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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 [W]

Aurine Verkest,[a] Carmem-Lara de O. Manes,[a,1] Steven Vercruysse,[a] Sara Maes,[a] Els Van Der Schueren,[a] Tom Beeckman,[a] Pascal Genschik,[b] Martin Kuiper,[a] Dirk Inzé,[a,2] and Lieven De Veylder[a]

[a] Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, B-9052 Gent, Belgium

[b] Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, F-67084 Strasbourg Cedex, France

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 A-type cyclin-dependent kinase CDKA;1 and its specific inhibitor, the Kip-related protein, 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 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 with 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 (Joubès et al., 1999; Kondorosi et al., 2000; Larkins et al., 2001). 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

[1] Current address: Observatoire Océanologique de Banyuls sur Mer, Laboratoire Arago, Centre National de la Recherche Scientifique, Avenue du Fontaulé, BP 44, F-66651 Banyuls sur Mer, France.

[2] To whom correspondence should be addressed. E-mail dirk.inze@psb.ugent.be; fax 32-9-3313809.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Lieven De Veylder (lieven.deveylder@psb.ugent.be).

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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 maize (*Zea mays*) endosperm and tomato (*Lycopersicon esculentum*) 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 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; Mironov et al., 1999; Sherr and Roberts, 1999). A likely candidate involved in CDK inhibitory phosphorylation during endoreduplication is the WEE1 kinase that is upregulated 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 from the mitotic cycle to the endocycle (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; Shcherbata 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, 1998, 2000; Lui et al., 2000; De Veylder et al., 2001; Zhou et al., 2002). Overproduction of KRPs in Arabidopsis resulted in plants with small

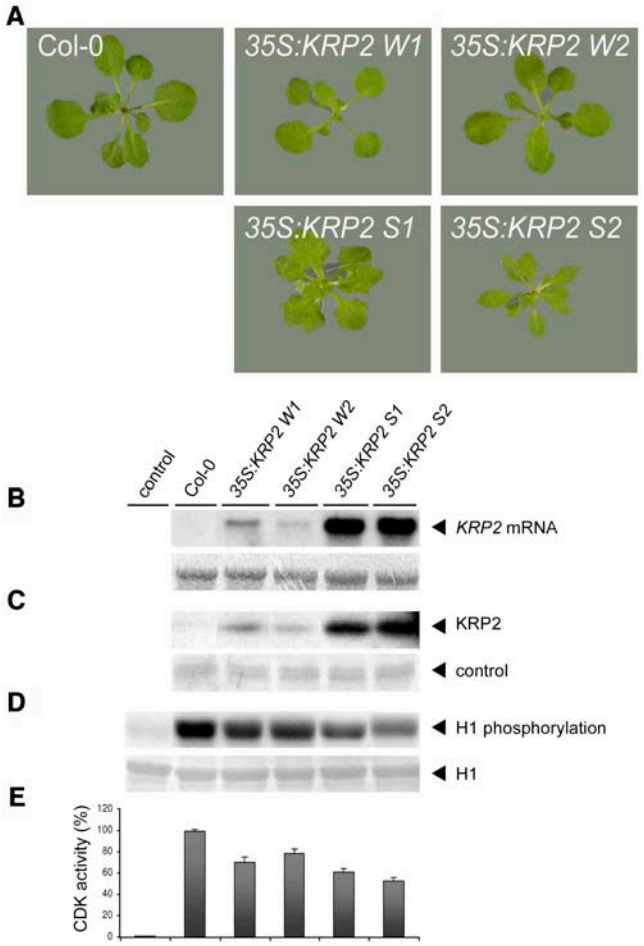

Figure 1. Phenotypic and Molecular Analysis of $KRP2^{OE}$ Arabidopsis Plants.

