D-type, but also A-type cyclin-harboring CDK complexes can be inhibited by KRPs *in vitro* (Coelho et al., 2005), so that the plant CKIs resemble the mammalian Kip/Cip inhibitors, which bind and inhibit a broad range of CDKs, including both A- and D-type cyclin containing CDK complexes.

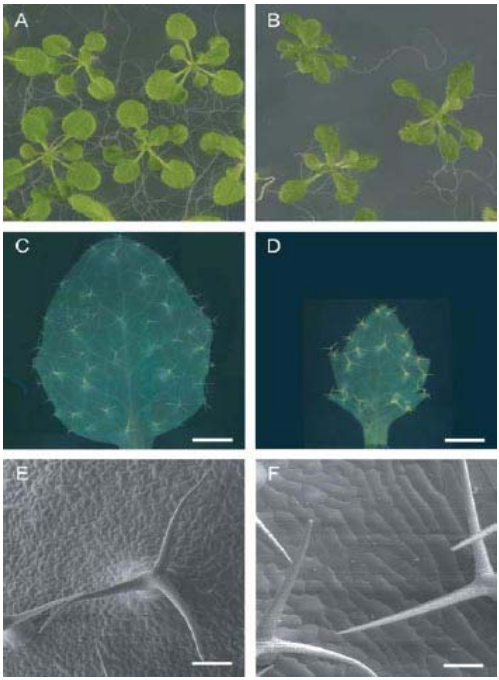


Figure 2. Phenotypes of *KRP*-misexpressing plants.
A. Wild-type Columbia plants.
B. Plants misexpressing the Arath;*KRP2* gene under the control of the shoot meristem-specific *STM* promoter, resulting into smaller and more elongated leaves than observed for wild-type plants. Plants are at the same developmental stage and magnification as in (A.)
C. and D., Rosette leaves from a wild-type (Col-0) and a transgenic plant misexpressing the Arath;*KRP1* gene under the control of the stomatal lineage-specific *TMM* promoter, respectively. Expression of Arath;*KRP1* in *TMM* cells results into an altered leaf morphology because of reduced leaf cell numbers.
E. and F., Close-up of the leaves shown in (C.) and (D.), respectively. Note the enlarged epidermal cells in (F). Bars = 1 mm (C. and E.) and 100 µm (D. and F.).

Structural organization

Like the mammalian Kip/Cip inhibitors, the plant CKIs have low sequence similarity to each other. Detailed analysis allowed the identification of several sequence elements shared by different KRPs, but only three C-terminally located motifs are conserved in all plant inhibitors (De Veylder et al., 2001). This region of the KRPs shows partial homology with the Kip/Cip protein domain necessary for interaction with the CDK subunit, suggesting that the plant CKI function resides at their C-terminus. Indeed, Wang et al. (1998) showed in a yeast-two hybrid interaction assay that the C-terminal domain of KRPs is sufficient for interaction of Arath;KRP1 with Arath;CDKA;1 and Arath;CYCD3;1. Moreover, the functionality of this domain for CDK binding and inhibition was proven *in vitro* and *in vivo* (Schnittger et al., 2003; Zhou et al., 2003a).
The role of the highly diverse N-terminal plant CKI sequences remains unclear, but could have additional functional implications and determine different regulatory mechanisms or a different specificity towards CDK-cyclin complexes. In Arath;KRP1, the N-terminal region was suggested to negatively regulate CKI function; deletion of this region increased the yeast two-hybrid physical interaction of Arath;KRP1 with CDKs and cyclins, and the enhanced the phenotype of Arath*;KRP1* overexpression in *Arabidopsis* (Wang et al., 1998; Schnittger et al., 2003; Zhou et al., 2003a). One possible function of the N-terminus could be the regulation of the KRP stability. Arath;KRP2 protein is highly unstable and its degradation depends on the proteasome (Verkest et al., 2005). Indeed, removal of the N-terminal region enhanced the Arath;KRP1 protein level. However, the regulating mechanism behind this protein stability remains unknown (Zhou et al., 2003a; Weinl et al. 2005).

Regulation of KRP activity at the transcript level

Yeast and mammalian CKIs are strictly regulated at the transcriptional, translational, and posttranslational level through mechanisms that rather affect their abundance than their intrinsic activity. Most plant tissues co-express various *KRPs*, but with different intensities in their mRNA levels, suggesting different transcriptionally regulatory mechanisms and possibly distinct roles for the plant CKIs within a single tissue (Wang et al., 1998; De Veylder et al., 2001, Jasinski et al., 2002b; Ormenese et al., 2004). A detailed spatial expression analysis by mRNA in situ hybridizations in the *Arabidopsis* shoot apex revealed different groups of KRPs with similar expression patterns. Whereas Arath*;KRP4* and Arath*;KRP5* expression was

confined to mitotically dividing tissues within the shoot apex, other *KRP* genes could be detected in both dividing and maturing cells (Arath*;KRP3*, Arath*;KRP6*, and Arath*;KRP7*) or exclusively in maturing cells (Arath*;KRP1* and Arath*;KRP2*) (Ormenese et al., 2004). These data hint at a function of Arath*;KRP1*, Arath*;KRP2*, Arath*;KRP3*, Arath*;KRP6*, and Arath*;KRP7* during the process of cell cycle exit and onset of differentiation, whereas Arath*;KRP4* and Arath*;KRP5* might direct specific aspects of the mitotic cell cycle, such as functioning of the checkpoints that control the correct timing of S- and M-phase onset.
A role for the KRPs during the regular cell cycle is also hinted by their observed cell cycle phase-dependent temporal regulation (Menges et al., 2005). Transcript levels peak during S-phase for Arath*;KRP3* and Arath*;KRP5*, in G2-phase for Arath*;KRP4*, during late G2-to-M for Arath*;KRP1*, and at M-to-G1 for Arath*;KRP6*. The expression of Arath*;KRP2* and Arath*;KRP7* is constitutive during the cell cycle.
Furthermore, transcript levels of the tobacco *NtKIS1a* and the *AtKRP1* accumulated with flower bud aging and leaf aging, respectively (Wang et al., 1998; Jasinski et al., 2002b). This temporal increase in transcripts during the course of cell cycle arrest and cellular differentiation suggests possible functions for these KRPs in development.
KRP mRNA is not only controlled in a spatial and temporal manner, but also through the generation of alternative splicing variants, as illustrated by the *NtKIS1* locus that generates two splice variants, *NtKIS1a* or *NtKIS1b* (Jasinski et al., 2002b). The splice variant *NtKIS1b* lacks the most C-terminal motif found in *NtKIS1a* and other plant CKIs. Consistently, NtKIS1b does not interact with A-type CDKs and D-type cyclins and is unable to inhibit CDK activity *in vitro* and *in vivo*.

Regulation of KRP activity at the posttranslational level

Currently, little is known about the regulation of plant CKIs at the protein level. In mammals, regulation of CKI activity is complex and is accomplished through several mechanisms. Kip/Cip inhibitors can be inactivated through out-titration by inert CDK-cyclinD complexes. Upon mitogenic stimuli, D-type cyclins accumulate and sequester the CKIs, resulting in their release from inhibited CDK-cyclinE complexes. Other mechanisms control the subcellular localization. Kip/Cip proteins have distinct nuclear and cytoplasmic functions and their cytoplasmatic compartmentalization releases and activates nuclear CDK-cyclin complexes (Coqueret, 2003). However, the best-studied posttranslationally regulatory mechanism of the mammalian CKIs affects their concentration through ubiquitin-dependent proteolysis. Two alternative proteolytic pathways control $p27^{Kip1}$ stability (Hengst, 2004). One pathway acts in

the nucleus and requires $p27^{Kip1}$ phosphorylation at Thr187 by CDK2-cyclinE complexes and subsequent recognition and degradation at the S-phase by the SCF^{Skp2} ubiquitin-ligase complex. The other one acts at the G1-phase, is independent of Skp2 and Thr187 phosphorylation, and involves cytoplasmic sequestration of $p27^{Kip1}$ and its degradation through the recently identified Kip ubiquitination-promoting complex (Hengst, 2004).
As evidenced, at least some plant KRPs are regulated through proteolysis. Functional analysis of the Arath;KRP1 domains indicated the presence of a regulatory motif for protein instability in its N-terminal domain (Zhou et al., 2003a; Weinl et al., 2005). Additionally, both Zeama;KRP2 and Arath;KRP2 are regulated at the posttranslational level during maize (Zea mays) endosperm and *Arabidopsis* leaf development, respectively, demonstrated by their alteration in protein levels while their transcript levels remain constant (Coelho et al., 2005; Verkest et al., 2005). In the case of Arath;KRP2, protein stability is regulated by the proteasome. Moreover, *in vitro* analysis illustrated that Arath;KRP2 is a CDK-cyclin substrate and that its phosphorylation is at least in part responsible for Arath;KRP2 proteolysis. Although both Arath;CDKA;1 and Arath;CDKB1;1 complexes phosphorylate Arath;KRP2, it is currently neither known at which cell cycle phase this event occurs nor which ubiquitin-ligase is responsible for Arath;KRP2 degradation.
Another mechanism of posttranslational regulation has been identified through comparative analysis of the *NtKIS1a* and *NtKIS1b* splice variants (Jasinski et al., 2002b). Even though the spliced form NtKIS1b does not interact with NtCDKA;1 and D-type cyclins, NtKIS1b counteracts the capacity of NtKIS1a to inhibit CDK activity *in vitro*. The two splice variants have a different transcriptional expression pattern: whereas *NtKIS1a* is constitutively present during the cell cycle, *NtKIS1b* transcript levels peak at G2-to-M. These data, together with their cooperative subcellular localization, suggest that NtKIS1b antagonizes NtKIS1a inhibition of CDK activity at the G2-to-M transition. However, the mechanism by which this occurs remains to be elucidated.

Intercellular and intracellular localization of KRPs

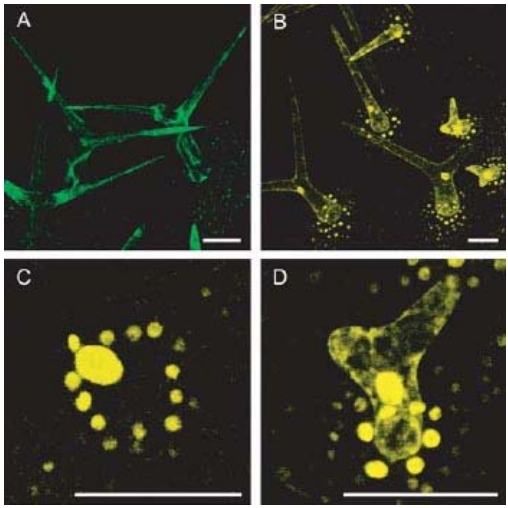
In animals, the CKI function depends on their intracellular localization. The CKI $p27^{Kip1}$ exerts its inhibitory function in the nucleus and entry into the nucleus appears to be used as a control mechanism for $p27^{Kip1}$. In addition, $p27^{Kip1}$ degradation is precisely regulated and is seemingly also connected, at least to some degree, with its intracellular localization pattern (see above).

Fusions of the green fluorescent protein (GFP) or the yellow fluorescent protein (YFP) and the Arath;KRP1 or the NtKIS1 have revealed a strict nuclear localization (Jasinski et al., 2002b; Zhou et al., 2003a; Weinl et al., 2005) (Figure 3). In addition, a putative nuclear localization signal has been identified in the protein sequences of Arath;KRP2, Arath;KRP5, and Arath;KRP7 (De Veylder et al., 2001).

In animals, regulation of nuclear import and export of $p27^{Kip1}$ is complex and involves phosphorylation at Ser10, Thr157, Thr187, and Thr198. None of these phosphorylation sites are conserved in plant KRPs. However, the stabilized N-terminally truncated Arath;KRP1 shows in addition to a nuclear, also a cytosolic localization (Fig. 3D). Thus, one likely possibility is that Arath;KRP1 becomes degraded in the cytoplasm and that a motif in the N-terminus of the protein is required for this degradation. It will be interesting to see to what degree the intracellular localization of CKIs is an important regulatory mechanism in plants as well.

Surprisingly, plant CKIs function in a non-cell-autonomous manner. Fusion proteins between YFP and Arath;KRP1 were found at least two to three cells away form their site of translation (Weinl et al., 2005) (Figure 3, B and C). Other plant cell cycle regulators have also been observed to travel between cells (Jakoby and Schnittger, unpublished data). Currently, it is still unclear whether it is the mRNA or the protein that moves or whether this movement is based on a targeted versus a non-targeted mechanism. So far, the functional relevance of this movement is unknown. On the one hand, the non-cell-autonomous behavior could simply be a consequence of overproduction of these proteins. On the other hand, the non-cell-autonomous action of Arath;KRP1 offers a possibility to link decisions on a cellular level with the supracellular division and growth pattern in tissues and organs. For instance, during leaf development, epidermal cells have been observed to exit the cell cycle before palisade cells do (Donnelly et al., 1999). A mechanism can be envisaged in which the diffusion of synthesized KRPs in the epidermis coordinates the initial cell cycle exit of the dermal layer with that of the palisade parenchyma layer later during development. In any case, the molecular nature of the non-cell-autonomous action of KRPs remains to be analyzed in detail.

Figure 3. Intercellular and subcellular localization of Arath;KRP1.
A. Trichome-specific expression of the *GLABRA 2* promoter as revealed by GFP fluorescence.
B. Protein fusions of Arath;KRP1 with YFP expressed from the *GLABRA 2* promoter spread from the trichome into the neighboring cells.
C. and D. Close-up of trichomes; the full-length Arath;KRP1-YFP fusion protein is found exclusively in the nuclei (C.); the N-terminally truncated Arath;KRP1D2-108 localizes to the nucleus and the cytoplasm (D.). Bars =50 µm.



KRPs and plant development

KRPs as integrators of developmental signals

The observation that several plant CKIs are transcriptionally regulated during development indicates that they share with the mammalian Kip/Cips the potential to integrate developmental signals into the core cell cycle machinery. Indeed, Arath;*KRP1* transcripts are induced by cold treatment, which correlates with a decrease in CDK activity. Furthermore, *KRP1* expression was found to be activated by the phytohormone abscisic acid (ABA) (Wang et al., 1998), suggesting that this particular KRP might be in part responsible for the growth inhibitory effect triggered upon ABA treatment. By contrast, the mitogenic hormone auxin repressed Arath;KRP2 transcription, both in cell cultures and in planta (Richard et al., 2001; Himanen et al., 2002). Downregulation of Arath;*KRP2* precedes the auxin-induced re-entry of quiescent root pericycle cells into the cell cycle. Interestingly, Arath;*KRP2* transcripts have been detected in young roots at the phloem but not the protoxylem poles of the pericycle, i.e. the sites at which lateral roots can initiate. In older root tissues, Arath;*KRP2* expression has been observed at both the phloem and

protoxylem poles, corresponding with the observation that new laterals mainly initiate at the young parts of the root. Curiously, upon the initiation of a lateral root primordium, Arath*;KRP2* expression is induced in cells opposite the developing new lateral root, implying a mechanism by which Arath;KRP2 prevents the formation of two opposing lateral roots. A role for KRPs in controlling root architecture has been confirmed by overexpression analysis, as illustrating by the observation that ectopic Arath*;KRP2* overexpression in *Arabidopsis* results in a dramatic decrease in the number of lateral roots (Himanen et al., 2002).

Control of endocycle onset

Mammalian Kip/Cip inhibitor gene expression has been found to correlate with the onset of endoreduplication (Bates et al., 1998; Hattori et al., 2000). The endocycle is an alternative cell cycle during which DNA replication is not followed by mitosis and cytokinesis, and often marks the onset of cell differentiation. Endoreduplication represents the most common mechanisms to increase the cellular DNA ploidy in plants and, although the physiological relevance of the endoreduplication process is still unresolved, there are several indirect reasons to believe that an increase in the DNA ploidy level supports cell growth and high metabolic activity (Schnittger et al., 2003; Sugimoto-Shirasu and Roberts, 2003).
In yeast and fruitfly (*Drosophila melanogaster*), the onset of endoreduplication corresponds with a decrease in CDK activity (Edgar and Orr-Weaver, 2001; Larkins et al., 2001). A similar mechanism is probably operational in plants, because the start of endoreduplication in *Arabidopsis* leaves, maize endosperm, and the fruit of tomato (Lycopersicon esculentum) is accompanied with a decline in extractable CDK activity (Grafi and Larkins, 1995; Joubès et al., 1999; Verkest et al., 2005). Recently, KRPs have been demonstrated to participate in the control of this decrease in CDK activity. In strong Arath*;KRP2*-overexpressing lines CDK activity is inhibited in both mitotically dividing and endoreduplicating leaf tissues. By contrast, in weak overexpressing lines only the mitotic CDK-cyclin complexes are affected, blocking entry into mitosis but still allowing the onset and progression through S-phase, resulting into an increase in DNA ploidy levels. The two apparently contradictive effects seen upon strong or weak *KRP* overexpression can be explained by assuming that KRPs show a binding preference toward the CDK-cyclin complexes that control the G2-M checkpoint or that higher levels of CDK-cyclin activity are required for entry into mitosis than for entry into S-phase. The capacity of KRPs to trigger the onset of the endocycle in dividing tissues was confirmed by the specific overexpression of Arath*;KRP2* in proliferating tissues, causing an

inhibition of mitotic CDK activity and a premature onset of endoreduplication (Verkest et al., 2005). Likewise, low levels of Arath*;KRP1* in trichome socket cells or its specific expression in the mitotically dividing stomatal precursor cells triggered increased ploidy levels (Weinl et al., 2005).
Interestingly, Arath;KRP2 protein is negatively regulated at the post-translational level by B-type CDK activity as seen by the increase in Arath;KRP2 abundance in transgenic plants with reduced Arath;CDKB1;1 activity (Verkest et al., 2005). B-type CDKs phosphorylate KRPs, marking them for protein destruction (see above). Previously, Arath;CDKB1;1 activity has been demonstrated to play an important role in the decision process of the cell to divide or to endoreduplicate: plants with reduced B-type CDK activity exit the mitotic cell cycle and enter the endocycle prematurely (Boudolf et al., 2004b). Because KRPs specifically inhibit A-type CDK activity, the controlled destruction of KRPs by B-type CDK complexes suggests a mechanism by which the entry into the endocycle is controlled by a sequential decrease of first B-type and then A-type CDK activities. In this model, A-type CDKs are protected from KRP2-mediated inhibition as long as cells possess a high level of B-type CDK activity, because CDKB1;1 marks the KRP proteins for destruction (Figure 4A). However, as cells enter the endocycle program they lose B-type CDK activity, with a stabilization of KRPs and a subsequent inhibition of A-type CDK activity as a result (Verkest et al., 2005) (Figure 4, B and C).

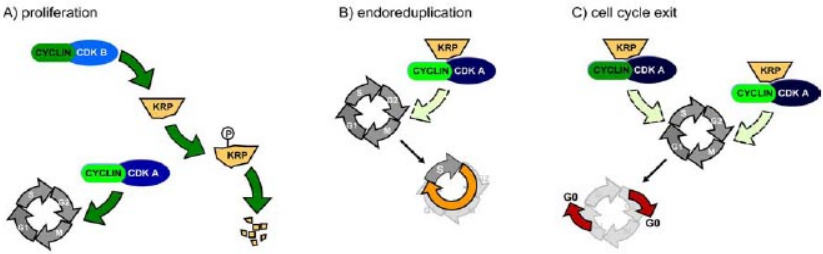


Figure 4. Model of KRPs controlling the switch between the different cell cycle programs.
A. In proliferating cells, B-type CDKs phosphorylate KRPs, triggering their destruction. In addition, phosphorylation might change the conformation of KRPs, interfering with their binding to A-type CDKs.
B. In cells triggered to endoreduplicate B-type CDK activity ceases, resulting into a stabilization of the KRPs, which now bind and inhibit A-type CDK/cyclin complexes with a role in mitosis. The KRP concentration, however, is probably not high enough to inhibit as well the CDK-cyclin complexes driving S-phase entry, allowing cells to re-enter the S-phase.
C. During cell cycle exit, *KRP* expression is upregulated. Now, besides blocking entry into mitosis also CDK-cyclin complexes controlling the entry into S-phase become inhibited, resulting in a complete cell cycle arrest.

Do KRPs function outside the cell cycle?

In animals, CKIs might also have functions outside the cell cycle, such as in differentiation, morphogenesis, and programmed cell death. So far, no clear evidence has been provided that plant CKIs also function outside the cell cycle. Strong and constitutive overexpression of Arath;*KRP2* did not alter cellular differentiation processes as illustrated by the unaltered timing of stomata differentiation patterns with respect to leaf development (De Veylder et al., 2001). Also premature onset of endoreduplication caused by Arath*;KRP1* expression in trichome neighboring cells does apparently not interfere with the adaptation of trichome socket cell fate (Weinl et al., 2005). However, misexpression of Arath*;KRP1* in *Arabidopsis* trichomes induces cell death (Schnittger et al., 2003). This phenotype seems to be linked to the developmental program of trichomes, because so far for other cell types no cell-death phenotypes have been observed upon *KRP* overexpression. At the moment, it is not clear whether the observed trichome cell death phenotype is linked with a compromised endoreduplication program, with cell death being indirectly initiated as a consequence of a discrepancy between DNA content and cell size. Clearly, additional experiments are required to conclude whether KRPs control cell survival.

Conclusions

The recent years have brought about a tremendous increase in our understanding of CKIs in plants. Some regulatory pathways are now emerging with KRPs

functioning as dose-dependent cell cycle regulators. KRPs might be important for adjusting CDK activity within dividing cells, as well as in facilitating the transition between different cell cycle programs, such as entering endoreduplication cycles or executing cell cycle exit (Figure 4). KRPs possibly play a central role in connecting cell cycle progression with developmental as well as environmental cues. Many questions, however, still need to be resolved. For instance, KRPs are very poorly conserved among species, thus do these proteins still share a common structure outside the CDK and cyclin binding motif? Is there a developmental or physiological need for the many different *KRP* genes within an organism (7 in *Arabidopsis*)? How is the KRP abundance and localization regulated? Many tools have been developed now that allow us to address these questions on a cellular, biochemical, and genetic level. In addition, KRPs from different plant species have been identified, setting the path for a comparative approach and leading to the anticipation of general principles in KRP function in plants.

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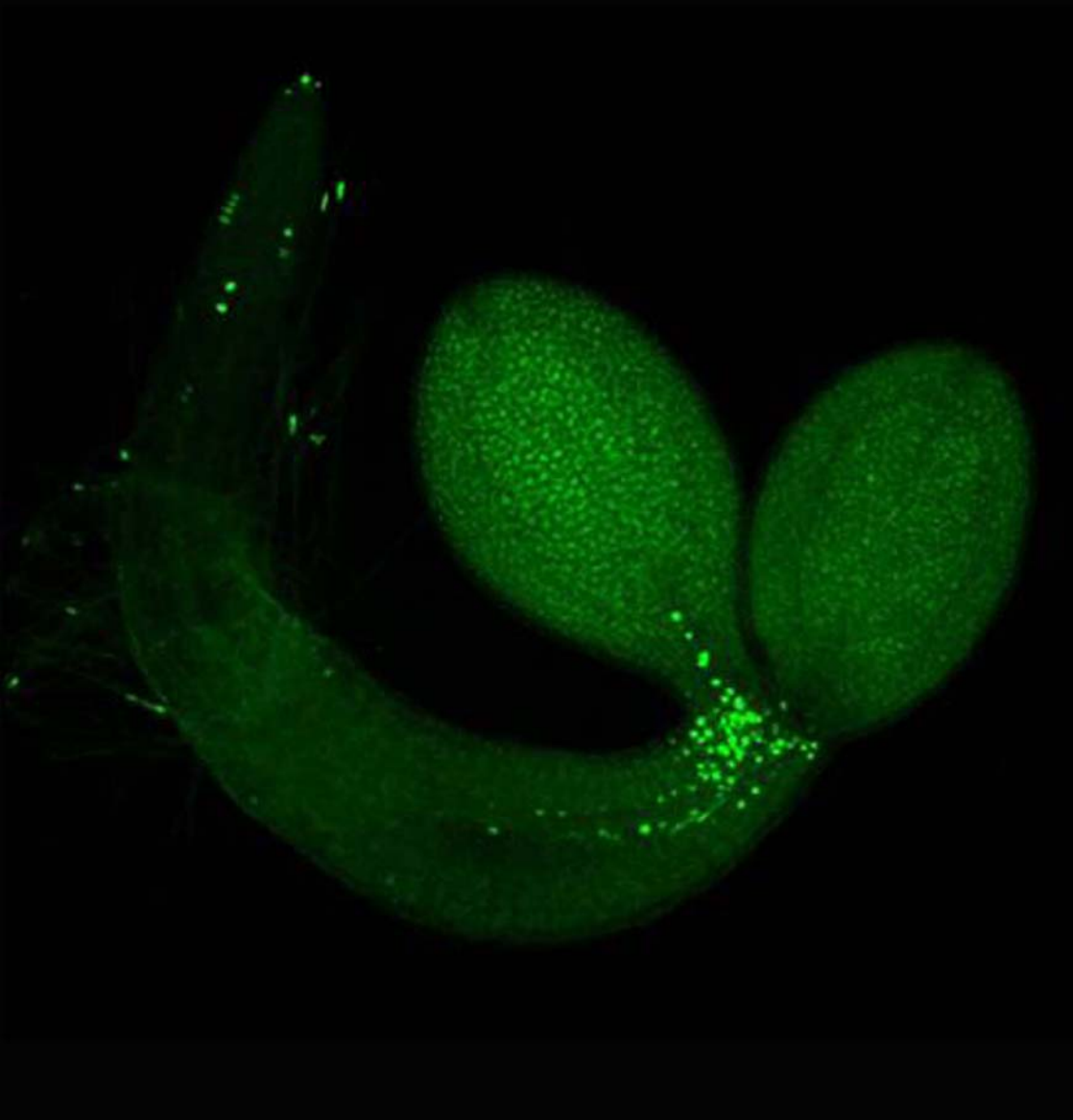
Chapter 3



Chapter page: Expression pattern of the *STM*-promoter as visualized by confocal microscopy on *STM:YFP-histoneH4* seedlings.

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The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during *Arabidopsis* leaf development through inhibition of mitotic CDKA;1 kinase complexes

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Abstract

Exit from the mitotic cell cycle and initiation of cell differentiation frequently coincides with the onset of endoreduplication, a modified cell cycle during which DNA continues to be duplicated in the absence of mitosis. Although the mitotic cell cycle and the endoreduplication cycle share much of the same machinery, the regulatory mechanisms controlling the transition between both cycles remain poorly understood. We show that the cyclin-dependent kinase CDKA;1 and its specific inhibitor KRP2 regulate the mitosis-to-endocycle transition during *Arabidopsis thaliana* leaf development. Constitutive overexpression of *KRP2* slightly above its endogenous level only inhibited the mitotic cell cycle-specific CDKA;1 kinase complexes, whereas the endoreduplication cycle-specific CDKA;1 complexes were unaffected, resulting in an increase in the DNA ploidy level. An identical effect on the endoreduplication cycle could be observed by overexpressing *KRP2* exclusively in mitotically dividing cells. In agreement with a role for KRP2 as activator of the mitosis-to-endocycle transition, KRP2 protein levels were more abundant in endoreduplicating than in mitotically dividing tissues. We illustrate that KRP2 protein

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abundance is regulated posttranscriptionally through CDK phosphorylation and proteasomal degradation. KRP2 phosphorylation by the mitotic cell cycle-specific CDKB1;1 kinase suggests a mechanism in which CDKB1;1 controls the level of CDKA;1 activity through regulating KRP2 protein abundance. In accordance to this model, KRP2 protein levels increased in plants with reduced CDKB1;1 activity. Moreover, the proposed model allowed a dynamical simulation of the *in vivo* observations, validating the sufficiency of the regulatory interactions between CDKA;1, KRP2, and CDKB1;1 in fine-tuning the mitosis-to-endocycle transition.

Introduction

Cells undergoing endoreduplication duplicate their genome in the absence of chromatin segregation and cytokinesis, with a progressive increase of their nuclear DNA content as a consequence. Endoreduplication is widespread among eukaryotes, although most prevailing in plants (Nagl, 1976). Despite its common nature, both the physiological role and the molecular control of endoreduplication are poorly understood. The endoreduplication level of a cell is often inversely correlated with genome size, which has led to the hypothesis that somatic polyploidy represents an evolutionary strategy to compensate for a lack of phylogenetic increase in nuclear DNA (Folkers et al., 1997; Traas et al., 1998; Kondorosi et al., 2000; Sugimoto-Shirasu and Roberts, 2003). Other hypotheses link endoreduplication with metabolic activity, maintenance of the optimal ratio between nuclear and organellar DNA, or protection against irradiation (Joubès and Chevalier 2000; Kondorosi et al., 2000; Larkins et al., 2001). Moreover, endoreduplication probably plays an important role in the differentiation process of post-mitotic cells because the onset of the endocycle often characterizes the switch between cell proliferation and differentiation, as observed during hypocotyl elongation, trichome growth, and fruit development (Kondorosi et al., 2000; Larkins et al., 2001; Joubès et al., 1999). As such, investigating how the endocycle onset is regulated might help to understand how cell differentiation is initiated.

The coincidence of the zone of meristematically dividing cells with the region of subsequent endoreduplication in the *Arabidopsis thaliana* shoot apex suggests that the endoreduplication cycle is initiated through a modification and exit of the mitotic cell cycle (Jacqmard et al., 1999). Progression through the mitotic cell cycle is mediated through sequential activation of S-phase- and M-phase-specific heterodimeric protein complexes, consisting of a catalytic subunit, the cyclin-

dependent kinase (CDK), and a regulatory cyclin subunit. In *Schizosaccharomyces pombe* and *Drosophila melanogaster*, transition from the mitotic cell cycle into the endoreduplication cycle has been suggested to involve nothing more than loss of M-phase CDK activity (Edgar and Orr-Weaver, 2001; Larkins et al., 2001). A similar mechanism probably operates in plant cells, because the onset of endoreduplication during endosperm and tomato fruit development correlates with the inhibition of M-phase-associated CDK activity (Grafi and Larkins, 1995; Joubès et al., 1999). Also during *Arabidopsis* leaf development, the start of endoreduplication coincides with the loss of mitotic activity (Boudolf et al., 2004a; Beemster et al., 2005). However, the identity of the CDK complexes and the mechanisms that account for the decrease in CDK activity are mostly unknown. Previously, we have shown that the G2-M-specific CDKB1;1 plays a determining role in whether *Arabidopsis* leaf cells divide or endoreduplicate, because overexpression of a dominant negative *CDKB1;1* allele triggered cells to enter the endoreduplication cycle prematurely (Boudolf et al., 2004a). A role for A-type CDK activity in the endocycle has recently been proven by the specific overexpression of a dominant negative allele of the maize (*Zea mays*) A-type CDK in endosperm cells, that inhibited endoreduplication (Leiva-Neto et al., 2004).

Modulations in CDK activity can be achieved through different mechanisms, including phosphorylation, cyclin degradation, or association with CDK inhibitory proteins (Nurse, 1994; Elledge, 1996; Nasmyth, 1996; Sherr and Roberts, 1999; Mironov et al., 1999). A likely candidate involved in CDK inhibitory phosphorylation during endoreduplication is the WEE1 kinase that is up-regulated in maize during endosperm development, coinciding with the onset of endoreduplication (Sun et al., 1999). Another well-documented mechanism for inhibition of M-phase CDK activity has been found in *Medicago truncatula* root nodules. CCS52, an activator of the anaphase-promoting complex involved in the degradation of mitotic cyclins, links cell proliferation to cell differentiation and is involved in the conversion of mitotic to endocycles (Cebolla et al., 1999; Vinardell et al., 2003; Tarayre et al., 2004).

CDK inhibitory proteins have been proven to be important regulators of the endoreduplication cycle in several organisms. Overexpression of *Rum1* in fission yeast induces polyploidy and nuclei enlargement through inhibition of M-phase CDKs (Moreno and Nurse, 1994). In mammalian trophoblasts, ectopic expression of $p57^{Kip2}$ promotes giant cell differentiation, whereas expression of a stable mutant form of the protein blocks endoreduplication (Hattori et al., 2000). Recently, it has been demonstrated that the CDK inhibitor Dacapo has a function in the mitosis-to-endocycle transition in Drosophila follicle cells and that it plays an important role in endocycling nurse cells (Hong et al., 2003; Scherbata et al., 2004).

Proteins related to the class of mammalian Kip/Cip CDK inhibitors have been identified in plants and designated Kip-related proteins (KRPs) in *Arabidopsis* (De Veylder et al., 2001; Vandepoele et al., 2002). The *Arabidopsis* genome encodes seven *KRP* genes. Despite the limited sequence homology with their mammalian counterparts, KRPs have been shown to be true functional homologs of the Kip/Cip proteins in inhibiting CDK activity both *in vitro* and *in vivo* (Wang et al., 1997; Lui et al., 2000; Wang et al., 2000; De Veylder et al., 2001; Zhou et al., 2002). Overproduction of KRPs in *Arabidopsis* resulted in plants with small and serrated leaves, due to a reduction in cell number as a consequence of an inhibition of the mitotic cell cycle. Moreover, these transgenic plants point to the involvement of KRPs and CDK activity in endoreduplication, because overproduction of the *Arabidopsis* KRPs or the tobacco (*Nicotiana tabacum*) KRP homolog NtKIS1a resulted in a decrease in the ploidy level in older leaves (De Veylder et al., 2001; Jasinski et al., 2002; Zhou et al., 2002).
Here, we investigate the physiological relevance of the interaction between KRP2 and CDKA;1 at the onset of endoreduplication. A specific inhibition of the mitotic CDKA;1 complexes through mild KRP2 overproduction was found to result into an increase in the DNA ploidy level. We propose a mechanism by which the level of CDKA;1 activity determines whether a cell divides or endoreduplicates. Moreover, we show that the KRP2 protein abundance is regulated posttranscriptionally through CDK phosphorylation and proteasomal degradation. The observation that CDKB1;1/cyclin complexes phosphorylate KRP2, whereas KRP2 protein levels are stabilized in plants overexpressing a dominant negative *CDKB1;1* allele, prompts us to postulate that CDKB1;1 regulates the level of CDKA;1 activity in dividing cells through the control of KRP2 protein abundance.

