Multiple mechanisms explain how reduced KRP expression increases leaf size of **Arabidopsis thaliana**

Multiple mechanisms explain how reduced *KRP* expression increases leaf size of *Arabidopsis thaliana*.

Bulelani L. Sizani[1,*], Shweta Kalve[1,*], Marios N. Markakis[1], Malgorzata A. Domagalska[1], Joanna Stelmaszewska[1,2], Hamada AbdElgawad[1,3], Xin'ai Zhao[4], Lieven De Veylder[5,6], Dirk De Vos[1,7], Jan Broeckhove[7], Arp Schnittger[4] and Gerrit T.S. Beemster[1]

[1]Laboratory for Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, 2020, Belgium

[2]Department of Reproduction and Gynecological Endocrinology Medical University of Bialystok, 15-089, Bialystok, Poland

[3]Department of Botany and Microbiology, Faculty of Science, Beni-Suef University, 62521 Beni-Suef, Egypt

[4]Department of Developmental Biology, University of Hamburg, 22609, Germany

[5]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052, Belgium

[6]VIB Center for Plant Systems Biology, Ghent, 6052, Belgium.

[7]Department of Mathematics and Computer Science, University of Antwerp, 2020, Belgium

[*]These authors contributed equally to this work

Author for correspondence:

Beemster, Gerrit TS

Tel: +32 3 265 34 21

gerrit.beemster@uantwerp.be

Acceptance date: 26 August 2018

Document statistics:

Total word count (excluding summary, references and legends):	6656
Summary:	198
No. of Figures:	10
No. of Tables:	2
No of Supporting Information files:	14 (Fig. S1-S9; Table S1-S2; Methods S1-S3)

SUMMARY

1. Although cell number generally correlates with organ size, the role of cell cycle control in growth regulation is still largely unsolved. We studied *kip related protein* (*krp) 4, 6 and 7* single, double and triple mutants of *Arabidopsis thaliana* to understand the role of cell cycle inhibitory proteins in leaf development.
2. We performed leaf growth and seed size analysis, kinematic analysis, flow-cytometery, transcriptome analysis by NGS and QRTPCR and mathematical modelling of G1/S and G2/M checkpoint progression of the mitotic and endoreplication cycle.
3. Double and triple mutants progressively increased mature leaf size, due to elevated expression of cell cycle and DNA replication genes stimulating progression through the division and endoreplication cycle. However, cell number was also already increased prior to leaf emergence, due to an increased cell number in the embryo. We show that increased embryo and seed size in *krp4/6/7* results from seed abortion, presumably reducing resource competition and that seed size differences contribute to the phenotype of several large-leaf mutants.
4. Our results provide a new mechanistic understanding of the role of cell cycle regulation in leaf development and highlight the contribution of the embryo to the development of leaves after germination in general.

Key words: Cell division, endoreplication, kinematic analysis, Kip Related Proteins, leaf development, seed abortion, *Arabidopsis thaliana*

INTRODUCTION

At molecular level, leaf growth is controlled by coordinated cell division and expansion activity (Kalve *et al.*, 2014). Leaf development is primary driven by cell proliferation that establishes cell number. Mature cell size is subsequently determined by cell expansion that in many species co-occurs with endoreplication (Schnittger *et al.*, 2003; Beemster *et al.*, 2005b; Gonzalez *et al.*, 2012). Consequently, smaller leaves are produced when the number and/or size of cells are reduced (Horiguchi *et al.*, 2005; Li *et al.*, 2005; Ferjani *et al.*, 2013). Frequently, defects in proliferation are associated with additional cell expansion. This phenomenon called compensation, as it reduces the effects of inhibited cell production on final organ size, and suggests the involvement of organ level growth control mechanisms (Beemster *et al.*, 2003; Ferjani *et al.*, 2007; Hisanaga *et al.*, 2015). Nevertheless, inhibiting cell cycle progression reduces leaf size (De Veylder *et al.*, 2001). Inversely, moderate stimulation increases leaf size (De Veylder *et al.*, 2002; Eloy *et al.*, 2012; Kumar *et al.*, 2015), whereas strong stimulation leads to over proliferation and stunted growth (De Veylder *et al.*, 2002).

Thus, although it is clear that cell proliferation is a major component of leaf growth, it is essential to understand how it is integrated in the developmental process. Leaves originate from stem cells at the shoot apical meristem. Developmental signals are perceived at the level of individual cells, where they feed into the control of cell proliferation, expansion (in conjunction with endoreplication) and differentiation (Beemster *et al.*, 2005a; Kalve *et al.*, 2014). Thus, a systems approach where cell cycle regulation is altered in combination with detailed developmental studies is required to gain a better mechanistic understanding of the role of cell proliferation in organ growth control.

Like in all other organisms, the plant cell cycle transitions between G_1 to S and G_2 to M are tightly controlled by cyclin-dependent kinase/cyclin (CDK/CYC) complexes, which initiate the onset of DNA synthesis and subsequently mitosis (Dewitte & Murray, 2003; Inzé & De Veylder, 2006). Among other factors, cell cycle progression is regulated at the posttranslational level by seven members of INHIBITOR/INTERACTION OF CYCLIN-DEPENDENT KINASE/KIP RELATED PROTEINS (ICK/KRPs) family that regulate CDK activity (Verkest *et al.*, 2005b). *KRPs* share a limited similarity to mammalian $p27^{Kip1}$ proteins (Wang *et al.*,

1997) and have been identified in various plant species, including Arabidopsis (Lui *et al.*, 2000; Zhou *et al.*, 2002) In Arabidopsis, *KRPs* share a conserved C-terminally located 31-amino-acid CDK-binding functional domain (Acosta *et al.*, 2011). Removal of this domain in *KRP1* leads to loss of CDK binding and inhibitory activity (Zhou *et al.*, 2003).

Domain studies demonstrated that *KRPs* have a CDK/CYC binding site to interact with A and B-type CDKs and D-type cyclins (Zhou *et al.*, 2002; Nakai *et al.*, 2006; Pettkó-Szandtner *et al.*, 2006; Van Leene *et al.*, 2010). In addition to the CDK/CYC binding site, *KRPs* contain several other functional domains and motifs, including a nuclear localization domain, a CDK phosphorylation site, protein degradation domain and coiled-coil domain (De Veylder *et al.*, 2001; Acosta *et al.*, 2011). Consistently, *KRPs* have been shown to localize in the nucleus (Jakoby *et al.*, 2006; Bird *et al.*, 2007). Putative *CDKA* phosphorylation sites were identified in *KRP3, 4, 6* and *7* (Acosta *et al.*, 2011), but their experimental verification and an assessment of their functionality is still pending. Several studies provided evidence that *KRPs* are regulated by protein turn-over (Kim *et al.*, 2008; Liu *et al.*, 2008; Ren *et al.*, 2008; Lai *et al.*, 2009). However, a possible degron has not been identified so far.