Three-week-old transgenic plants were compared with untransformed control plants (Columbia-0 [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. Ribulose-1,5-bisphosphate carboxylase/oxygenase protein levels visualized by Ponceau S staining act as a 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	13,532 ± 875	1,160 ± 33
CaMV 35S:KRP2 W1	11.08 ± 0.38	11,104 ± 861	1,033 ± 78
CaMV 35S:KRP2 W2	10.25 ± 0.30	10,712 ± 898	968 ± 74
CaMV 35S:KRP2 S1	7.68 ± 0.37	1,895 ± 205	4,330 ± 494
CaMV 35S:KRP2 S2	8.98 ± 0.23	1,770 ± 93	5,250 ± 358

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

and serrated leaves because of 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 in 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 an 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 HA-KRP2 levels 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 to 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).

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 with 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 coimmunoprecipitated with KRP2 correlated with that of the

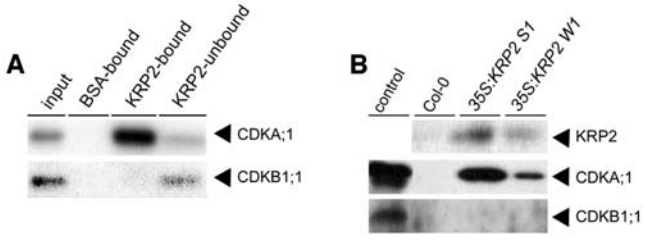

Figure 2. Specific in Vitro and in Vivo CDKA;1 Binding by KRP2.

(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 one-thirtieth 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 (one-tenth of the amount loaded) from untransformed plants was used as control.

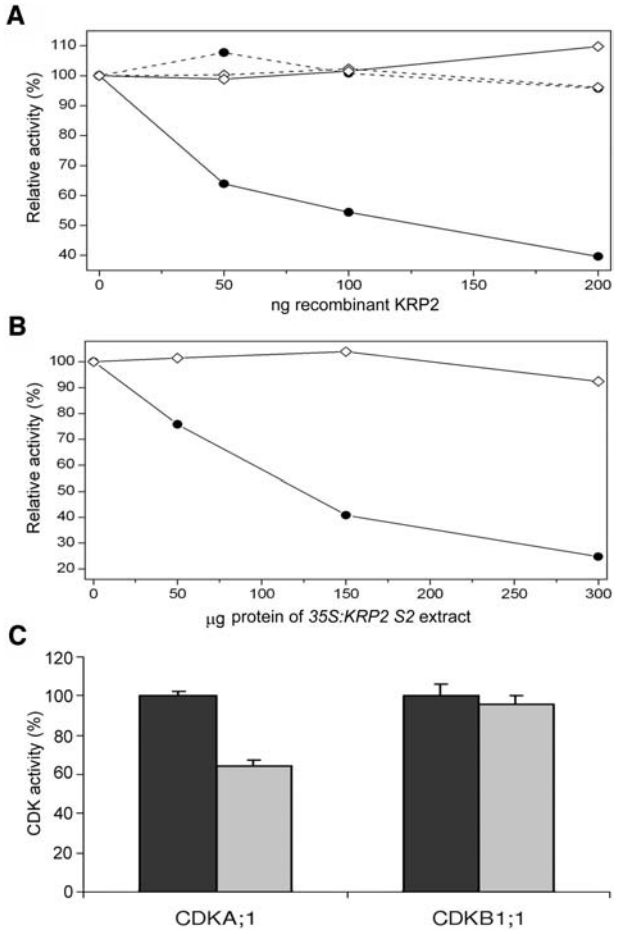

Figure 3. Specific in Vitro and in Vivo Inhibition of CDKA;1 Kinase Activity by KRP2.

(A) Effect of recombinant KRP2 protein on A- and B-type CDK activity. KRP2 (solid 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-d-old wild-type (Col-0) plants. Kinase activity was measured with histone H1 as substrate.

(B) Kinase activity measurements of immunoprecipitated CDKA;1 (filled dots) and CDKB1;1 (open diamonds) 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) (gray) 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 ($n = 3$).