Results

KRP2 specifically binds and inhibits CDKA;1 and not CDKB1;1

Previously, the interaction of KRP proteins with the archetypical A-type CDKA;1 but not with the B-type CDKB1;1 has been demonstrated by yeast two-hybrid analysis (Lui et al, 2000; De Veylder et al., 2001; Zhou et al., 2002). To analyze the CDK-binding specificity of KRP2 *in vivo*, transgenic lines were generated that overexpressed a N-terminal hemagglutinin(HA)-tagged *KRP2* gene under control of the constitutive Cauliflower Mosaic virus *(CaMV) 35S* promoter. Several independent

lines were obtained. For molecular analysis, two lines (S1 and S2) homozygous for one T-DNA locus with high and two lines (W1 and W2) with low HA-KRP2 levels were selected, referred hereafter as strong and weak *KRP2*^{OE} lines, respectively (Figure 1A). Differences in transgene expression were reflected in the KRP2 mRNA and protein levels and the strength of CDK activity inhibition (Figures 1B-1E). Although CDK activity was more severely inhibited in the strong than in the weak *KRP2*^{OE} lines, no linear correlation was found between the amount of transgenic KRP2 protein and the level of CDK inhibition. This observation points toward the presence of a KRP2-resistant fraction of CDK/cyclin complexes that cannot be inhibited by KRP2, even at high levels.

As reported previously, strong *KRP2*^{OE} lines had a reduced leaf size compared with that of wild-type plants and a distinct morphology (De Veylder et al., 2001) (Figure 1A). The observed decrease in leaf size was due to a strong reduction in cell number resulting from an inhibition of the mitotic cell cycle (Table 1) (De Veylder et al., 2001). Also for the weak *KRP2*^{OE} lines, a negative effect on the mitotic cell cycle was seen, as illustrated by the reduction in leaf size and cell number, although the phenotype was less pronounced than that of the strong *KRP2*^{OE} lines (Table 1). Weak *KRP2*^{OE} lines differed from the strong lines in the lack of a serrated leaf phenotype (Figure 1A).

Figure 1. Phenotypic and molecular analysis of *KRP2*^{OE} *Arabidopsis* plants.
Three-week-old transgenic plants were compared with untransformed control plants (Col-0) of the same age.
A. Phenotypes of wild-type (Col-0) plants and two independent weak (35S:KRP2 W1 and W2) and strong (35S:KRP2 S1 and S2) *KRP2*^{OE} lines.
B. RNA gel blot analysis of wild-type and *KRP2*^{OE} plants. Equal loading of the gel was confirmed by methylene blue staining of the membrane (bottom panel).
C. Immunoblot analysis of the same lines as in (B.) using an anti-KRP2 antibody. Rubisco protein levels visualized by Ponceau S staining act as loading control.
D. p10^{CKS1At}-associated kinase activity in transgenic plants. Autoradiogram representing a typical result from CDK activity assays with histone H1 as substrate. The first lane is a background control, in which the activity of BSA-Sepharose bound Col-0 extract is shown. Coomassie blue staining of the electrophoresis gel area with histone H1 was used as a control of equal substrate quantity per phosphorylation reaction.
E. Relative quantification of three independent kinase activity measurements as depicted in (D.). The control was arbitrarily set at 100%.

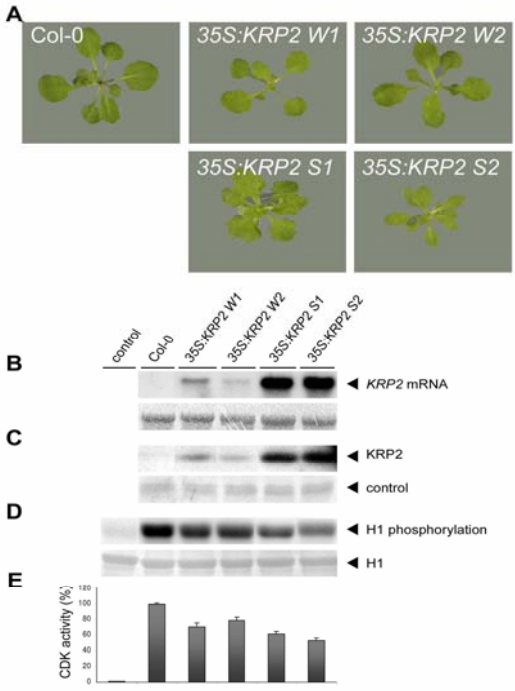


Table 1. Abaxial epidermis cell size and cell number in leaves of wild type and *KRP2*^{OE} plants

Line	Leaf size (mm^2)	Abaxial Epidermal Cells	
		Estimated number	Size (μm^2)
Col-0	15.03±1.02	13532±875	1160±33
CaMV 35S:KRP2 W1	11.08±0.38	11104±861	1033±78
CaMV 35S:KRP2 W2	10.25±0.30	10712±898	968±74
CaMV 35S:KRP2 S1	7.68±0.37	1895±205	4330±494
CaMV 35S:KRP2 S2	8.98±0.23	1770±93	5250±358

All measurements were performed on 3-week-old mature first leaves. The indicated values are means ± SE (n = 6 to 10).

The CDK binding-specificity of KRP2 was initially tested by an *in vitro* binding assay. Recombinant KRP2 proteins were coupled to Sepharose beads that were used to make an affinity column. To this column *Arabidopsis* protein extracts were applied, and the bound and unbound fractions were probed for the presence of CDKA;1 or CDKB1;1 by protein blot analysis with specific antibodies (Hemerly et al., 1995; Porceddu et al., 2001). As a control, a BSA-Sepharose affinity column was used. The CDKA;1 protein was found to bind specifically to the KRP2 column (Figure 2A). Interestingly, not all CDKA;1 protein retained on the column, because a portion of CDKA;1 was found in the unbound fraction. In contrast to CDKA;1, no CDKB1;1 associated with the KRP2-Sepharose column (Figure 2A).
To analyze the *in vivo* binding-specificity of KRP2, HA-KRP2-containing complexes were immunoprecipitated from total protein extracts prepared from strong and weak *KRP2*^{OE} lines. Subsequently, the pulled-down material was probed with CDKA;1- and CDKB1;1-specific antibodies. For both cell extracts, a clear CDKA;1 signal was detected. The amount of CDKA;1 protein that co-immunoprecipitated with KRP2 correlated with that of the KRP2 protein, although not all CDKA;1 proteins were pulled-down. CDKB1;1, on the other hand, did not co-immunoprecipitate with KRP2 (Figure 2B).

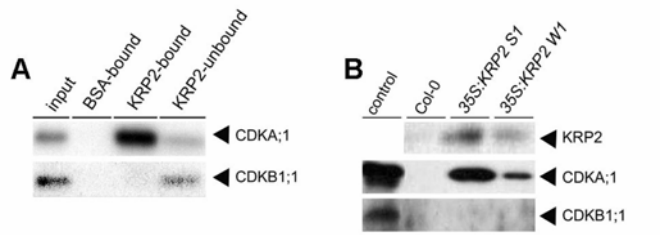
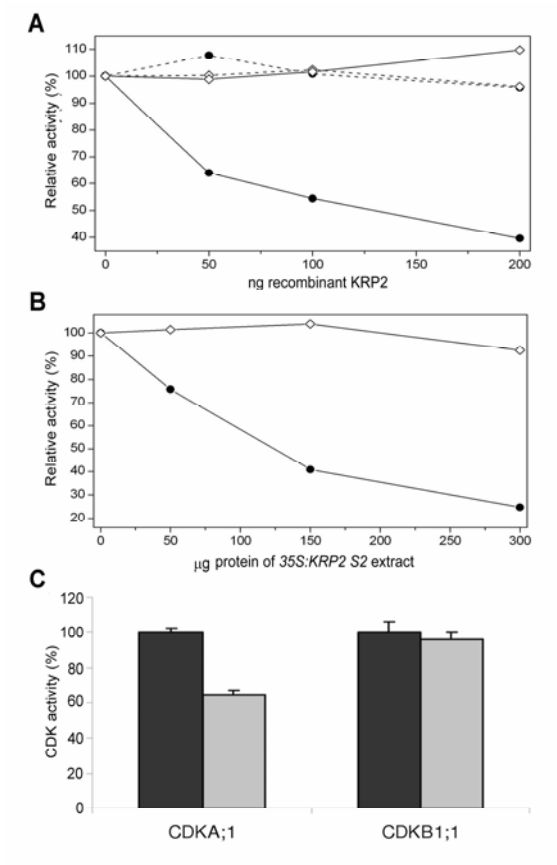


Figure 2. Specific *in vitro* and *in vivo* CDKA;1 binding by KRP2.
A. *In vitro* interaction of KRP2 with CDKA;1. Protein extracts were loaded onto a BSA-Sepharose or KRP2-Sepharose column, and bound and unbound fractions were tested for the presence of CDKA;1 or CDKB1;1 with specific antibodies. Input and unbound fractions represent 1/30th of the amount of protein loaded on the beads.
B. *In vivo* interaction of KRP2 with CDKA;1. Immunoprecipitated HA-KRP2 complexes from total protein extracts of 3-week-old wild-type (Col-0) and strong (35S:KRP2 S1) and weak (35S:KRP2 W1) *KRP2*^{OE} plants were analyzed by immunoblot analysis with anti-HA, anti-CDKA;1, and anti-CDKB1;1 antibodies. Total protein extract (1/10th of the amount loaded) from untransformed plants was used as control.

The overexpression of *KRP* genes has been demonstrated to result in a decrease in extractable CDK activity (Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2001). To test whether the observed decrease in kinase activity was due to a specific inhibition of the CDKA;1 kinase, the effect of increasing amounts of recombinant KRP2 and of extracts of a strong *KRP2*^{OE} plant on the immunoprecipitated CDKA;1 and CDKB1;1 kinase activity of wild-type plants was analyzed (Figure 3A and 3B). In both cases a similar inhibition profile was obtained showing specific inhibition of the kinase activity of CDKA;1, and not of CDKB1;1 complexes. Specific inhibition of CDKA;1 activity by KRP2 was also confirmed *in vivo* through comparison of the immunoprecipitated CDKA;1- and CDKB1;1-associated kinase activity from an untransformed control line and a strong *KRP2*^{OE} line. In comparison to the control plants, CDKA;1 activity was reduced by almost 40% in the *KRP2*^{OE} line, whereas no significant inhibition of CDKB1;1 activity was observed (Figure 3C).
Together, the interaction data and kinase activity measurements demonstrated that CDKA;1, but not CDKB1;1, was inhibited by KRP2 *in vitro* and *in vivo*. A systematic two-hybrid interaction screen between all *Arabidopsis* CDKs and KRPs further confirmed that CDKA;1 was the only CDK to associate with KRPs (data not shown). Therefore, CDKA;1 is very probably the only CDK to be targeted in the *KRP2*^{OE} lines.

Figure 3. Specific *in vitro* and *in vivo* inhibition of CDKA;1 kinase activity by KRP2.
A. Effect of recombinant KRP2 protein on A- and B-type CDK activity. KRP2 (full line) or BSA (dashed line) were incubated with the indicated amounts to CDKA;1 (filled dots) and CDKB1;1 (open diamonds) complexes immunoprecipitated from 12-day-old wild-type (Col-0) plants. Kinase activity was measured with histone H1 as substrate.
B. Kinase activity measurements of immunoprecipitated CDKA;1 and CDKB1;1 complexes from untransformed plants (Col-0) in the presence of increasing amounts of extract from a strong *KRP2*^{OE} line (35S:KRP2 S2).
C. Specific inhibition of CDKA;1/cyclin complexes by KRP2 *in vivo*. Kinase activity assays of immunoprecipitated CDKA;1 and CDKB1;1 complexes of wild-type (Col-0)(black) and strong *KRP2*^{OE} (35S:KRP2 S1)(grey) plants. Relative CDK activity was measured using histone H1 as substrate. For quantification, the control was arbitrary set at 100%. The indicated values are means ± SD (n = 3).



KRP overexpression triggers a dose-dependent endoreduplication phenotype

To analyze the effects of *KRP2* overexpression on the DNA ploidy distribution, the DNA content of 3-week-old first leaves of wild-type and transgenic lines was measured by flow cytometry (Table 2). At this stage of development, the leaf is mature: no mitotic divisions can be detected and DNA ploidies have reached a steady-state level (De Veylder et al., 2001; Boudolf et al., 2004a; Beemster et al.,

2005; Vlieghe et al., 2005). Surprisingly, contrasting results were obtained for strong and weak *KRP2*^{OE} lines. In the strong *KRP2*^{OE} lines, an increase in the 2C population was observed, correlated with a decrease in the number of nuclei with a 4C and 8C DNA content, illustrating an inhibition of the endoreduplication cycle (Table 2). This inhibition of the endoreduplication cycle is in agreement with previous reports on *KRP* overexpression in *Arabidopsis* (De Veylder et al., 2001; Jasinski et al., 2001; Zhou et al., 2002; Schnittger et al., 2003). In contrast, in independent weak *KRP2*^{OE} lines, a reproducible increase in the DNA ploidy level was observed, as seen by the decrease in number of cells with a 2C DNA content and the increase in the 8C and 16C cell populations (Table 2). This effect on the endoreduplication cycle correlated with the *KRP2* expression level and was also seen in cotyledons (data not shown).

Table 2. DNA ploidy levels in 3-week-old mature first leaves of wild-type and *KRP2*^{OE} transgenic lines

Line	2C (%)	4C (%)	8C (%)	16C (%)
Col-0	31.2 ± 0.9	52.8 ± 0.5	15.5 ± 0.6	
CaMV 35S:KRP2 W1	24.7 ± 0.9	51.1 ± 1.0	21.9 ± 0.4	0.9± 0.1
CaMV 35S:KRP2 W2	20.8 ± 1.1	36.6 ± 1.2	36.9 ± 1.1	2.7 ± 0.2
CaMV 35S:KRP2 S1	47.8 ± 0.9	37.4 ± 0.6	13.2 ± 0.1	
CaMV 35S:KRP2 S2	49.5 ± 0.2	39.1 ± 0.1	10.9 ± 0.1	

Data represent average ± SD (n= 4 to 8).

CDKA;1 transcripts can be detected in both dividing and endoreduplicating cells of the shoot apex and leaf (Jacqmard et al., 1999; Beemster et al., 2005), suggesting a role for CDKA;1 in both the mitotic cell cycle and endoreduplication cycle. Distinct CDKA;1/cyclin complexes have been demonstrated to regulate the mitotic cell cycle and endoreduplication cycle and endoreduplication has been suggested to involve loss of M-phase CDK activity (Grafi and Larkins, 1995; Larkins et al., 2001). As such, the different effect on the endoreduplication cycle observed for strong and weak *KRP2*^{OE} lines is best explained by a specific inhibition of the mitotic CDKA;1/cyclin complexes in the weak *KRP2*^{OE} lines, whereas in the strong *KRP2*^{OE} lines both the CDKA;1/cyclin complexes with a role in the mitotic cell cycle and the endoreduplication cycle are targeted. To test this hypothesis, CDKA;1 kinase activity was measured in the leaf tissue of the first leaf pair harvested 9 and 15 days after sowing (DAS). Leaves of nine-day-old plants are predominantly mitotically dividing, whereas those of 15-day-old plants mainly endoreduplicate (Boudolf et al., 2004a; Beemster et al., 2005; Vlieghe et al., 2005). For wild-type leaves, CDKA;1-associated kinase activity was lower at 15 than at 9 DAS, illustrating that dividing

cells possess more CDKA;1 activity than endoreduplicating cells (Figure 4A-4B). In mitotic cells (9 DAS) a decrease in CDKA;1 activity was observed for both the strong and the weak *KRP2*^{OE} lines by approximately 40% and 20%, respectively (Figure 4A). In contrast, at 15 DAS, kinase activity was inhibited by approximately 40% in the strong *KRP2*^{OE} lines, whereas in the weak lines CDKA;1 activity was unaffected (Figure 4B). These data indicate that in the strongest *KRP2*^{OE} lines CDKA;1/cyclin complexes with both a role in the mitotic cell cycle and endocycle were inhibited, whereas in the weak *KRP2*^{OE} lines mainly the mitotic CDKA;1 complexes were targeted.

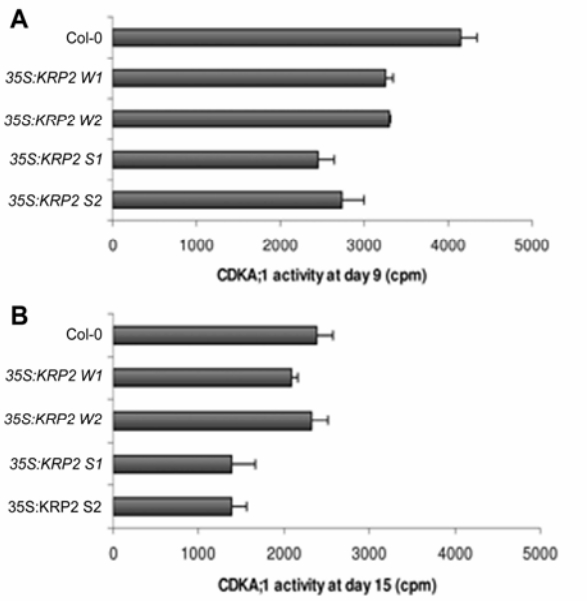


Figure 4. KRP2 dose-dependent control of the endoreduplication cycle.
Quantitative analysis of immunoprecipitated CDKA;1 kinase activity in the first leaf pair of wild-type (Col-0) and weak (35S:KRP2 W1 and W2) and strong (35S:KRP2 S1 and S2) *KRP2*^{OE} plants, using histone H1 as substrate.
A. Nine-day-old mitotically dividing leaves.
B. Fifteen-day-old endoreduplicating leaves.
Data represent average ± SD (n=2).

Inhibition of the cell cycle in mitotically dividing cells triggers the mitosis-to-endocycle transition

To confirm that a specific inhibition of the mitotic cyclin/CDKA;1 complexes accounts for the premature onset of endoreduplication, transgenic lines were generated that expressed the *KRP2* gene under the control of the promoter of the meristem-specific *SHOOTMERISTEMLESS* (*STM)* gene (Barton and Poethig, 1993; Long et al., 1998; Byrne et al., 2002). The expression pattern of the *STM* promoter was analyzed using transgenic plants harboring a *STM:YFP-histone H4* promoter construct. In 5-day-old seedlings, *STM* promoter activity was restricted to the shoot apical meristem (Figure 5A). At an earlier growth stage, additional expression was detected in the hypocotyl in the cells just underneath the shoot apical meristem, whereas in older leaf primordia *STM* activity could be detected as well in the young vascular cells of the leaf primordia (data not shown). At any stage during leaf development the *STM* promoter activity was restricted to mitotically dividing cells and not observed in endoreduplicating cells.

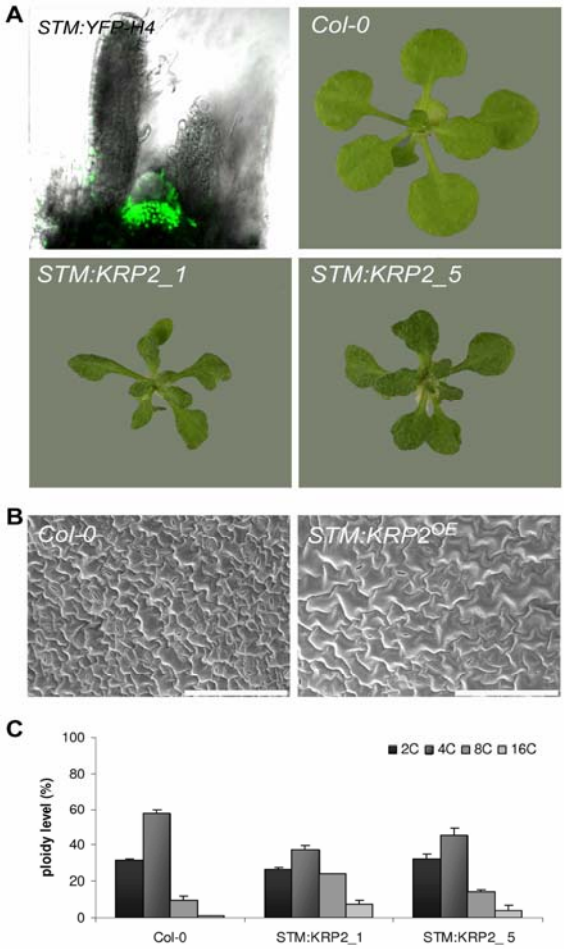
STM:KRP2^{OE} lines had malformed, elongated leaves that were smaller than those of wild-type plants (Figure 5A). Scanning electron microscopy pictures revealed that the decrease in leaf size was accompanied with an increase in cell size (Figure 5B). This reduction in the total leaf cell number is due to an inhibition of the mitotic cell cycle and is similar to that observed in plants overexpressing the *KRP2* gene under the control of the *CaMV 35S* promoter. However, in contrast to the strong *KRP2*^{OE} lines, plants expressing the *KRP2* gene under the control of the *STM* promoter displayed a different DNA ploidy distribution, as illustrated by the increase in number of cells with an 8C and 16C DNA content (Figure 5C).

Figure 5. Meristem-specific overexpression of *KRP2*.

A. Expression pattern of the *STM* promoter as visualized by confocal microscopy on 5-day-old *STM:YFP-histone H4* seedlings, and phenotypes of 3-week-old wild-type (Col-0) and two independent *STM:KRP2*^{OE} plants.

B. Scanning electron microscopy analysis of the adaxial epidermis of the first leaf pair of a 3-week-old wild-type (Col-0) and *STM:KRP2*^{OE} (line 5) plant. Scale bar 100 µM.

C. Ploidy level distribution of the first leaves of 3-week-old untransformed (Col-0) and *STM:KRP2*^{OE} plants as measured by flow cytometry. The indicated values are means ± SE (n = 3 to 5).



Previously, we have illustrated that the timing of endoreduplication cycle onset is developmentally regulated during leaf development (Boudolf et al., 2004a; Beemster et al., 2005; Vlieghe et al., 2005). Cells of the first leaf pair were found to divide until approximately 10 DAS during which most cells had a 2C DNA content and the remaining predominantly a 4C. After 10 DAS, the leaf cells exit the mitotic division program and enter the endoreduplication cycle, which was correlated with an

increase in the 4C DNA population. In the *STM:KRP2*^{OE} lines, the DNA ploidy distribution of the first leaves at 8 DAS was found to be identical to that of wild-type plants (Figure 6A). In contrast, at 10 DAS, the 4C DNA population was significantly higher in the transgenic lines (Figure 6B). Similarly, at 12 DAS, *STM:KRP2*^{OE} lines had an advanced endoreduplication cycle, as illustrated by the higher number of cells with an 8C DNA content than that observed in wild-type plants (Figure 6C). These data indicate that inhibition of CDKA;1 activity in mitotically dividing cells results into a more rapid entry into the endoreduplication cycle.

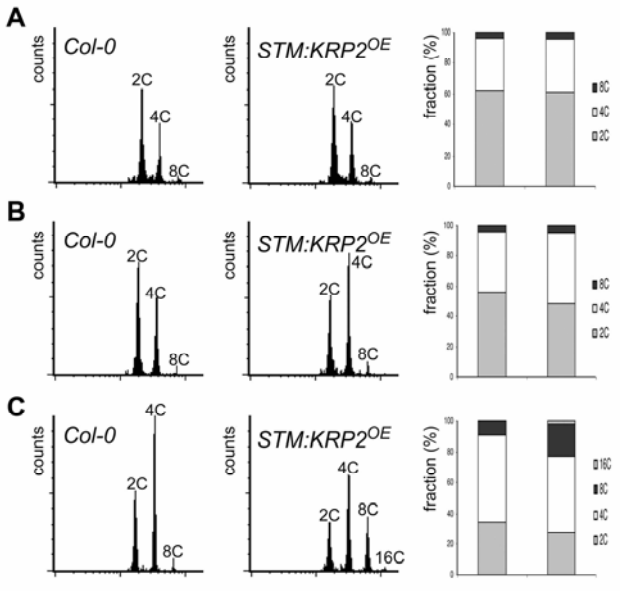


Figure 6. Ploidy level distribution of the first leaves of wild-type (Col-0) and *STM:KRP2*^{OE} (line 5) plants during development as measured by flow cytometry.
A. 8 DAS.
B. 10 DAS.
C. 12 DAS.
Histograms represent average data of two to four independent measurements.

KRP protein abundance is regulated at the posttranscriptional level during leaf development

To analyze the *KRP2* mRNA and protein levels during leaf development, the first leaf pair of untransformed plants was harvested at two developmental stages, representing mitotically dividing (9 DAS) or endoreduplicating (15 DAS) cells, for semiquantitative reverse trancription (RT)-PCR and protein blot analysis. CDKA;1 and CDKB1;1 were included as controls in the analysis. Both the CDKA;1 transcript level and protein abundance were relatively constant during development, whereas the mRNA and protein levels of the mitosis-specific CDKB1;1 were clearly most abundant at 9 DAS. In contrast, KRP2 protein levels had an inverse abundance pattern with low levels at 9 DAS and accumulation at 15 DAS. The corresponding *KRP2* transcript level remained relatively constant, illustrating that the KRP2 protein abundance is regulated at the posttranscriptional level (Figures 7A-7B).

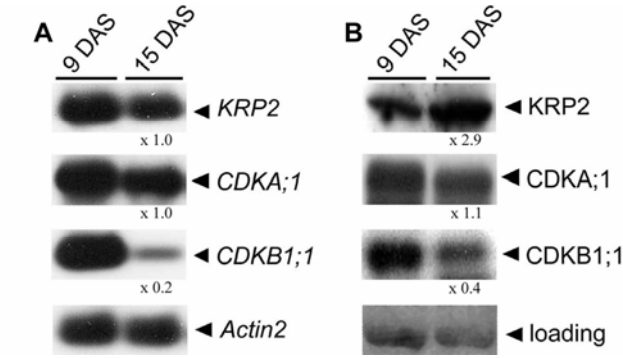


Figure 7. Expression analysis of *KRP2* during leaf development.
cDNA and total protein were prepared from the first leaf pair of wild-type (Col-0) plants at two developmental stages representing mitotically dividing (9 DAS) and endoreduplicating (15 DAS) cells.
A. Semiquantitative RT-PCR analysis with gene-specific primers. Transcript levels of *KRP2*, *CDKA;1,* and *CDKB1;1* were analyzed. The *actin2* gene was used as loading control.
B. Immunoblot analysis with anti-KRP2, anti-CDKA;1 and anti-CDKB1;1 antisera. Equal loading of the gel was confirmed by visualizing the Rubisco protein levels by Ponceau S staining of the membrane.
Results of relative quantification using the respective loading controls are indicated below each gel image.

KRP2 is subjected to proteolysis in a phosphorylation-dependent manner

Mammalian and yeast CDK inhibitors are phosphorylated by CDKs, after which they are recognized and degraded by the proteasome (Verma et al., 1997; Vlach et al., 1997; Nishizawa et al., 1998; Tomoda et al., 1999). To test whether a similar mechanism might be operational in plants, recombinant KRP2 protein was incubated with $p10^{CKS1At}$-purified CDK complexes in the presence of radioactively labeled ATP. As a control, histone H1 was included, a well-known *in vitro* substrate of CDKs. KRP2 was found to be phosphorylated by CDKs, albeit with less intensity than the histone H1 protein, possibly as a consequence of the presence of more potential CDK phosphorylation sites in histone H1 than in the KRP2 protein (Figure 8A). Incubation with CDKA;1- and CDKB1;1-immunoprecipitates illustrated that KRP2 is a substrate of both CDKs (Figure 8B).

To analyze whether KRP2 is regulated by proteasomal destruction, recombinant KRP2 protein was added to a protein extract of dividing cells in the absence or presence of the proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132; Planchais et al., 2000). Immediately after incubation, half of the fraction was collected and frozen. The remaining half was harvested 2 h later. Protein gel blotting showed a clear decrease in KRP2 abundance after 2 h of incubation with control extract, corresponding to protein degradation (Figure 8C). In contrast, protein abundance did not decrease in the MG132-containing extract, illustrating that the observed KRP2 destruction is mediated through the proteasome. Proteasome-dependent degradation was confirmed *in vivo*, by the increase in KRP2 protein abundance in plants treated with MG132 (Figure 8D).

Proteolysis of the KRP2 protein depended on CDK activity, because no KRP2 destruction was observed in extracts pre-incubated with olomoucine, a strong and specific inhibitor of CDK activity (Planchais et al., 2000; Figure 8E). Because B-type CDKs phosphorylated KRP2, the KRP2 protein abundance in the first leaf pair of 12-day-old seedlings was compared between wild-type plants and plants harboring reduced CDKB1;1 activity due to the presence of a dominant negative allele (Boudolf et al., 2004a, 2004b). The KRP2 protein level was clearly higher in plants with reduced CDKB1;1 activity, strongly indicating that KRP2 stability is controlled through CDKB1;1 phosphorylation (Figure 8F).

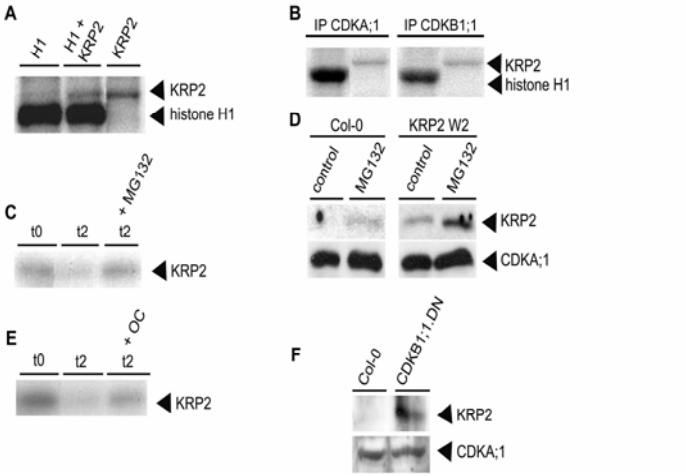


Figure 8. CDK-dependent phosphorylation and proteasome-mediated degradation of KRP2.
A. KRP2 phosphorylation by CDKs. $p10^{CKS1At}$ bound CDK activity recognizes both histone H1 and KRP2 as substrates.
B. Phosphorylation of KRP2 by CDKA;1 and CDKB1;1 complexes. CDKA;1 and CDKB1;1 immunoprecipitated kinase activity was tested with histone H1 and KRP2 as substrates.
C. Immunoblot analysis with anti-KRP2 antiserum of recombinant KRP2 protein added to protein extracts of dividing cell suspensions. KRP2 protein stability was monitored during a period of 2 h in the presence or absence of 100 µM MG132.
D. Immunoblot analysis with anti-KRP2 antiserum of *in vivo* levels of KRP2 protein in whole cell extracts of 5-day-old *Arabidopsis* seedlings of wild-type (Col-0) and weak $KRP2^{OE}$ plants (35S:KRP2 W2) grown in the absence (control) or presence of MG132. CDKA;1 protein levels determined with anti-CDKA;1 antiserum act as control.
E. Analysis of the stability of recombinant KRP2 protein in the presence or absence of 10 µM olomoucine. Samples were handled as described in (C).
F. Protein gel blot with anti-KRP2 and anti-CDKA;1 antisera to analyze the KRP2 and CDKA;1 protein abundance in 12-day-old first leaf pairs of wild-type (Col-0) and $CDKB1;1.N161^{OE}$ (*CDKB1;1.DN*) plants.

Discussion

Although our knowledge on how the different cell cycle transitions are regulated has

increased dramatically over the last years (De Veylder et al., 2003), it is still unclear how a dividing cell exits its division program and enters the differentiation pathway. A major cause for the absence of information on this important aspect of development is the lack of good differentiation markers. Recently, we have demonstrated by a kinematic growth analysis that the exit of the mitotic cell cycle of *Arabidopsis* leaf cells coincides with the onset of endoreduplication (Boudolf et al., 2004a; Beemster et al., 2005; Vlieghe et al., 2005). As such, insight into how the mitosis-to-endocycle transition is regulated could help us understand how a proliferating cell exits the cell cycle and starts to differentiate.