Ectopic expression of *KRP* genes in Arabidopsis results in small, serrated leaves with reduced cell number, but increased cell size (De Veylder *et al.*, 2001; Bemis & Torii, 2007; Jun *et al.*, 2013). Strong overexpression of *KRPs* inhibits cell cycle progression by blocking both G_1/S and G_2/M transition, resulting in plants with large cells and reduced endoreplication (Kumar & Larkin, 2017) as well as induction of cell death (Schnittger *et al.*, 2003). Conversely, weaker ectopic expression of *KRPs*, inhibits only mitotic entry and promotes endoreplication (Kumar & Larkin, 2017). When *KRP4* was overexpressed in the central domain of the shoot apex using the *CLV3* promoter, cell size was strongly increased, but cell-size homeostasis was restored once cells exited the expression domain (Serrano-Mislata *et al.*, 2015).

Despite a comprehensive understanding of the effects of *KRP* overexpression, only few studies have examined the effect of loss of *KRP* function, presumably because *KRPs* act redundantly and may compensate the loss of individual *KRP* genes (Cheng *et al.*, 2013; Schiessl *et al.*,

2014). In the present study, we describe the effect of concurrent knockout of *KRP4*, *6* and *7* on leaf growth. Our results show enhanced leaf growth in the *krp4/6/7* triple mutant due to enhanced cell proliferation and endoreplication. We also demonstrate that seed development in *krp4/6/7* is stimulated by the induction of seed abortion and that increased seed and embryo size in general contributes to enhanced seedling growth.

MATERIALS AND METHODS

Plant material and growth conditions

The T-DNA insertion lines *krp4* (At2g32710, SAIL_248_B06), *krp6-1* (At3g19150, SAIL_548_B03), *krp7-1* (At1g49620, GK_841D12) and *sgo1-1* (SK35523), were obtained from NASC and Zamariola *et al.*, (2013) respectively. *AVP1*, *GA20OX1*, *GRF5, APC10, AN3, SAUR19, 35S:ami-ppd*, *ARG* overexpressing lines and *samba, da1-1, eod1-1*, *gra2-D* were a kind gift from Dr. N. Gonzalez (Hu *et al.*, 2003; Horiguchi *et al.*, 2009; Gonzalez *et al.*, 2010; Eloy *et al.*, 2011; Vanhaeren *et al.*, 2014). Double and triple mutants were created by crossing as previously described by (Zhao *et al.*, 2017). Seeds were sterilized in 70% ethanol for 1 min and in 5% bleach for 10 min, rinsed three to four times with distilled water and sown on half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 1% sucrose and 0.8% plant tissue agar. After three days of stratification (DAS) at 4°C, to synchronize germination, plates were placed horizontally on benches cooled to 19°C in a growth chamber with an air temperature of 22°C, under long-day conditions (16 h : 8 h, light : dark, 80-90 $\mu mols^{-1}m^{-2}s^{-1}$ photosynthetically active radiation, supplied by fluorescent tubes (Osram Lumilux, Cool white).

Genotyping, RT-qPCR and gene constructs

Plants homozygous for *krp4*, *krp6-1* and *krp7-1* were identified by PCR (primers listed in Table S1). Total RNA was extracted from the whole rosette at the 1.04 stage (Boyes, 2001) using Trizol Reagent (Life Technologies) and purified with the RNeasy mini kit (Quiagen). The quantity of RNA was determined with a nanodrop ND 1000 (Thermo Scientific) and qualified using QIAxcel RNA (Qiagen). For RT-PCR, first-strand cDNA was synthesized from 2 µg of

total RNA according to "Maxima® First Strand cDNA Synthesis Kit" (Fermentas, Cambridgeshire). Construction and selection of transgenic lines is descibed in Methods S1.

Leaf size measurements

The first leaves to emerge (leaf one & two) were harvested at 26 days after sowing (DAS) for analysis of mature leaf size. Leaves were fixed overnight in absolute ethanol, cleared and mounted in 100% lactic acid (Emprove, Merck) for microscopy. A binocular microscope (Nikon SMZ1000) connected with a digital camera (Zeiss AxioCam Cc1) was used to photograph the leaf blade area under dark field illumination using Axiovision (Rel.4.8. Zeiss) image analysis software. Leaf blade area was measured manually using ImageJ v 1.48 (http://rsbweb.nih.gov/ij).

Flow cytometry

Flow cytometry was performed on the first leaf pair, dissected with microscissors, immediately frozen in liquid nitrogen and stored at -80°C. After thawing, leaves were chopped with a razor blade in 200 µl of Cystain UV-Precise P Nuclei extraction buffer, supplemented with 800 µl of staining buffer. The mixture was filtered through a 50 µm filter and analyzed with a Cyflow MB flow cytometer (Partec). The DNA content distribution of the nuclei was analyzed using Cyflogic software (v1.2.1) and the endoreduplcation index (EI) was calculated as EI = 0 * %2C + 1 * %4C + 2* %8C + 3 * %16C+ 4 * %32C.

Kinematic analysis

Cellular measurements were performed on three to six average leaves from six until 26 days after sowing (DAS). Because cells were not clearly visible in the youngest leaves (6-10 DAS), they were stained with propidium iodide (Wuyts *et al.*, 2010) and images of the abaxial epidermis were taken with a Nikon C1 confocal microscope (Nikon Eclipse E600). Leaves of 11 to 26 DAS were cleared with 100% ethanol and subsequently stored and mounted in 100% lactic acid for microscopy. Abaxial epidermal cells were photographed by DIC optics on a

Zeiss Axio Scope A1 fitted with a digital camera (Zeiss AxioCam Cm1) at 20x magnification. Approximately 60-190 cells per leaf were drawn on an LCD tablet (Wacom drawing pad) using ImageJ image analysis software (http://imagej.nih.gov/ij/). Cell analysis was done with automated image analysis software (Andriankaja *et al.*, 2012).

From these data we calculated average cell area and estimated the number of cells per leaf by dividing leaf blade area by average cell area. Stomatal index was calculated as the fraction of stomata in the total of epidermal cells. We calculated cell division rates as the relative rate of increase in cell number over time. For this, the logarithmic values of mean of cell number were locally fitted with a five point quadratic function of which the first derivative was used as the division rate.

Transcriptome analysis

Total RNA (100 ng) of the first leaf pair was isolated on 9 and 15 DAS using Trizol Reagent (Life Technologies). Two and three biological replicates were used on 9 and 15 DAS, respectively and the quantity and integrity of the RNA was measured spectrophotometrically on a QIAxcel system (Qiagen). Library preparation was done using the TruSeq® Stranded mRNA sample preparation 96 rcx kit (Illumina™) following the low sample protocol according to Illumina™ guidelines. Subsequently, the library was quantified using PicoGreen® dye (Life Technologies™). In order to accurately quantify the fragment concentration in each pool, the concentration of all indexed fragments was determined using the KAPA SYBR® FAST universal qPCR kit (KAPA Biosystems™) for Illumima™ sequencing and the average fragment size of the pool was calculated using the Bioanalyzer® (Agilent Technologies™). 12 samples were pooled and sequenced in each flow cell lane on an Illumina HiSeq 1500 sequencer obtaining 50-base pair single end sequences for each fragment. Transcriptome data analysis was performed according to Methods S2.