KRP2 protein, although not all CDKA;1 proteins were pulled down. CDKB1;1, on the other hand, did not coimmunoprecipitate with KRP2 (Figure 2B).

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., 2002). 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 (Figures 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 with 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.

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., 2002; Zhou et al., 2002; Schnittger et al., 2003). By contrast, a reproducible increase in the DNA ploidy level was observed in independent weak *KRP2*^{OE} lines, 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 was also seen in cotyledons (data not shown).

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

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).

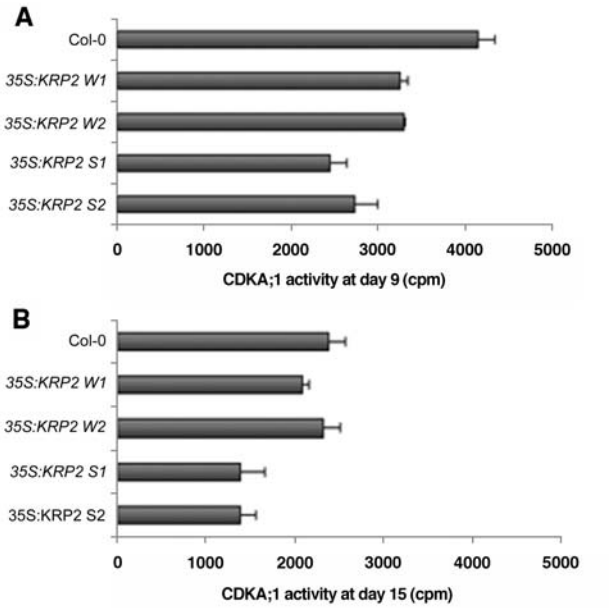

Figure 4. KRP2 Dose-Dependent Control of the Endoreduplication Cycle.

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. Data represent average ± SD ($n = 2$).

(A) Nine-day-old mitotically dividing leaves.

(B) Fifteen-day-old endoreduplicating leaves.

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 d after sowing (DAS). Leaves of 9-d-old plants predominantly divide mitotically, whereas those of 15-d-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 (Figures 4A and 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 ~40 and 20%, respectively (Figure 4A). By contrast, at 15 DAS, kinase activity was inhibited by ~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 a role in both the mitotic cell cycle and endocycle were inhibited, whereas in the weak *KRP2*^{OE} lines, mainly the mitotic CDKA;1 complexes were targeted.

Inhibition of the Cell Cycle in Mitotically Dividing Cells Triggers the Mitosis-to-Endocycle Transition

To confirm that a specific inhibition of the mitotic CDKA;1/cyclin complexes accounts for the premature onset of endoreduplication,