Here, we showed that KRP2 gain-of-function plants display a positive or negative effect on the DNA ploidy level, depending on the level of *KRP2* overexpression. The inhibition of the endocycle in strong *KRP2*^{OE} plants is in agreement with previous reported *KRP*^{OE} studies (De Veylder et al., 2001; Jasinski et al., 2001; Zhou et al., 2002; Schnittger et al., 2003), but is apparently in contradiction with the observed stimulation of the endoreduplication cycle in weak *KRP2*^{OE} plants. However, all previously published analyses were focused on the strongest expression lines, in which the amount of transgenic KRP protein exceeds far above the endogenous level. KRP2 protein abundance in wild-type plants is very low (unpublished results). Although the different spatial expression patterns of the *CaMV 35S* and the endogenous *KRP2* promoters do not allow us to quantify the level of *KRP2* overexpression at the cellular level in the weak *KRP2*^{OE} plants, we may reasonably assume that the level of KRP2 in the weakest *KRP2*^{OE} lines is closer to its physiological level than in strong *KRP2*^{OE} plants. Even though both the strong and weak *KRP2*^{OE} lines specifically target CDKA;1, the phenotypes observed for the weak *KRP2*^{OE} lines might relate more to the natural situation. We hypothesize that under these close-to-natural situations KRP2 preferentially targets mitotic cell cycle-specific cyclin/CDKA;1 complexes. By contrast, in the strong *KRP2*^{OE} lines, cyclin/CDKA;1 complexes with a role in the endoreduplication cycle might be inhibited as well. KRP2 specificity toward mitotic cell cycle complexes was confirmed by kinase activity measurements, demonstrating that in the weak *KRP2*^{OE} plants the CDKA;1 complexes purified from dividing leaf cells were much more inhibited than those isolated from endoreduplicating tissues. In contrast, in the strong *KRP2*^{OE} lines, both mitotic cell cycle and endoreduplication cycle CDKA;1 complexes were inhibited to the same degree.

In mammals, the Kip/Cip inhibitors only bind and inhibit a subset of the CDK/cyclin complexes. A similar situation probably exists in plants because KRP2 does not bind and inhibit all CDKA;1 proteins in *KRP2*^{OE} plants. Although the three-dimensional structure of p27^{Kip1} in complex with CDK2/cyclinA revealed that Kip/Cip binding

involved interaction with both the CDK and cyclin subunit, the binding specificity of the inhibitors has only recently been proven to rely solely on their interaction with the cyclin (Russo et al., 1996; Lacy et al., 2004). *In vivo* binding specificity between KRPs and different D-type cyclins has been demonstrated by Schnittger et al. (2003), who showed that the trichome endoreduplication phenotype resulting from *KRP1* overexpression can be complemented by overexpression of *CYCD3;1*, but not of *CYCD4;1*. In mammalian trophoblasts, the transition of mitotic cell division to endoreduplication is accompanied by a switch of D-type cyclin isoform expression (MacAuley et al., 1998). Expression of *CYCD3* was high in proliferating cells, but decreased significantly with the onset of differentiation, whereas expression of *CYCD1* was only low in proliferating cells, but was induced at the start of endoreduplication correlated with differentiation. Similarly, CDKA;1 might change cyclin partner at the mitosis-to-endocycle transition, resulting in the shift from a KRP-sensitive to a KRP-insensitive CDKA;1/cyclin complex.

Endoreduplicating leaf tissue was found to have less CDKA;1 activity than mitotically dividing leaves, illustrating that the onset of endoreduplication is correlated with a decrease in CDK activity. Also in maize endosperm and tomato fruits, the onset of endoreduplication is accompanied with a decrease in CDK activity (Grafi and Larkins, 1995; Joubès et al., 1999). Start of DNA replication requires the assembly of a protein complex at the origins of replication, called the pre-replication complex (Bryant et al., 2001; Nishitani and Lygerou, 2002). At the onset of S phase, pre-replication complexes are activated, resulting in DNA replication. For a number of species, high CDK activity has been demonstrated to ensure that chromosomes are only replicated once per cell cycle by inhibiting reactivation of the replication origins in G2 cells. Only when CDK activity drops at the end of S phase, cells are reset for replication, resulting in endopolyploidy (Hayles et al., 1994; Moreno and Nurse, 1994; Itzhaki et al., 1997). The underlying principle of activation of DNA replication is conserved in plants (Castellano et al., 2001, 2004; Masuda et al., 2004). As such, it is reasonable to assume that also in plants a reduction in CDK kinase activity is required for cells to endoreduplicate. The observation that CDKA;1 activity is not totally inhibited in endoreduplicating cells suggests an active role for this CDK in the endoreduplication cycle. Such a role has recently been elegantly demonstrated by the specific overexpression of a dominant negative allele of the maize A-type CDK in endosperm cells, resulting in inhibition of endoreduplication, correlated with reduction in CDKA;1 activity (Leiva-Neto et al., 2004).

Specific inhibition of the mitotic cell cycle CDKA;1 complexes, through overexpression of the *KRP2* gene in the meristem, causes a premature start of the endoreduplication cycle. Therefore, we propose a model in which the level of CDK

activity determines whether a cell divides mitotically or endoreduplicates (Figure 9). We assume that CDK activity gradually decreases as cells move away from the meristem. As long as CDK activity is above a certain threshold, cells divide, but once kinase activity drops below the threshold, cells stop proliferating and start to endoreduplicate. The overproduction of KRP2 probably results in an overall reduction of CDKA;1 activity, by which plants reach the endoreduplication threshold earlier during development. This model is a confirmation and refinement of that previously proposed by Yamaguchi et al. (2003), who proposed that the CDK activity level at early organogenesis controls the differentiation state of leaf callus cells and as such that CDK activity is a major determinant of cell differentiation to accomplish proper organ development. What causes CDKA;1 activity to decrease as cells move away from the meristem? Our data suggest a prominent role for the KRP2 proteins, because KRP2 protein levels increase in differentiating cells. Also in mammals, Kip/Cip inhibitors are up-regulated as cells exit the mitotic cycle and begin to differentiate (Polyak et al., 1994; Parker et al., 1995). In $p27^{Kip1}$- and $p57^{Kip2}$-deficient mice abnormalities in cell number and organ size were observed and many tissues had a delayed cell differentiation and an inappropriate continuation of cell proliferation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Yan et al., 1997; Durand et al., 1998). However, in all cases, cells eventually differentiated, suggesting that CDK inhibitors are not critical, but rather are involved in the correct timing of cell cycle exit. This result has also been demonstrated by studies on the function of the Drosophila Kip/Cip homolog, Dacapo (de Nooij et al., 1996; Lane et al., 1996).

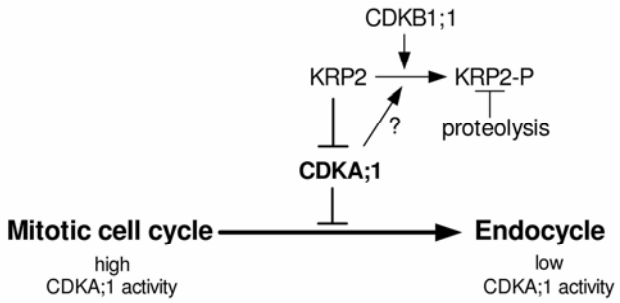


Figure 9. Model illustrating the role of CDK activity in controlling the onset of endoreduplication. For details, see text.

KRP2 expression is at least partially regulated at the posttranscriptional level, through its phosphorylation by A- and B-type CDKs. In mammals, the phosphorylation of Kip/Cip inhibitors results in their destruction through the ubiquitination pathway (Vlach et al., 1997; Tomoda et al., 1999). Similarly, the yeast Sic1/Rum1 inhibitors are substrates of Sic1/Rum1-resistant CDKs, with consequent ubiquitin-dependent degradation (Verma et al., 1997; Nishizawa et al., 1998). We have demonstrated that KRP2 is an unstable protein and that its degradation depends on CDK phosphorylation and the presence of an active proteasome. Interestingly, *KRP2* was found to be an efficient CDKB1;1 substrate and to be stabilized in plants overexpressing a dominant negative *CDKB1;1*. Previously, we showed that CDKB1;1 activity plays an important role in determining whether cells divide or endoreduplicate, because overexpression of a dominant negative *CDKB1;1* caused cells to enter the endoreduplication cycle prematurely (Boudolf et al., 2004a). It is tempting to speculate that CDKB1;1 controls the level of CDKA;1 activity through the phosphorylation of KRPs. In such a model, CDKB1;1 activity in dividing cells would prevent CDKA;1 inhibition through the phosphorylation and destruction of KRPs. However, when cells enter the endoreduplication cycle they lose CDKB1;1 activity, resulting in increased KRP stability and inhibition of CDKA;1 activity. This hypothesis implies that the factors that regulate the mitosis-to-endocycle transition through inactivation of CDKB1;1 activity, simultaneously control the amount of CDKA;1 activity (Figure 9). Such a coordinated decrease in activity of both types of CDKs might be essential to warrant a correct timing of mitotic cell cycle exit.

To validate that the model presented in Figure 9 is sufficient to control the timing of the mitosis-to-endocycle transition, we performed a number of dynamical simulations using the SIM-plex toolpack (Vercruysse and Kuiper, 2005). This software uses the PLDE mathematical framework (De Jong et al., 2004), an approach that approximates regulatory interactions between proteins as operating in a switch-like manner, with activation events depending on concentrations reaching a critical threshold (Glass and Kauffman, 1973). The activity of the CDKB1;1 complex was predefined as peaking at 4 h in a complete cell cycle of 20 h, as determined by Menges and Murray (2002). The KRP2 protein was assumed to be continuously produced during the cell cycle and to be degraded when CDKB1;1 activity exceeded a critical threshold. When KRP2 reached a specific threshold value, it inhibited CDKA;1 activity. In wild-type cells the KRP2 protein level continuously increased during a cell division cycle, but was repressed by the peak of CDKB1;1 activity. As a result, CDKA;1 activity oscillated during the cell cycle, but remained high enough to enable mitotic divisions (Figure 10A). Only when CDKB1;1 activity dropped, KRP2 protein accumulated above a level high enough to inhibit mitotic

CDKA;1 activity. Simulating a faster decrease in CDKB1;1 activity yielded a pattern with a more rapid increase in KRP2 protein levels, and, as such, a faster entry into the endoreduplication cycle (Figure 10B). This result supports the observation that overexpression of a dominant negative CDKB1;1 allele results in a premature onset of the endoreduplication cycle (Boudolf et al., 2004a). Similarly, simulating a scenario with an increased net synthesis of KRP2 results in an earlier mitosis-to-endocycle transition, due to a faster decrease in CDKA;1 activity (Figure 10C). All simulation scenarios can be viewed and tested at http://www.psb.ugent.be/cbd/krp2sim. The capacity to mimic true *in vivo* situations through dynamical modeling allows us to hypothesize that the interactions as presented in Figure 9 are an important part of the core mechanism controlling the mitosis-to-endocycle transition during *Arabidopsis* leaf development.

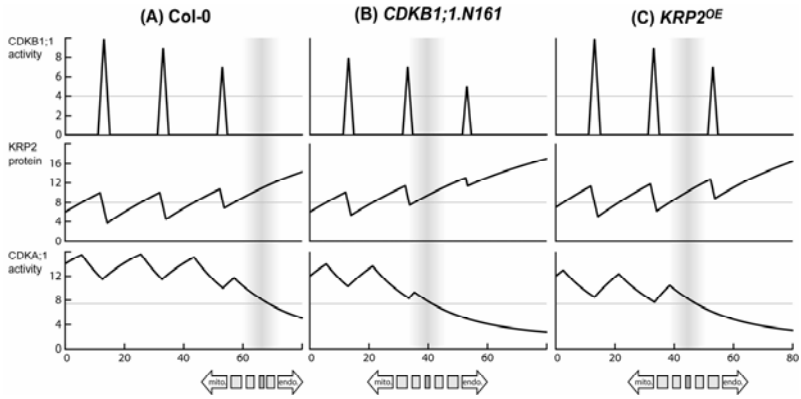


Figure 10. Simulation of the onset of endoreduplication.
Simulation of KRP2 protein abundance and CDKA;1 activity as based on predefined CDKB1;1 activity. Activities were simulated in arbitrary units. CDKB1;1 initiates KRP2 phosphorylation when it reaches the activity threshold of 4 units. KRP2 inhibits CDKA;1 activity above its threshold 8. CDKA;1 activity first remains above a level that is assumed to be necessary to maintain mitotic division. In the grey-colored zone, CDKA;1 activity drops below this critical level, marking the onset of the endoreduplication cycle. Note the remaining CDKA;1 activity at this stage to keep the endoreduplication cycle running.

Methods

Regeneration and molecular analysis of transgenic lines and growth conditions

The coding region of KRP2 was amplified by polymerase chain reaction (PCR) and cloned in the pREP42-HA vector (Craven et al., 1998). A HA-tagged version of KRP2 was obtained by NcoI and BamHI digestion of the pREP42-HA_N.KRP2 construct. Subsequently, the restriction fragment was cloned between the *CaMV 35S* promoter and the nopaline synthase (*NOS*) 3' untranslated region of PH35S (Hemerly et al., 1995). The *CaMV35S/HA.KRP2/NOS* cassette was released of the pH35S.HA.KRP2 constructs and cloned into the *EcoRI* and *SalI* sites of pBinPLUS (van Engelen et al., 1995) to obtain the pBIN.HA.KRP2 vector.

The *STM* promoter was amplified from the full bacterial artificial chromosome clone, BAC F2401, as a 4.5-kb fragment. A single ATG site was generated at its 3' end, creating a NcoI restriction site through PCR amplification. The KRP2 insert was obtained as described previously (De Veylder et al., 2001). The enhancer trap binary vector pTer-9 was a kind gift from Prof. I. Negrutiu (Lyon, France). pSTM was cloned into pTer-9 at the KpnI/BamHI sites to verify the tissue specificity of this promoter. For the transcriptional fusions, pSTM was first cloned into the pBinPLUS vector. Thereafter, the cassette containing KRP2 together with a 3´NOS terminator was introduced behind pSTM.

Both pBIN.HA.KRP2 and pSTM.KRP2 were mobilized by the helper plasmid pRK2013 into the *Agrobacterium tumefaciens* C58C1Rif^R harboring the plasmid pMP90 (Koncz and Schell, 1986). *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia-0 (Col-0) was transformed by the floral dip method (Clough and Bent, 1998).

Transgenic plants were selected on kanamycin-containing medium. For all analyses, plants were grown under a 16-h light/8-h dark photoperiod at 22°C on germination medium (Valvekens et al., 1988). Molecular analysis of the obtained transformants was performed by RNA gel blot analysis and semiquantitative RT-PCR as described before (De Veylder et al., 1999; Boudolf et al., 2004a). Information about the primer set sequences used for RT-PCR can be given upon request. Plants harboring the mutant *CDKB1;1.N161* gene under the control of the *CaMV 35S* promoter (Boudolf et al., 2004b) were constructed as described previously.

Microscopy and flow cytometric analysis

Leaves were harvested 21 DAS, cleared overnight in ethanol, stored in lactic acid for microscopy, and observed under a microscope fitted with differential interference contrast optics (Leica, Wetzlar, Germany). The total (leaf) area was determined from pictures digitized directly with a digital camera (Axiocam; Zeiss, Jena, Germany), mounted on a binocular (Stemi SV11; Zeiss). From scanned drawing-tube images of outlines of at least 30 cells of the abaxial epidermis located 25% and 75% from the distance between the tip and the base of the leaf, halfway between the midrib and the leaf margin, the following parameters were determined: total area of all cells in the drawing and total numbers of pavement and guard cells, from which the average cell area was calculated and the total number of cells per leaf estimated by dividing the leaf area by the average cell area. Confocal microscopy was performed on 2-day-old seedlings as described by Autran et al. (2002), and scanning electron microscopy samples were prepared as described by Traas et al. (1995).
For flow cytometric analysis, leaves were chopped with a razor blade in 300 µl of 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7), and 1% Triton X-100 (Galbraith et al., 1991). From a stock of 1 mg/ml 4,6-diamidino-2-phenylindole, 1 µl was added to the filtered supernatants. The nuclei were analyzed with the BRYTE HS or CyFlow flow cytometer with Win-Bryte (Bio-Rad, Hercules, CA) or FloMax (Partec, Münster, Germany) software, respectively.

Semi-quantitative RT-mediated PCR analysis

RNA was extracted from leaves of *Arabidopsis* (Col-0) with TriZol reagent (Amersham Biosciences, Little Chalfont, UK). First-strand cDNA synthesis was performed on 3 µg of total RNA with the Superscript RT II kit (Invitrogen, Carlsbad, CA) and oligo(dT)18 according to the manufacturer's instructions. A 1-µl aliquot of the total RT reaction volume (20 µl) was used as a template in semiquantitative RT-mediated PCR analysis, ensuring that the amount of amplified product remained in linear proportion to the initial template present in the reaction. Ten microliters from the PCR reaction was separated on a 0.8% agarose gel and transferred onto Hybond N+ membranes (Amersham Biosciences). The membranes were hybridized at 65°C with fluorescein-labeled probes (Gene Images random prime module; Amersham Biosciences). The hybridized bands were detected with the CDP Star detection module (Amersham Biosciences). Primers used were GGCTCCTCTTAACCCAAAGGC and CACACCATCACCAGAATCCAGC for *actin2*

(At3g18780); CGGAATAAGTTGTTGGAATGTTCTATGAAGTGT and GGCGGATCCTCATGGATTCAATTTAACCC for *KRP2* (At3g50630); CCTAGGATCTCATCATTACTCTACACC and CCATGTATCCTCGTACGGAGTTCC for *CDKA;1* (At3g48750); and GGTGGTGACATGTGGTCTGTTGG and CGCAGTGTGGAAACACCCGG for *CDKB1;1* (At3g54180).

Preparation of recombinant KRP2 protein and KRP2 antibody, *in vitro* KRP2 binding assay, immunoprecipitations, and immunoblotting

For *KRP2* expression and purification, a His-tagged fusion protein was generated. The KRP2 coding region was PCR amplified and cloned into the GATEWAY pDONR207 vector (Invitrogen). After recombination with pDEST17, the obtained expression vector pDEST17.KRP2 was transformed in the *Escherichia coli* BL21-CodonPlus™(DE3)-RIL strain (Novagen, Madison, WI). *E. coli* cells were grown to an A600 nm of 0.5-0.7 at 37°C in LB+ medium and the expression of *KRP2* was induced by addition of 0.2 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C. The cells were lyzed with lysozyme in buffer 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 2 mM imidazole, 0.5% Triton X-100, 0.5 mM DTT, and complete protease inhibitor without EDTA (Roche, Diagnostics, Brussels, Belgium). The protein was purified on Ni-NTA resin (Qiagen, Hilden, Germany) according to the manufacturers instructions and dialyzed against buffer 50 mM Tris–Cl, pH 7.5, 15 mM MgCl2, 5 mM EGTA, and 1 mM DTT.

To raise a KRP2-specific antibody, a peptide of 20 amino acids located in the C-terminal domain of the KRP2 protein [(C)-FEKDEPLGGG RYEWVKLNP, with C indicating an extra cysteine] was synthesized, linked to keyhole limpet hemocyanin carrier protein, and used to immunize rabbits. The antiserum was immuno-affinity purified against the same peptide bound to a Sepharose matrix (Amersham Biosciences). CDKA;1 and CDKB1;1 specific antisera were described before (Hemerly et al., 1995; Porceddu et al., 2001).

Purified recombinant KRP2 protein or BSA (Sigma-Aldrich, St. Louis, MO) was coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences) at a concentration of 5 mg/ml according to the manufacturer's instructions. Two-day-old MM1 cell suspension culture extracts (900 µg) (Menges and Murray, 2002) in a total volume of 200 µl homogenization buffer was loaded onto 50 µl of 50% (v/v) KRP2- or BSA-Sepharose and incubated on a rotating wheel for 2 h at 4°C. The unbound proteins were collected and the bead-bound fractions were washed three

times with bead buffer. The beads were resuspended in 20 µl of SDS loading buffer and boiled.
For immunoprecipitations, 300 µg of total protein in homogenization buffer (25 mM Tris-Cl, pH 7.6, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM p-nitrophenylphosphate, 60 mM β-glycerophosphate, 1 mM DTT, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail P9599 (Sigma-Aldrich) were precleared with 30 µl of 50% (v/v) protein A-sepharose beads (Amersham Biosciences) for 1 h at 4°C or immediately incubated with 30 µl of 50% (v/v) anti-HA Affinity Matrix (Roche Diagnostics). After a short centrifugation, the precleared supernatants were transferred to new Eppendorf tubes containing CDKA;1 (1/250) or CDKB1;1 (1/100) antibodies and incubated at 4°C for 2 h. In the following step, 30 µl of 50% (v/v) protein A-Sepharose was added, and the tubes were incubated for 1 h at 4°C on a rotating wheel. Thereafter, beads were washed three times with RIPA buffer (20 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 300 µM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin and pepstatin), and used for CDK activity reactions or protein gel blot analysis.
Proteins were separated by 12% SDS-PAGE and blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Filters were blocked in 3% (v/v) milk powder in 25 mM Tris-Cl (pH 8), 150 mM NaCl, 0.05% Tween 20 for at least 1 h at room temperature and incubated overnight at 4°C with CDKA;1 (1/5000), CDKB1;1 (1/1000), KRP2 (1/1000), or HA (1/1000) (Roche Diagnostics) antibody in blocking buffer. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1/10000 (Amersham Biosciences) with a chemiluminescence system (Perkin Elmer, Norwalk, CT).

Protein extraction, CDK activity and phosphorylation assays, and degradation assays

Arabidopsis plants or tissues and 2-day-old MM1 cell suspension cultures (Menges and Murray, 2002) were harvested, used immediately or snap-frozen in liquid nitrogen, and stored at –70°C. Proteins were extracted by grinding cells with quartz sand in homogenization buffer. The protein content was determined by using the Bio-Rad protein assay kit.
Equal amounts of total protein were incubated with $p10^{CKS1At}$- or BSA-Sepharose beads (De Veylder et al., 1997) or used for immunoprecipitations. Kinase assays were performed as described by De Veylder et al. (1997) with histone H1 and/or recombinant KRP2 as CDK substrates.

For the *in vitro* degradation assay, mixtures contained 100 µg of *Arabidopsis* cell suspension culture MM1 protein extract (20 mM HEPES, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$) supplemented with an ATP-regenerating system (35 mM phosphocreatine, 50 µg/ml creatine kinase), 1 mM ATP, 1/15 (v/v) wheat germ lysate (Promega, Madison, WI), and 50 ng substrate. The reaction mixtures were incubated at 30°C for 2 h and the reactions were stopped by the addition of SDS sample buffer. Either 10 µM olomoucine (Alexis, San Diego, CA) or 100 µM carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) (Affiniti Research, Exeter, UK) were preincubated with the extract for 15 min at room temperature before the addition of the substrate. *In vivo* degradation was assayed by MG132 treatment (100 µM) of 5-day-old seedlings for 12 h. All control samples were treated with dimethyl sulfoxide.

Network simulations

For the dynamical simulations we used the SIM-plex software (Vercruysse and Kuiper, 2005) that allows the specification of a regulatory network in a series of 'if-then' statements. Network definitions can be found at http://www.psb.ugent.be/cbd/krp2sim. For wild-type conditions, the first program line predefines a fixed CDKB1;1 activity profile. Peak duration is approximately one-fifth of the duration of a full cell cycle according to Menges and Murray (2002). The next statements define the different components of the model. Initial KRP2 protein level and CDKA;1 activity were set so that, from the onset, the simulation immediately displayed a robust oscillating profile that assumed the occurrence of cyclical state transitions prior to the start of the simulation, as expected during normal mitotic division. KRP2 degradation (0.02) was set below that of the standard degradation in SIM-plex (0.05) to emphasize the active effect of CDK phosphorylation. The "time points" statement primed SIM-plex to simulate the time required for four normal mitotic divisions (4 x 20 h). The two "if-true-then" statements ensured constant creation of KRP2 and CDKA;1 proteins, by a cause not further specified. Natural degradation eventually puts a saturation effect on protein creation. The final two statements modeled the phosphorylation of KRP2 under the influence of CDKB1;1, and the deactivation of CDKA;1 complexes by KRP2. Thresholds and creation rates were chosen so that simulated profiles matched reality most closely, but taking into account all known data on transcript and protein concentrations during the cell cycle. The network definition of the dominant negative *CDKB1;1* allele was changed from the wild-type settings in the CDKB1;1 definition and also in the specification of the initial level of CDKA;1 (12

instead of 14, because of the higher KRP2 activity prior to time = 0). The network definition of the *KRP2*-overexpressing line differed from the wild-type settings in the initial level of KRP2 protein (7 instead of 6), the constant creation rate of KRP2 (0.55 instead of 0.5), and the initial level of CDKA;1 activity (12 instead of 14). The simulation results were exported into Adobe Illustrator for optimal presentation. Simulation conditions can be altered at http://www.psb.ugent.be/cbd/krp2sim.

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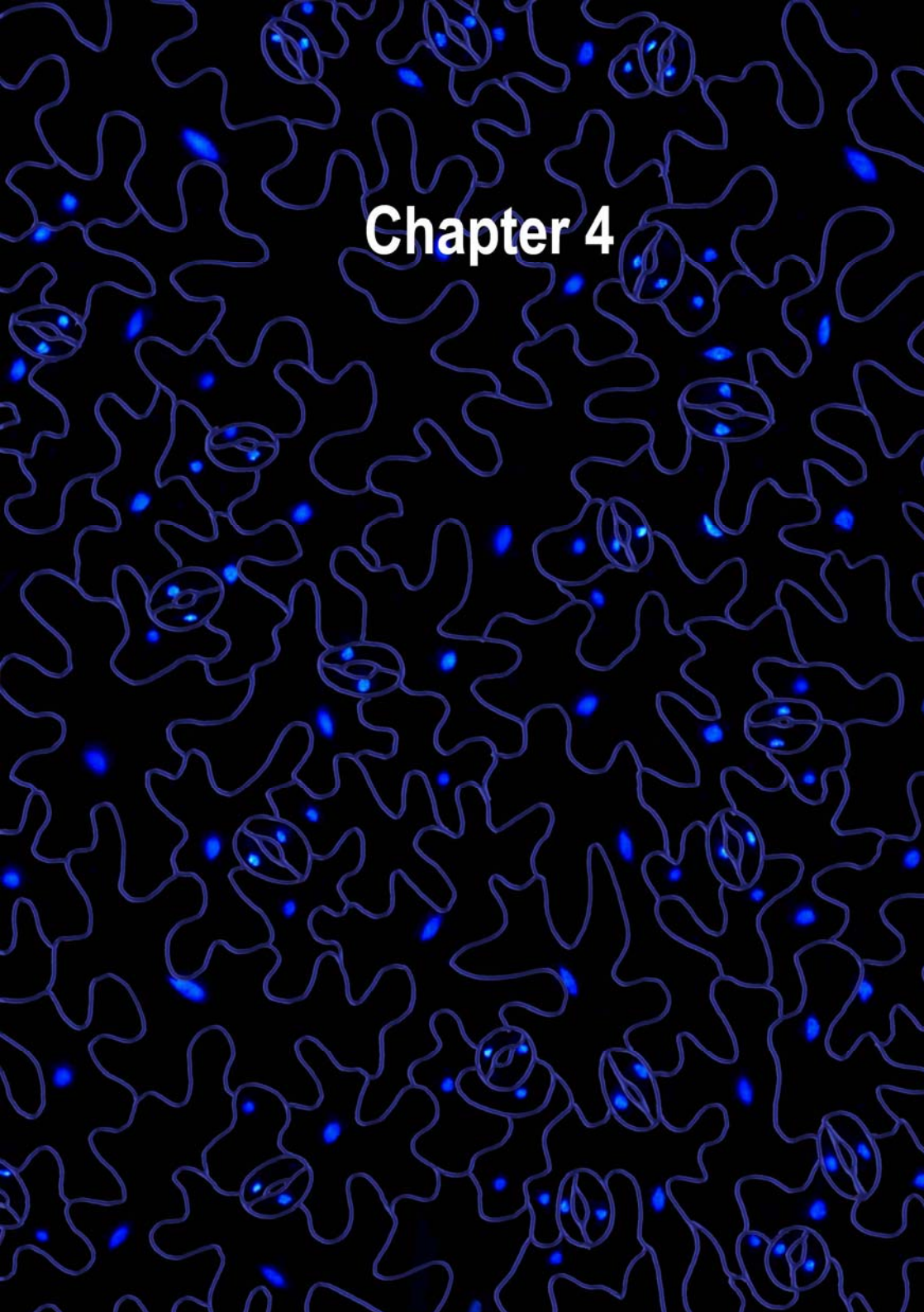
Chapter 4

Chapter page: Abaxial epidermal peel of the first leaf of a wild-type *Arabidopsis* plant stained with 4,6-diamino-2-phenylindole (DAPI).

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Cyclin-dependent kinase inhibitor regulation through CDK kinase phosphorylation and CDK subunit CKS1

Aurine Verkest, Dirk Inzé, Lieven De Veylder

Abstract

Selective ubiquitin-mediated protein degradation controls the availability, and therefore the activity, of several cell cycle proteins. Previously, we showed that the *Arabidopsis* CDK inhibitor KRP2 is regulated through proteasomal degradation and CDK phosphorylation. In yeast and mammalia, Suc1/Cks1 proteins play distinct roles in cell cycle regulated proteolysis. Here, we show that *Arabidopsis* CKS1 regulates KRP2 inhibitory activity. Overexpression of *CKS1* resulted in plants with reduced KRP protein levels due to posttranslational regulation. We illustrate that KRP2 phosphorylation by CDKs enhances its degradation and results in loss of its binding and inhibitory specificity towards CDKA;1 kinases. Interestingly, mitotic CDKB1;1/cyclin complexes do not merely phosphorylate KRP2, marking it for destruction, but also only bind phosphorylated KRP2. In agreement with a role for CKS1 in KRP2 proteolysis, CKS1 enhanced the CDKB1;1-dependent phosphorylation of KRP2. In addition, by competing for CDKA;1 binding, CKS1 counteracts KRP2-mediated CDKA;1 inhibition. We postulate that the combination of these functions of CKS1 triggers KRP2 degradation by a yet to be identified ubiquitin-ligase.

Manuscript in preparation

Introduction

Cyclin-dependent kinases (CDKs) regulate progression through the cell cycle in all eukaryotes. These Ser/Thr kinases are composed of a catalytic CDK subunit and a regulatory cyclin subunit. A third partner often found in complex with CDK/cyclins is a small protein known as the Cdc kinase subunit (Cks). Suc1/Cks1 proteins were originally identified through their ability to genetically suppress defective alleles of the CDKs of both fission and budding yeast (Hayles et al. 1986; Hadwiger et al. 1989; Reed et al. 1989). Subsequent investigations demonstrated that these small proteins are ubiquitously present in eukaryotes and bind directly to CDK/cyclin complexes. Although the precise functions of the Cks proteins have been elusive, they were shown to be essential for viability in both fission and budding yeast (Pines, 1996).

Most studies point to a mitotic role for Cks proteins, although other cell cycle functions have been suggested (Tang and Reed 1993; Reynard et al., 2000). Loss of Suc1/Cks1 function in both fission yeast (*S. pombe*) and *S. cerevisiae* results in a M phase arrest, correlated with elevated levels of CDK/cyclinB kinase activity (Moreno et al., 1989; Tang and Reed, 1993). In *Xenopus*, immunodepletion of the Cks homolog Xe-p9 from interphase egg extracts prevents entry into mitosis, whereas its immunodepletion from mitotic extracts leads to a M phase arrest with elevated levels of cyclinB and CDK1/cyclinB kinase activity (Patra and Dunphy, 1996). Taken together, these results suggest that the Cks proteins may be required both for entry into and progression through mitosis. It has been demonstrated that Xe-p9 enhances *in vitro* phosphorylation of various mitotic CDK substrates, including Cdc25 and Wee1, consistent with its observed role in mitotic entry (Patra et al., 1999). Precisely how Cks1 facilitates phosphorylation of CDK substrates is not known, but the presence of a phosphate-binding surface on Cks1 suggests that the targets of Cks1 are prephosphorylated (Avrai et al., 1995; Bourne et al., 1996). Consistently, Suc1 was shown to bind a phosphorylated Cdc25 peptide and suggested to stimulate Cdc25 phosphorylation by CDK/cyclin complexes (Landrieu et al., 2001).

Cks was shown to have also a G_1 function in budding yeast, as certain Suc1 mutations lead to a cell cycle arrest in G1 due to a defect in G1 CDK activity towards important substrates such as the CDK inhibitors Sic1 and Far1 (Tang and Reed, 1993; Reynard et al., 2000). Phosphorylation of Sic1 and Far1 by G1 CDKs is required for their degradation and entry into S phase (Henchoz et al., 1997; Verma et al., 1997; Nishizawa et al., 1998). Similarly, CDK

phosphorylation of mammalian Kip/Cip CDK inhibitors results in their destruction through the ubiquitination pathway (Sheaff et al., 1997; Vlach et al., 1997). One of the two mammalian Cks proteins, Cks1, was shown to facilitate the ubiquitin-mediated proteolysis of the Kip/Cip CDK inhibitors, revealing its role in controlling the G0–G1 transition (Ganoth et al., 2001; Spruck et al., 2001; Bornstein et al., 2003; Kamura et al., 2003). Interestingly, the mechanism of mammalian Cks1-assisted Kip/Cip degradation is different than in yeast. Whereas in yeast Suc1 accelerates the phosphorylation of CDK inhibitors, marking them for destruction, mammalian Cks1 triggers Kip/Cip degradation by enhancing the interaction between phosphorylated $p27^{Kip1}$ and the SCF^{Skp2} ubiquitin ligase (Ganoth et al., 2001; Spruck et al., 2001). Moreover, Cks1 was recently shown to negatively regulate Skp2 auto-ubiquitination and proteolysis (Wang et al., 2004).