Seed and embryo size measurements

Seeds of wild type (Col-0) and the *krp4/6/7* were sown in 5×5 cm pots filled with a mixture (1:1, v/v) of loamy soil and organic compost (Tref EGO substrates, Moerdijk, The Netherlands) at a humidity of 0.30 g water/g dry soil, stratified in the dark at 4°C for three days. Pots were transferred to a growth chamber with an air temperature of 21/18°C and 50-55% humidity under 16 h day (90-100 $\mu mols^{-1}m^{-2}\,s^{-1}$ PAR) and 8 h night regime. Siliques from the primary inflorescence were harvested after the first one senesced, fixed in ethanol:acetic acid (9:1) overnight, washed with 90% and 70% ethanol, stored and mounted in 100% lactic acid (Emprove, Merck) for microscopy (Chen *et al.*, 2015). Seeds within the prepared siliques were viewed and counted under a Zeiss Stemi 2000 binocular microscope to estimate total number of aborted seeds.

For embryo size analysis, matured dry seeds were imbibed for 2 hours and dissected under a binocular microscope to isolate embryos. Embryos were treated for 1 h in Hoyer's solution as described by Ohto *et al.* (2005). Images of about 30-40 embryos were obtained with DIC microscopy and projected areas were measured by ImageJ (v 1.48; http://rsbweb.nih.gov/ij).

Statistical analysis

Statistical analysis of leaf area, cell number, cell size, endoreplication index, leaf series, seed and embryo size were conducted by t-test in Microsoft Excel or by using R (v 3.3.2; https://www.r-project.org/).

RESULTS

Knockdown of multiple *KRPs* increases leaf size by increased cell number

To investigate the relationship between cell cycle regulation and leaf development, we aimed accelerate cell cycle progression by reducing the expression of multiple *KRP* gene family members. *KRP4, 6* and *7* genes act redundantly and in contrast to single (*krp4*, *krp6* and *krp7*) and double (*krp4/6, krp4/7* and *krp6/7*), the triple mutant (*krp4/6/7*), induced a strong defect in meiotic division, resulting in embryonic abortion (Zhao *et al.*, 2017). We therefore decided to

analyze the effect of the same mutant combinations on vegetative development. To this end, we first measured leaf blade area and epidermal cell area of the first leaf pair at maturity, 26 days after sowing (DAS), in single, double and triple mutants. Leaf blade area of *krp6* and *krp7* single mutants was not affected, while the blade area in *krp4* was significantly smaller than the WT. In all double mutants, however, leaf size was increased between 10-15% (*krp4/6* and *krp6/7)* and 23% (*krp4/7*) compared to the WT. Down-regulation of all three *KRPs* (*krp4/6/7)* had the most pronounced effect, increasing mature leaf blade area by 33% (Fig. 1a).

To quantify the cellular basis for the leaf size differences, we determined cell size and number in the abaxial epidermis. In addition to the absence of an effect on leaf size, no notable change in cell size or number were observed in *krp6* and *krp7*. The reduced leaf size of *krp4* was due to smaller cells, while cell number was unaffected. Cell number increased *krp4/6, krp4/7* and *krp6/7* (by 35-45%), explaining the increased leaf area. However, there was also a decline in cell size in the double mutants (by 12-22%), partly counteracting the impact of the increased cell number on mature leaf size. The same phenomenon was more pronounced in the triple *krp4/6/7*, where the cell number was increased by 77% compared to the WT, which was partly offset by a 25% reduction in mature cell size (Fig. 1b,c). The stomatal index, the number of guard cells as a fraction of total number of cells, showed only a small, albeit significant, increase in *krp4/6, krp4/7* and *krp6/7* compared to the WT (Fig. 1d), indicating that divisions leading to the formation of stomata are stimulated slightly more than the proliferative divisions forming pavement cells.

Kinematic analysis shows increased cell division rates

To understand how *krp4/6/7* affects cell division and expansion during the development of the first pair of leaves, we performed kinematic analysis. From 6 to 12 DAS, leaf blade area increased exponentially in both WT and *krp4/6/7* (Fig. 2a). Interestingly, leaves of *krp4/6/7* were already 37% larger on six DAS than those of the WT ($p = 0.046$). Initially, on 6 and 7 DAS leaf expansion rates were significantly higher in *krp4/6/7* ($p < 0.01$), but from 8 DAS leaf expansion rates were similar in *krp4/6/7* and WT and gradually decreased until they reached maturity around 22 DAS (Fig. 2b). Cellular measurements showed that *krp4/6/7* leaves contained 45% more cells than the WT at 6 DAS ($p = 0.033$). From 6 to 12 DAS, cell number

increased exponentially in both the WT and *krp4/6/7* (Fig. 2c). Cell division rates decreased in the WT and *krp4/6/7* from 6 to 13 DAS (Fig. 2d). The rate of change in cell number over the period of most rapid cell proliferation, between 6 and 10 DAS, was marginally higher (4%; $p = 0.019$) in *krp4/6/7* compared to the WT, corresponding to a shorter cell cycle duration (39.8 ± 0.6 vs 38.2 ± 0.3 hr).

Average cell size depends on the balance between division and expansion rates (Green, 1976). Our results showed an exponential increase in cell area in the WT and *krp4/6/7* from 6 to 12 DAS (Fig. 2e). Average epidermal cell area was reduced in *krp4/6/7* from D10 onwards, which indicates that the enhanced cell division is not balanced by an equal increase of cell expansion during this phase (Fig. 2e). From 6 to 8 DAS, very few stomata were present, and there was no significant difference in stomatal index. However, from 9 to 15 DAS the increase of the stomatal index in *krp4/6/7* lagged behind that of the WT, reaching slightly elevated levels by extended duration of stomatal formation (Fig. 2f). As the timing of expansion and division are not perturbed, this pattern suggests a specific delay in this developmental process in *krp4/6/7* leaves. Overall, our kinematic analysis shows that increased leaf size is due to increased cell division during early leaf development, part of which occurred prior to the start of the analysis at 6 DAS.

Down regulation of *KRPs* stimulates endoreplication in leaves

To gain insight into the role of *KRP* genes in cell cycle progression underlying the cellular phenotype (faster cell division and smaller cell size than the WT), we measured ploidy levels in mature leaves at 26 DAS. Single mutants *krp4*, *krp6* and *krp7* showed no differences in endoreplication index compared to the WT. However, *krp4/6*, *krp4/7* and *krp6/7* double and the *krp4/6/7* triple mutants had significantly higher ploidy levels than the WT (Fig. 3a). To see where these differences originate, we performed a time course analysis of the WT and *krp4/6/7* throughout leaf development (9 to 26 DAS). At 9 DAS, when leaves are actively proliferating (Fig. 2c,d), 70% of the cells has a 2C and 30% a 4C DNA content, indicating the dominance of the G1 phase. No significant differences were found between mutant and WT, suggesting that the reduced cell cycle duration in *krp4/6/7* is caused by a roughly equal reduction of G1/S and G2/M phases (Fig. 3b). When cells exit from mitosis, the fraction of cells with a 2C DNA

content rapidly decreases. In contrast, a cohort of cells progresses to 4C at day 11 (*krp4/6/7*) and 13 (WT) and subsequently to 8C at day 17 (*krp4/6/7*) and 19 (WT) and even partly to 16C (Fig. 3c-f), indicating the occurrence of endoreplication, coinciding with the expansion phase (Fig. 2a,b; (Beemster *et al.*, 2005b). In comparison to the WT, endoreplication starts earlier in *krp4/6/7* and progresses faster (c.f. the slopes in Fig. 3b,e,f).