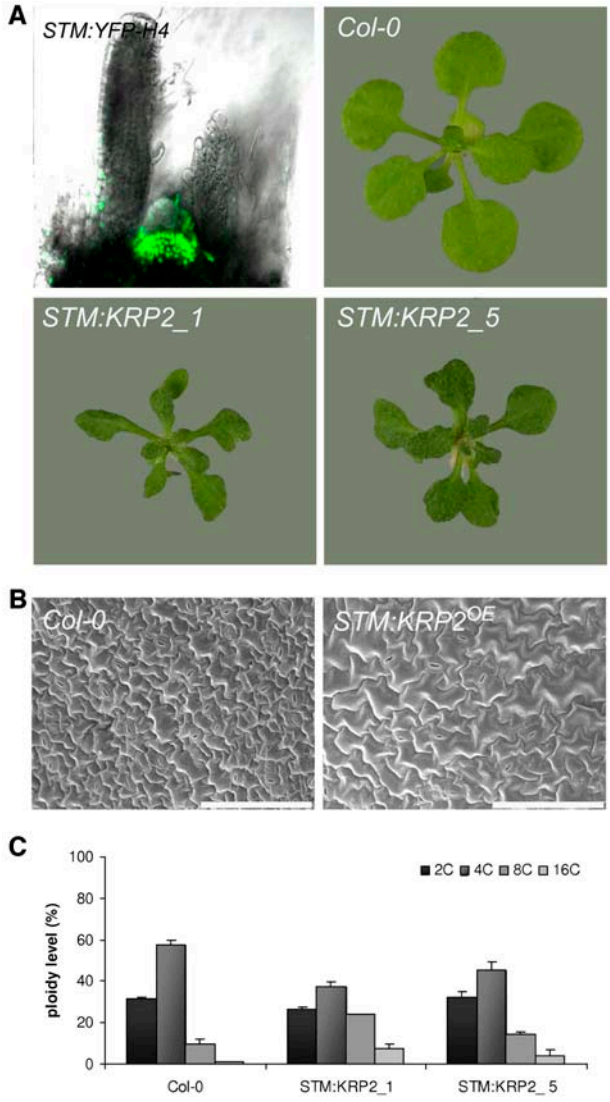

Figure 5. Meristem-Specific Overexpression of *KRP2*.

(A) Expression pattern of the *STM* promoter as visualized by confocal microscopy on 5-d-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. 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 ± SD ($n = 3$ to 5).

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 and Barton, 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-d-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 cells of the hypocotyl 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 by 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 with the strong *CaMV 35S 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).

Previously, we have illustrated that the onset of the endoreduplication cycle in the leaf is developmentally regulated (Boudolf et al., 2004a; Beemster et al., 2005; Vlieghe et al., 2005). Cells of the first leaf pair were found to divide until ~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 is 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 identical to that of wild-type plants (Figure 6A). By 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 in a more rapid entry into the endoreduplication cycle.

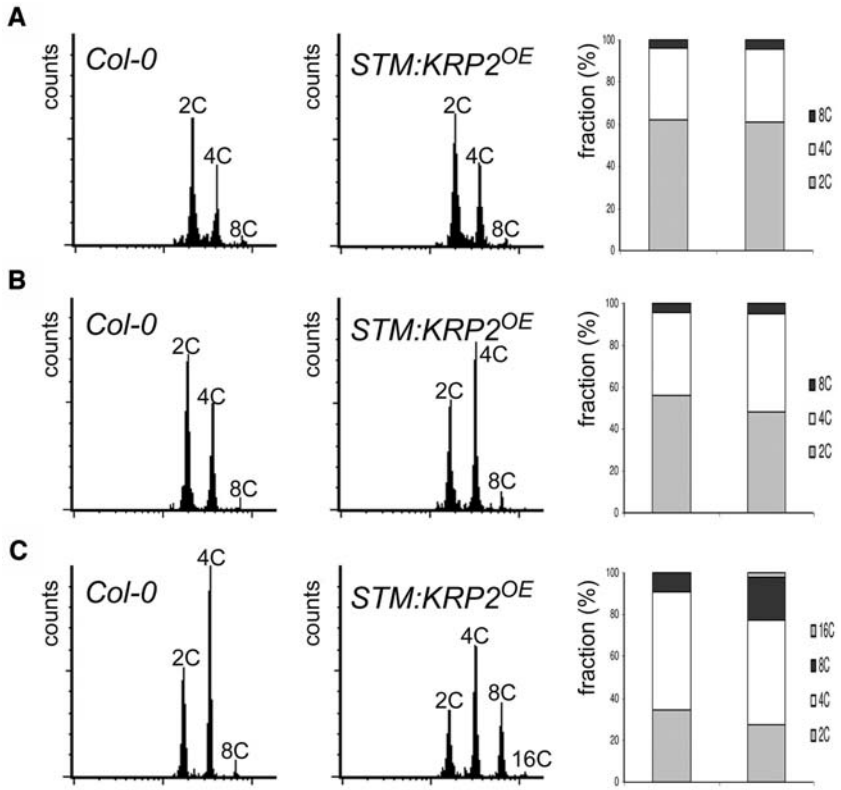

Figure 6. Ploidy Level Distribution of the First Leaves of Wild-Type (Col-0; Left) and *STM*:*KRP2*^{OE} (Line 5; Right) Plants during Development as Measured by Flow Cytometry.