Whereas lower eukaryotes express only one Cks gene, *Arabidopsis thaliana*, like mammals, expresses two orthologs, Arath;*CKS1* and Arath;*CKS2* (Richardson et al., 1990; Vandepoele et al., 2002). In plants, biochemical and genetic data concerning CKS function is scarce. *Arabidopsis* CKS1 was shown to bind CDKs and is sufficiently conserved at the structural and functional level that it can functionally substitute for Cks1 in yeast (De Veylder et al., 1997; Boudolf et al., 2001). Overexpression of Arath;*CKS1* in *Arabidopsis* caused a prolongation of cell cycle duration and strongly reduced meristem size (De Veylder et al., 2001a). To further characterize the function of Arath;*CKS1* we investigated its role in KRP2 CDK inhibitor regulation. The *Arabidopsis* genome encodes seven CDK inhibitor genes, designated *KRPs* (Kip related protein) because of their homology with the mammalian Kip/Cip CDK inhibitors (De Veylder et al., 2001b; Vandepoele et al., 2002). KRPs have been shown to be true functional orthologs of the Kip/Cip proteins in binding and inhibiting CDK activity (reviewed in Verkest et al., 2005b). Recently, Arath;KRP1 and Arath;KRP2 have been demonstrated to participate in the exit of mitosis and the onset of endoreduplication (Verkest et al., 2005a; Weinl et al., 2005). Interestingly, Arath;KRP2 was shown to be posttranslationally regulated through CDK phosphorylation and proteasomal degradation (Verkest et al., 2005a). Here, we demonstrate that CDK phosphorylation of KRP2 interferes with its association and inhibition of CDKA;1 complexes and accelerates its degradation. Moreover, we reveal that CKS1 negatively regulates KRP2 activity by competing for binding of CDKA;1, and by enhancing CDKB1;1-mediated KRP2 phosphorylation, marking KRP2 for its degradation.

Results

KRP2 phosphorylation by CDKs alters its CDK affinity and decreases its stability

It was shown recently that the *Arabidopsis* CDK inhibitor KRP2 is subjected to CDK-dependent phosphorylation (Verkest et al., 2005a). Intriguingly, both A- and B-type CDKs phosphorylate KRP2, even though KRP2 was shown to bind exclusively CDKA;1 in yeast two-hybrid, and in *in vitro* and *in vivo* interaction assays (Lui et al., 2000; De Veylder et al., 2001b; Verkest et al., 2005a). To analyze the effect of CDK-mediated KRP2 phosphorylation upon CDK binding-specificity, CDKA;1 and CDKB1;1 protein complexes were immunoprecipitated using specific antibodies from protein extracts of dividing cells preincubated with recombinant KRP2 or CDK phosphorylated KRP2 (Hemerly et al., 1995; Porceddu et al., 2001). Subsequently, the bound fractions were analyzed for the presence of KRP2 by autoradiography or protein blot analysis. As expected, non-phosphorylated KRP2 specifically bound CDKA;1 but not CDKB1;1 complexes (Figure 1A). In contrast, phosphorylated KRP2 changed its binding specificity and interacted exclusively with CDKB1;1 complexes (Figure 1A).

KRP2 has been verified to inactivate CDK activity *in vitro* and *in vivo* due to a specific inhibition of the CDKA;1 kinase (Lui et al., 2000, De Veylder et al., 2001b; Verkest et al., 2005a). To test the consequence of its phosphorylation on CDK activity, the effect of recombinant KRP2 and phospho-KRP2 on immunoprecipitated CDKA;1 and CDKB1;1 kinase activity was analyzed. Whereas non-phosphorylated KRP2 inhibited CDKA;1 activity, its phosphorylated isoform had no inhibitory effect, most probably as a result of its inability to bind CDKA;1/cyclin complexes (Figure 1B). Curiously, phosphorylated KRP2, as its nonphosphorylated form, did not inhibit but instead activated CDKB1;1 kinase activity (Figure 1B).

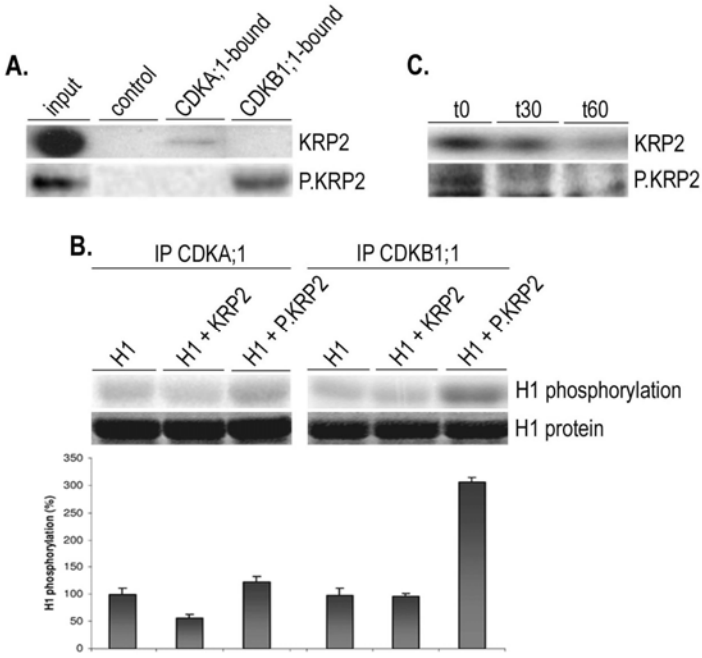

Figure 1. Analysis of the effect of KRP2 phosphorylation by CDKs.
A. *In vitro* CDK binding-specificity of KRP2 and phosphorylated KRP2 (P.KRP2) towards CDKA;1 and CDKB1;1 kinases.
B. Effect of KRP2 phosphorylation on A- and B-type CDK activity. Recombinant KRP2 or phospho-KRP2 were added to CDKA;1 and CDKB1;1 kinase complexes immunoprecipitated from actively dividing *Arabidopsis* MM1 cell suspension extracts. Kinase activity was tested towards histone H1 as substrate. Coomassie blue staining of the electrophoresis gel area with histone H1 was used as a control of equal substrate quantity per phosphorylation reaction. The diagram represent the relative quantification of two independent kinase activity measurements with the controls arbitrarily set at 100%.
C. Immunoblot analysis with anti-His antiserum of recombinant KRP2 and phospho-KRP2 protein added to protein extracts of dividing cell suspensions. KRP2 protein stability was monitored after 30 and 60 minutes of incubation.

Previously, we showed that KRP2 is an unstable proteasome-dependent regulated protein, whose protein abundance is stabilized both *in vitro* and *in planta* in the presence of the proteasome inhibitor carbobenzoxyl-leucinyl-

leucinyl-leucinal (MG132). Interestingly, *in vitro* KRP2 degradation was found to be diminished by olomoucine, a strong and specific inhibitor of CDK activity. Moreover, KRP2 protein levels were shown to be higher in plants harboring reduced CDKB1;1 activity (Verkest et al., 2005a). These data suggest that KRP2 phosphorylation by CDKs is at least in part responsible for its proteolysis. To confirm this hypothesis, we compared the *in vitro* stability of recombinant KRP2 and its phosphorylated form. The recombinant proteins were added to protein extracts of dividing cells and fractions were collected and frozen at the indicated time points. Protein gel blotting showed a decrease in KRP2 abundance after incubation with control extract (Figure 1C). Analysis of the phospho-KRP2 protein level by autoradiography showed an even faster decrease in protein abundance, compared to KRP2 (Figure 1C), illustrating that CDK phosphorylation lowers KRP2 protein stability.

CKS1 regulates KRP2 protein abundance at the posttranslational level

In mammalia, ubiquitin-dependent degradation of the Kip/Cip CDK inhibitors is regulated through two alternative proteolytic pathways (Hengst, 2004). The best studied pathway requires phosphorylation of $p27^{Kip1}$ by CDK2/cyclinE complexes prior to recognition and subsequent degradation by the SCF^{Skp2} ubiquitin-ligase complex (Carrano et al., 1999; Montagnoli et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). CKS1 plays an important and necessary function in Kip/Cip degradation, as an adaptor that binds phosphorylated $p27^{Kip1}$ and Skp2 (Ganoth et al., 2001; Spruck et al., 2001; Hao et al., 2005). To investigate whether CKS1 plays a similar function in plants, we analyzed *KRP2* mRNA and protein levels in wild-type versus $CKS1^{OE}$ plants by Northern and protein blot analysis, respectively. *CDKA;1* was included as a control in the analysis. Whereas *CDKA;1* transcipts were slightly reduced, CDKA;1 protein levels were unaffected in $CKS1^{OE}$ plants. By contrast, KRP2 protein levels were found to be lower in the $CKS1^{OE}$ plants, whereas the corresponding *KRP2* transcript level slightly increased (Figure 2A and B). These data illustrate that CKS1 regulates KRP2 protein abundance at the posttranslational level.

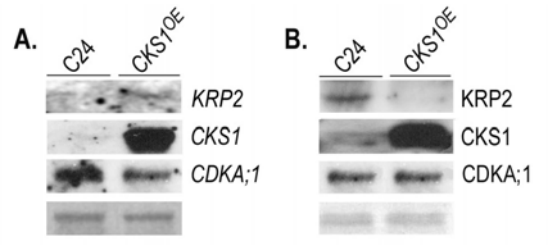

Figure 2. Analysis of *KRP2* levels in *CKS1* overexpressing plants.
RNA and total protein were prepared from three week old wild-type (C24) and *CKS1*^{OE} plants.
A. RNA gel blot analysis of *KRP2*, *CDKA;1* and *CKS1* transcript levels. Equal loading of the gel was confirmed by methylene blue staining of the membrane (bottom panel).
B. Immunoblot analysis with anti-KRP2, anti-CDKA;1 and anti-CKS1 antisera. Equal loading of the gel was confirmed by visualizing the Rubisco protein levels by Ponceau S staining of the membrane (bottom panel).

CKS1 relieves CDKA;1 inhibition by KRP2 and enhances KRP2 phosphorylation by CDKB1;1 kinase complexes

In order to unravel the mechanism of KRP2 regulation by CKS1 kinase assays were performed. First, we examined the *in vitro* effect of CKS1 on CDK kinase activity. Addition of recombinant CKS1 to immunoprecipitated CDK complexes showed that CKS1 does not affect CDKA;1, nor CDKB1;1 kinase activity towards histone H1 (Figure 3A). In contrast, KRP2 exhibits an inhibitory activity specifically towards CDKA;1 complexes, confirming previously published data (Figure 3A; Verkest et al., 2005a). To investigate the possible role of CKS1 as regulator of KRP2 activity, we subsequently analyzed the combinatorial effect of recombinant CKS1 and KRP2 on CDKA;1- and CDKB1;1-immunoprecipitated kinase activity, using both histone H1 and KRP2 as substrates. Interestingly, addition of both CKS1 and KRP2 partially relieved KRP2-mediated inhibition of CDKA;1 activity (Figure 3A). Moreover, CKS1 assisted the phosphorylation of KRP2 by CDKB1;1 complexes (Figure 3B). No such enhanced phosphorylation of KRP2 was observed when CKS1 was added to CDKA;1 complexes (Figure 3B).

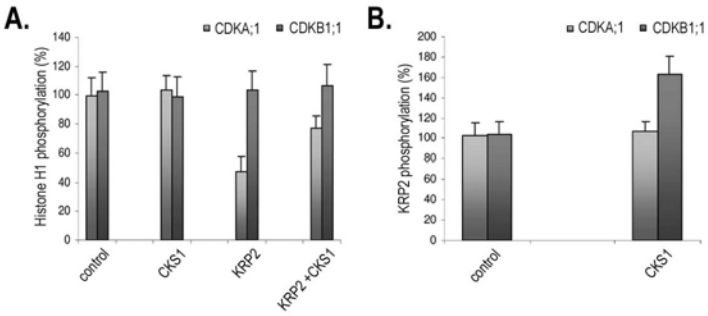

Figure 3. CKS1 regulation of KRP2 inhibition of CDKA;1 activity and of KRP2 phosphorylation by CDKB1;1 kinases.
A. Effect of recombinant CKS1 and/or KRP2 on A- and B-type CDK activity. Kinase activity was measured using histone H1 as substrate.
B. Analysis of immunoprecipitated CDKA;1 and CDKB1;1 activity towards KRP2 in the absence or presence of CKS1. For quantification, the control was arbitrary taken at 100%. The indicated values are means ± SD (n = 2).

CKS1 is a competitor of KRP2 for CDKA;1 interaction and binds KRP2

To comprehend the observed effect of CKS1 on lowering the KRP2 inhibitory activity on CDKA;1 we performed several binding assays. We initially analyzed the effect of adding increasing amounts of recombinant CKS1 protein on the ability of KRP2-coupled Sepharose beads to pull down CDKA;1 complexes from *Arabidopsis* extracts (Figure 4A). Also the reciprocal experiment was conducted, by adding increasing amounts of recombinant KRP2 protein to protein extracts incubated with $p10^{CKS1}$-beads (Figure 4B). In both cases, weaker CDKA;1 interaction to KRP2- or CKS1-beads was observed when increasing amounts of recombinant CKS1 or KRP2 protein were added, respectively. Moreover, analysis of the bound fraction revealed interaction between CKS1 and KRP2.
We next checked whether the observed weakening of CDKA;1 binding to CKS1 upon the addition of KRP2, and vice versa, is the result of competition between KRP2 and CKS1 for CDKA;1 binding, or merely is caused by mutual binding of these proteins in the absence of CDKA;1. Different concentration

ratios of recombinant KRP2/CKS1 were pre-incubated with total protein extracts of dividing *Arabidopsis* cells, and CDKA;1 complexes were immunoprecipitated. Analysis of the CDKA;1-immunoprecipitated fraction by protein blotting using CKS1 and KRP2 antibodies illustrated the lack of association between CDKA;1 and KRP2 in the presence of relative high CKS1 concentrations. Vice versa, in the presence of high KRP2 protein levels, no association between CDKA;1 and CKS1 was observed. These data suggest that the KRP2 and CKS1 proteins compete for CDKA;1 interaction (Figure 4C).

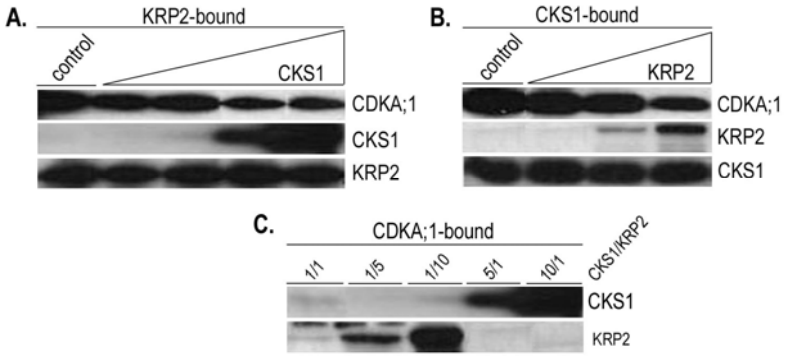

Figure 4. CKS1 and KRP2 compete for CDKA;1 interaction.
A. Protein extracts were pre-incubated with increasing amounts of recombinant CKS1 and loaded onto a KRP2-Sepharose column. Bound fractions were analyzed with CKS1- and CDKA;1-specific antibodies.
B. Reciprocal experiment of (A.) analyzing CDKA;1 and KRP2 binding to CKS1-Sepharose in the presence of increasing amounts of recombinant KRP2 protein.
C. Analysis of the amount of CDKA;1 co-immunoprecipitated CKS1 and KRP2 protein in extracts of dividing cell suspensions preincubated with different concentration ratios of recombinant CKS1/KRP2.

To confirm *in vivo* interaction between CKS1 and KRP2, KRP2-bound proteins were immunoprecipitated from total protein extracts of *CKS1*^{OE} plants treated with MG132 using a specific anti-KRP2 antibody (Verkest et al., 2005a). Subsequently, the pulled-down material was probed with CKS1 and CDKA;1 specific antibodies (Hemerly et al., 1995; De Veylder et al., 1999). As a control, binding to protein A-Sepharose beads was checked. CDKA;1 as well as CKS1 specifically co-immunoprecipitated with KRP2 (Figure 5A).

To characterize whether KRP2 directly interacts with CKS1, we pulled-down KRP2 or CKS1 out of a mixture of recombinant KRP2 and CKS1 in the absence or presence of *Arabidopsis* protein extract (Figure 5B). CKS1 bound KRP2 preferentially in the presence of cell extract. Interestingly, KRP2 was found to bind CKS1 in the presence of CDKA;1-depleted *Arabidopsis* extract, illustrating that CKS1 binds KRP2 in the absence of CDKA;1 (Figure 5B).

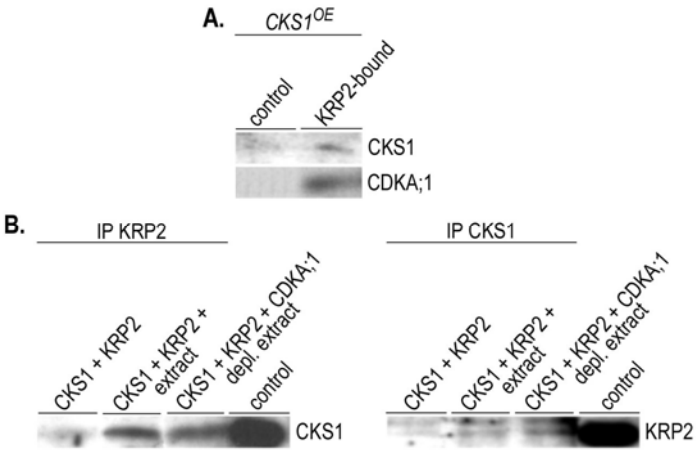

Figure 5. *In vivo* and *in vitro* KRP2 binding by CKS1.
A. *In vivo* interaction of KRP2 with CKS1. KRP2 immunoprecipitated complexes from total protein extracts of 3-week-old *CKS1*^{OE} plants were analyzed for the presence of CKS1 and CDKA;1 with specific antibodies. As a control binding to protein A-Sepharose was checked.
B. *In vitro* interaction of KRP2 with CKS1. Recombinant CKS1 and KRP2 were incubated in the absence or presence of total and CDKA;1-depleted protein extracts and subjected to KRP2- or CKS1-immunoprecipitation. Immunoblot analysis of the immunoprecipitated fractions tested the presence of CKS1 or KRP2, respectively.

Discussion

Because of the significance of linking cell division with growth and development, negative control of cell cycle progression has always received

extensive attention, especially in animal systems where the inability to correctly exit the cell cycle can eventually result in the formation of tumors. CDK inhibitors are important negative regulators that delay or arrest cell cycle progression through binding and inhibiting CDK complexes (reviewed in Peter, 1997; Mendenhall, 1998; Verkest et al., 2005b). Accordingly, yeast and mammalian CDK inhibitor activities are strictly regulated at the transcriptional, translational and posttranslational level. In plants currently little is known about the regulation of CDK inhibitors. Recently, we have demonstrated that the CDK inhibitor KRP2 is posttranslationally regulated during *Arabidopsis* leaf development (Verkest et al., 2005a). KRP2 was shown to be an unstable protein and to be regulated by proteolysis and CDK phosphorylation. Our data indicated that KRP2 phosphorylation by CDKs is at least in part responsible for its proteolysis.

Here, we provide further evidence that CDK phosphorylation of KRP2 triggers its degradation. *In vitro* degradation analysis of KRP2 demonstrated that it has a lower stability when phosphorylated by CDKs. This confirms our previous observation that inhibition of KRP2 phosphorylation through inhibition of CDK activity by olomoucine enhances KRP stability (Verkest et al., 2005a). Moreover, kinase assays showed that phosphorylated KRP2, in contrast to its unphosphorylated form, does not inhibit CDKA;1/cyclin complexes. This loss of binding-specificity towards CDKA;1 complexes implies the possibility that CDK phosphorylation changes the conformation of KRP2, thereby interfering with its binding to A-type CDKs. Indeed, co-immunoprecipitation assays demonstrated the loss of CDKA;1 binding of phosphorylated KRP2. Curiously, phosphorylated KRP2 interacted with CDKB1;1 protein complexes, a feature not shared by its unphosphorylated isoform. Moreover, phosphorylated KRP2 stimulated CDKB1;1 kinase activity towards histone H1. The exact mechanism of this activation remains to be studied in more detail but could be artificial due to contaminating residual CDK kinases present in the prepared CDK phosphorylated KRP2. Consistent with this, addition of very high amounts of phosphorylated KRP2 resulted in the enhancement of CDKA;1 activity (data not shown). However, the extent of CDKA;1 activation is much lower than observed for CDKB1;1 which indicates that phosphorylated KRP2 possibly stabilizes, and as such activates, CDKB1;1/cyclin complexes. Similarly, the mammalian Kip/Cip inhibitors were shown to function as assembly factors of certain CDK/cyclins (LaBaer et al., 1997; Cheng et al., 1999). Previously, we demonstrated that both A- and B-type CDKs phosphorylate KRP2 *in vitro* (Verkest et al., 2005a). Moreover, KRP2 protein abundance was shown to be negatively regulated at the posttranslational level by B-type CDK activity, as seen

by the increase in KRP2 abundance in transgenic plants with reduced Arath;CDKB1;1 activity. This suggests that phosphorylation by B-type CDKs marks KRP2 for protein destruction. The observed switch in CDK specificity upon KRP2 phosphorylation indicates that CDK activity possibly controls KRP2 binding to CDKB1;1. In such a model, CDKA;1/cyclin phosphorylation of KRP2 would target it to CDKB1;1/cyclin complexes for secondary phosphorylation, triggering its degradation (Figure 6). The observed CDKB1;1 kinase activation by phosphorylated KRP2 possibly exemplifies a negative feedback mechanism, enhancing KRP proteolysis. Multisite phosphorylation has been recognized as a general mechanism to set thresholds in regulated protein-protein interactions. The yeast CDK inhibitor Sic1, for example, requires phosphorylation of at least six of its nine CDK sites prior to recognition and degradation by its proteasomal pathway (Nash et al., 2001).

Further investigation suggested that CKS1 has a role in posttranslational regulation of KRP proteins as indicated by the decreased KRP2 protein levels in *CKS1* overexpressing plants and by the observed *in vivo* interaction between CKS1 and KRP2. In yeast and animals, Cks proteins are known to function in cell cycle specific proteolysis (Patra and Dunphy, 1998; Kaiser et al., 1999; Shteinberg and Hershko, 1999; Ganoth et al., 2001; Spruck et al., 2001; Morris et al., 2003). Interestingly, CKS1 enhanced the phosphorylation of KRP2 by CDKB1;1/cyclin complexes, further emphasizing that it contributes to KRP2 proteolysis (Figure 6). In budding yeast, Suc1 was similarly shown to activate the ability of CDK complexes to phosphorylate the CDK inhibitors Sic1 and Far1 (Reynard et al., 2000). In contrast to the situation seen with CDKB1;1, CKS1 did not enhance CDKA;1-dependent KRP2 phosphorylation. Surprisingly, CKS1 diminished the extent of the *in vitro* KRP2 inhibition of CDKA;1 kinase activity towards histone H1. This effect is the result of an *in vitro* competition between CKS1 and KRP2 for CDKA;1 interaction. The structural basis for this competition is unclear. The crystal structures of the mammalian Kip/Cip CDK inhibitor $p27^{Kip1}$ bound to CDK2/CyclinA and that of the CksHs1/CDK2 complex do not exclude mutual binding of $p27^{Kip1}$ and Cks proteins to CDKs, because both proteins interact with different sites of CDK2 (Bourne et al., 1996; Russo et al., 1996). Previously, we suggested that KRPs, like the mammalian Kip/Cip inhibitors, might only bind and inhibit a subset of the plant CDK/cyclin complexes (Verkest et al., 2005a). Indeed, *in vitro* assays illustrated that B-type CDK kinases are KRP2-insensitive and that CDKA;1/cyclin complexes could only be inhibited partially upon the addition of high doses of recombinant KRP2. Moreover, only mitotic cell cycle CDKA;1 complexes, and not endocycle CDKA;1 kinases, were inhibited in weak *KRP2*

overexpressing *Arabidopsis* plants. Similarly, Suc1/Cks1 proteins, although widely used as affinity matrix for the purification of CDKs, do not bind all CDK complexes. In synchronized fission yeast cells Cdc2 binding to Suc1 was only observed during mitosis, correlating with the peak in cdc13 cyclin levels and the associated peak in Cdc2/cdc13 kinase activity (Booher et al., 1989). In agreement, Nigg et al. (1991) detected no Suc1 precipitation of Cdc2 from G1 lysates of synchronized chicken cells. Similarly, Suc1 could not bind all plant CDK kinases of maize endosperm extracts (Grafi and Larkins, 1995). Interestingly, CksHs1 and Suc1 were shown not to bind CDK4 and CDK6 (Azzi et al., 1994; Vogel et al., 2002), two well known Kip/Cip CDK targets (Harper et al., 1995). Together this could imply that CDK inhibitor-bound CDKs do not bind Cks and vice versa, which fits with our observation that CKS1 and KRP2 work antagonistic (Figure 6). Although binding assays indicated that CKS1 only binds recombinant KRP2 efficiently in the presence of *Arabidopsis* extract, this could be due to the requirement of posttranslational modifications of either one of these components. The observation that KRP2 also binds CKS1 in CDKA;1-immunodepleted extracts and that phosphorylated KRP2 is unable to interact with CDKA;1 kinases, strengthens our hypothesis that KRP2 bound and inhibited CDKA;1/cyclin complexes do not contain CKS1 (Figure 6).

The identified role of CKS1 in enhancing KRP2 phosphorylation by CDKB1;1 complexes, however, implies the existence of CDKB1;1/cyclin/CKS1 complexes bound to phosphorylated KRP2. Similar complexes were suggested to exist in mammals, where Cks1 functions as a cofactor for SCF^{Skp2}-dependent Kip/Cip inhibitor ubiquitination and degradation (Ganoth et al., 2001; Spruck et al., 2001; Bornstein et al., 2003; Kamura et al., 2003). Assembly of $p27^{Kip1}$ with the Cdk2/cyclinA/E complex has been reported to stimulate its ubiquitination (Montagnoli et al., 1999; Nguyen et al., 1999) and binding of Cks1 to this trimeric complex via its Cdk-binding site was shown to increase the affinity of SCF^{Skp2} to the $p27^{Kip1}$ substrate (Sitry et al., 2002). This has been suggested to reflect a recruitment effect, because compared to free $p27^{Kip1}$, Cdk2/cyclinA bound $p27^{Kip1}$ could in principle benefit from two additional interactions with the SCF^{Skp2}/Cks1 complex, one between Cks1 and Cdk2 (Bourne et al., 1996) and the other between Skp2 and cyclinA (Zhang et al., 1995). Consistent with this, the ability of Cdk2/cyclinA to stimulate $p27^{Kip1}$ ubiquitination is abolished by the deletion of the cyclinA binding site of Skp2 (Zhu et al., 2004) and is reduced by mutations in the Cdk2 binding site of Cks1 (Sitry et al., 2002). Moreover, Hao et al. (2005) recently demonstrated the co-existence of Cks1, $p27^{Kip1}$, Cdk2/cyclinA, and SCF^{Skp2} in one complex. Interestingly, *in vitro* kinase assays and the three-dimensional structure of the

p27/Cdk2/cyclinA complex showed that $p27^{Kip1}$ binding inhibits Cdk2/cyclinA/E catalytic activity (Polyak et al., 1994; Toyoshima et al., 1994; Harper et al., 1995; Russo et al., 1996). How $p27^{Kip1}$ can be regulated by the Cdk/cyclin complex that it targets for inhibition was explained by kinetic analysis experiments suggesting that $p27^{Kip1}$ binds to Cdk2/cyclinE in at least two ways: first as a substrate (loose binding), and then as an inhibitor (tight binding) (Sheaff et al., 1997). Recently, $p27^{Kip1}$ binding to Cdk/cyclin complexes was shown to occur through a sequential mechanism initiated by cyclinA binding and followed by a slower binding to Cdk2 (Lacy et al., 2004 and 2005). Possibly $p27^{Kip1}$ phosphorylation makes it an inefficient inhibitor that interacts loosely with the Cdk2/cyclinA/E complex, allowing binding of Cks. Consistent with this, $p27^{Kip1}$ needs to be phosphorylated prior to Cks1 and Skp2 binding and degradation (Ganoth et al., 2001). This suggests that the Kip/Cip proteins have two interaction sites: one on the CDK/cyclin complex; and one, when phosphorylated, for CKS binding even when bound on the CDK since the Cks phospho-binding site is free in the CDK/cyclin/Cks complex. In plants similar theories could explain phosphorylation of KRP2 by 'inhibited' CDKA;1 complexes and, although phosphorylated KRP2 does not bind CDKA;1, the occurrence of CKS1 bound CDKB1;1/cyclin/phospho-KRP2 complexes.

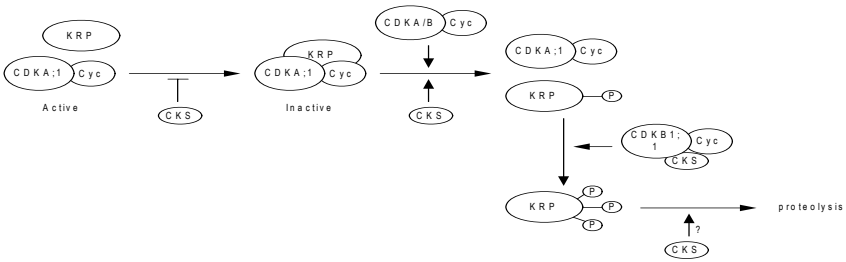

Figure 6. Schematic overview of the role of CKS1 in regulating KRP2 function and stability. By competing with KRP2 for CDKA;1/cyclin interaction CKS1 counteracts KRP2-mediated inhibition of CDKA;1 kinases. In addition, this might enhance *in vivo* KRP phosphorylation by A-type CDKs. Once phosphorylated, KRP2 changes its binding-specificity from A- to B-type CDKs. Multisite CKS1 enhanced CDKB1;1-dependent phosphorylation of KRP2 finally triggers its degradation. Whether CKS1 also plays a direct role in the proteolytic process of KRP2 remains to be determined.

Although the function of CKS1 in enhancing the phosphorylation of KRP2 is not conserved in mammalia (Ganoth et al., 2001), the indication that it binds phosphorylated KRP2 suggests the possibility that *Arabidopsis* CKS1, like CksHs1, directly activates KRP degradation (Figure 6). Several SCF ubiquitin-ligase complexes were shown to play important functions in plants (Nemhauser and Chory, 2005). Moreover, two F-box proteins, similar to the metazoan Skp2, have been identified in *Arabidopsis thaliana*. Previously, the role of the plant SCF^{Skp2} in regulating the cell cycle was shown by controlling the stability of the E2Fc transcription factor (del Pozo et al., 2002). However, experimental proof that the plant SCF^{Skp2} is regulating KRP protein levels is still missing and the ubiquitin-ligase involved in KRP degradation remains to be identified. Whether CKS1 directly regulates this complex awaits future investigation, but it is at least indirectly enhancing KRP proteolysis (Figure 6).

Methods

Regeneration and molecular analysis of transgenic lines

Plants overexpressing the *CKS1* gene (De Veylder et al., 2001a) under the control of the 35S promoter of the cauliflower mosaic virus (CaMV35S) were constructed as described previously. Molecular analysis of the transgenic plants by RNA and protein gel blotting was performed as described by De Veylder et al. (1999) and Verkest et al. (2005a).

Antibodies, protein extraction, recombinant proteins, kinase assays, and degradation assays

CDKA;1, CDKB1;1, CKS1, and KRP2 specific antisera were described before (Hemerly et al., 1995; Porceddu et al., 2001; De Veylder et al., 1999; Verkest et al., 2005a).

Arabidopsis plants and 2-day-old MM1 cell suspension cultures (Menges and Murray, 2002) were harvested, used immediately or snap-frozen in liquid nitrogen, and stored at –70°C. Proteins were extracted by grinding cells with quartz sand in homogenization buffer (25 mM Tris-Cl, pH 7.6, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM p-nitrophenylphosphate, 60 mM β-

glycerophosphate, 1 mM DTT, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail P9599 (Sigma-Aldrich)). The protein content was determined by using the Bio-Rad protein assay kit.
His-tagged KRP2 was expressed in *Escherichia coli* and purified by nickel-agarose chromatography, as described previously (Verkest et al., 2005a). For GST-tagged KRP2 expression and purification the *KRP2* coding region was PCR amplified and cloned into the GATEWAY pDONR207 vector (Invitrogen). After recombination with pDEST15, the obtained expression vector pDEST15.KRP2 was transformed in the *Escherichia coli* BL21-CodonPlus™(DE3)-RIL strain (Novagen, Madison, WI). *E. coli* cells were grown to an A600 nm of 0.5-0.7 at 37°C in LB^+ medium and the expression of *KRP2* was induced by addition of 0.2 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C. The cells were lyzed with lysozyme in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and the protein was purified on Glutathione Sepharose-4B (Amersham Biosciences) according to the manufacturers instructions. Recombinant CKS1 was obtained as described by Landrieu et al. (1999).
For preparation of phosphorylated KRP2, purified recombinant His.KRP2 was incubated for 60 min at 30°C in a kinase reaction mixture. All other *in vitro* kinase assays were performed as described by De Veylder et al. (1997) with histone H1 as CDK substrate and recombinant KRP2 and/or CKS1.
For the *in vitro* degradation assay, mixtures contained 100 µg of *Arabidopsis* cell suspension culture MM1 protein extract (20 mM HEPES, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$) supplemented with an ATP-regenerating system (35 mM phosphocreatine, 50 µg/ml creatine kinase), 1 mM ATP, 1/15 (v/v) wheat germ lysate (Promega, Madison, WI), and 50 ng substrate. The reaction mixtures were incubated at 30°C for the indicated times and the reactions were stopped by the addition of SDS sample buffer.