To determine the mechanism causing increased ploidy levels in mature mutant leaves and the difference in the temporal patterns of the ploidy levels of WT and *krp4/6/7*, we constructed a mathematical model describing the dynamics of progression between ploidy fractions (2, 4, 8 and 16C; Methods S3). Through experimental data fitting (Fig. S1) we estimated the kinetic parameters for ploidy conversion rates. Interestingly, the rate constants decrease with increasing ploidy levels (Table 1), implying that the endocycle progressively slows down at higher DNA contents. The rate constants for *krp4/6/7* indicate a doubling of all values compared to those of the WT (Table 1). Thus, next to an advanced onset of endoreplication, faster rates of endoreplication contribute to the increased ploidy levels in mature leaves of *krp4/6/7* compared to the WT (Fig. 3b).

Transcriptome analysis reveals upregulation of genes related to cell cycle and DNA synthesis in leaves of *krp4/6/7*

To understand the molecular mechanism responsible for the observed cellular changes, we performed a genome wide transcript profiling of proliferating and expanding leaves of *krp4/6/7* and WT by mRNA sequencing. Overall, 425 genes were differentially expressed (FDR < 0.05; $\log_2$ FC > 0.75) between the mutant and WT. On 9 DAS 232 genes were up and 91 down regulated, whereas on 15 DAS 76 genes were up and 26 down regulated (Fig. 4). To identify the major biological functions affected in the mutant, we performed an over-representation analysis of the differentially expressed genes. Generally, over-represented categories were related to cell cycle and DNA synthesis, repair and replication (Fig. S2a,b), explaining the enhanced cell cycle activity at both 9 and 15 DAS.

We then focussed in more detailed on cell cycle gene expression. As expected, the mRNA seq data showed reduced expression of *KRP4* and *KRP6* transcripts in *krp4/6/7* relative to the WT. However, *KRP7* expression levels appeared to be elevated in both proliferating and differenting cells (Table S2). RT-PCR in contrast showed downregulation of all three genes (Fig. S4a-c). A closer analysis of the NGS read mapping showed an absence of reads from the first exon of *KRP4* (Fig. S3a) and a strong reduction along the entire coding region of KRP6 (Fig. S3a). Reads from the 3rd and 4th exon of *KRP7*, downstream of the T-DNA insert, where upregulated, whereas exon 1 reads were transcribed from the opposite strand (Fig. S3c). This implies that, although total *KRP7* transcript reads were higher, they are nonsense transcripts. One of the primers used for RT-PCR targets the first exon of *KRP7*, explaining the discrepancy between NGS and RT-PCR data and providing a quantification of the functional expression levels.

Because the mutant phenotypes suggest that *KRPs* are redundantly acting as inhibitors of CDKs, we hypothesized that the loss of function of single or multiple *KRP* genes would be partly compensated by enhanced transcription of the remaining non-mutated *KRP* genes. Indeed, there is an overall tendency for the non-mutated genes (*KRP1-3* and *5*) to be upregulated in *krp4/6/7* on both D9 and 15, although the difference is only significant in proliferationg cells D9 ($P = 0.001$; Fig. S5a). Making the assumption that all *KRP* transcripts translate equally well into functional proteins, we then used the NGS transcript levels of all *KRP* genes combined to assess the total *KRP* levels in *krp4/6/7* and WT. Despite the compensation of the WT alleles and an increased number of reads for *KRP7*, total *KRP* transcripts decreased by at least 35% in proliferating cells (D9) and 40% in expanding cells (D15) in *krp4/6/7* (Fig. S5b), consistent with increased cell cycle activity in both proliferating and endoreplicating cells.

Next, we investigated the effect of *krp4/6/7* on the expression of other cell cycle regulators. The data showed that most of the positive cell cycle regulators (CDKs, cyclins, DNA synthesis machinery) were upregulated (Table S2). This suggests that down regulation of *KRP4*, *6* and *7* enhances cell cycle progression by a global upregulation of cell cycle gene expression.

The cumulative effect of concurrent down regulation of *KRP* family members with an increase of cell cycle activity (this study and Cheng *et al.*, 2013) and the compensatory upregulation of the remaining wild type *KRP* genes, led to the question if down regulating additional family members would lead to a further increase in leaf size. To investigate this, we transformed the *krp4/6/7* mutant with an ami-RNA targeting *KRP1*, *3* and *5* to further increase the number of down regulated *KRP* family members. This construct effectively down regulated the expression of *KRP1, 3* and *5* in the WT background (Fig. S6). Instead of causing an additional increase of leaf blade area, one of the constructs led to a small decrease (Fig. S7a,b), suggesting that basal levels of KRPs are needed for optimal development.

Effect of seed on leaf development in *krp4/6/7* mutant

The kinematic analysis (Fig. 2) suggested that part of the increased cell number and leaf area of *krp4/6/7* was already established shortly after germination. Therefore, we investigated the possibility that the difference was already established during seed development. To evaluate this, we first compared seed size of WT and *krp4/6/7*. Indeed, *krp4/6/7* had 25% larger seeds than the WT ($p < 0.001$; Fig. 5a). Moreover, distribution of seed size revealed an overall shift in the seed size; the median seed size was 100 μm^2 and 130 μm^2 in WT and *krp4/6/7*, respectively (Fig. 6a). To further investigate the possible relationship between seed and leaf size, WT and *krp4/6/7* seeds were selected across the size range and grown *in vitro*. In both WT and mutant there was a positive correlation between seed size and leaf area (Fig 6b). These results were not limited to the first leaf pair as a positive correlation between seed size and rosette area could still be observed at 26 DAS, when 12-13 leaves have appeared (Fig. S8). When we eliminated seed size differences by selecting seeds of 120 μm^2 prior to sowing, WT and *krp4/6/7* seedlings had a nearly equal leaf area (Fig. 6b). As expected, plants grown exclusively from median size (100 μm^2 for WT; 130 μm^2 for *krp4/6/7*) seeds produced significantly larger leaves in *krp4/6/7* compared to WT (30%; $p < 0.001$; Fig. 6b).

To investigate the mechanism by which larger seed size caused increased leaf size after germination, we investigated the size of the embryos in the seeds. Because the embryo takes up most of the seed volume (Li & Li, 2016), embryo size correlates strongly with seed size. Consequently, the embryos of *krp4/6/7* were on average 19% bigger than those of the WT (p

390 < 0.001; Fig. 5b). We then analyzed if the increased size of the embryo, was associated with
391 increased cell number or cell size. As the primordia of the first leaf pair were difficult to
392 visualize, we focused on the cotyledons. As expected, *krp4/6/7* cotyledons were significantly
393 larger than WT ($p < 0.001$; Fig. 7a). The 30% increase in *krp4/6/7* cotyledon area was due to a
394 40% increase in cell number (Fig. 7b). In contrast to our observations in the leaves, there was
395 no significant effect on epidermal cell area of the embryonal cotyledons (Fig. 7c).