(A) Eight DAS.
(B) Ten DAS.
(C) Twelve 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 semi-quantitative 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. By 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 and 7B).

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). By 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 preincubated 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-d-old seedlings was compared between wild-type plants and plants harboring reduced CDKB1;1 activity as a result of 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).

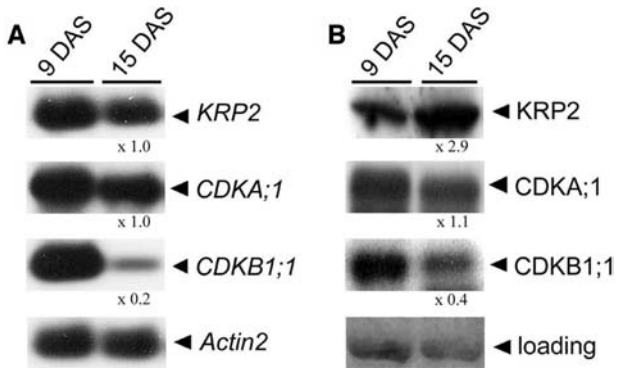

Figure 7. Expression Analysis of *KRP2* during Leaf Development.

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. Results of relative quantification using the respective loading controls are indicated below each gel image.

(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 ribulose-1,5-bisphosphate carboxylase/oxygenase protein levels by Ponceau S staining of the membrane.

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 previously reported KRP^{OE} studies (De Veylder et al., 2001; Jasinski et al., 2002; 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 previous 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 (our unpublished results). Although the

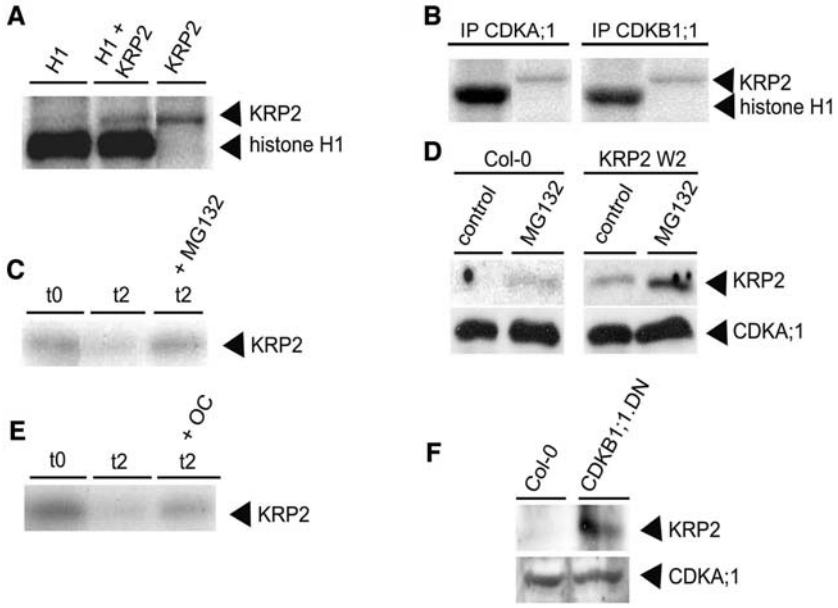

Figure 8. CDK-Dependent Phosphorylation and Proteasome-Mediated Degradation of KRP2.

(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-d-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-d-old first leaf pairs of wild-type (Col-0) and *CDKB1;1.N161*^{OE} (*CDKB1;1.DN*) plants.