Binding assays, immunoprecipitations, and immunoblotting

When used for ligand affinity purification recombinant KRP2 or CKS1 was coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences) at a concentration of 5 mg/ml according to the manufacturer's instructions. Non-reacted groups of the resin were blocked adding 1M ethanolamine pH 8.0. Different concentrations of purified recombinant CKS1 or KRP2 were added to MM1 cell suspension culture extracts (300 µg) (Menges and Murray, 2002) in a total volume of 200 µl homogenization buffer, loaded onto 40 µl of 50% (v/v) KRP2- or CKS-Sepharose, respectively and incubated on a

rotating wheel for 2 h at 4°C. The bead-bound fractions were washed three times with RIPA buffer (20 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 300 µM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin and pepstatin) and resuspended in 20 µl of SDS loading buffer.

For immunoprecipitations, MM1 extracts containing the indicated amounts of recombinant CKS1 and KRP2 or phospho-KRP2 protein in homogenization buffer were precleared before incubation at 4°C for 2 h with CDKA;1 (1/250), CDKB1;1 (1/100), CKS1 (1/100), KRP2 (1/100), GST (1/1000) (Amersham Biosciences) or penta-His (1/200)(Qiagen) antibodies. After incubation for 1 h at 4°C with 30 µl of 50% (v/v) protein A-Sepharose and extensive washes in RIPA buffer the beads were used for CDK activity reactions or protein gel blot analysis.

Proteins were separated by SDS-PAGE and blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Filters were blocked in 3% (v/v) milk powder in 25 mM Tris-Cl (pH 8), 150 mM NaCl, 0.05% Tween 20 for at least 1 h at room temperature and incubated overnight at 4°C with CDKA;1 (1/5000), CDKB1;1 (1/1000), KRP2 (1/1000), CKS1 (1/1000), GST (1/1000) (Amersham Biosciences) or penta-His (1/2000) (Qiagen) antibody in blocking buffer. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1/10000 (Amersham Biosciences) with a chemiluminescence system (Perkin Elmer, Norwalk, CT).

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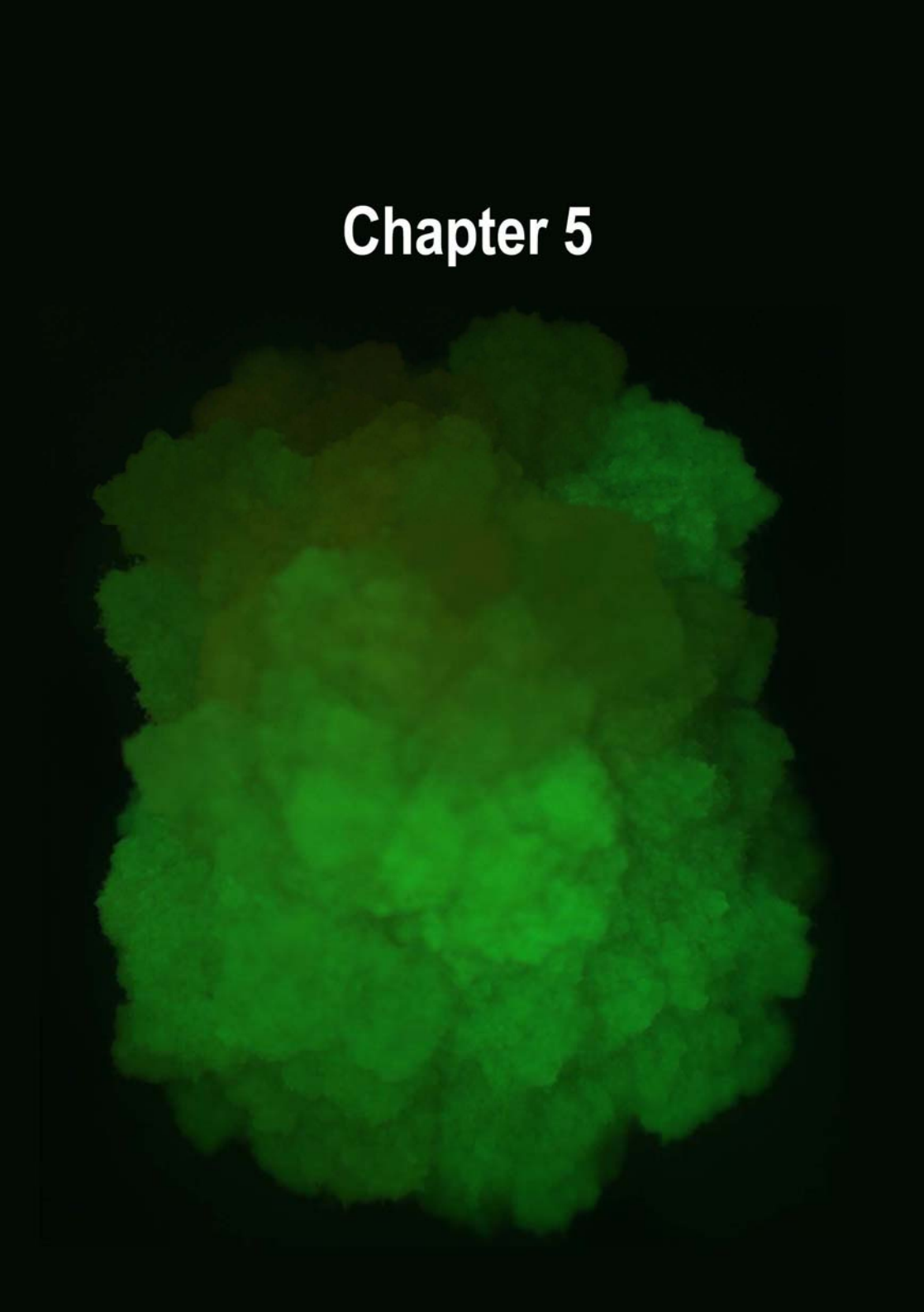
Chapter 5



Chapter page: Transgenic *Arabidopsis* callus tissue overexpressing GFP-KRP2 as screened using fluorescence microscopy.

Table of contents

Identification of functional and regulatory domains of the plant cyclin-dependent kinase inhibitor KRP2

Aurine Verkest, Dirk Inzé, Lieven De Veylder

Abstract

The ability of the plant Kip-related proteins (KRPs) to act as cyclin-dependent kinase (CDK) inhibitors and repressors of the cell cycle is becoming well established, yet little information is available regarding their mechanism of CDK binding and inhibition. The *Arabidopsis thaliana* CDK inhibitor KRP2 is known to inhibit mitotic cell cycle progression and to function in the mitosis-to-endocycle transition. To identify the protein domains within the KRP2 protein that account for KRP2 activity, a mutational analysis was performed. Deletion of its N-terminal domain enhanced the cell cycle inhibitory effects of KRP2 in transgenic plants. Moreover, a role was demonstrated for this N-terminal sequence in regulating KRP protein stability and CDKA;1 affinity. In contrast, deletion of the C-terminal domain completely abolished the CDK binding activity of KRP2. Directed point mutagenesis within the CDK binding domain of KRP2 confirmed its importance in CDK inhibition. The centrally located cyclin-binding domain, which is partially overlapping with the CDK-binding site, efficiently complexes CDKA;1/cyclin complexes, but is on its own insufficient for inhibiting CDKs. The observation that the KRP2 CDK binding domain solely does not affect kinase activity, further strengthens our hypothesis that CDK inhibition requires binding of KRP2 to both the cyclin and CDK subunit.

Manuscript in preparation

Introduction

In all eukaryotes progression through the cell cycle requires the activity of a family of protein kinase complexes known as cyclin-dependent kinases (CDKs). CDK catalytic subunits are inactive as monomers and require heterodimerization with activating regulatory subunits known as cyclins (Pines, 1994). The assembly of these CDK/cyclin complexes is regulated by the temporal cell cycle-dependent expression and proteolytical destruction of different cyclins (Morgan, 1995; Peters, 1998). In addition, the activity of these CDK/cyclin complexes is regulated by positive and negative phosphorylation events executed by the CDK activating kinase (CAK), by the WEE1 kinase and the CDC25 phosphatase, respectively (Dunphy, 1994). Furthermore, kinase activity is indirectly regulated by binding with small proteins, known as CDK inhibitors (CKIs), which either physically block CDK activation or block CDK/cyclin substrate/ATP access.

In mammals, CKIs have been divided into two families, the INK4 and the Kip/Cip class (Sherr and Roberts, 1995). The CKIs from different families differ in their protein structure, mode of action and substrate specificity. Members of the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) are characterized by the presence of ankyrin-type repeats for CDK binding and bind and inhibit only a small subset of CDKs that are primarily responsible for passage through G1. Binding of INK4 proteins to monomeric CDKs or CDK/cyclin complexes causes either allosteric changes that impair cyclin binding or lead to the dissociation of the CDK/cyclin complex, respectively. In contrast, inhibitors of the Kip/Cip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) bind and inhibit a broader range of CDKs and function in dimeric as well as heterotrimeric complexes with CDKs and cyclins. Kip/Cip binding occurs through a conserved inhibitory domain at their N-terminus that does not dissociate the CDK-cyclin complex, but distorts the catalytic ATP-binding center of the CDK subunit.

Proteins related to the class of mammalian Kip/Cip CDK inhibitors have been identified in plants, designated Kip-related proteins (KRPs) in *Arabidopsis* (De Veylder et al., 2001; Vandepoele et al., 2002). Overall, the plant KRPs display only low sequence identity to each other and the non-plant CKIs. Despite this limited sequence homology, KRPs have been shown to be true functional homologs of the Kip/Cip proteins in binding and inhibiting CDK/cyclin complexes both *in vitro* and *in vivo* (Wang et al., 1997; Lui et al., 2000; Wang et al., 2000; De Veylder et al., 2001; Zhou et al., 2002; Jasinski et al, 2002a and b; Coelho et al., 2005; Verkest et al., 2005a). Overproduction of KRPs in *Arabidopsis* results in plants with small and serrated leaves, due to a reduction in cell number as a consequence of an inhibition

of the cell cycle. Interaction analysis demonstrated that the plant KRPs bind A-type CDKs and D-type cyclins (reviewed by Verkest et al., 2005b), suggesting that they inhibit CDKA;1/cyclinD complexes. Consistently, Arath;KRP2 and Zeama;KRP1 and KRP2 were shown to inhibit CDKA;1- and cyclinD5;1-immunoprecipitated kinase complexes, respectively (Coelho et al., 2005; Verkest et al., 2005a). *In vivo* binding specificity between the plant KRPs, CDKA;1, and D-type cyclins has been demonstrated by the observation that *in planta* the aberrant cell and leaf phenotypes seen upon KRP overexpression can be complemented by the co-overexpression of CDKA;1 and D-type cyclins (Jasinski et al., 2002a, Schnittger et al., 2003; Zhou et al., 2003b; Cho et al., 2004). However, still little is known about the molecular mechanism of KRP interaction and inhibition of CDK/cyclin complexes. Here, a deletion and mutational analysis of the *Arabidopsis* KRP2 allowed the identification of important domains and amino acid residues required within KRP2 for specific functions and regulation in plants. We demonstrate that CDK inhibition requires the presence of both the CDK- and cyclin-binding domains of KRP2, suggesting that CDK/cyclin binding by KRPs mainly occurs through a sequential mechanism initiated by cyclin interaction. Curiously, analysis of KRP2 mutants with impaired CDK inhibitory activity suggests a possible dual role for KRP2 as negative and positive CDK regulator.

Results

KRP2 binds both monomeric CDKA;1 and CDKA;1/cyclinD complexes *in vitro*

In mammals, Kip/Cip CDK inhibitors were shown to bind both monomeric cyclins and CDKs and CDK/cyclin complexes (Hall et al., 1995; Harper et al., 1995; Lin et al., 1996). By yeast two-hybrid analysis it was previously shown that all *Arabidopsis* KRPs interact with D-type cyclins and CDKA;1, but not with B-type CDKs (De Veylder et al., 2001; Zhou et al., 2002). Independently, binding assays confirmed some of these interactions for KRP1, KRP2 and KRP3 (Wang et al., 1997; De Veylder et al., 2001; Verkest et al., 2005a). However, it is not known whether the plant KRPs bind monomeric CDKs and cyclins or only interact with CDK/cyclin complexes.

Using a coupled transcription-translation system we generated both S^{35}-labelled and cold c-myc-tagged CYCD4;1, HA-tagged CDKA;1 and His-tagged KRP2, respectively (Figure 1A). Different combinations of translation products were

mixed, subjected to immunoprecipitation with a specific antibody against the tagged cold protein in the mixture, and subsequently analyzed by autoradiography (Figure 1A). In the absence of cold translation product, no S^{35}-labelled proteins were precipitated by the anti-c-myc, anti-HA or anti-His antibodies (data not shown). However, both S^{35}-HA-CDKA;1 and S^{35}-c-myc-CYCD4;1 co-precipitated with His-KRP2 (Figure 1A). Identical results were obtained in a reciprocal experiment, confirming that KRP2 interacts with CDKA;1 and CYCD4;1. We also observed the previously reported interaction of CDKA;1 with CYCD4;1 (Kono et al., 2003). Moreover, our data also shows CDKA;1/CYCD4;1/KRP2 complex formation, since equal amounts of CDKA;1 and CYCD4;1 can be co-precipitated with KRP2 (Figure 1A).

To analyze the KRP2 binding to monomeric CDKA;1, we pulled-down a mixture of purified recombinant His-KRP2 and MBP or MBP-CDKA;1 with amylose beads. The fraction bound to the amylase-agarose was, after extensive washes, subsequently analyzed for the presence of His-KRP2 by protein blot analysis using an anti-His antibody (Figure 1B). A small fraction of KRP2 was detected on the MBP-CDKA;1 bound beads, but not on the MBP beads, suggesting only a weak binding of KRP2 with monomeric CDKA;1.

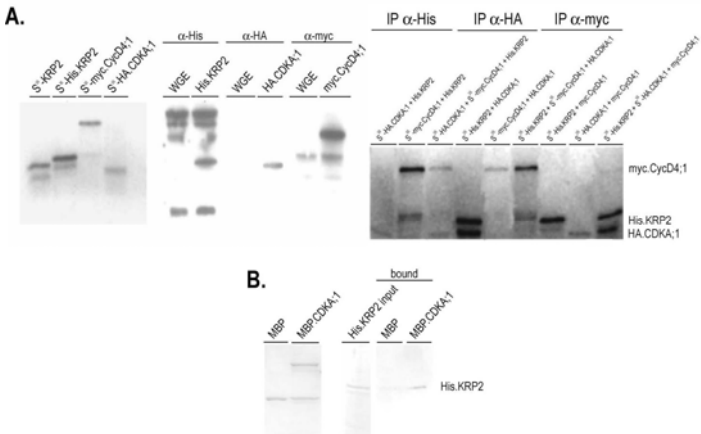


Figure 1. *In vitro* binding assays.
A. Analysis of the interaction between KRP2, CDKA;1, and CycD4;1 using *in vitro* translated His-, HA- , and myc-tagged cold and S^{35}-labelled proteins. The first panels show autoradiograph and protein blot analyses of the produced proteins (WGE = wheat germ extract). The right panel shows the α-His, α-HA, and α-myc immunoprecipitated proteins as visualized by autoradiography.
B. *In vitro* binding of KRP2 to monomeric CDKA;1. Purified recombinant MBP or MBP.CDKA;1 was incubated with 50 times the amount of His.KRP2 input.

Identification of KRP2 CDK/cyclin binding and inhibiting domains

In order to identify the KRP domains involved in the binding and inhibition of CDK/cyclin complexes we generated KRP2 deletion mutants. Figure 2 shows the different KRP2 domains that were generated and their initial evaluation to interact with CDKA;1 and CYCD4;1 by yeast two-hybrid analysis. The deletion mutants were generated based on sequence homology of KRP2 with the other KRPs and the Kip/Cip proteins. Previously, it was shown that the C-terminal domains of KRP1/ICK1 and KRP2/ICK2 interact with CDKA;1 and D-type cyclins (Wang et al., 1998; Lui et al., 2000). We confirmed these results showing that the N-terminal domain of KRP2 (KRP2.N) is dispensable for CDK and cyclin binding. By contrast, the C-terminal domain of KRP2 (KRP2.C/K) is needed for CDKA;1 interaction. Similarly, the KRP2.C/K domain is sufficient for D-type cyclin interaction with CYCD3;1. To uncouple the CDK and cyclin binding motives, extra deletion mutants were generated and tested for their interaction with CDKA;1 and CYCD3;1 (Figure 2).

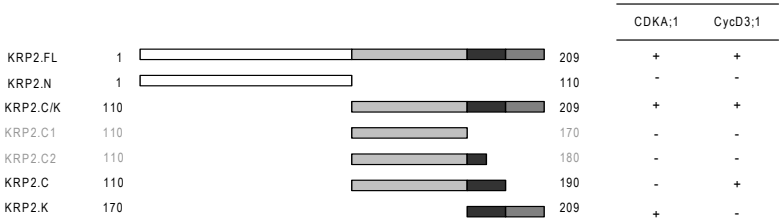


Figure 2. Schematic presentation of the KRP2 domains and their yeast two-hybrid interaction with CDKA;1 and CYCD3;1 (FL = full length protein, N = N-terminal sequence, C/K = CDK and cyclin binding domain, C = cyclin binding domain, K = CDK binding domain).

To investigate the functionality of the different KRP2 domains *in vivo*, transgenic plants were generated overexpressing N-terminal fusion proteins with hemagglutinin (HA) of the full-length and deleted forms of the *KRP2* gene under control of the constitutive Cauliflower Mosaic virus *(CaMV) 35S* promoter (used KRP2 domains are depicted in black in Figure 2). Several independent transgenic lines were analyzed for gene and protein expression levels. RNA and protein gel blot analysis revealed variation in expression among different lines harboring the same construct (data not shown). Comparison of the RNA with the protein levels within each class of transgenics ectopically expressing a specific gene construct, indicated differences in protein stability for the different KRP2 constructs (Figure 3). In comparison with wild-type plants, clear accumulation of both transcript and protein levels for the *KRP2.FL*^{OE} and *KRP2.N*^{OE} plants was detected. By contrast, although the *KRP2.C/K*^{OE}, *KRP2.C*^{OE} and *KRP2.K*^{OE} plants clearly expressed the transgenes (Figure 3B), little or no protein was detected, suggesting that the N-terminal domain plays a regulatory role in protein stability (Figure 3C).

As reported previously, strong *KRP2.FL*^{OE} plants are characterized by a striking serrated leaf phenotype and a reduced plant size (De Veylder et al., 2001; Verkest et al., 2005a) (Figure 3A). By contrast, all *KRP2.N*^{OE}, *KRP2.C*^{OE} and *KRP2.K*^{OE} plants displayed a wild-type like phenotype. Compared to *KRP2.FL*^{OE} lines, a higher percentage of *KRP2.C/K*^{OE} transgenics showed a strong phenotype with serrated leaves.

Previously, the overexpression of *KRP* genes has been demonstrated to result in a decrease in extractable CDK activity (Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002a). In this study we could confirm via analysis of the p10^{CKS1At}-purified CDK kinase activity in the transgenic plants that KRP2.FL and KRP2.C/K are potent inhibitors; yet KRP2.N, and KRP2.K have no effect on CDK activity (Figure 3D and E). Curiously, *KRP2.C*^{OE} plants displayed a somewhat reduced level of CDK activity. Together, these results indicate that full KRP2 inhibition of CDK activity requires the presence of both the cyclin and CDK binding domain.

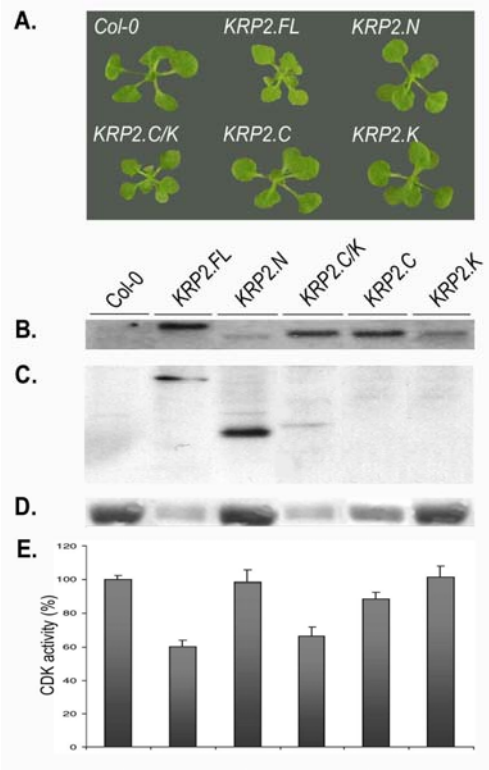


Figure 3. Phenotypic and molecular analysis of transgenic *Arabidopsis thaliana* plants. Three-week-old transgenic plants were compared with untransformed control plants (Col-0) of the same age.
A. Phenotypes of wild-type (Col-0) and transgenic plants
B. RNA gel blot analysis
C. Immunoblot analysis with an anti-HA antibody
D. $p10^{CKS1At}$-associated kinase activity in transgenic plants. Autoradiogram representing a typical result from CDK activity assays with histone H1 as substrate
E. Relative quantification of three independent kinase activity measurements as depicted in D. The control was arbitrarily set at 100%.

KRP2 overexpression in *Arabidopsis thaliana* was recently shown to trigger a dosis-dependent endoreduplication phenotype (Verkest et al., 2005a). As such, we compared the DNA ploidy level distribution among different transgenic lines with high, similar levels of transgene expression of the different KRP2 deletion variants in 3-week-old cotyledons (Table 1). Similar effects on the endocycle were seen in leaves (data not shown). As reported previously, high levels of KRP2.FL resulted into a partial inhibition of the endocycle, as seen by the strong increase in the 2C DNA population, accompanied with a decrease in the number of cells with a 8C or 16C DNA content. Similarly, KRP2.C/K overexpression resulted into an inhibition the endocycle, whereas the N-terminal and the separate cyclin and CDK binding domains have no effect (Table 1).

Table 1. DNA ploidy levels in 3-week-old cotyledons of wild-type (Col-0) and transgenic lines.

Line	2C (%)	4C (%)	8C (%)	16C (%)	32C (%)
Col-0	23.1 ± 1.8	26.7 ± 0.4	30.2 ± 2.3	19.1 ± 0.9	0.8 ± 0.1
KRP2 FL	33.5 ± 4.4	25.2 ± 0.7	25.0 ± 2.2	15.9 ± 0.6	0.4 ± 0.2
KRP2 N	23.1 ± 2.4	26.0 ± 0.4	31.4 ± 2.6	19.3 ± 0.1	-
KRP2 C/K	31.3 ± 2.5	22.2 ± 1.4	26.6 ± 2.4	16.7 ± 0.9	0.8 ± 0.4
KRP2 C	24.6 ± 1.5	25.8 ± 0.9	28.3 ± 1.9	19.9 ± 0.5	1.4 ± 0.1
KRP2 K	26.0 ± 0.9	24.6 ± 0.1	29.6 ± 1.7	19.0 ± 0.9	0.7 ± 0.1

Data represent average ± SD (n = 2).

The *in vivo* binding-specificity of the different KRP2 domains towards CDKA;1 was analyzed by immunoprecipitation of HA-KRP2-containing complexes from total protein extracts prepared from the same transgenic lines as depicted in Figure 3B and C. Subsequently, the pulled-down material was probed with CDKA;1-specific antibodies (Hemerly et al., 1995). In both KRP2.FL and KRP2.C/K cell extracts, CDKA;1 was co-precipitated with KRP2 (Figure 4A). Remarkable, relatively more CDKA;1 was pulled-down with KRP2.C/K, suggesting that this deletion mutant of KRP2 has a higher affinity for CDKA;1 than the full-length protein. Interestingly, although in the yeast two-hybrid system only an interaction with cyclins was observed, immunoprecipitation of HA-KRP2.C could pull-down CDKA;1 as well, suggesting that KRP2 binds both monomeric cyclins and cyclin/CDK complexes *in vivo*. On the other hand, no CDKA;1 signal was obtained in the KRP2.K pulled-down material, illustrating no or very weak *in vivo* interaction of KRP2 with monomeric CDKA;1. No CDKA;1 interaction was observed with KRP2.N. This is in agreement with the observed yeast two-hybrid results of KRP2.N and confirms that,

as was previously shown for KRP1/ICK1 (Wang et al., 1998; Zhou et al., 2003a), the N-terminal domain of the *Arabidopsis* KRPs is not involved in CDK binding.
To analyze the functionality of the different KRP2 domains towards CYCD3;1, we crossed the different transgenic *Arabidopsis* plants overexpressing *KRP2* domains with *CYCD3;1* overexpressing plants. As a control all the transgenic lines were crossed with wild-type plants. The fidelity of the crosses was confirmed by PCR analysis (data not shown). Crossing *CYCD3;1*^{OE} with *KRP2.FL*^{OE} plants resulted in the expected restoration of a wild-type phenotype (Jasinski et al., 2002a; Zhou et al., 2003b; Cho et al., 2004; Figure 4B). Similarly, co-expression of *KRP2.C/K* with *CYCD3;1* complemented the KRP2 and CYCD3;1 phenotype. Crosses between *CYCD3;1* ^{OE} plants and *KRP2.N* ^{OE} or *KRP2.K* ^{OE} plants showed the typical *CYCD3;1* overexpression phenotype with undulated and curled leaves (Rhiou-Khamlichi et al., 1999; DeWitte et al.,2003), indicating the lack of complementation. Interestingly, ectopic expression of *KRP2.C* and *CYCD3;1* resulted in a different phenotype with larger leaves, suggesting that KRP2 has an additional cyclin regulatory role besides its inhibitory function.

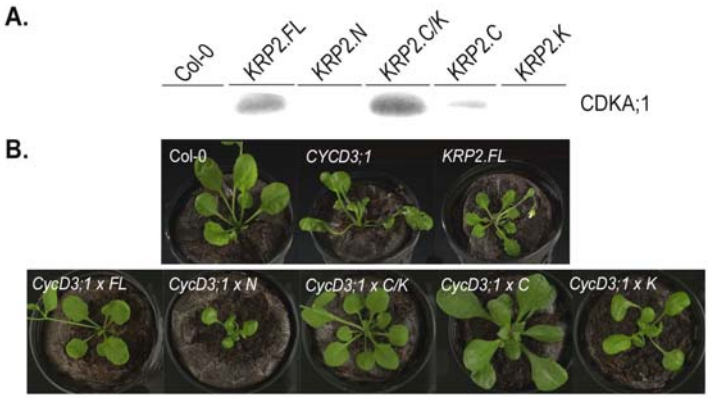


Figure 4. CDK and cyclin specificity of full-length and truncated KRP2.
A. *In vivo* interaction of KRP2 domains with CDKA;1. HA-immunoprecipitated complexes from total protein extracts of 3-week-old wild-type (Col-0) and transgenic plants were analyzed by immunoblot analysis with anti-CDKA;1 antibody. B. *In vivo* specificity of KRP2 for CYCD3;1. Phenotypes of 6-week-old *Arabidopsis thaliana* plants co-overexpressing CYCD3;1 and KRP2 domains.

KRP2 CDK-binding domain is critical for CDK inhibition

Considering the importance for KRP2 functioning of the CDK- and cyclin-binding domains we performed a combined random mutagenesis of its C-terminal domain (KRP2.C/K) and reverse two-hybrid screen to identify mutants for CDKA;1 or CYCD3;1 binding. No KRP2.C/K mutants for CYCD3;1 binding were isolated. In contrast, we identified several KRP2 mutants with altered CDKA;1 binding affinity. Most of these mutants showed point mutations of residues that are conserved in all *Arabidopsis* KRPs (Figure 5). Interestingly, Kip/Cip homologues of some of the isolated mutants were described previously and analyzed in mammalia, where they analogously were demonstrated to lack CDK-binding affinity (Goubin et al., 1995; Welcker et al., 1998). To further analyze these mutants we performed a site-directed mutagenesis on KRP2. Figure 5 depicts the different KRP2 mutants tested for the interaction with CDKA;1 and CYCD3;1 by yeast two-hybrid analysis. Mutation of strictly conserved residues in the CDK binding domain of all plant KRPs and animal Kip/Cips abolished CDKA;1 interaction, corroborating our reverse two-hybrid data.

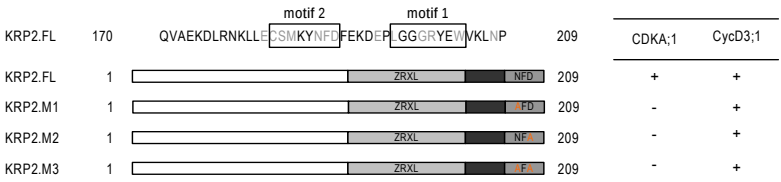
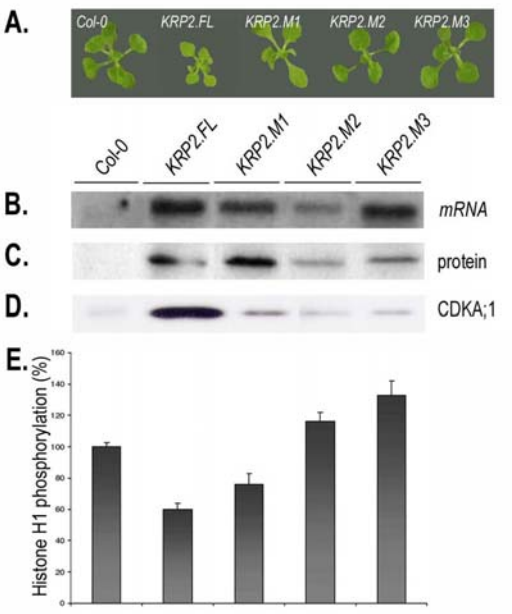


Figure 5. KRP2 mutants with altered CDKA;1 binding affinity. Sequence of the wild-type KRP2 CDK binding domain with motifs 1 and 2 boxed and the reverse two-hybrid isolated point mutations indicated in grey. Yeast two-hybrid interaction analysis of full length and motif 2 mutated KRP2 with CDKA;1 and CYCD3;1. (M1 = N188 → A, M2 = D190 → A, M3 = N188, D190 → A).

To analyze the functionality of the KRP2.M1, KRP2.M2 and KRP2.M3 mutants *in vivo*, transgenic plants were generated overexpressing N-terminal hemagglutinin(HA)-tagged mutant forms of the *KRP2* gene under control of the constitutive Cauliflower Mosaic virus *(CaMV) 35S* promoter. Several independent lines were obtained. For phenotypic analysis, wild-type and mutant *KRP2* overexpressing lines with similar levels of transgene expression were selected. No clear differences in protein stability were detected by analyzing the RNA and protein levels of the different KRP2 constructs (Figure 6B and C).

In comparison with *KRP2.FL* overexpressing plants, only a minority of the transgenic *Arabidopsis* plants overexpressing *KRP2.M1* showed limited serration of the leaves and reduced growth, whereas *KRP2.M2* and *KRP2.M3* overexpression were indistinguishable from untransformed control lines in respect to their growth rate and leaf morphology (Figure 6A).
The CDK binding-specificity of these three mutants was analyzed through immunoprecipitation of HA-KRP2-containing complexes. The results show that, in contrast to the yeast two-hybrid data, all mutant forms bind CDKA;1. Probably this interaction is indirect and occurs through the cyclin partner of CDK complexes, as the amount of CDKA;1 protein that co-immunoprecipitated with the KRP2 mutants was lower than seen for wild-type KRP2 (Figure 6D).
Purification of active CDK/cyclin complexes with $p10^{CKS1At}$-beads allowed analysis of the effect of full-length and mutant *KRP2* overexpression on the CDK activity towards histone H1. *KRP2.M1* overexpression resulted in a decrease of extractable CDK activity (Figure 6E). However, the extent of CDK inhibition was less severe than seen for the $KRP2.FL^{OE}$ plants with the same level of transgene expression, suggesting that the mutated KRP2 is a less efficient inhibitor than the wild-type protein. In contrast, overexpression of *KRP2.M2* or *KRP2.M3* did not result in CDK inhibition, but curiously seemed to activate CDK/cyclin complexes.

Figure 6. Phenotypic and molecular analysis of transgenic *Arabidopsis* plants overexpressing *KRP2.FL*, *KRP2.M1*, *KRP2.M2*, and *KRP2.M3*.
A. Phenotypes
B. RNA gel blot analysis
C. Immunoblot analysis with an anti-HA antibody
D. Analysis of the CDK binding specificity. HA-immunoprecipitated complexes from total protein extracts of 3-week-old wild-type (Col-0) and transgenic plants were analyzed by immunoblot analysis with anti-CDKA;1
E. Relative quantification of three independent $p10^{CKS1At}$-associated kinase activity measurements towards histoneH1. The control was arbitrarily set at 100%.



KRP2.M3 enhances endocycle onset through activation of endocycle-specific CDKA;1 complexes

To comprehend the observed stimulation of CDK activity observed for the *KRP2.M3*^{OE} plants in more detail, the DNA content of 3-week-old first leaves of independent wild-type, *KRP2.FL*^{OE} and *KRP2.M3*^{OE} lines was measured by flow cytometry. At this stage of development the leaf is mature: no mitotic divisions can be detected and DNA ploidies have reached a steady-state level (De Veylder et al., 2001; Verkest et al., 2005a; Vlieghe et al., 2005). Contrasting results were obtained for *KRP2.FL*^{OE} and *KRP2.M3*^{OE} plants. Whereas full-length *KRP2* overexpression triggered a dosis-dependent endoreplication phenotype (Verkest et al., 2005a), *KRP2.M3* overexpression was always correlated with enhanced DNA ploidy levels (Figure 7B). These effects on the endocycle correlated with the *KRP2.FL and KRP2.M3* expression levels (Figure 7A) and were also seen in cotyledons (data not shown).