396

397 **Concurrent mutation of *KRP4*, *KRP6* and *KRP7* genes causes seed abortion**

398 Combining our observations that *krp4/6/7* induces early differences in leaf development (Fig.
399 2), increased seed and embryo size (Fig. 7) and causes seed abortion by arresting ovule
400 development (Zhao *et al.*, 2017), we hypothesized a causal relationship between these
401 phenotypes. According to this hypothesis seed abortion provides extra resources to the
402 remaining seeds, stimulating cell division in developing embryos. This leads to an increased
403 cell number in young leaves that contributes to increased leaf size at maturity. To validate this
404 hypothesis, we compared the phenotype of *krp4/6/7* with *shugoshin-1* (*sgo1*), a mutant that
405 induces seed abortion by an independent mechanism, causing a high level of aneuploidy due
406 to unbalanced segregation of chromosomes (Cromer *et al.*, 2013; Zamariola *et al.*, 2013, 2014).
407 Both *sgo1* and *krp4/6/7* plants produced roughly the same number of siliques as the WT (Table
408 2). However, in both mutants the siliques where significantly shorter than those of WT (Fig 8;
409 Table 2), which correlated with a high percentage of seed abortion, 54% and 30% in *sgo1* and
410 *krp4/6/7*, respectively, compared to only 10% in the WT (Table 2). Consistent with our
411 hypothesis, seed size was inversely proportional to the percentage of seed abortion, as seeds of
412 *sgo1* and *krp4/6/7* were 1.5x and 1.3x bigger than WT seeds, respectively. Seed size of *krp6/7*
413 was slightly bigger than WT, consistent with *krp6/7* effect on megaspore mother cell formation
414 (Zhao *et al.*, 2017), while there was no noticeable increase in *krp4/6* and *krp4/7* (Fig. S9)

415

416 As a final step, we analyzed the effect of *sgo1* on leaf development. Although *sgo1* has a
417 specific function in meiosis (Cromer *et al.*, 2013; Zamariola *et al.*, 2013), the blade area of first
418 leaf pair was increased to a similar extend as in the *krp4/6/7* (Fig. 9a). Similar to our findings
419 for *krp4/6/7* (Fig 6b), leaf area of WT and *sgo1* seedlings grown from the same seed size were
420 not significantly different (Fig. 9d). Cellular analysis demonstrated that, although *sgo1* is not

involved in mitotic cell divisions, the increased leaf area of *sgo1* was due to an increased cell number (Fig. 9b). However, unlike in *krp4/6/7*, mature cell area was not affected in *sgo1* (Fig. 9c), indicating that the stimulation of cell cycle activity throughout the development of the leaf results in smaller cells in *krp4/6/7*.

Seed size differences contribute to the phenotype of well-known leaf size mutants

The importance of embryo and seed size on leaf development, led us to question whether seeds size could also explain (part of) the large leaf phenotype of mutants reported in the literature. To investigate this possibility, we measured seed size and leaf blade area of 12 published mutants with increased leaf sizes (Hu *et al.*, 2003; Horiguchi *et al.*, 2009; Gonzalez *et al.*, 2010; Eloy *et al.*, 2011; Vanhaeren *et al.*, 2014). In our conditions, we obtained a significantly increased blade area for the first leaf pair for 8 of the 12 lines (Fig. 10a), 4 of which also had increased seed size (Fig. 10b), suggesting that enhanced seed size could indeed contribute to the increased leaf size of these four mutants. To test this, we measured leaf blade area for these mutants and WT on seedlings grown from the same seed size (160 μm^2). Although in *samba* there was no significant effect on leaf size, *35S:GRF5*, *gra2-D* and *da1-1* lines still formed larger leaves than the WT (Fig. 10c), albeit less so than observed when their average sized seeds were used. By comparing leaf size at average seed size for each genotype (Fig. 10a), with those obtained from a uniform seed size across genotypes (Fig. 10c), we estimated that increased seed size contributed between 25% and 90% to the leaf size phenotype of these 4 mutants (Fig. 10d).

DISCUSSION

KRPs as a means to understand organ growth regulation

By studying the effect of reducing the number of functional *KRP* genes we investigated the role of cell cycle regulation in leaf size control in Arabidopsis seedlings. Earlier, we pointed out that, in addition to effects of altered cell cycle gene expression on duration and rate of mitotic and endoreplication activities during organ development, also effects on earlier events, such as quiescence and organ initiation could play an important role (Beemster *et al.*, 2005b).

We also demonstrated that a simple mechanistic model of cell cycle regulation during the expansion and endoreplication phase of leaf growth could explain the phenotype of *KRP2* overexpression on cell number, but not mature cell size (Beemster *et al.*, 2006). The results described in this manuscript provide new insight in the role of cell cycle regulation during organ growth. At first sight the phenotype of reduced *KRP* expression is opposite to that observed in *KRP* overexpressing lines, which have a reduced leaf size, enlarged cells and reduced endoreplication (De Veylder *et al.*, 2001). However, kinematic analysis of *KRP2* overexpressing lines show that at emergence the cell number of these lines is not affected, whereas inhibition of cell cycle activity during leaf growth is very strong (De Veylder *et al.*, 2001). Our study shows that reduced expression of multiple *KRP* genes does have the opposite effect on division and endoreplication, but in addition it unexpectedly stimulates the earlier stages of development.

The observed opposite effects on cell number and size in response to both up and down regulated *KRP* expression provides evidence to link between cell division and whole organ growth. Conceptually, division only subdivides volume created by cell expansion and does not contribute to growth directly (Green, 1976). By means of modelling we recently demonstrated that cell division and expansion cannot be regulated cell autonomously, but require chemical signaling (De Vos *et al.*, 2017). Possible links downstream such signals include cell division being upstream or downstream of cell expansion or regulated independently, with a feedback mechanism between the two (Kaplan & Hagemann, 1991). Our results are consistent with the latter, as they show that division and expansion can be decoupled, resulting in cell size differences. However, this uncoupling is not limitless, so that a feedback mechanism reducing cell expansion and hence overall organ size must also exist.

One global mechanism by which cell cycle activity and mature cell size could be coupled is a potential dependence of post meristematic cell expansion on DNA content, determined by endoreplication. A striking correlation between mature cell size and endoploidy levels has often been observed (Melaragno *et al.*, 1993; Schnittger *et al.*, 2003; Boudolf *et al.*, 2004). However, our earlier results (De Veylder *et al.*, 2001) and the work presented here provide evidence that by altered *KRP* gene expression endoreplication can be up and downregulated in opposite

direction of mature cell size. These results are consistent with earlier studies showing that while it remains a possibility that cell growth can drive the endocycle, the inverse is very unlikely (Massonnet *et al.*, 2011; Tsukaya, 2013, 2014).