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 CDKA;1/cyclin complexes, whereas in the strong *KRP2*^{OE} lines, CDKA;1/cyclin 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. By contrast, in the strong *KRP2*^{OE} lines, both the mitotic cell cycle and the 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/cyclin A 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

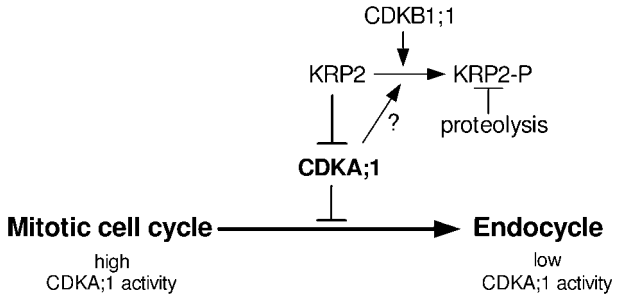

Figure 9. Model Illustrating the Role of CDK Activity in Controlling the Onset of Endoreduplication.

For details, see text.

of endoreduplication is correlated with a decrease in CDK activity. Also, in maize endosperm and tomato fruits, the onset of endoreduplication is accompanied by 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 prereplication complex (Bryant et al., 2001; Nishitani and Lygerou, 2002). At the onset of S-phase, prereplication complexes are activated, resulting in DNA replication. For several 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 the 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 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 the 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 the CDK activity gradually decreases as cells move away from the meristem. As long as the CDK activity is above a certain threshold, cells divide, but once the kinase activity drops below the threshold, cells stop proliferating

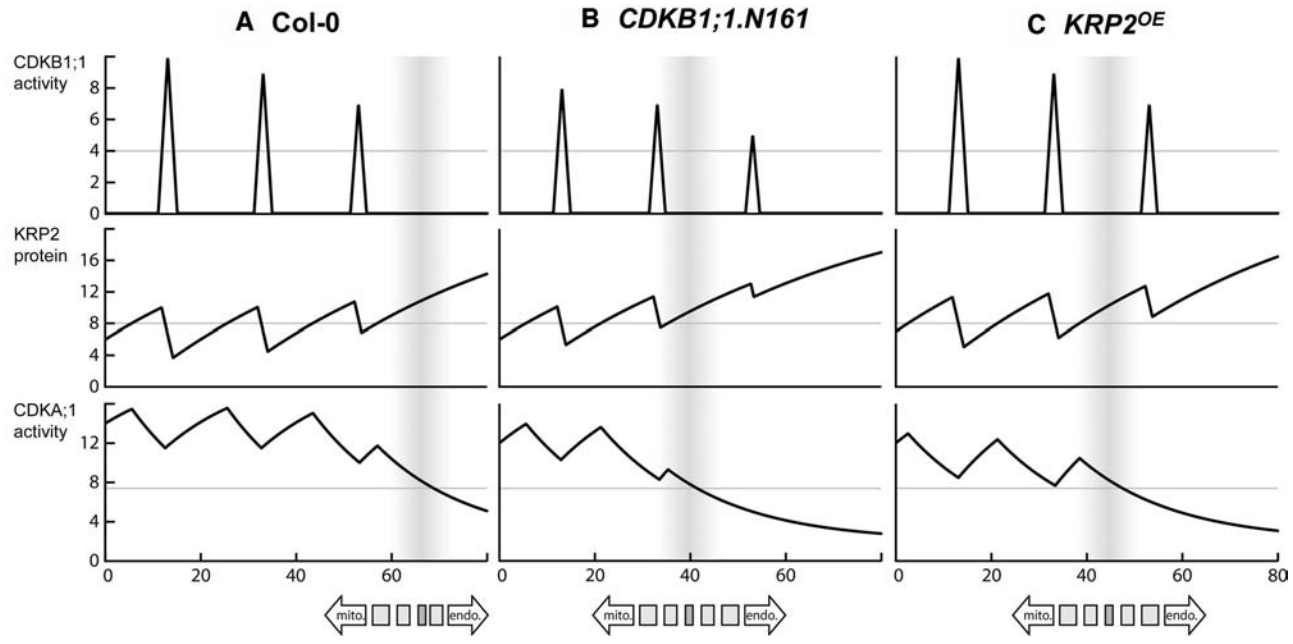

Figure 10. Simulation of the Onset of Endoreduplication.