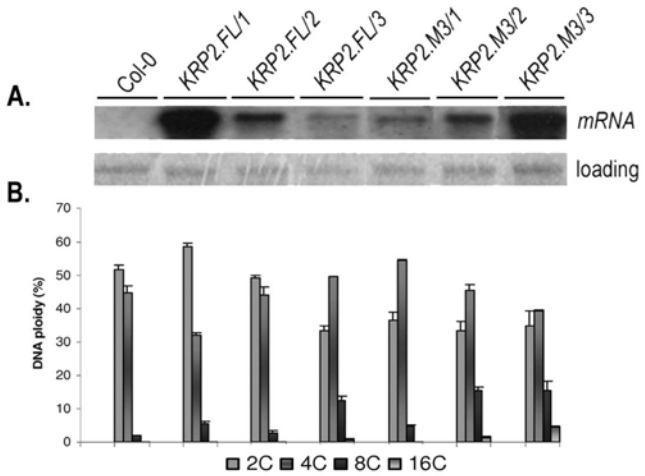


Figure 7. Analysis of the effect of wild-type and mutant *KRP2* overexpression on the DNA ploidy distribution.

A. RNA gel blot analysis of 3-week-old wild-type and transgenic lines. Equal loading of the gel was confirmed by methylene blue staining of the membrane (bottom panel).

B. Ploidy level distribution of the first leaves of 3-week-old wild-type (Col-0) and independent *KRP2.FL* and *KRP2.M3* overexpressing plants as measured by flow cytometry. The indicated values are means ± SE (n = 2 to 4).

The timing of endoreplication onset is developmentally regulated during leaf development (Boudolf et al., 2004; Beemster et al., 2005; Vlieghe et al., 2005). Cells of the first leaf pair primarily divide at 9 DAS (days after sowing) during which most cells have a 2C DNA content and the remaining predominantly 4C. At 15 DAS, leaf cells have exited the mitotic division program and are mainly endoreduplicating, correlated with an increase in the 4C DNA population and the presence of cells with an 8C DNA content. When comparing the DNA ploidy distribution at these developmental stages in wild-type, strong *KRP2.FL*^{OE}, and *KRP2.M3*^{OE} plants, young *KRP2.FL*^{OE} leaf cells (9 DAS) showed no altered ploidy distribution when compared to wild-type plants, and clear inhibition of the endocycle was only seen in older tissue (21 DAS; Figure 7B and 8A; De Veylder et al., 2001). In contrast, *KRP2.M3*^{OE} tissues showed elevated ploidy levels at the mitotically dividing developmental stage (9 DAS), illustrating a premature onset of the endocycle.

Distinct CDKA;1/cyclin complexes have been demonstrated to regulate the mitotic cell cycle and the endocycle (Grafi and Larkins, 1995; Verkest et al., 2005a). Consistently, in strong *KRP2* overexpressing lines CDK activity was inhibited in both mitotic dividing and endoreplicating leaf tissues; whereas in the weak lines only the mitotic CDK/cyclin complexes were affected. To test the effect of *KRP2.M3* overexpression, CDKA;1 kinase activity was measured in the leaf tissue of the first leaf pair harvested at 9 and 15 DAS. Preliminary data show that in mitotic cells (9 DAS) CDKA;1 activity was unaffected or weakly inhibited compared to wild-type plants (Figure 8B). Absent or weak inhibition of the mitotic cell cycle was also demonstrated by analyzing leaf epidermal cell numbers and sizes in three-week old leaves (Table 2). Surprisingly, at 15 DAS, kinase activity was activated in *KRP2.M3*^{OE} plants (Figure 8B). These data suggest that the increased CDK activity observed in *KRP2.M3*^{OE} plants and the enhanced endocycle onset results from the activation of endocycle CDKA;1 complexes.

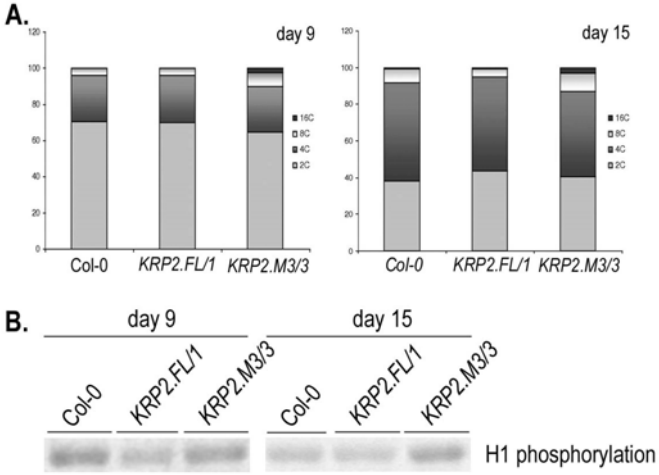


Figure 8. KRP2.M3 enhances endocycle onset.
A. Ploidy level distribution of the first leaves of wild-type (Col-0) and strong *KRP2.FL* and *KRP2.M3* overexpressing plants during development as measured by flow cytometry,
B. Autoradiograph analysis of immunoprecipitated CDKA;1 kinase activity in the first leaf pair of wild-type (Col-0) and strong *KRP2.FL* and *KRP2.M3* overexpressing plants, using histone H1 as substrate.

Table 2. Abaxial epidermis cell size and cell number in leaves of wild-type, *KRP2.FL* and *KRP2.M3* plants

Line	Leaf size (mm^2)	Abaxial Epidermal Cells Estimated number	Size (μm^2)
Col-0	15.03±1.02	13532±875	1160±33
KRP2.FL/1	9.84±0.93	2789±204	3897±417
KRP2.M3/3	15.42±0.94	11682±691	1345±89

All measurements were performed on 3-week-old mature first leaves. The indicated values are means ± SE (n = 6 to 10).

Discussion

Since the isolation of the first plant CDK inhibitory gene *ICK1/KRP1* (Wang et al., 1997), a family of related genes has been identified (reviewed by Verkest et al., 2005b). Whereas the ability of the plant KRPs to act as CDK inhibitors and repressors of cell proliferation is becoming well established, little information is available regarding their molecular mechanism of CDK inhibition. Here, a deletion and mutational analysis of the *Arabidopsis thaliana* CDK inhibitor KRP2 allowed the identification of important domains and amino acid residues defining KRP2 function.

The high diversity of the N-terminal domain among KRP sequences suggests that this particular region is not involved in CDK binding or inhibition. Previously, it was shown that deletion of the N-terminal region of KRP1/ICK1 indeed does not impede its interactions with either CDKA;1 and CYCD3;1 (Wang et al., 1998). The N-terminal domain of KRP2 similarly does not bind CDKA;1 nor CYCD3;1. The observation that *Arabidopsis* plants overexpressing the N-terminal domain of *KRP1* or *KRP2* do not exhibit lowered CDK kinase activities (Zhou et al., 2003a; this study) and that *KRP2.N*, in contrast to *KRP2.FL*, did not complement the *CYCD3;1* overexpression phenotype, indeed illustrates that the N-terminal KRP domain does not function in CDK/cyclin targeting. Interestingly, the N-terminal sequences of KRP1 and KRP2 appear to have opposing functions in regulating KRP protein stability: the N-terminus of KRP1 was found to destabilize this plant CDK inhibitor (Zhou et al., 2003a), whereas the N-terminal domain of KRP2

stabilizes the KRP2 protein. The specific amino-acid sequence conferring KRP2 stability still remains to be identified.

A variety of techniques, including: sequence analysis, deletions, mutations and peptides allowed the mapping of the CDK and cyclin binding sites of the mammalian Kip/Cip proteins. A conserved N-terminal-located region of 65 amino-acids was shown to be necessary and sufficient for Kip/Cip binding and inhibition of CDK/cyclin complexes (Luo et al., 1995; Chen et al., 1995, Goubin et al., 1995, Nakanishi et al., 1995). The crystal structure of the $p27^{Kip1}$ N-terminal CDK inhibitory domain bound to the cyclinA/CDK2 complex showed that this peptide spans and interacts with a large surface area of the CDK/cyclin complex causing conformational changes in and around the catalytic cleft of CDK2 (Russo et al., 1996). In this complex the $p27^{Kip1}$ domain has a nonglobular, extended structure that consists sequentially of a rigid coil, an amphiphatic α-helix, a β-hairpin, a β-strand and a 3_{10}-helix (Figure 9). The $p27^{Kip1}$-cyclinA interaction predominantly involves the $p27^{Kip1}$ coil-located ZRXLFG motif that binds a shallow groove of cyclinA lined with highly conserved cyclin-box amino acids. Interaction with the CDK subunit occurs through the β-hairpin, the β-strand, and the 3_{10}-helix. The first two structures associate with the N-terminal lobe of the CDK, resulting in the destabilization of the ATP binding site, whereas the 3_{10}-helix binds to the catalytic cleft and occupies the ATP binding site (Russo et al., 1996). Despite their low sequence identity, the conserved C-terminal domains of the plant KRPs show partial homology with the Kip/Cip protein sequence necessary for interaction with the CDK subunit, suggesting that the plant CKI inhibitory function resides at their C-terminus. Indeed, yeast-two hybrid interaction assays demonstrated that the C-terminal domains of KRP1 and KRP2 are sufficient for interaction with CDKA;1 and CYCD3;1 (Wang et al., 1998; Lui et al., 2000; this study). Moreover, the functionality of these domains for CDK binding and inhibition (Schnittger et al., 2003; Zhou et al., 2003a; this study) and the specificity of KRP2.C/K for CYCD3;1 was proven *in vivo*.

Figure 9. Comparison of the KRP2 protein sequence with its mammalian $p27^{Kip1}$ homologue. Plant KRP conserved motifs 1, 2, and 3 are indicated in boxes, the cyclin and CDK binding domains of KRP2 are underlined. For more details, see text.

The sequence homology of the conserved KRP motifs 1 and 2 with part of the CDK binding domain of the Kip/Cips suggests that this domain is involved in plant CDKA;1 interaction (Figure 9). In agreement, yeast two-hybrid interaction analysis demonstrated that the KRP2 deletion mutant harboring this domain (KRP2.K) could bind CDKA;1, but does not complex CYCD3;1. Although the plant KRPs display no region of similarity to the cyclin binding domain of the mammalian Kip/Cip CKIs, further deletion mapping allowed us to identifiy a KRP2 motifs 2 and 3 containing cyclin binding domain (Figure 9). Curiously, motif 2 of the CDK binding domain is necessary for interaction of KRP2.C with CYCD3;1. This partial overlap between the CDK and cyclin binding domains was also suggested to occur in KRP1 (Wang et al., 1998), but is not reported in Kip/Cip inhibitors. Analysis of the *in vivo* functionality of the separate KRP2 CDK and cyclin binding domains suggested that the inhibiting function of KRP2 depends on both domains since *KRP2.C* nor *KRP2.K* overexpression resulted in an alteration of the wild-type phenotype. Moreover $KRP2.K^{OE}$ plants displayed wild-type CDK activity levels. The weak inhibition of CDK activity which was measured in the $KRP2.C^{OE}$ plants is in agreement with the observation that peptides from the cyclin-binding domain of $p21^{Cip1}$ partially inhibit CDK activity through blocking CDK/cyclin accessibility of other CDK substrates containing cyclin-binding domains (Chen et al., 1996). *In vivo* binding assays demonstrated that KRP2.K could not complement the *CYCD3;1 Arabidopsis* phenotype and did not bind CDKA;1, when compared to KRP2.FL. This suggests that KRP2 preferentially associates CDK/cyclin complexes and does not or very weakly binds monomeric CDKA;1. *In vitro* binding assays demonstrated weak interaction of full-length KRP2 with CDKA;1. Similarly, Kip/Cip interaction with isolated CDK-subunits is weak and appreciable CKI binding is only found when the kinase is associated to a cyclin partner (Harper et al., 1995; Lin et al., 1996). In contrast, Kip/Cip CKIs show a high affinity for monomeric cyclins as was illustrated by Hall et al. (1995) with a mutant cyclinD1 construct, which whilst retaining the ability to interact with Kip/Cips, was inactive for CDK binding. Analysis of the CDK binding-specificity of KRP2.C showed that, in contrast to the yeast two-hybrid data, it binds CDKA;1. Probably this interaction is indirect and occurs through the cyclin partner of CDK complexes. Moreover, even though we did not analyze KRP binding to monomeric cyclins, the observation that co-expression of *KRP2.C* and *CYCD3;1*

results in an additional plant phenotype suggests that KRPs, like the Kip/Cip proteins, bind monomeric cyclins.
In mammalia, the ZRXL motif was identified as involved in cyclin binding in several cell cycle proteins, including E2F1, Cdc25a, p107 and the Kip/Cip inhibitors (Vlach et al., 1997; Lin et al., 1996; Chen et al., 1996; Welcker et al., 1998). Although this motif does not appear in other *Arabidopsis* KRPs it is present in the analyzed cyclin binding domain of KRP2 (Figure 9). Yeast two-hybrid interaction analysis of ZRXL mutants of KRP2 however showed that it does not function in CYCD3;1 binding (data not shown). This observation further suggests that plant KRPs use a distinct cyclin-binding mechanism. However, we can not exclude that this sequence motif has no other cell cycle function since even in mammalia the nature of the ZRXL motif is not completely understood. Whereas it functions in the interaction with A-, E- and D-type cyclins in $p21^{Cip1}$, it binds cyclinA and cyclinE, but not D-type cyclins in p107 (Ewen et al., 1992; Li et al., 1993). Moreover, the ZRXL motif of $p21^{Cip1}$ was shown to be necessary for its interaction with monomeric A- and E-type cyclins and for the interaction and inhibition of cyclinE/CDK2, but not of cyclinA/CDK2 complexes (Wohlschlegel et al., 2001).
Random mutagenesis and reverse two-hybrid analysis allowed the fine-mapping of the KRP2 CDK binding site. All KRP2.C/K mutants with altered CDKA;1 binding affinities showed amino-acid mutations in or near motifs 1 and 2, further emphasizing their sufficiency and necessity for CDKA;1 binding. Furthermore, this illustrated that the KRPs utilize the homologous $K/RWNFDFX_4PLEG$ sequence for CDK interaction, like observed for their mammalian counterparts (Figure 9). Based on these results and reports from the Kip/Cip inhibitors we rationally designed three KRP2 point mutants in the conserved motif 2 for *in vivo* analysis (Goubin et al., 1995; Nakanishi et al., 1995). Yeast two-hybrid analysis demonstrated that the KRP2.M1, KRP2.M2 and KRP2.M3 mutants kept the KRP2 ability to bind CYCD3;1, but they could not interact with CDKA;1. By contrast, immunoprecipitation of HA-KRP2 from plant extracts overexpressing *KRP2.M1*, *KRP2.M2* and *KRP2.M3* illustrated that all of them pulled down CDKA;1, although less efficiently than KRP2.FL. In agreement with this observed altered CDKA;1 affinity, *KRP2.M1* overexpression in plants resulted in a less dramatic phenotype and less efficient CDK inhibition, when compared to *KRP2.FL* plants. Overexpression of *KRP2.M2* and *KRP2.M3* even stimulated CDK activity. Similarly, some Kip/Cip mutants in CDK binding were reported to be less efficient inhibitors, whereas others activated CDK complexes (Welcker et al., 1998). These results confirm that tight binding of the KRPs with both the cyclin and CDK subunit is a prerequisite for CDK inhibition. Combined, our data indicate that,

similar to the Kip/Cip inhibitors, the cyclin-binding motif is involved in the docking of the CDK/cyclin complex, but that the CDK binding domain accounts for the inhibitory activity of KRPs (Russo et al., 1996; Lacy et al., 2004 and 2005).
The observed stimulation of CDK activity upon KRP2.M2 or KRP2.M3 overexpression indicated that both alleles might encode dominant negative forms of the wild-type KRP2 protein. To test this hypothesis, we crossed *KRP2.FL*^{OE} and *KRP2.M3*^{OE} plants (data not shown). As a control both lines were separately crossed with wild-type plants. The fidelity of the crosses was confirmed by PCR analysis with dCAPS primers. Phenotypical analysis of the crossed lines, however, showed that the observed effects of *KRP2.M3* overexpression did not result from an obstruction of CDKA;1 binding with wild-type KRP2 through KRP2.M3, as crossed plants still exhibited the *KRP2.FL* overexpression phenotype, suggesting that the activation of CDK activity in *KRP2.M3*^{OE} plants occurs through another unidentified mechanism. Previously, we demonstrated that wild-type KRP2 overexpression triggers a dose-dependent endoreplication phenotype (Verkest et al., 2005a). Whereas the overexpression of KRPs at high levels induces an inhibition of the endocycle (De Veylder et al., 2001; Jasinski et al., 2002a; Zhou et al., 2002), mild overexpression leads to increased DNA ploidy levels (Verkest et al., 2005a; Weinl et al., 2005). In contrast, both weak and strong *KRP2.M3* overexpressing plants exhibited increased ploidy levels. The apparently contradicting results in *KRP2.FL*^{OE} plants were shown to result from an inhibition of both CDKA;1/cyclin complexes with a role in the mitotic cell cycle and endocycle in strong *KRP2.FL*^{OE} lines, whereas in weak *KRP2.FL*^{OE} lines mainly the mitotic CDKA;1 complexes were targeted (Verkest et al., 2005a). Curiously, the enhanced endocycle onset in *KRP2.M3*^{OE} plants was shown to result from an activation of endocycle CDKA;1 complexes. This suggests that KRP2, besides having a role in the mitotic cell cycle and mitosis-to-endocycle transition, also functions in the endocycle. Consistent with this KRP2 was shown to be expressed in both mitotically dividing and endoreplicating tissues (Ormenese et al., 2004; Verkest et al., 2005a). In mammals, Kip/Cips display besides their inhibitory function also a role as assembly and targeting factors of CDK/cyclin complexes (Sherr and Roberts, 1999). These contradicting functions are the consequence of binding of Kip/Cip CKIs to both sensitive and insensitive CDK complexes (Blain et al., 1997). Whereas interaction of Kip/Cip inhibitors to susceptible CDK complexes results in their inhibition, binding to inert CDK/cyclins stabilizes these complexes (LaBaer et al., 1997; Cheng et al., 1999). Moreover, Kip/Cips have been implicated as nuclear import factors for CDK/CycD complexes and were shown to increase the stability of D-type cyclins (LaBaer et al., 1997; Cheng et al., 1999; Alt et al., 2002). *In vivo* inhibitory specificity between KRPs and different D-type cyclins has

been demonstrated by Schnittger et al. (2003), who showed that the trichome endoreduplication phenotype resulting from *KRP1* overexpression can be complemented by overexpression of *CYCD3;1*, but not of *CYCD4;1*. Previously, the ability of modest levels of KRP2 to inhibit mitotic cell cycle CDKA;1 complexes, but not endocycle complexes was suggested to possibly result from a shift in CDKA;1 cyclin partners at the mitosis-to-endocycle transition since this transition in mammalian trophoblasts is accompanied by a switch of D-type cyclin isoform expression (MacAuley et al., 1998; Verkest et al., 2005a). Another possibility is that this switch to KRP-inert CDKA;1 complexes is accompanied by their activation by KRP2 and masks the inhibition of KRP-sensitive CDKA;1 complexes. Although further investigations are required to identify a possible function of KRP2 as assembly or targeting factor, this could explain how KRP2 mutants with weakened or impaired CDK inhibitory function activate endocycle CDKA;1 complexes.

Methods

Construction of KRP2 domains and mutants – Plasmids

The pGAD.KRP2.FL, pGBT.CDKA;1, pGBT.CYCD3;1 and pDEST17.KRP2 constructs were obtained as described before (De Veylder et al., 2001; Verkest et al., 2005a). The different KRP2 domains were amplified by polymerase chain reaction (PCR) with specific primers and cloned in the EcoR1 and BamH1 sites of pGAD424 (Clontech) or in the Nde1 and BamH1 sites of pREP42-HA_N (Craven et al., 1998). Information about the primer set sequences can be given upon request. Site-directed KRP2 mutants were generated by PCR of the KRP2.FL sequence in pGEM-T using the following pre-phosphorylated primers: 5'-CTTCGATTTCGAGAAAGATGAGCCACTTGGTGGAGG –3' and 5'-GCATACTTCATAGAACATTCCAACAACTTATTCCG –3' for substitution of N186 → A186, 5'- TTTCGAGAAAGATGAGCCACTTGGTGGAGGAAGATACG -3' and 5'- GCGAAGTTATACTTCATAGAACATTCCAACAACTTATTCCG –3' for substitution of D188 → A188 in pGEM-T.KRP2.FL or pGEM-T.KRP2.N186 → A186. Information about the primer set sequences for ZRXL motif mutations can be given upon request. The linear PCR products were circularized by ligation and the resulting plasmids were sequenced to confirm if the correct residue substitution took place. The mutants were amplified with the KRP2.FL primers and subcloned in the pGAD424 and pREP42-HA_N vectors.

HA-tagged versions of the KRP2 domains and mutants were obtained by Nco1 and BamH1 digestion of the pREP42-HA_N.KRP2 constructs. Subsequently, the restriction fragments were cloned between the *CaMV35S* promoter and the nopaline synthase (*NOS*) 3' untranslated region in the NcoI and BamHI sites of PH35S (Hemerly et al., 1995). The *CaMV35S/HA.KRP2/NOS* cassettes were released of the pH35S.HA.KRP2 constructs by EcoRI and SalI and cloned in pBinPLUS (van Engelen et al., 1995) to obtain the pBIN.HA.KRP2 vectors.
Random mutagenesis of pGAD.KRP2.C/K was performed by error-prone PCR with modified dNTP concentrations and Mn^{2+} with primers 5'-AGGGATGTTTAATACCACTAC –3' and 5'- GCACAGTTGAAGTGAACTTGC –3' (Miyazaki and Arnold, 1999). A reaction contained 1 × PCR buffer (Roche, with $MgCl_2$), 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.5 mM $MnCl_2$, 1 μM of both primers, 30 ng template and 5 U Taq DNA polymerase (Boehringer Mannheim). PCR was carried out at 94°C for 5 min (denaturation), 40 cycles: 94°C for 1 min, 40°C for 1 min, 72°C for 2 min, and 72°C for 5 min.
HA- and MBP-tagged versions of CDKA;1 were constructed by cloning, CDKA;1 digested out of the EcoR1 and BamH1 sites of pGAD.CDKA;1 (De Veylder et al., 2001), into pBluescript plasmid (Stratagene) containing a HA-tag (HA-pSK) or pMal2c (New England Biolabs), respectively. CYCD4;1 was obtained by PCR and cloned into the EcoR1 and Pst1 sites of the pBluescript plasmid (Stratagene) containing a double c-myc tag. All cloning steps were carried out according to standard procedures, and the reading frames were verified by direct sequencing.

Yeast two-hybrid and reverse two-hybrid experiments

The vectors pGAD424 and pGBT9 harboring the GAL4 activation domain and the GAL4 binding domain respectively, and the yeast strain AH109 (Clontech) were used for yeast two-hybrid interactions. Yeast cells were transformed according to the Matchmaker two-hybrid system protocol (Clontech) and assayed for their ability to grow on $Leu^-/Trp^-/His^-$ minimal medium supplemented with 10 mM 3-aminotriazole (3-AT) after 3 days of incubation at 30°C. Bait plasmids co-transformed with empty pGAD424 and prey plasmids co-transformed with empty pGBT9 were assessed along as controls for the specificity of the interaction.
For reverse two-hybrid screening the yeast strain MaV203 (Life Technologies) was sequentially transformed with pGBT.CDKA;1 and a library of mutated KRP2.C/K constructed in pGAD424 by gap repair cloning (Fusco et al., 1999). For gap repair cloning 250 ng linearized pGAD424 and 750 ng PCR fragment were used to

transform MaV203.pGBT.CDKA;1 with the lithium acetate method. Transformed yeasts were plated on Leu^-/Trp^- minimal medium to evaluate the overall efficiency of the transformation and on Leu^-/Trp^- minimal medium supplemented with 0.2% 5-fluoro-orotic acid to screen for KRP2.C/K mutants with impaired ability to bind CDKA;1. 3×10^4 transformants were screened to yield approximately 40 RTH positive clones. PCR on spheroplasted yeast colonies was performed to check insert size and nucleotide sequence.

In vitro transcription/translation of proteins, production of recombinant proteins, and *in vitro* binding assays

In vitro transcription and translation experiments were performed using the TnT T7-coupled wheat germ extract kit (Promega) primed with the appropriate plasmids for 90 min at 30 °C in the presence or absence of 35S-methionin.

For MBP.CDKA;1 expression and purification the pMal2c.CDKA;1 vector was transformed in the *Escherichia coli* BL21-CodonPlus™(DE3)-RIL strain (Novagen, Madison, WI) and after induction purified by amylose affinity chromatography according to the manufacturer's instructions (New England BioLabs). His-tagged KRP2 was expressed in *Escherichia coli* and purified by nickel-agarose chromatography as described previously (Verkest et al., 2005a).

For immunoprecipitations 10 μl of each *in vitro* translated protein was mixed and diluted at 1:5 in Nonidet NP40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM PMSF, 10 μg/ml leupeptin/aprotinin/pepstatin) and incubated for 2 h at 4°C with anti-HA (16B12; BabCo), anti-c-myc (9E10; BabCo) or penta-His (Qiagen) antibodies, respectively. Protein-A-Sepharose (40 μl 25% [v/v]) was added and incubated for 1 h at 4°C, beads were washed four times with Nonidet NP40 buffer and immune complexes were eluted with 10 μl 2× SDS sample buffer and analyzed by 13% SDS-PAGE and by autoradiography.

Binding of KRP2 to monomeric CDKA;1 was analyzed by incubation of 500ng recombinant His.KRP2 and MBP or MBP.CDKA;1 in RIPA buffer (20 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 300 μM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and pepstatin). After addition and incubation with amylose resin, the bead bound fractions were washed three times with RIPA buffer and eluted by boiling the samples in SDS loading buffer for subsequent gel blot protein analysis with a penta-His antibody (Qiagen).

Regeneration and analysis of transgenic lines

The pBIN.KRP2 plasmids were mobilized by the helper plasmid pRK2013 into the *Agrobacterium tumefaciens* C58C1Rif^R harboring the plasmid pMP90 (Koncz and Schell, 1986). *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia was transformed by the floral dip method (Clough and Bent, 1998). Transgenic *CaMV35S-KRP2* plants were obtained on kanamycin-containing medium. To minimize the effects of copy numbers on gene expression, transgenic plants with one insert were selected by the segregation ratio of kanamycin resistance on selective medium. Double *KRP2.FL-KRP2.M3*^{OE} plants were obtained by crossing homozygous *KRP2.FL*^{OE} plants with homozygous *HA.KRP2.M3* overexpressing plants with similar levels of transgene expression. Similarly crossing of homozygous *CycD3;1*^{OE} plants with plants overexpressing the *KRP2* domains resulted in the regeneration of double transformants. The fidelity of the crosses was confirmed by PCR analysis. For all analyses, plants were grown under a 16 h light/8 h dark photoperiod at 22°C on germination medium (Valvekens et al., 1988).

Analysis of the transgenic plants by RNA and protein gel blotting was performed as described by Verkest et al. (2005a). For flow cytometric analysis, leaves or cotyledons were chopped with a razor blade in 300 µl of 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7), and 1% Triton X-100 (Galbraith et al., 1991). From a stock of 1 mg/ml 4,6-diamidino-2-phenylindole, 1 µl was added to the filtered supernatants. The nuclei were analyzed with the BRYTE HS or CyFlow flow cytometer with Win-Bryte (Bio-Rad, Hercules, CA) or FloMax (Partec, Münster, Germany) software, respectively. Analysis of leaf epidermal cell numbers and sizes of the transgenic lines was performed as described previously (Verkest et al., 2005a).

For immunoprecipitations, 300 µg of total protein extract in homogenization buffer (25 mM Tris-Cl, pH 7.6, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM p-nitrophenylphosphate, 60 mM β-glycerophosphate, 1 mM DTT, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail P9599 (Sigma-Aldrich)) were incubated with 30 µl of 50% (v/v) anti-HA Affinity Matrix (Roche Diagnostics) for 1 h at 4°C on a rotating wheel. Thereafter, beads were washed three times with RIPA buffer (20 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 300 µM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin and pepstatin), and used for CDK activity reactions or protein gel blot analysis. Kinase assays were performed as described by De Veylder et al. (1997) and Verkest et al. (2005a).

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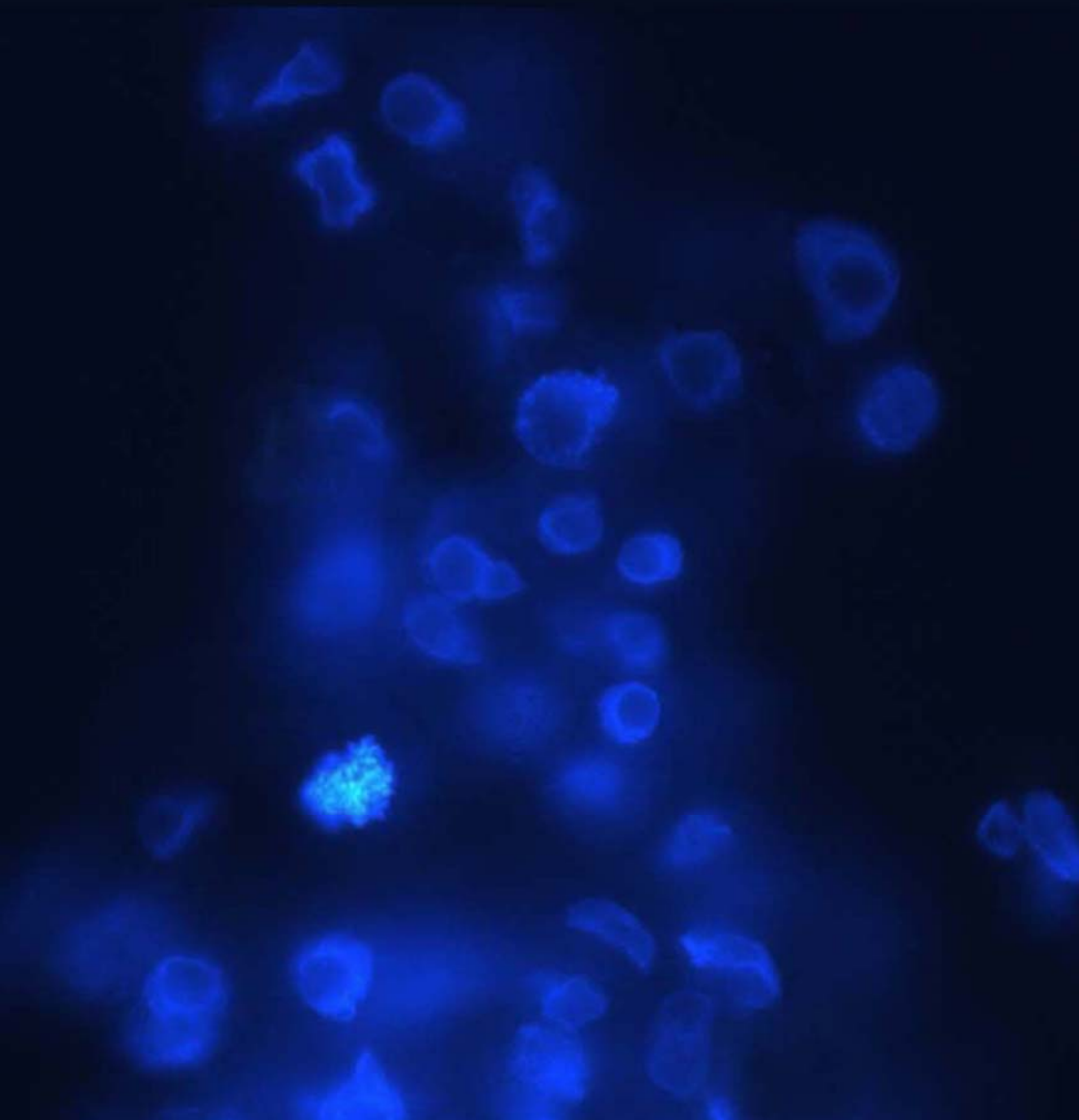
Chapter 6



Chapter page: Fluorescence microscopy image of nuclear, DAPI stained *Arabidopsis* cell suspension cultures.