The role of *KRP*s in cell cycle regulation

Ectopic expression of individual *KRP* genes reduces cell proliferation by inhibiting CDKs, resulting in small plants with large cells that resemble mutants with reduced CDK activity (Lui *et al.*, 2000; De Veylder *et al.*, 2001). Studying the loss of *KRP* gene function to assess if it can induce the inverse, enhanced cell cycle activity and organ growth, is challenging due to functional redundancy between the 7 members of this gene family (Acosta *et al.*, 2011; Cheng *et al.*, 2013; Zhao *et al.*, 2017) Consistently, we found no significant increase of cell number or leaf size in single mutants. In *krp4* we even observed a reduced leaf area due to smaller cells. However, concurrent loss of two *KRP*s did result in increased cell number and leaf size and this effect became even more prominent in *krp4/6/7*, suggesting a gradual stimulation of cell cycle activity with gradually decreasing *KRP* levels. Cheng *et al*., (2013) found a similar leaf phenotype for the quintuple *krp1/2/3/4/7* mutant and demonstrated reduced *KRP* levels correlated with enhanced CDK activity.

Our results also show that in addition to progressively stimulating cell division, mutating multiple *KRPs* also progressively enhances endoreplication. This is in contrast with the observation that weak ectopic expression of *KRP*s only blocks mitosis, resulting in decreased number of cells with elevated DNA content, but consistent with the observation that strong ectopic expression inhibits both mitosis and endoreplication, resulting in fewer cells with a reduced DNA content (Verkest *et al.*, 2005a; Weinl *et al.*, 2005).

We attributed the absence of phenotypes in single mutants not only with redundant functionality of *KRPs*, but also with the possibility of a compensation between *KRP* family members. Indeed, we demonstrated a significantly increased expression of the four remaining non-mutated *KRP1-3* and *5* in *krp4/6/7*. Likewise, quintiple *krp1/2/3/4/7* mutant showed slight

increase in levels of the two remaining *KRP5* and *KRP6* transcripts (Cheng *et al.*, 2013). Overall, *KRP* transcripts decreased by at least 35% and 40% on 9D and 15D respectively, providing a basis for increased CKD activities upon downregulation of *KRP* genes (Cheng *et al.*, 2013) and high endoreplication levels we observed. Interestingly, by comparing transcripts of proliferating cells with expanding cells, we observed that although individual transcripts varied, the total amount of *KRP* transcripts remained essentially unchanged throughout leaf development at least in the WT (total *KRP* transcripts of the WT decreased by only 8%), suggesting that, consistent with the effect of overexpressed *KRP2* (De Veylder *et al.*, 2001) *KRPs* regulate cell cycle rates, but not the transition between proliferation and expansion/endocycle activity.

Transcriptome analysis of dividing and expanding cells showed differential expression of core cell cycle genes (Table S2). This explains the increased cell division and endoreplication in *krp4/6/7* and is most likely mediated by increased *CDKA* kinase activity. This is consistent with the observation that quintuplet *krp1/2/3/4/7* enhances *CDKA* activity, increases the levels of phosphorylated *RBR1* and expression of *E2F/DP* target genes (Cheng *et al.*, 2013). Consistently, in our study cell cycle genes associated with DNA synthesis, controlled by E2F (Vandepoele *et al.*, 2005) were significantly upregulated both in proliferating and expanding cells (Table S2), correlating with flow cytometry results of increased rates of endoreplication. Because upregulation of *E2Fa/DPa* levels stimulates both the mitotic and endoreduplication cycle (De Veylder *et al.*, 2002), it is most likely that the effect of *krp4/6/7* is mainly mediated by enhanced *E2F/DPa* activity as a result of enhanced *CKDA* activity.

Mathematical modelling of cell cycle progression

The model we presented, similar to that proposed by Kawade & Tsukaya (2017), but including mitosis (the reverse 2 to 4C transition), describes cell cycle dynamics. Its main use is to integrate the complex temporal ploidy data by a simple set of equations describing the transition between ploidy levels. This approach allowed us to draw profound conclusions about cell cycle progression and the role of *KRPs*. The first conclusion is that cell cycle progression rates slowly decline with progressing age. This corresponds well with progressively reducing CDK activity in developing leaves, analogous to observations in the developing maize leaf

(Granier *et al.*, 2000). The second conclusion is that *KRPs* apparently inhibit cell cycle progress to the same extent in G1/S and G2/M of the mitotic and during the endocycle. In this respect they apparently operate as global moderators rather than specific modifiers. These conclusions are not intuitively obvious from the experimental data and therefore demonstrate the importance of an integrated model-based approach to cell cycle regulation. This could probably be achieved by adapting detailed molecular models developed for single cell organisms with higher level approaches as the one developed in this study.

Seed abortion affects organ size.

Compared to the WT, *krp4/6/7* had fewer and smaller siliques containing less seeds (Fig. 9; Table 2). Likewise, *sgo1* plant had small siliques with few seeds due high levels of aneuploidy in the gametes causing seed abortion (Cromer *et al.*, 2013; Zamariola *et al.*, 2013, 2014). Our analysis indicated that *sgo1* plants had 54% aborted seeds. Consistent with the previous results (Zhao *et al.*, 2017), we found that reduced silique length of *krp4/6/7* is a consequence of 30% aborted seeds. This abortion results from the formation of multiple embryo sacs in *krp4/6/7* ovules due to failures in restricting meiotic fate to a single megaspore mother cell (Zhao *et al.*, 2017).

This implies that increased size of *krp4/6/7* seeds are probably due to allocation of resources to fewer developing seeds. Several studies have shown a correlation between accumulation of resources and seed size. Plants mutant for *AP2* have large seeds with increased amounts of sugars, proteins and oils (Jofuku *et al.*, 2005; Ohto *et al.*, 2005), and expression of *KLUH/CYP78A5* in integuments (*pINO::KLU*) resulted in large seeds with high oil content (Adamski *et al.*, 2009). Seed size is determined by the growth of the embryo, the endosperm, and the integuments. Our embryonic analysis indicated that the enlarged seeds of *krp4/6/7* are due to increased embryo size and an increased number of embryonic cells. Alteration of sugar composition (hexose to sucrose) has been implicated to control mitotic activity during seed development (Weber *et al.*, 1996). Ohto *et al*., (2005) found that the large seeds of *ap2* had a high hexose to sucrose ratio, which was suggested to extend the duration rather than the rate of cell division. In fission yeast, growth in limited nutrients increased the fraction of cells in G_1/S at the expense of the fraction in G_2/M phase (Jorgensen & Tyers, 2004) as they require time to

grow to G_1/S cell size threshold before the cell cycle progresses. However, in Arabidopsis SAM, G_1/S and G_2/M transitions are size-dependent (Jones *et al.*, 2017). Presumably both G_1/S and G_2/M size thresholds can be reached faster in the presence of a high supply of resources, reducing the length of cell cycle significantly. Taken together, this suggest that *krp4/6/7* embryos contain more cells, possibly due to extended period of cell division and increased rate of cell cycle progression facilitated by extra availability of sugars during their development.

An important conclusion from the kinematic analysis was that part of the *krp4/6/7* phenotype originated prior to our analysis shortly after germination. We then demonstrated a direct correlation between increased seed size in *krp4/6/7* and increased leaf size after germination. Using the *sgo1* mutant that also have large seeds owing to a different mechanism, we illustrated that this is a general mechanism, and it is not limited to *KRP* or mitotic cell cycle regulators. Finally, we confirmed that seed size of several mutants and transgenics significantly contributes to their leaf size phenotype. Although the contribution of seed size to leaf blade area explained between 25 and 90% for half (4/8) of the genotypes with larger leaves in this study, the contribution of seed size to final leaf size is nevertheless evident. Overall our studies therefore show that seed size has a strong impact on leaf size, even in a single genotype. Superimposed on that genetic effects also play a role in leaf developmental after germination and growth regulating genes may affect both seed and leaf development in parallel. Hence, our results imply that the contribution of seed size needs to be routinely incorporated in phenotyping studies in order to draw reliable conclusions about the underlying molecular and developmental mechanisms controlling organ growth.