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 of 8. CDKA;1 activity first remains above a level that is assumed to be necessary to maintain mitotic division. In the gray-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.

(A) Wild-type (Col-0) plants.

(B) *CDKB1;1.N161*-overexpressing plants.

(C) *KRP2*^{OE} plants.

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 the 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 upregulated as cells exit the mitotic cycle and begin to differentiate (Polyak et al., 1994; Parker et al., 1995). In mice deficient in $p27^{Kip1}$ and $p57^{Kip2}$, 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 Cip/Kip homolog, Dacapo (de Nooij et al., 1996; Lane et al., 1996).

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 have shown that the 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 the 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 several dynamical simulations using the SIM-plex toolpack (Vercruysse and Kuiper, 2005). This software uses the piecewise linear differential equations 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, the CDKA;1 activity oscillated during the cell cycle, but remained high enough to enable mitotic divisions (Figure 10A). Only when the CDKB1;1 activity dropped, KRP2 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 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 because of a faster decrease in CDKA;1 activity (Figure 10C). All simulation scenarios can be viewed and tested at http://www.psb.ugent.be/cbd/papers/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.

METHODS

Regeneration and Molecular Analysis of Transgenic Lines and Growth Conditions

The coding region of KRP2 was amplified by PCR and cloned in the pREP42-HA vector (Craven et al., 1998). A HA-tagged version of KRP2 was obtained by *Nco*I and *Bam*HI 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 from the pH35S.HA.KRP2 constructs and cloned into the *Eco*RI and *Sal*I sites of pBinPLUS (van Engelen et al., 1995) to obtain the pBIN.HA.KRP2 vector.

The *STM* promoter was amplified from the full BAC clone, BAC F2401, as a 4.5-kb fragment. A single ATG site was generated at its 3′ end, creating a *Nco*I 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 I. Negrutiu (Ecole Normale Supérieure, Lyon, France). pSTM was cloned into pTer-9 at the *Kpn*I/*Bam*HI 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* ecotype 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-d-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.

Semiquantitative 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 were 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 5′-GGCTCCTCTTAACCCAAAGGC-3′ and 5′-CACACCATCACCAGAATCCAGC-3′ for *actin2* (At3g18780); 5′-CGGAATAAGTTGTTGGAATGTTCTATGAAGTGT-3′ and 5′-GGCGGATCCTCATGGATTCAATTTAACCC-3′ for *KRP2* (At3g50630); 5′-CCTAGGATCTCATCATTACTCTACACC-3′ and 5′-CCATGTATCCTCGTACGGAGTTCC-3′ for *CDKA;1* (At3g48750); and 5′-GGTGGTGACATGTGGTCTGTTGG-3′ and 5′-CGCAGTGTGGAAACACCCGG-3′ 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 A_{600} nm of 0.5 to 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 lysed 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 nickel-nitrilotriacetic acid agarose resin (Qiagen, Hilden, Germany) according to the manufacturer's instructions and dialyzed against buffer 50 mM Tris-Cl, pH 7.5, 15 mM $MgCl_2$, 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 Cys] was synthesized, linked to keyhole limpet hemocyanin carrier protein, and used to immunize rabbits. The antiserum was immunoaffinity 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 tubes (Eppendorf, Hamburg, Germany) 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, and 0.05% Tween 20 for at least 1 h at room temperature and incubated overnight at 4°C with a 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-d-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 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, and 5 mM $MgCl_2$) supplemented with an ATP-regenerating system (35 mM phosphocreatine and 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) was 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-d-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 before 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 × 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 before 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 (San Jose, CA) for optimal presentation. Simulation conditions can be altered at http://www.psb.ugent.be/cbd/papers/krp2sim.

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