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Chapter 6: Molecular analysis of an *Arabidopsis* cyclin-dependent kinase inhibitor interacting KBR1 RING finger protein

Molecular analysis of an *Arabidopsis* cyclin-dependent kinase inhibitor interacting KBR1 RING finger protein

Aurine Verkest, Kristiina Himanen, Mirande Naudts, Dirk Inzé, Lieven De Veylder

Abstract

The ubiquitin-proteasome pathway drives the cell division cycle by the timely destruction of numerous regulatory proteins. The two main activities that catalyze substrate ubiquitination in the cell cycle, the SCF complexes and the APC/C, define a superfamily of E3 ubiquitin-ligases, all based on related cullin and RING finger protein subunits. Furthermore, some RING finger proteins can act as single subunit E3s. The *Arabidopsis* genome contains more than 400 RING finger proteins and several studies have implicated single and multisubunit RING finger E3s in a number of processes in plants. However, at present, control of the plant cell cycle by ubiquitin-dependent proteolysis is poorly understood. A two-hybrid screen using the *Arabidopsis thaliana* CDK inhibitor KRP5 as a bait led to the identification of a 33.5 kDa protein containing a RING finger motif, named KBR1. KBR1 displays *in vitro* E3 ubiquitin ligase activity and is shown to interact with all different KRPs tested. In addition, KBR1 was found to associate with CDKA;1. Although overexpression of the wild-type *KBR1* gene in *Arabidopsis* appears to be lethal, the analysis of transgenic plants overexpressing dominant negative *KBR1* alleles suggests a function for KBR1 in the regulation of cell cycle and plant growth during stress. Moreover, a screening for putative KBR1 substrates led to the isolation of several stress regulated interactors. Finally, the protein level of the CDK inhibitor KRP2 was elevated in transgenic *Arabidopsis* plants with reduced KBR1 activity, illustrating that KBR1 is part of an E3 complex regulating the KRP proteolysis.

Introduction

Selective protein degradation by the ubiquitin-proteasome pathway has emerged as a powerful regulatory mechanism in a wide variety of cellular processes including cell cycle progression, morphogenesis and signal transduction. Ubiquitin conjugation requires the sequential activity of three enzymes or protein complexes called the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3) (Hershko & Ciechanover, 1998). Once a polyubiquitin chain is assembled on a substrate, the substrate is quickly captured and degraded by the 26S proteasome (Smalle & Vierstra 2004). The cardinal regulatory step of substrate recognition is performed by the E3 ligases (Jackson et al., 2000). Recent findings reveal that all known E3s utilize one of two possible catalytic domains, a HECT domain or a RING finger domain. The zinc binding RING finger domain, constituting the largest class of E3s, are formed by either a single polypeptide containing both RING finger and substrate binding domains, or by a multi-component complex with separate polypeptides containing these domains (Ciechanover et al., 2000). Several types of multisubunit E3s are known in which a small RING finger protein is an essential component. All single-subunit RING E3s examined so far bind either directly or as a homodimer or heterocomplex with their E2 and their substrate, catalyzing substrate ubiquitination and sometimes their own (E2-dependent) ubiquitination.

The proportion of the genome that is devoted to ubiquitination is larger in plants than in animals or fungi (Moon, 2004). Cross-species comparisons show a good conservation of genes encoding ubiquitin, E1 and E2. In contrast, plant proteins with a suspected E3 function are more diverged, and homologues can be identified only in a minority of cases. A database search indicates more than 400 RING finger domains in the *Arabidopsis* genome (Kosarev et al., 2002, Vierstra 2003, Stone et al., 2005). Although it is currently unclear whether all RING finger domains mediate interaction with an E2, it is possible that each RING finger protein is part of a distinct E3. Studies have implicated single and multisubunit RING finger E3s in a number of processes in plants. However, only for few RING proteins functional interactions with their substrates have been characterized (Matsuda et al., 2001, Hardtke et al., 2002, Holm et al., 2002, Xie et al., 2002, Stary et al., 2003).

During cell division the unidirectional cell cycle progression is in part controlled by the ubiquitin-mediated proteolysis of several cell cycle regulatory proteins. In mammalia, two main types of multisubunit E3s are involved in cell cycle regulation. Skp1-Cdc53/cullin-Fbox protein (SCF) complexes are responsible for

transition from G1 to S phase by catalyzing the phosphorylation-dependent ubiquitination of G1 cyclins, CDK inhibitors, transcription factors and many other proteins (Patton et al., 1998). The anaphase-promoting complex/cyclosome (APC/C) is active from the beginning of anaphase untill the end of G1, a window during which it eliminates anaphase inhibitors, mitotic cyclins and components of the mitotic spindle (Zachariae et al., 1999). Both SCF and APC/C multisubunit RING enzymes are conserved in plants, but the identity of their substrates and their importance in cell cycle regulation is largely unknown (Capron et al., 2003a; Viestra et al., 2003). Several plant cell cycle proteins were shown to be regulated by proteasomal degradation (Criqui et al., 2000 and 2001; Castellano et al., 2001 and 2004; del Pozo et al., 2002; Planchais et al., 2004; Verkest et al., 2005). The mechanisms that regulate the ubiquitination of these proteins constitute essential components of cell cycle control, but are poorly understood at present.

CDK inhibitory proteins are important cell cycle regulators and have been proven to be regulated by ubiquitin-dependent degradation in several organisms (Henchoz et al., 1997; Feldman et al., 1997; Carrano et al., 1999; Bornstein et al., 2003; Kamura et al., 2003; Kamura et al., 2004). Proteins related to the class of mammalian Kip/Cip CDK inhibitors have been identified in plants and designated Kip-related proteins (KRPs) in *Arabidopsis* (De Veylder et al., 2001; Vandepoele et al., 2002). The *Arabidopsis* genome encodes seven *KRP* genes. Despite the limited sequence homology with their mammalian counterparts, KRPs have been shown to be true functional homologs of the Kip/Cip proteins in inhibiting CDK activity both *in vitro* and *in vivo* (Wang et al., 1997; Wang et al., 2000; De Veylder et al., 2001; Zhou et al., 2002). Currently, little is known about the regulation of the KRPs at the posttranslational level but preliminary evidence shows that at least some of them are regulated through proteolysis. Functional analysis of KRP1 deletion mutants indicated the presence of a regulatory motif for protein instability in its N-terminal domain (Zhou et al., 2003; Weinl et al., 2005). Recently, we have shown that KRP2 protein stability is regulated by the proteasome, as KRP2 protein levels become stabilized in cells treated with the proteasome inhibitor MG132 (Verkest et al., 2005). Moreover, *in vitro* analysis illustrated that KRP2 is a CDK/cyclin substrate and that its phosphorylation is at least in part responsible for KRP2 proteolysis. However, it is currently not known which ubiquitin-ligase is responsible for KRP degradation.

Here, we report the isolation and partial characterization of a RING finger protein, designated Arath;KBR1, that interacts with KRPs and CDKA;1 and has *in vitro* E3 ligase activity. The results presented indicate that the KBR1 ubiquitin-ligase possibly targets a broad range of substrates involved in the regulation of cell cycle and plant growth during stress.

Results

Isolation of the *KBR1* gene from *Arabidopsis*

To identify putative KRP5 interacting proteins, a two-hybrid screen was performed based on GAL4 recognition sites to regulate the expression of both *his3* and *lacZ* reporter genes (Chien et al., 1991; Fields and Sternglanz, 1994). The pGBT.KRP5 vector, encoding a fusion between the C-terminus of the GAL4 DNA-binding domain and KRP5 was constructed by cloning the full-length coding region of KRP5 into the pGBT9 vector. The screening was performed with a GAL4 activation domain cDNA fusion library constructed from mRNA of *Arabidopsis thaliana* cell suspension cultures harvested at different growth stages, including the early exponential, exponential, early stationary and stationary phases. The HF7c yeast reporter strain was co-transformed with the pGBT.KRP5 vector and the library, and 10^6 independent transformants were screened for growth on histidine free medium and lacZ gene activation.

At4g19700
MAVQAHHMNIFSQFISPNRDCVKFQENMNHGEFEFTGGEVPLITGESFAVEPLAAKANFN
KAESGLSYNFTVPPLSTKRQRDFQFSDSNAPVKRRSVAFDSSSPSLINVELVSQIQNQQQ
SEIDRFVAQQTEKLRIEIEARQQTQTRMLASAVQNVIAKKLKEKDDEIVRIRNLNWVLQERV
KSLYVENQIWRDIAQTNEANANTLRTNLDQVLAQLETFPTASAVVEDDAESSCGSCCGDG
GGEAVTAVGGGCKRCGEREASVLVLPCRHLCLCTVCGGSALLRTCPVCDMVMNASVHV
NMSS

Figure 1. Sequence of KBR1. The RING-HCa motif with its Cys and His Zinc-binding reisdues is indicated in black and red.

One of the interacting clones (At4g19700) encoded a putative 33.5kDa protein with a C-terminal located Zinc-finger motif and was named KRP Binding RING finger protein 1 (Arath;KBR1). The RING domain is similar to the DNA binding zinc fingers with Cys and/or His residues coordinating the attachment of two zinc ions, but is rather involved in protein-protein interactions because the zinc ions are coordinated in a cross-brace structure (Barlow et al., 1994). The Cys-rich RING domain was initially identified in a protein encoded by the Really Interesting New Gene (Freemont et al., 1991). RING protein genes encode candidate ubiquitin E3 ligases functioning in E2-dependent protein degradation. The RING domains that are essential for E3 ligase activity of RING containing proteins have either a Cys or His residue at metal ligand position 5 as C3HC4 for RING–HC

type and C3H2C3 for RING–H2 type domains. Sequence analysis of KBR1 detected a RING-HC motif belonging to the HCa-class (Figure 1; Stone et al., 2005).

KBR1 interacts with KRPs and CDKA;1

To assess whether the binding of KBR1 with KRP5 is specific or accounts for all KRPs, additional yeast two-hybrid interaction assays were performed. The full-length cDNA of the *KBR1* gene was isolated and cloned into an appropriate yeast two-hybrid vector, resulting in a transcriptional fusion between KBR1 and the GAL4 transcriptional activation domain (pGAD.KBR1). In a yeast reporter strain, the pGAD.KBR1 plasmid was co-transformed with vectors encoding a fusion between different KRPs, CDKA;1 and CDKB1;1 cDNAs and the GAL4 DNA-binding domain (pGBT). As a control empty pGBT and pGAD vectors were used. Transformed strains were plated on medium without His, which allows growth of yeast only when the two proteins interact. Although pGBT.KRP2 showed transactivation, all other KRP transformed yeasts only grew on His-lacking medium in the presence of KBR1, demonstrating that KBR1 interacts with all *Arabidopsis* KRPs tested (Table 1). Furthermore, KBR1 was found to interact as well with CDKA;1, but not with the G2/M-specific CDKB1;1, implying a specific role of KBR1 in cell cycle.

Table 1. Two-hybrid interaction of KBR1 with different KRPs and CDKs

	KRP2	KRP3	KRP4	KRP5	KRP6	KRP7	CDKA;1	CDKB1;1
KBR1	TA	+	+	+	+	+	+	-

+ growth on medium -His; - no growth on medium –His; TA transactivation

To confirm the observed interaction of KBR1 with KRPs and CDKA;1 *in vitro* pull down assays were performed. Although several attempts were undertaken, we were unsuccessful in confirming the yeast two-hybrid interaction *in vitro* (data not shown). E3 ligase substrate recognition is often dependent on phosphorylation modifications and E3 activity on homo- or heterodimerization of RING proteins. Possibly the lack of necessary posttranslational modifications and/or additional cofactors prevented the *in vitro* functional interaction observed in living yeast cells. In agreement with this, kinase assays performed with $p10^{CKS1At}$ affinity-

purified CDK/cyclin complexes isolated from actively dividing cell suspensions demonstrated that KBR1 is phosphorylated by CDKs, suggesting interaction of KBR1 with CDKA;1 (Figure 2).

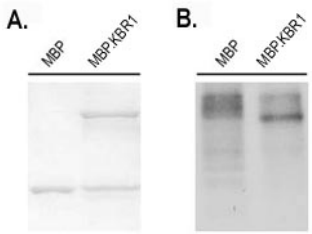


Figure 2. *In vitro* phosphorylation of KBR1 by CDKs.
A. Purified recombinant MBP and MBP.KBR1.
B. $\gamma^{32}P$ incorporation in MBP.KBR1 by active CDK complexes.

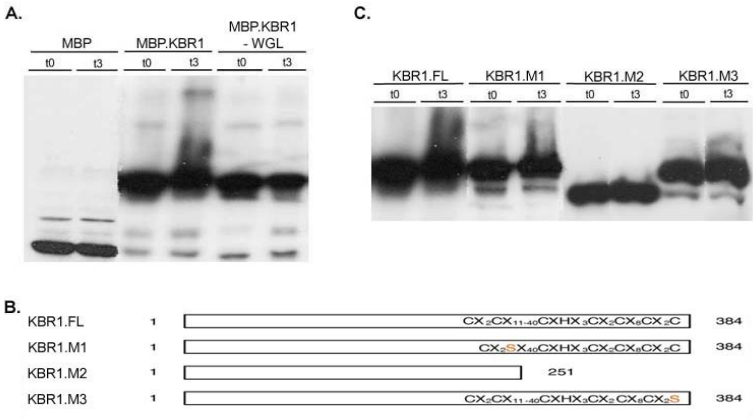
KBR1 processes *in vitro* ubiquitin-ligase activity

To investigate whether KBR1 has E3 ligase activity, *in vitro* ubiquitylation experiments were performed. These assays typically rely on the function of RING protein E3s to bring their substrate in close vicinity with the E2 ubiquitin-conjugate. E3 activity can be reconstituted *in vitro* by providing to the putative E3 enzyme ubiquitin, an ubiquitin-activating E1, a conjugating E2 enzyme, ATP, and the target protein. A successful reaction results in an increased molecular weight of the target protein, caused by poly-ubiquitylation. In the absence of a trans-target, various E3s have been shown to self-ubiquitylate (Bays et al., 2001; Fang et al., 2000; Lorick et al., 1999; Nuber et al., 1998). In our particular experiment, wheat germ lysate was used as a source of E1 and E2, because it possesses a high ubiquitin-dependent proteolysis activity (Hatfield and Vierstra, 1989). Recombinant MBP-KBR1, being a fusion between the maltose-binding protein and KBR1, was incubated at 30°C with ATP, ubiquitin and the wheat germ lysate in the presence of the proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), which was used to block proteolysis, allowing the detection of poly-ubiquitylated protein. Immunoblotting using an anti-MBP antibody of fractions collected at the indicated times showed that prolonged incubation of MBP-KBR1 led to the formation of slower-migrating ladders (Figure 3A), illustrating KBR1 E3

ligase activity. MBP alone or the omission of wheat germ lysate did not result in high molecular mass modifications.
We next tried to reconstitute self-ubiquitination of MBP-KBR1 by purified components only using ubiquitin, MBP-KBR1 and HsUbcH5a (E2) and mammalian E1 in an *in vitro* assay mixture as described by Seo et al. (2003). Incubation of MBP-KBR1 did not result in high molecular mass ladders (data not shown). The reason for the inability of KBR1 to auto-ubiquitinate in the absence of applied plant extract is not known but suggests the necessity of cofactors/additional activating proteins.

It has been shown that the RING finger motif is required for the ubiquitin-ligase activity of almost all RING protein containing E3 enzymes. To test whether this also holds true for KBR1 we analyzed the *in vitro* ubiquitylation of deletion and point mutants of MBP-KBR1. Abrogation of the intact RING domain by mutation, deletion or addition of Zinc chelators often impairs E2-dependent ubiquitylation. Figure 3B shows the RING-HC type domain of KBR1 and the KBR1 deletion and point mutants generated for subsequent characterizations. In the KBR1.M2 mutant the complete C-terminal located RING-HC domain was deleted. Two different point mutation alleles were constructed, because KBR1 possesses two possible RING domain configurations. In KBR1.M1, the Cys2 residue of motif 1 was mutated into a Ser residue, whereas in KBR1.M3 the mutation of Cys7 should abrogate both possible RING motifs. Deletion of the RING motif of KBR1 diminished the appearance of a smear when MBP-KBR1.M2 was incubated with ubiquitin, ATP, wheat germ lysate and MG132 (Figure 3C), indicating that the RING motif is necessary for E3 activity. Curiously, incubation of the point mutants MBP-KBR1.M1 and MBP-KBR1.M3 still resulted in the generation of high molecular mass ladders (Figure 3C).

Figure 3. E3 ubiquitin-ligase activity of wild-type and mutant KBR1.
A. MBP.KBR1 was assayed for E3 activity in the presence or absence of wheat germ lysate (WGL). MBP was used as negative control.
B. Schematic overview of the generated wild-type and mutant KBR1 proteins.
C. Assay of self-ubiquitination of MBP.KBR1.FL and mutants.



Overexpression of *KBR1* in *Arabidopsis*

To investigate the role of KBR1 in plant development, transgenic plants were generated harboring the wild-type and mutant *KBR1* alleles under the control of the constitutive cauliflower mosaic virus 35S promoter. No transgenic lines were obtained for *KBR1* and *KBR1.M1*, suggesting that *KBR1* overexpression is lethal and that the KBR1.M1 is still functional. In contrast, multiple independent transgenic lines were obtained for the KBR1.M2 and KBR1.M3 constructs. Two independent lines overexpressing *KBR1.M2* and *KBR1.M3* were selected in which the mRNA levels exceeded those found in untransformed plants (Figure 4D). *KBR1.M2*^{OE} plants were smaller when compared to wild-type plants (Figure 4A). By contrast, *KBR1.M3* overexpressing plants did not reveal any obvious phenotypical changes (Figure 4A). Mutant seedlings and soil grown plants looked similar in size to untransformed control plants. Stems, cauline and rosette leaves, flowers, siliques appeared simultaneously in wild-type and *KBR1.M3*^{OE} plants and their morphology was comparable. However, when grown in continuous light conditions these transgenic plants displayed altered phenotypes. When initially, in Petri dishes and normal light conditions, grown wild-type looking *KBR1.M3* seedlings were transferred to soil in pots and grown in continuous light the transgenic plants showed reduced growth phenotypes (Figure 6B). Furthermore, when *KBR1.M3* seedlings were grown in continuous light young plants showed a senescence phenotype and displayed at a later stage a high lethality in

comparison with wild-type plants (Figure 6C). These data suggest that functional KBR1 is required for normal plant growth and has a role in light stress control.

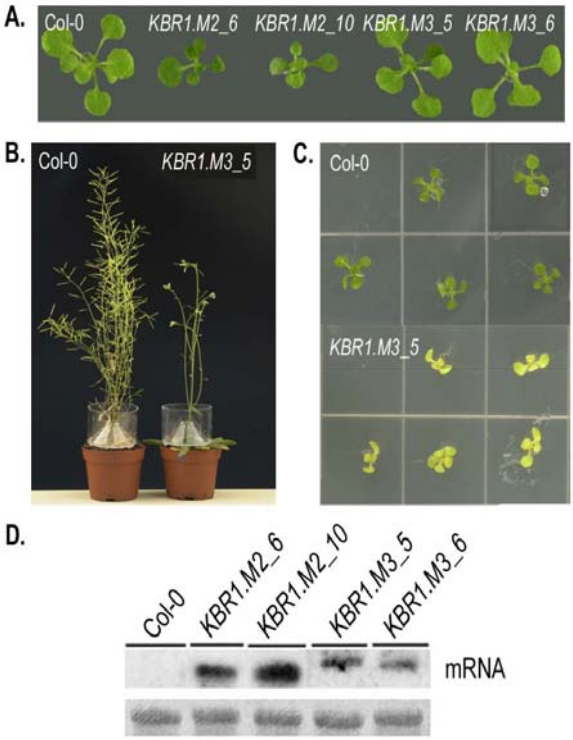


Figure 4. Phenotypic and molecular analysis of *KBR1* overexpressing *Arabidopsis* plants.
A. Phenotypes of 3-week-old wild-type (Col-0) plants and two independent *KBR1.M2* and *KBR1.M3* overexpressing plants.
B. Reduced growth phenotypes of *KBR1.M3* overexpressing plants when transferred to continuous light.
C. Phenotypes of 7-day-old *KBR1.M3* overexpressing seedlings grown in continuous light.
D. RNA gel blot analysis of wild-type and *KBR1.M* overexpressing plants. Equal loading of the gel was confirmed by methylene blue staining of the membrane (bottom panel).

KRP protein levels in transgenic lines

The measured lack or weakened *in vitro* E3 activity of KBR1.M2 and KBR1.M3 combined with the phenotypes observed for plants overexpressing these *KBR1* mutants suggests that these mutant alleles might operate as dominant negatives. E3 ubiquitin-ligase proteins mediate substrate recognition and bring them in contact with E2 ubiquitin-conjugating enzymes. The RING domain is directly binding the E2 enzymes while the substrate recognition sites usually lie outside the RING domain. As such, abrogation of the intact RING domain impairs or reduces E3 activity. These dominant negative E3 ligases are still able to interact with their substrates but their degradation is reduced due to the titration effect caused by the binding with ectopically expressed dominant negative E3 ligases (Seo et al., 2003). In this case the phenotypes are expected to be milder than those of knockout of the E3 ligase. The observed *KBR1* phenotypes are in accordance with this hypothesis since there are no *KBR1* knockout lines available, suggesting gametophytic lethality, while *KBR1.M2* and *KBR1.M3* overexpressing plants show lethality at a later stage in development. As such, KBR1 proteolytic substrates are expected to accumulate in KBR1.M2 and KBR1.M3 overexpressing plants.

To investigate whether KRPs are KBR1 substrates we compared the KRP2 protein levels in wild-type, *KBR1.M2*^{OE}, and *KBR1.M3*^{OE} plants. Protein blot analysis using a specific antibody against KRP2 (Verkest et al., 2005) showed accumulation of KRP2 in the transgenic lines when compared to wild-type plants (Figure 5). This suggests that KBR1 functions in the degradation of KRPs.

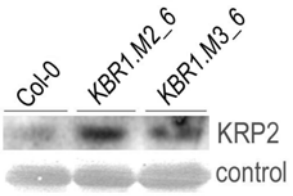


Figure 5. Immunoblot analysis on protein extracts from three-week old wild-type (Col-0) and transgenic plants using an anti-KRP2 antibody. Rubisco protein levels visualized by Ponceau S staining act as loading control.

Identification of KBR1 interactors

The suggested lethality of wild-type *KBR1* overexpression in plants indicates an important role for this particular E3 ligase in *Arabidopsis*. Possibly KBR1 targets multiple substrates for degradation and/or functions in complex with several different ubiquitination involved proteins. In order to identify regulation partners and putative additional substrates of KBR1 a second two-hybrid screen was performed, using the KBR1 itself as a bait. The pGBT.KBR1 vector was co-transformed with the library in the yeast HF7c reporter strain. A total of 10^7 independent cotransformants were screened for their ability to grow on histidine free medium. A 4-day incubation at 30°C yielded 129 colonies that were tested for LacZ gene activation and used for plasmid DNA preparation for sequencing. Many different candidate interactors were isolated (Supplementary table). Confirmation of these interaction partners of KBR1 awaits retransformation experiments.

Among the different KBR1 interacting proteins 26S proteasome subunits and an ubiquitin-activating domain containing protein were found, corresponding with the proposed function for KBR1 in ubiquitin-mediated protein degradation. In addition, KBR1 showed interaction with several other RING finger motif containing proteins (RFMs). Moreover, KBR1 was found to homodimerize. These data suggest that KBR1 may form homo- and/or heterocomplexes for activation and/or recognition of a broad range of substrates. To investigate the mechanism of KBR1 dimerization in more detail, we analyzed the yeast two-hybrid interaction of KBR1 with its mutated forms. Also we tested the necessity of the intact RING domain for KRP and CDKA;1 interaction. All KBR1 alleles were found to interact with full length KBR1, the KRPs, and CDKA;1 (Table 2), indicating that the RING motif is dispensable for KBR1 oligomerization and binding to target proteins.

Table 2. Two-hybrid interaction of KBR1 mutants with KBR1, KRP5 and CDKs

	KBR1.FL	KBR1.M2	KBR1.M3	KRP5	CDKA;1	CDKB1;1
KBR1.FL	+	+	+	+	+	-
KBR1.M2	+	+	+	+	+	-
KBR1.M3	+	+	+	+	+	-

+ growth on medium –His (10 mM 3-AT); - no growth on medium –His (10 mM 3-AT)

Furthermore, several transcription factors (TF) were found to interact with KBR1 in the yeast two-hybrid screen. These were often isolated more than once, indicating that they may represent biological significant interaction partners and as such putative substrates of KBR1 (Table
3). All transcription factors were found to be induced by stress conditions according to the Digital Northerns provided by Genevestigator (Zimmermann et al., 2004), further implying a role for KBR1 in stress.

Table 3. Digital Northern (Genevestigator) of KBR1 interactors

KBR1 interacting Transcription Factors (TF)	Conditions inducing TF expression according to Genevestigator
MYB92 (At5g10280)	Every stress(root specific + cells)
Zn Finger (At1g34370)	Every stress (root specific)
TCP20 (HLH TF) (At3g27010)	Dormancy (potato buds), CHX
Nuclear protein (At3g09980)	Heat stress (SAM, flower)
TCP (At3g47620)	Every stress (shoot specific), light signaling
C2H2 Zn Finger (At2g02080)	Dormancy, senescence, seeds, UV light

CHX cycloheximide; SAM shoot apical meristem

Discussion

Ubiquitination plays a central role in regulating protein turnover and modification of proteins by the attachment of the polypeptide ubiquitin. The diversity and number of proteins regulated by ubiquitination predicts the existence of a high diversity of E3 ligases. Indeed, more than 5% of predicted *Arabidopsis* genes are proposed to encode ubiquitination and 26S proteasome activities related proteins, and numerous genes encoding RING Finger motif proteins (RFMs) have been isolated or predicted in higher plants (Kosarev et al., 2002, Vierstra 2003, Stone et al., 2005). RING domains are Zn^{2+}-binding structures that were first recognized for their ability to mediate protein-protein interactions (Saurin et al., 1996). More recently, they have been shown to be integral parts of a second major class, in addition to HECT-domain proteins, of ubiquitin-ligases in which the RING is essential for function (Borden, 2000; Freemont, 2000; Lorick et al., 1999). Several *Arabidopsis* RFMs are proven to mediate ubiquitination (Stone et al., 2005), but only for few of these biological functions and/or substrates are identified.

KBR1 possesses E3 ligase activity

Although future investigations are required to unravel the complex composition and activity mechanism of the KBR1 E3 ligase, our data suggest that KBR1 meets the criteria of a genuine E3 enzyme. Crude *in vitro* ubiquitylation assays indicated that the 304 amino-acid and RING-HC motif containing *Arabidopsis* protein KBR1 indeed harbors ubiquitin-ligase activity. However, the inability to reconstitute self-ubiquitylation of MBP-KBR1 with purified recombinant E1 and E2 enzymes indicates that an additional unknown factor, present in the wheat germ lysate, is necessary for its E3 function. KBR1 is a small protein and sequence analysis revealed no known protein-protein interaction domains, suggesting that KBR1 functions in complex with other proteins. Besides as single-polypeptide RING E3s, RING proteins exist as part of several types of multisubunit E3s and in complex with other RING proteins (Jackson et al., 2000). Although the performed yeast two-hybrid screen with KBR1 may not have revealed all interactions, no known subunits of multicomponent E3s were isolated, whereas many different associating RFMs were identified. Di- or polymerization of RING proteins often occurs as a general regulatory mechanism of RING E3 ligases. For example, the heterodimer mammalian BRCA1-BARD1 RING finger complex, involved in ubiquitination of RNA polymerase II, contains significant E3 activity, whereas individually BRCA1 and BARD1 display almost no ubiquitin-ligase activity *in vitro* (Hashizume et al., 2001; Starita et al., 2005). Similarly, KBR1 might require polymerization with other RFMs to be active. Alternatively, the KBR1 might need prior activation by posttranslational modifications. KBR1 was found to associate with CDKA;1 and was efficiently phopshorylated by CDKs. There are several examples where CDK phosphorylation plays regulatory roles in RFM E3 function (Elias et al., 2005; Hayami et al., 2005).

It is believed that part of the RFM function is simply to bind and activate E2s. *In vitro* enzymatic systems showed that single-subunit or heterodimeric RING proteins are sufficient and necessary to catalyze formation of multi-ubiquitin chains (Lorick et al., 1999). The multicomponent SCF and APC/C RING E3s also require the RING components of ROC1/Rbx1 and APC11 for ubiquitin-ligase activity. E2 binding of RFMs was shown to occur through their RING motif. As such, deletion or mutation of this motif results in loss of catalytic activity of most RFMs. A complete deletion of the RING motif of KBR1 indeed resulted in loss of E3 activity, suggesting that this motif is possibly involved in E2 binding. However, mutation of Zinc-chelating Cys residues had no effect on KBR1 *in vitro* ubiquitin-ligase activity. In the case of KBR1.M1 this can be explained by the fact that this mutation does not necessarily abrogate the RING domain, since KBR1 has two

possible RING motifs. Overexpression of this mutant allele of *KBR1* did not result in any transgenics, just as observed for the wild-type *KBR1* gene, indicating that *KBR1.M1* encodes still an active protein and that the overexpression of active *KBR1* interferes with the regeneration of transgenic lines. The observed *in vitro* activity of KBR1.M3 is more puzzling, as in this mutant allele both possible RING finger configurations should be abrogated. The viability of *KBR1.M3*^{OE} plants, however, indicates that KBR1.M3 is less active than wild-type KBR1. Indeed, mutations of Cys residues in RING domains do not always result in a total inactivation of E3 activity. E.g. one mutation of ROC1/Rbx1 destroyed ubiquitin-ligase activity, whereas another one only reduced it (Ohta et al., 1999). Evaluation of other KBR1 mutants could indicate a similar mechanism. Moreover, oligomerization of KBR1 could be required for the surface scaffold necessary for E2 binding. Although our data indicates that KBR1 dimerization occurs independent from the RING motif, other RFMs were reported to hetero-oligomerize through their RING motif (Wu et al., 1996; Stad et al., 2000). Perhaps heterodimerization of KBR1 with another RFM through its RING motif is not completely impaired by the KBR1.M3 mutation. In such a scenario, the residual E3 activity observed when evaluating KBR1.M3 results from intermolecular trans-ubiquitylation of KBR1 by E2-interaction of its complexing RFM.

KBR1 E3 activity regulates KRP abundance

In both fungi and animal cells, single- and multi-subunit RING E3s were shown to play critical roles during the cell cycle by promoting degradation of regulatory proteins (Tyers and Jorgensen, 2000; Vodermaier, 2004). In plants, the control of the cell cycle by RING E3 enzymes has not yet been established, but accumulating data points to their importance in this process. Down-regulation of Arath;Rbx1, the RFM component of the SCF complex, resulted in the accumulation of CYCD3;1 protein in *Arabidopsis* (Lechner et al., 2002). Consistent with a role for SCF E3s in cell cycle control *Arabidopsis* cullin *Cul1* loss-of-function mutants arrest in early embryogenesis at the zygote stage (Shen et al., 2002). Similarly, APC/C subunits *apc2* and *apc6/nomega Arabidopsis* knock-out lines are impaired in female gametophyte development, after meiosis (Capron et al., 2003b; Kwee et al., 2003). Interestingly, these arrested mutant gametophytes failed to degrade mitotic cyclin reporter proteins. Another example of cell cycle control was found in alfalfa where the APC/C activator CCS52 was shown to control the switch from the mitotic cycle to endoreduplication, an

alternative cell cycle during which DNA replication is not followed by mitosis and cytokinesis (Cebolla et al., 1999).

Because of their importance in cell cycle regulation and exit, CDK inhibitory proteins are strictly regulated. Yeast and mammalian inhibitors are mainly regulated through mechanisms that affect their abundance rather than their intrinsic activity. In mammalia the concentration of the CDK inhibitor $p27^{Kip1}$ is thought to be regulated predominantly by ubiquitin-dependent degradation. The best studied degradation pathway acts in the nucleus and requires $p27^{Kip1}$ phosphorylation at Thr187 by cyclinE/CDK2 complexes and subsequent recognition and degradation at the S-G2 phase by Skp2 (S-phase kinase associated protein2), a member of the F-box family of proteins that associates with Skp1, Cul1 and the RING finger protein ROC1/Rbx1 to form an SCF E3 complex (Montagnoli et al., 1999; Carrano et al., 1999; Tsvetkov et al., 1999). Moreover CKS1, which belongs to the highly conserved Suc1/Cks1 family of proteins that bind to some CDKs and phosphorylated proteins and are essential for cell cycle progression, is required for and enhances SCF^{Skp2} mediated ubiquitination of p27 (Ganoth et al., 2001; Spruck et al., 2001). More recently, the existence of a second ubiquitin-proteasome dependent pathway of $p27^{Kip1}$ degradation was identified (Malek et al., 2001; Hara et al., 2001). This Skp2- and Thr187 independent mechanism operates in the cytoplasm and degrades $p27^{Kip1}$ at the G0-G1 transition (Hara et al., 2001). Purification of the SCF^{SKP2}-independent E3 activity identified a RING protein containing E3 enzyme, designated KPC (Kip1 ubiquitylation-promoting complex) responsible for CDK phosphorylation independent ubiquitylation of $p27^{Kip1}$ (Kamura et al., 2004).

In plants little is known about the posttranslational regulation of CDK inhibitors. Previously, we demonstrated that the *Arabidopsis* CDK inhibitor KRP2 is an unstable protein and that its degradation depends on the presence of an active proteasome (chapter 3; Verkest et al., 2005). *In vitro* analysis illustrated that several KRPs are CDK substrates and KRP2 phosphorylation by CDKs was shown to be at least in part responsible for Arath;KRP2 proteolysis. Moreover, Arath;CKS1 seems to negatively regulate KRP2 function and protein stability (chapter 4). These data suggest the possibility that the *Arabidopsis* KRPs are degraded by a plant SCF^{Skp2}-like ubiquitin-ligase. Two F-box proteins, similar to the metazoan Skp2, have been identified in *Arabidopsis*. The plant SCF^{Skp2} functions in regulating the cell cycle by controlling the abundance of the E2Fc transcription factor (del Pozo et al., 2002). However, the demonstration that any KRP protein is degraded by this ubiquitin-ligase is still missing. The isolation and identification of the KRP interacting and ubiquitin-ligase activity containing RFM KBR1 indicated that it might control SKP-independent ubiquitin-dependent degradation of the *Arabidopsis* CDK inhibitors.