Acknowledgments

We thank Sevgi Oden for the help in drawing cells for kinematic analysis. This work was supported by a concerted research activity (GOA) research grant, the MARS Inter University Attraction Poles project (IAP7/29) funded by the Belgian Federal Science Policy Office (BELSPO). D.D. was funded by a return grant from BELSPO, S.K. by training grant from the Department of Science and Education of the Flemish Government and B.L.S. by National Research Foundation-SA fellowship and Erasmus Mundus-EUROSA fellowship.

Author Contribution

B.L.S. and S.K. performed experimental work, analyzed data, and wrote the article. M.N.M. performed RNA sequencing and bioinformatics analysis of the RNA sequencing data. M.A.D., J.S., H.A. X.Z. D.D. designed, analyzed and interpreted data. L.D., D.D., A.S., and G.T.S.B contributed to the writing of the manuscript. J.B. and G.T.S.B supervised the project.

REFERENCES

Acosta JAT, Fowke LC, Wang H. **2011**. Analyses of phylogeny, evolution, conserved sequences and genome-wide expression of the ICK/KRP family of plant CDK inhibitors. *Annals of Botany* **107**: 1141–1157. doi: 10.1093/aob/mcr034.

Adamski NM, Anastasiou E, Eriksson S, O'Neill CM, Lenhard M. **2009**. Local maternal control of seed size by KLUH/CYP78A5-dependent growth signaling. *Proceedings of the National Academy of Sciences, USA* **106**: 20115–20120. doi: 10.1073/pnas.0907024106.

Andriankaja M, Dhondt S, DeBodt S, Vanhaeren H, Coppens F, DeMilde L, Mühlenbock P, Skirycz A, Gonzalez N, Beemster GTS, *et al.* 2012. Exit from proliferation during leaf development in *Arabidopsis thaliana*: A not-so-gradual process. *Developmental Cell* **22**: 64–78. doi:10.1016/j.devcel.2011.11.011.

Beemster GTS, Fiorani F, Inzé D. **2003**. Cell cycle: The key to plant growth control? *Trends in Plant Science* **8**: 154–158. doi: 10.1016/S1360-1385(03)00046-3.

Beemster GTS, Mironov V, Inzé D. **2005a**. Tuning the cell-cycle engine for improved plant performance. *Current Opinion in Biotechnology* **16**: 142–146. doi: 10.1016/j.copbio.2005.01.006.

Beemster GTS, Vercruysse S, De Veylder L, Kuiper M, Inzé D. **2006**. The Arabidopsis leaf as a model system for investigating the role of cell cycle regulation in organ growth. *Journal of Plant Research* **119**: 43–50. doi: 10.1007/s10265-005-0234-2.

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D, Vuylsteke M. **2005b**. Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. *Plant Physiology* **138**: 734–743. doi: 10.1104/pp.104.053884.

Bemis SM, Torii KU. **2007**. Autonomy of cell proliferation and developmental programs during Arabidopsis aboveground organ morphogenesis. *Developmental Biology* **304**: 367–381. doi:10.1016/j.ydbio.2006.12.049.

Bird DA, Buruiana MM, Zhou Y, Fowke LC, Wang H. **2007**. Arabidopsis cyclin-dependent kinase inhibitors are nuclear-localized and show different localization patterns within the nucleoplasm. *Plant Cell Reports* **26**: 861–872. doi: 10.1007/s00299-006-0294-3.

Boudolf V, Vlieghe K, Beemster G. **2004**. The plant-specific cyclin-dependent kinase CDKB1;1 and transcription factor E2Fa-DPa control the balance of mitotically dividing and endoreduplicating cells in Arabidopsis. *Plant Cell* **16**: 2683–2692.doi: 10.1105/tpc.104.024398.

Boyes DC. **2001**. Growth stage-based phenotypic analysis of Arabidopsis: A model for high throughput functional genomics in plants. *Plant Cell* **13**: 1499–1510. doi: 10.1105/TPC.010011.

Chen L-Q, Lin IW, Qu X-Q, Sosso D, McFarlane HE, Londoño A, Samuels AL, Frommer WB. **2015**. A cascade of sequentially expressed sucrose transporters in the seed coat and endosperm provides nutrition for the Arabidopsis embryo. *Plant Cell* **27**: 607–619. doi: 10.1105/tpc.114.134585.

Cheng Y, Cao L, Wang S, Li Y, Shi X, Liu H, Li L, Zhang Z, Fowke LC, Wang H, *et al.* 2013. Downregulation of multiple CDK inhibitor ICK/KRP genes upregulates the E2F pathway and increases cell proliferation, and organ and seed sizes in Arabidopsis. *Plant Journal* **75**: 642–655. doi: 10.1111/tpj.12228.

Cromer L, Jolivet S, Horlow C, Chelysheva L, Heyman J, De Jaeger G, Koncz C, De Veylder L, Mercier R. **2013**. Centromeric cohesion is protected twice at meiosis, by SHUGOSHINs at anaphase I and by PATRONUS at interkinesis. *Current Biology* **23**: 2090–2099. doi:

10.1016/j.cub.2013.08.036.

De Vos D, Dzhurakhalov A, Klosiewicz P, Broeckhove J, Beemster GTS. **2017**. Modeling the foundations of plant organ growth regulation. In: Plant Physiology and Function (Clemens S., Ed.). New York, NY: Springer. doi: 10.1007/978-1-4614-7611-5_4-1.

De Veylder L, Beeckman T, Beemster GTS, de Almeida Engler J, Ormenese S, Maes S, Naudts M, Van der Schueren E, Jacqmard A, Engler G, *et al.* 2001. Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. *Plant Cell* **13**: 1653–1667. doi: 10.1105/TPC.010087.

De Veylder L, Beeckman T, Beemster GTS, De Almeida Engler J, Ormenese S, Maes S, Naudts M, Van der Schueren E, Jacqmard A, Engler G, *et al.* 2002. Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. *EMBO Journal* **21**: 1360–1368. doi: 10.1093/emboj/21.6.1360.

Dewitte W, Murray JAH. **2003**. The plant cell cycle. *Annual Review of Plant Biology* **54**: 235–264. doi: 10.1146/annurev.arplant.54.031902.134836.

Eloy NB, de Freitas Lima M, Van Damme D, Vanhaeren H, Gonzalez N, De Milde L, Hemerly AS, Beemster GTS, Inzé D, Ferreira PCG. **2011**. The APC/C subunit 10 plays an essential role in cell proliferation during leaf development. *Plant Journal* **68**: 351–363. doi: 10.1111/j.1365-313X.2011.04691.x.