Although we were unable to prove *in vitro* binding and ubiquitylation of KRPs by KBR1 (data not shown), overexpression of dominant negative forms of the *KBR1* allele resulted in the accumulation of KRP2 protein levels in *Arabidopsis*. Further investigations are however required to verify that this increase in KRP2 protein level is the direct consequence of its lowered ubiquitin-dependent proteolysis.

KBR1 function links stress – cell cycle regulation?

Because of their sessile lifestyle, plant survival largely depends on their ability to adjust to a plethora of environmental conditions. Response to the environment is mediated by a myriad of regulatory pathways which integrate both intra- and extracellular signals to the regulation of plant growth. Stress responses can be divided into early and late responses. Upon perception of stress, plant growth is arrested to allow acclimation to the new conditions. After the transient growth arrest follows a recovery phase and growth is resumed. During these responses, the plant cell cycle is regulated through the levels and activities of key cell cycle regulators (Logemann et al., 1995; Schuppler et al., 1998; Burssens et al., 2000; Granier et al., 2000; West et al., 2004). For the moment it is, however, not known which mechanisms link the perception of stress directly to the cell cycle. Hormones do play roles in regulating cell cycle gene expression, but negative regulators also need to be removed before positive responses can re-occur. In mammalia, recent studies identified RFMs as candidates in mediating external signals to the cell cycle (Casanovas et al., 2000; Chou, 2004). Arath;*KBR1* may have a similar function.

In plants, it is suggested that D-type cyclins operate as primary sensors of external conditions (De Veylder et al., 2003; Dewitte and Murray, 2003). In such a model, CDK/CycD complexes might be important complexes to be inhibited upon the perception of stress signals. Interestingly, KRPs were demonstrated to inhibit CycD/CDK complexes (Jasinski et al., 2002, Schnittger et al., 2003; Zhou et al., 2003b; Cho et al., 2004; Coelho et al., 2005). The *Arabidopsis* KRPs moreover share the potential of the Kip/Cip inhibitors to respond to intrinsic and extracellular antimitogenic signals to arrest the cell cycle. *KRP1* transcripts are induced by cold treatment, which correlates with a decrease in CDK activity (Wang et al., 1998). Furthermore, *KRP1* expression was found to be induced by the phytohormone abscisic acid (ABA) (Wang et al., 1998), suggesting that this particular KRP might be in part responsible for the growth inhibitory effect triggered upon ABA treatment. By contrast, the mitogenic hormone auxin repressed *KRP2* transcript levels, both in cell cultures and in planta (Richard et al., 2001; Himanen et al., 2002). This means

that the conditional phenotypes of reduced growth and early senescence observed in plants overexpressing dominant negative forms of KBR1 could result in part from the accumulation of KRPs. Although, misexpression of *KRP1* in *Arabidopsis* trichomes induces cell death (Schnittger et al., 2003), and high overexpression of KRPs results in a yet to be analyzed senescence-like phenotype, no straightforward similarities are observed between *KBR1.M* and *KRP* overexpressing plants.

However, in addition to KRPs, KBR1 may target other substrates. A yeast-two hybrid screen using KBR1 as bait identified a number of stress-induced transcription factors as KBR1 interacting proteins, suggesting that these TFs might represent potential substrates for ubiquitin-dependent proteolysis. Since stress and cell division are mutually exclusive, KBR1 might ensure cell cycle activity by mediating degradation of these stress-induced TFs. As such, the hypersensitivity of *KBR1.M3*^{OE} plants to light stress might be due to the inability to resume cell division after stress adaptation as a consequence of the inability to degrade the TFs (Figure 6). Confirmation of this hypothesis, however, awaits extra experimentation. Also, the precise role of the stress-induced KBR1 interacting proteins in cell cycle regulation remains to be determined. Their tissue specificity indicates that they may mediate growth arrest in the meristem upon a broad range of stresses with some organ specificity (Table 3). A role in cell cycle regulation for other Myb TFs was proven (Ito et al., 2001). TCP20 is a transcription factor regulating *CYCB1;1* gene expression. Moreover, one of the KBR1 interactors, a nuclear protein with unknown function was independently identified as a yeast two-hybrid KRP interactor (Cropdesign), suggesting a possible interconnection between KRP and TF accumulation.

In conclusion, these data support a function of KBR1 in stress responses and imply a role for KBR1 as a positive regulator of the cell cycle by mediating degradation of negative cell cycle regulators (Figure 6). Even though further stress assessments are required to refine the conditions where KBR1 could be active we suggest a role for KBR1 in signaling with the environment.

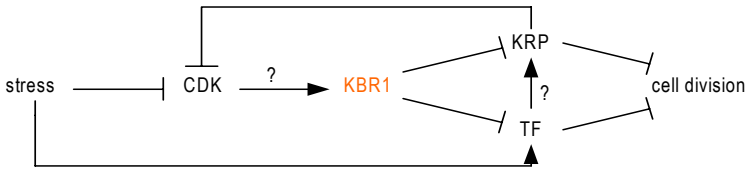


Figure 6. Hypothetical model of the role of KBR1 as positive regulator of the cell cycle through degradation of stress induced negative cell cycle regulators. For details, see text.

Methods

Yeast two-hybrid system

Vectors and strains used were provided with the Matchmaker Two-Hybrid System (Clontech, Palo Alto, CA). Baits were constructed by inserting PCR fragments into the pGBT9 vector. The pGAD424 and pGBT9 vectors containing KRP2, KRP4, KRP5, KRP6, KRP7, CDKA;1 and CDKB1;1 were constructed as described previously (De Veylder et al., 2001). The KBR1 cDNA was isolated using RNA prepared from *Arabidopsis thaliana* seedlings (ecotype Columbia (Col-0)) and the Superscript RT II kit (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. Primer sequences used can be given upon request. The KBR1 PCR fragment was cut with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of pGBT9 and pGAD424.
The GAL 4 activation domain cDNA fusion library was obtained from Clontech from mRNA of *Arabidopsis thaliana* cell suspensions harvested at various growth stages (De Veylder et al., 1999).
For the screenings a 500mL culture of the *Saccharomyces cerevisiae* strain HF7c (*MAT*$_a$ *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 URA3::GAL4*$_{17mers(3x)}$*-CyC1*$_{TATA}$*-LacZ*) was cotransformed with 250 µg pGBTKRP5 or pGBTKBR1, 500 µg DNA of the library, and 20 µg salmon sperm carrier DNA using the lithium acetate method (Gietz et al., 1992). To estimate the number of independent cotransformants, 1/1000 of the transformation mix was plated on Leu^- and Trp^- medium. The rest of the transformation mix was plated on medium to select for histidine prototrophy (Trp^-, Leu^-, His^-). After 4 days of growth at 30°C, the colonies larger than 2 mm were streaked on histidine-lacking medium supplemented with 10 mM 3-amino-1,2,4-triazole (Sigma, St. Louis, MO).
Of the His^+ colonies the activation domain plasmids were isolated as described (Hoffman and Winston, 1987). The pGAD10 inserts were PCR amplified using the primers 5'-ATACCACTACAATGGATG-3' and 5'-AGTTGAAGTGAACTTGCGGG-3'. Plasmid DNA was electroporated into *Escherichia coli* XL1-Blue, and the DNA sequence of the inserts was determined.

Generation of wild-type and mutant KBR1 proteins

The KBR1 coding region was PCR amplified with primers 5'-AAAAAGCAGGCTTCACAATGGCTGTTCAAGCTCAT -3' and 5'-AGAAAGCTGGGTCAAGAAGACATGTTAACATGC -3', and cloned into the GATEWAY pDONR207 vector (Invitrogen). Site-directed KBR1 mutants were generated by PCR of the KBR1 sequence in pDONR207 using the following pre-phosphorylated primers: 5'- CGATATGGTCATGAACGCTAGTGTGC -3' and 5'-GAAACCGGACAAGTCCGTAACAAAGC -3' for KBR1.M3 and 5'-CGGTGAGAGAGAAGCGAGTGTGTGGG -3' and 5'-GACCGTTTACAACCACCACCAACC -3' for KBR1.M1. The linear PCR products were circularized by ligation and the resulting plasmids were sequenced to confirm if the correct residue substitution took place. The wild-type and point mutated KBR1 sequences were amplified with primers 5'-GGGAATTCATGGCTGTTCAAGCTCATCACATGAAC –3' and 5'-GGGGATCCTCAAGAAGACATGTTAACATGCACACTAGC –3' and the truncated KBR1.M2 with primers 5'- GGGAATTCATGGCTGTTCAAGCTCATCACATGAAC –3' and 5'-AGAAAGCTGGGTCGGATCCTCAACTCGATTCCGCATCGTCTTCTACAACGG –3'. All PCR fragments were subsequently cloned in the EcoR1 and BamH1 sites of the pMal-2c vector (New England Biolabs).
The pMALKBR1.FL, pMALKBR1.M1, pMALKBR1.M2 and pMALKBR1.M3 plasmids were transformed in the *Escherichia coli* BL21-CodonPlus™(DE3)-RIL strain (Novagen, Madison, WI) to produce the recombinant proteins and were purified by amylose affinity chromatography according to the manufacturer's instructions (New England BioLabs).

In vitro ubiquitination experiments

Using the wheat germ lysate (Promega) as a source of E1 and E2, the ubiquitination assay of MBPKBR1 and its derivates was performed as followed. 150 ng purified recombinant MBP, MBPKBR1, MRPKBR1.M1, MBPKBR1.M2 or MBPKBR1.M3 was brought to 150 µl containing 300 ng/µl ubiquitin (Sigma, St Louis, Missouri, USA), 100 µM MG132 (Affiniti Research, Exeter, UK) and 10 µl wheat germ lysate in a ubiquitination buffer (40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT). The reaction mixture was incubated at the 30°C and 50 µl

aliquots were taken at the appropriate times for analysis by immunoblotting with an anti-MBP antibody (New England BioLabs).
For reconstitution of auto-ubiquitylation with E1 and E2, purified recombinant mouse E1 and HsUbcH5a were obtained from Biotrend. Ubiquitination assays were carried out in 30 µl reaction mixtures containing 20 ng each of E1 and UbcH5a, 500 ng MBP-KBR1.WT, and 10 µg ubiquitin in a 50 mM Tris pH 7.4, 2 mM ATP, 5 mM $MgCl_2$ and 2 mM DTT buffer.

Regeneration and molecular analysis of transgenic lines and growth conditions

The KBR1, KBR1.M1, KBR1.M2 and KBR1.M3 coding regions were PCR amplified and cloned into the GATEWAY pDONR207 vector (Invitrogen). Information about the primer sequences used can be given. After recombination with pH7WG2D, the obtained plasmids were mobilized by the helper plasmid pRK2013 into the *Agrobacterium tumefaciens* C58C1Rif^R harboring the plasmid pMP90 (Koncz and Schell, 1986). *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia-0 (Col-0) was transformed by the floral dip method (Clough and Bent, 1998).
Transgenic plants were selected on hygromycin-containing medium. Plants were grown under a 16-h light/8-h dark photoperiod at 22°C on germination medium, unless otherwise stated (Valvekens et al., 1988). Molecular analysis of the obtained transformants was performed by RNA gel blot analysis and protein blotting as described before (Verkest et al., 2005).

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Supplementary table.

Binding clone	Function	Isolated (×)
At1g45976	Putative S-ribonucl. bind. prot. SBP1,RFM	17
At4g19700	KBR1	10
At5g47050	Unknown, RFM	6
At1g43700	VirE2-interacting protein VIP1	6
At4g24690	Putative protein (Ub-associating domain)	4
At1g60610	Unknown, RFM	4
At1g34370	Zn finger protein	4
At3g27010	Unknown protein, TCP (HLH TF)	4
At5g45100	Unknown, RFM	3
At3g09980	Unknown, nuclear protein	2
At5g10280	MYB TF	2
At2g02080	Putative C2H2-type Zn finger protein	2
At5g13920	Putative protein, TCP	2
At5g42980	Thioredoxin	1
At1g13440	Glyceraldehyd-3-P dehydrogenase	1
At3g03460	Hypothetical	1
At3g27360	HistonH3	1
At3g27960	Hypothetical	1
At5g58290	26S proteasome AAA-ATPase subunit RPT3	1
At2g37290	Hypothetical	1
At3g12920	Expressed protein, RFM	1
At4g25530	Homeobox protein FWA	1
At1g52410	Myosin-like protein	1
At5g49160	DNA-methyltransferase	1
O04905	Uridylate kinase	1
O64903	Nucleoside diphosphate kinase	1
At1g31780	Unknown	1
At3g13670	Casein kinase, putative	1
At3g47620	Putative protein	1
At1g20640	Nodule inception protein, putative	1
At1g28210	Putative mitochondrial protein AtJ1	1
At5g51110	Putative protein	1
At1g28290	Prolin-rich protein, putative	1
AC009322_7	Putative splicing factor Prp8	1
At2g05650	En/Spm-like transposon protein	1
At5g16720	Putative protein	1
At1g51950	Aux. regul. protein IAA18 putat.	1

Summary/Samenvatting

Summary

In eukaryotic cells, progression through the cell cycle is governed by a suite of kinase complexes, which in their minimal configuration consist of a Ser/Thr kinase (the cyclin-dependent kinase; CDK) and a regulating cyclin subunit. CDKs phosphorylate a plethora of substrates, as such triggering the transition from one cell cycle phase into the next one. The sequential and transient activation of different CDK/cyclin complexes dictates the unidirectional progression through the cell cycle (chapter 1). Because of its importance in growth and development, CDK/cyclin activity must be strictly controlled. Negative control of cell cycle progression has always received extensive attention, especially in animal systems where the inability to correctly exit the cell cycle can eventually result in the formation of tumours. CDK activity can be inhibited by several mechanisms. Indirectly, kinase activity is inhibited by the controlled degradation of cyclin subunits. Direct inhibition of the catalytic activity occurs through the phosphorylation of specific residues located in the ATP-binding region of the CDK protein. Another active method of CDK inhibition involves the docking of mainly small proteins, known as CDK inhibitors (CKIs), which induce a cell cycle arrest or delay cell cycle progression in response to intracellular or extracellular signals.
The *Arabidopsis thaliana* genome contains seven *CKI* genes called *KRPs* (Kip related proteins), because of their homology with the mammalian CKIs of the Kip/Cip family (chapter 2). Overall, the KRPs display only low sequence identity to each other and the Kip/Cip inhibitors. Nevertheless, the plant *KRP* genes encode functional CDK inhibitors. Interaction analysis demonstrated that the KRPs bind D-type cyclins and A-type, but not B-type, CDKs. In addition, the KRPs were shown to be true biochemical homologues of the Kip/Cip proteins since recombinant KRPs are able to inhibit CDK activity *in vitro*, whereas their overexpression in plants results in a decrease in kinase activity *in vivo*. Also, they share the potential of the Kip/Cips to integrate developmental signals with the core cell cycle machinery as illustrated by their transcriptional regulation by plant hormones. Further transcriptional regulation of the plant inhibitors occurs during development and the cell cycle. However, despite accumulating information, little is known about the regulation of the plant CDK inhibitors at the post-transcriptional and post-translational level. Moreover, even though the ability of KRPs to act as CDK inhibitors is becoming well established, information is lacking regarding their mechanism of CDK binding and inhibition and possible other KRP functions.

Here, we identified a new role for KRP2 as activator of the mitosis-to-endocycle transition (chapter 3). *Arabidopsis* KRP2 gain-of–function plants display a positive or negative effect on the DNA ploidy level, depending on the level of *KRP2* overexpression. The inhibition of the endocycle in strong *KRP2*^{OE} plants is in agreement with previous reported *KRP*^{OE} studies, but is apparently in contradiction with the observed stimulation of the endocycle in weak *KRP2*^{OE} plants. We showed that constitutive expression of *KRP2* slightly above its endogenous level only inhibited the mitotic cell cycle-specific CDKA;1 kinase complexes, whereas the endocycle-specific CDKA;1 complexes were unaffected, resulting in an increase in the DNA ploidy level. An identical effect on the endocycle could be observed by overexpressing *KRP2* exclusively in mitotically dividing cells. In agreement with a role for KRP2 as activator of the mitosis-to-endocycle transition, KRP2 protein levels were more abundant in endoreduplicating than in mitotically dividing tissues. We illustrated that KRP2 protein abundance is regulated posttranscriptionally through CDK phosphorylation and proteasomal degradation. KRP2 phosphorylation by the mitotic cell cycle-specific CDKB1;1 kinase suggests a mechanism in which CDKB1;1 controls the level of CDKA;1 activity through regulating KRP2 protein abundance. In accordance to this model, KRP2 protein levels increased in plants with reduced CDKB1;1 activity. Moreover, the proposed model allowed a dynamical simulation of the *in vivo* observations, validating the sufficiency of the regulatory interactions between CDKA;1, KRP2, and CDKB1;1 in fine-tuning the mitosis-to-endocycle transition.

More detailed analysis of the effect of CDK phosphorylation on KRP2 function and stability illustrated that KRP2 phosphorylation by CDKs enhances its degradation and results in loss of its binding and inhibitory specificity towards CDKA;1 kinases (chapter 4). Interestingly, mitotic CDKB1;1/cyclin complexes do not merely phosphorylate KRP2, marking it for destruction, but also only bind phosphorylated KRP2. This suggests a mechanism where multisite phosphorylation by CDK complexes followed by B-type CDKs enforces KRP2 proteolysis. We have shown as well that *Arabidopsis* CKS1 regulates KRP2 inhibitory activity. Overexpression of *CKS1* resulted in plants with reduced KRP protein levels due to posttranslational regulation. In agreement with a role for CKS1 in KRP2 proteolysis, CKS1 enhanced the CDKB1;1-dependent phosphorylation of KRP2, marking it for destruction. In addition, by competing for CDKA;1 binding, CKS1 counteracts KRP2-mediated CDKA;1 inhibition, suggesting that KRP2 bound CDKA;1/cyclin complexes do not contain CKS1 and vice versa. In contrast, phosphorylated KRP2 is able to bind trimeric B-type, but not A-type, CDK/cyclin/CKS1 complexes. Although the structural basis for these different

complexes still needs to be examined, we suggest that KRP2 associates CDK/cyclin complexes differentially as inhibitor than as substrate, and hypothesize that CKS1-containing CDK/cyclin complexes only bind KRP2 as a substrate. As such, CKS1 helps in phosphorylating KRP2 by CDKs and triggers KRP2 degradation by a yet to be identified ubiquitin-ligase.

To identify the active region(s) of KRP2, a deletion and mutational analysis was performed (chapter 5). Deletion of the N-terminal domain enhanced the effects of *KRP2* in transgenic plants and illustrated a role for the N-terminal sequence in regulating KRP protein stability and CDKA;1 affinity. In contrast, deletion of the C-terminal domain completely abolished the CDK binding activity of KRP2, demonstrating its necessity for kinase inhibition. The identification of the plant-specific cyclin binding domain showed that, even though it efficiently complexes CDKA;1/cyclin complexes, it is insufficient for kinase inhibition. Likewise, point mutations made in the CDK binding domain of KRP2 confirmed the importance of this domain in CDK inhibition. Combined these data demonstrate that CDK inhibition by KRP2 requires the presence of both the CDK and cyclin binding domains of KRP2 and suggests that CDK/cyclin binding by KRPs, similarly to the mammalian Kip/Cip CKIs, mainly occurs through a sequential mechanism initiated by cyclin interaction. Analysis of KRP2 mutants with impaired CDK inhibitory activity indicated a possible dual role for KRP2 as both negative and positive CDK regulator. Overexpression of *KRP2.M3* resulted in *Arabidopsis* plants with a prolonged endocycle program due to an activation of endocycle CDKA;1 complexes, illustrating a role of KRP2 in the endocycle. The mechanism of CDK activation still needs to be investigated but KRP2 possibly acts as targeting and/or assembly factor of “insensitive” endocycle-specific CDK complexes.

Although KRP2, among other plant cell cycle proteins, was shown to be regulated by proteolysis it is currently not known which ubiquitin-ligase is responsible for KRP degradation. A two-hybrid screen using the *Arabidopsis* KRP5 led to the identification of a RING finger motif containing protein, designated KBR1 (chapter 6). RING protein genes encode candidate ubiquitin-ligases functioning in protein degradation. We show that KBR1 displays *in vitro* ubiquitin-ligase activity and interacts with different KRPs. Moreover, KBR1 was found to associate CDKA;1 and to be an *in vitro* CDK substrate, further empathizing that KBR1 functions in cell cycle regulation. Analysis of the KRP protein level in *Arabidopsis* plants overexpressing truncated and mutated negative forms of the *KBR1* allele demonstrated accumulation of KRP2, illustrating that KBR1 regulates KRP proteolysis. The identity of the KBR1 containing ubiquitin-ligase awaits further examination. Interestingly, dominant negative *KBR1* transgenic plants point to a

role of KBR1 in the regulation of plant growth during stress. Accordingly, a screening for putative KBR1 substrates led to the isolation of several stress-induced interactors. Based on our data we propose a role for KBR1 as positive regulator of the cell cycle by mediating degradation of negative cell cycle regulators. Also we suggest that KBR1 might provide a possible interconnection between signalling with the environment and the cell cycle.

Samenvatting

In eukaryote cellen is de progressie doorheen de celcyclus gereguleerd door een groep kinase complexen bestaande uit een Ser/Thr kinase (het cycline-afhankelijke kinase; CDK) en een regulerende cycline sub-unit. De transitie van de ene naar de andere celcyclus fase wordt veroorzaakt door de fosforylatie van verschillende CDK substraten. Aldus wordt de unidirectionele doorgang doorheen de celcyclus gedicteerd door de sequentiële en transiente activatie van verschillende CDK/cycline complexen (hoofdstuk 1). CDK/cycline activiteit is strikt gereguleerd. Negatieve controle van celcyclus progressie is sterk onderzocht, vooral in dierlijke systemen waar incorrecte celcyclus exit kan resulteren in tumorgenesis. De activiteit van CDKs wordt negatief gereguleerd door verschillende mechanismen. De celcyclus-afhankelijke degradatie van cyclines inhibeert CDK activiteit op een indirecte wijze, terwijl directe inhibitie van de katalytische activiteit geschiedt door fosforylatie van specifieke aminozuur residuen in het ATP-bindend domein van de CDKs. Een andere actieve methode van CDK inhibitie wordt veroorzaakt door binding van laag moleculair gewicht eiwitten, gekend als CDK inhibitoren (CKIs), die in respons op intra- of extracellulaire signalen een celcyclus arrest of vertraging veroorzaken.
Het *Arabidopsis thaliana* genoom bevat zeven *CKI* genen, die wegens hun homologie met de zoogdier CKIs van de Kip/Cip familie KRPs genoemd worden (Kip related proteins) (hoofdstuk 2). De KRPs vertonen slechts een lage sequentie identiteit onderling en tot de Kip/Cip inhibitoren. Niettemin, coderen ze functionele CDK inhibitoren. Interactie analyse toonde aan dat de KRPs D-type cyclines en A-type, maar niet B-type, CDKs binden. Bovendien werd aangetoond dat ze biochemische homologen zijn van de Kip/Cip eiwitten, daar recombinante KRPs *in vitro* CDK activiteit inhiberen, terwijl hun overexpressie in planten resulteert in een daling van de *in vivo* kinase activiteit. Eveneens delen de KRPs het potentieel van Kip/Cips om ontwikkelingssignalen te integreren in de celcyclus progressie, daar ze transcriptioneel gereguleerd worden door planthormonen. Verdere transcriptionele regulatie van de KRP inhibitors geschiedt tijdens plant ontwikkeling en gedurende de celcyclus. Ondanks deze kennis is er echter weinig geweten over de regulatie van de plant CDK inhibitoren op het posttranscriptioneel en posttranslationeel niveau. Voorts ontbreekt er informatie betreffende hun mechanisme van CDK binding en inhibitie en, alhoewel hun rol als CDK inhibitors reeds gevestigd is, over mogelijk andere functies van de KRPs.

Hoofdstuk 3 beschrijft de identificatie van een nieuwe rol voor KRP2 als activator van de transitie van de mitotische celcyclus naar de endocyclus. *Arabidopsis KRP2* overexpressie planten vertonen een positief of negatief effect op het DNA ploidy niveau, afhankelijk van het niveau van *KRP2* overexpressie. De inhibitie van de endocyclus in sterke *KRP2*^{OE} planten is in overeenstemming met andere gepubliceerde *KRP* overexpressie studies, maar is blijkbaar in tegenspraak met de waargenomen stimulatie van endocyclus in zwakke *KRP2*^{OE} planten. We toonden aan dat zwakke ectopische expressie van *KRP2* boven het endogeen niveau slechts de mitotische celcyclus-specifieke CDKA;1 kinase complexen inhibeert en de endocyclus-specifieke CDKA;1 kinase activiteit niet beinvloedt, wat resulteert in een toename van het DNA ploidy niveau. Een identiek effect op endocyclus werd waargenomen in planten die *KRP2* specifiek overexpresseren in mitotisch delende weefsels. In overeenstemming met een rol voor KRP2 als activator van de mitose-endocyclus overgang zijn KRP2 eiwit niveaus hoger in endoreduplicerende dan in mitotische weefsels. We illustreerden dat het KRP2 proteine posttranslationeel gereguleerd wordt door CDK fosforylatie en proteasomale degradatie. Fosforylatie van KRP2 door mitotische celcyclus-specifieke CDKB1;1 kinasen suggereert bovendien een mechanisme waarin CDKB1;1 het niveau van CDKA;1 activiteit controleert via regulatie van het proteine niveau van KRP2. In overeenstemming met dit model, accumuleert KRP2 in planten met verminderde CDKB1;1 activiteit. Eveneens liet het voorgestelde model een dynamische simulatie van de *in vivo* observaties toe, wat de rol van de regulerende interacties tussen CDKA;1, KRP2 en CDKB1;1 in de mitose-endocyclus transitie verder confirmeert.

Een meer gedetailleerde analyse van het effect van CDK fosforylatie op de KRP2 functie en stabiliteit illustreerde dat KRP2 fosforylatie door CDKs diens stabiliteit verlaagt en resulteert in verlies van diens bindings- en inhibitorische specificiteit voor CDKA;1 kinasen (hoofdstuk 4). Bovendien blijken mitotische CDKB1;1/cycline complexen niet enkel KRP2 te fosforyleren, maar binden ze eveneens gefosforyleerd KRP2. Dit suggereert een mechanisme waar multi-site fosforylatie door CDKA;1 complexen gevolgd door B-type CDK kinasen resulteert in KRP2 proteolyse. Eveneens toonden we aan dat *Arabidopsis CKS1* de KRP2 activiteit reguleert. Overexpressie van *CKS1* resulteerde in planten met lagere KRP eiwitniveaus ten gevolge van posttranslationele regulatie. In overeenstemming met een rol voor CKS1 in KRP2 proteolyse, verhoogde CKS1 de CDKB1;1-afhankelijke fosforylatie van KRP2, wat resulteert in KRP2 degradatie. Bovendien competeteert CKS1 met KRP2 voor CDKA;1 interactie wat de inhibitie van CDKA;1 complexen door KRP2 vermindert en suggereerd dat KRP2 gebonden CDKA;1/cycline complexen geen CKS1 binden en vice versa.

Gefosforyleerd KRP2 kan daarentegen wel trimere B-type, en niet A-type, CDK/cycline/CKS1 complexen binden. Hoewel de structurele basis voor deze verschillende complexen nog moet worden onderzocht, suggereren we dat KRP2 CDK/cycline kinasen verschillend bindt als inhibitor dan als substraat en stellen we dat CKS1 gecomplexeerde CDK/cycline complexen KRP2 enkel als substraat binden. Zodoende helpt CKS1 in de fosforylatie van KRP2 door CDKs en veroorzaakt het de degradatie van KRP2 door een nog niet geidentificeerd ubiquitine-ligase.

Teneinde de actieve regios van KRP2 te identificeren werd een deletie en mutationele analyse van KRP2 uitgevoerd (hoofdstuk 5). De deletie van het N-terminaal KRP2 domein versterkte het effekt van *KRP2* in transgene lijnen en suggereert een rol voor de N-terminale sequentie in de regulatie van KRP proteine stabiliteit en CDKA; 1 affiniteit. In tegenstelling, veroorzaakte de deletie van het C-terminaal domein het volledig verlies van de CDK bindende en inhiberende functie van KRP2. De identificatie van het plant-specifieke KRP2 cycline bindende domein toonde aan dat het, alhoewel het efficient CDKA;1/cyclines bindt, onvoldoende is voor kinase ihibitie. Eveneens bevestigden gerichte mutaties van residuen in de CDK bindende sequentie van KRP2 het belang van dit domein voor CDK inhibitie. Deze resultaten tonen aan dat CDK inhibitie de aanwezigheid van zowel het CDK als cycline bindende domein van KRP2 vereist. Dit suggereert dat CDK/cycline binding door KRPs, zoals de dierlijke Kip/Cip CKIs, voornamelijk geschiedt via een sequenteel mechanisme dat door cycline interactie geinitieerd wordt. De analyse van KRP2 mutanten met gewijzigde CDK inhiberende functies wijst bovendien op een mogelijke duale rol voor KRP2 als zowel negatieve als positieve regulator van CDKs. Overexpressie van *KRP2.M3* resulteerde in *Arabidopsis* planten met een verlengd endocyclus programma ten gevolge van de activatie van endocyclus-specifieke CDKA;1 complexen. Dit illustreert een regulerende functie van KRP2 in de endocyclus. Alhoewel het mechanisme van CDK activatie nog moet worden onderzocht functioneert KRP2 eventueel als targeting- en/of assemblagefactor van niet inhibeerbare endocyclus-specifieke CDK complexen.

Hoewel KRP2, net als andere plant celcyclus proteinen, gereguleerd wordt door proteolyse is het momenteel niet geweten welk ubiquitine-ligase de degradatie van KRPs veroorzaakt. Een gist two-hybrid screening met *Arabidopsis* KRP5 leidde tot de identificatie van een RING finger motief bevattend eiwit genaamd KBR1 (hoofdstuk 6). RING finger proteinen zijn kandidaat ubiquitine-ligasen die in eiwitdegradatie functioneren. We toonden aan dat KBR1 *in vitro* ubiquitine-ligase activiteit bezit en interageert met verschillende KRPs. Voorts bindt KBR1 CDKA;1 en is het een *in vitro* CDK substraat, wat verder suggereert dat KBR1 een rol in

de celcyclus heeft. Analyse van het KRP eiwitniveau in *Arabidopsis* planten die negatieve vormen van het KBR1 alleel overexpresseren toonden accumulatie van KRP2 aan en illustreren dat KBR1 KRP proteolyse regelt. Bovendien, suggereren deze transgene planten dat KBR1 een functie heeft in de regulatie van plantengroei tijdens stress. Dusdanig, resulteerde een screening naar vermeende KBR1 substraten tot de isolatie van verscheidene stress geinduceerde interactors. Gebaseerd op onze gegevens stellen we een functie van KBR1 als positieve regulator van de celcyclus voor, door degradatie van de negatieve regulators van de celcyclus te bemiddelen. Ook suggereren we dat KBR1 een mogelijke interconnectie vormt tussen het signaleren met de omgeving en de celcyclus.

Additional publications

Conditional, recombinase-mediated expression of genes in plant cell cultures

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Abstract

In plant cells, overexpression of critical genes can be hampered by deleterious effects on development that results in a counterselection of transgenic cells harboring the gene of interest. Inducible expression systems have been reported, but many of them show unwanted leaky expression. To circumvent this potential problem, a novel inducible system was developed based on two previously characterized systems: the CRE-*loxP* site-specific recombination system of bacteriophage P1 and the subcellular targeting of proteins by a mammalian glucocorticoid receptor (GR). By fusing the receptor domain of the rat GR to the carboxyl terminus of the CRE recombinase, a double-lock conditional transcriptional induction system was created that is highly useful to overexpress genes whose expression may block transgenic regeneration. Furthermore, because the designed vector utilizes the GATEWAY™ recombination technology, cloning was restriction- and ligation-free, thus rendering the vector suitable for high-throughput research. The system was tested in *Nicotiana tabacum* bright yellow-2 (BY-2) cells and its efficiency was demonstrated for the controlled overexpression of the *gus* reporter gene and a mutant allele of the A-type cyclin-dependent kinase (CDKA), which is known to be a potent inhibitor of the cell cycle.

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B1-type cyclin-dependent kinases are essential for the formation of stomatal complexes in Arabidopsis thaliana

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Abstract

Cyclin-dependent kinases (CDKs) are key regulators of the cell cycle. In yeast, only one CDK is sufficient to drive cells through the cell cycle, whereas higher eukaryotes developed a family of related CDKs. Curiously, plants contain a unique class of CDKs (B-type CDKs), whose function is still unclear. We show that the *CDKB1;1* gene of *Arabidopsis* (*Arabidopsis thaliana*) is highly expressed in guard cells and stomatal precursor cells of cotyledons, suggesting a prominent role for B-type CDKs in stomatal development. In accordance, transgenic *Arabidopsis* plants with reduced B-type CDK activity had a decreased stomatal index because of an early block of meristemoid division and inhibition of satellite meristemoid formation. Many aberrant stomatal cells were observed, all of them blocked in the G2 phase of the cell cycle. Although division of stomatal precursors was inhibited, cells still acquired stomatal identity, illustrating that stomatal cell differentiation is independent of cellular and nuclear division.

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