Eloy NB, Gonzalez N, Van Leene J, Maleux K, Vanhaeren H, De Milde L, Dhondt S, Vercruysse L, Witters E, Mercier R, *et al.* 2012. SAMBA, a plant-specific anaphase-promoting complex/cyclosome regulator is involved in early development and A-type cyclin stabilization. *Proceedings of the National Academy of Science, USA* **109**: 13853–13858. doi: https://doi.org/10.1073/pnas.1211418109.

Ferjani A, Horiguchi G, Yano S, Tsukaya H. **2007**. Analysis of leaf development in fugu mutants of Arabidopsis reveals three compensation modes that modulate cell expansion in determinate organs. *Plant Physiology* **144**: 988–999. doi: 10.1104/pp.107.099325.

Ferjani A, Ishikawa K, Asaoka M, Ishida M, Horiguchi G, Maeshima M, Tsukaya H. **2013**.

683 Enhanced cell expansion in a krp2 overexpressor is mediated by increased v-atpase activity.
684 *Plant and Cell Physiology* **54**: 1989–1998. doi: 10.1093/pcp/pct138.

685 **Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L,**
686 **Weigel D, Kamiya Y, *et al.* 2010**. Increased leaf size: Different means to an end. *Plant*
687 *Physiology* **153**: 1261–1279. doi: 10.1104/pp.110.156018.

688 **Gonzalez N, Vanhaeren H, Inzé D**. **2012**. Leaf size control: Complex coordination of cell
689 division and expansion. *Trends in Plant Science* **17**: 332–340. doi:
690 10.1016/j.tplants.2012.02.003.

691 **Granier C, Inze D, Tardieu F**. **2000**. Spatial distribution of cell division rate can be deduced
692 from that of p34(cdc2) kinase activity in maize leaves grown at contrasting temperatures
693 and soil water conditions. *Plant Physiol* **124**: 1393–1402. doi: 10.1104/pp.124.3.1393.

694 **Green PB**. **1976**. Growth and cell pattern formation on an axis: Critique of concepts,
695 terminology, and modes of study. *Botanical Gazette* **137**: 187–202.

696 **Hisanaga T, Kawade K, Tsukaya H**. **2015**. Compensation: A key to clarifying the organ-level
697 regulation of lateral organ size in plants. *Journal of Experimental Botany* **66**: 1055–1063. doi:
698 10.1093/jxb/erv028.

699 **Horiguchi G, Gonzalez N, Beemster GTS, Inzé D, Tsukaya H**. **2009**. Impact of segmental
700 chromosomal duplications on leaf size in the grandifolia-D mutants of *Arabidopsis thaliana*.
701 *Plant Journal* **60**: 122–133. doi: 10.1111/j.1365-313X.**2009**.03940.x.

702 **Horiguchi G, Kim GT, Tsukaya H**. **2005**. The transcription factor AtGRF5 and the
703 transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis*
704 *thaliana*. *Plant Journal* **43**: 68–78. doi: 10.1111/j.1365-313X.2005.02429.x.

705 **Hu Y, Xie Q, Chua NH**. **2003**. The Arabidopsis Auxin-Inducible Gene. *Plant Cell* **15**: 1951–
706 1961. doi: 10.1105/tpc.013557.

707 **Inzé D, De Veylder L**. **2006**. Cell cycle regulation in plant development. *Annual Review of*
708 *Genetics* **40**: 77–105. doi: 10.1146/annurev.genet.40.110405.090431.

Jakoby MJ, Weinl C, Pusch S, Kuijt SJH, Merkle T, Dissmeyer N, Schnittger A. **2006**. Analysis of the subcellular localization, function, and proteolytic control of the Arabidopsis cyclin-dependent kinase inhibitor ICK1/KRP1. *Plant Physiology* **141**: 1293–1305. doi: 10.1104/pp.106.081406.

Jofuku KD, Omidyar PK, Gee Z, Okamuro JK. **2005**. Control of seed mass and seed yield by the floral homeotic gene APETALA2. *Proceedings of the National Academy of Sciences, USA* **102**: 3117–22. doi: 10.1073/pnas.0409893102.

Jones AR, Forero-Vargas M, Withers SP, Smith RS, Traas J, Dewitte W, Murray JAH. **2017**. Cell-size dependent progression of the cell cycle creates homeostasis and flexibility of plant cell size. *Nature Communications* **8**: 1–13. doi: 10.1038/ncomms15060.

Jorgensen P, Tyers M. **2004**. How cells coordinate growth and division. *Current Biology* **14**: 1014–1027.

Jun SE, Okushima Y, Nam J, Umeda M, Kim GT. **2013**. Kip-related protein 3 is required for control of endoreduplication in the shoot apical meristem and leaves of Arabidopsis. *Molecules and Cells* **35**: 47–53. doi: 10.1007/s10059-013-2270-4.

Kalve S, De Vos D, Beemster GTS. **2014**. Leaf development: a cellular perspective. *Frontiers in Plant Science* **5**: 1–25. doi: 10.3389/fpls.2014.00362

Kaplan DR, Hagemann W. **1991**. The relationship of cell and organism in vascular plants: Are cells the building blocks of plant form? *BioScience* **41**: 693–703. doi: 10.2307/1311764.

Kawade K, Tsukaya H. **2017**. Probing the stochastic property of endoreduplication in cell size determination of *Arabidopsis thaliana* leaf epidermal tissue. *PLoS ONE* **12**: 1–18. doi: 10.1371/journal.pone.0185050.

Kim HJ, Oh SA, Brownfield L, Hong SH, Ryu H, Hwang I, Twell D, Nam HG. **2008**. Control of plant germline proliferation by SCFFBL17 degradation of cell cycle inhibitors. *Nature* **455**: 1134–1138. doi: 10.1038/nature07289.

Kumar N, Harashima H, Kalve S, Bramsiepe J, Wang K, Sizani BL, Bertrand LL, Johnson MC, Faulk C, Dale R, *et al.* 2015. Functional conservation in the SIAMESE-RELATED family of

cyclin-dependent kinase inhibitors in land plants. *Plant Cell* **27**: 3065–3080. doi: 10.1105/tpc.15.00489

Kumar N, Larkin JC. **2017**. Why do plants need so many cyclin-dependent kinase inhibitors? *Plant Signaling and Behavior* **12**: 1–4. doi: 10.1080/15592324.2017.1282021.

Lai J, Chen H, Teng K, Zhao Q, Zhang Z, Li Y, Liang L, Xia R, Wu Y, Guo H, *et al.* 2009. RKP, a RING finger E3 ligase induced by BSCTV C4 protein, affects geminivirus infection by regulation of the plant cell cycle. *Plant Journal* **57**: 905–917. doi: 10.1111/j.1365-313X.2008.03737.x.

Li N, Li Y. **2016**. Signaling pathways of seed size control in plants. *Current Opinion in Plant Biology* **33**: 23–32. doi: 10.1016/j.pbi.2016.05.008.

Li J, Yang H, Peer WA, Richter G, Blakeslee J, Bandyopadhyay A, Titapiwantakun B, Undurraga S, Khodakovskaya M, Richards EL, *et al.* 2005. Arabidopsis H+-PPase AVP1 regulates auxin-mediated organ development. *Science* **310**: 121–125. doi: 10.1126/science.1115711.

Liu J, Zhang Y, Qin G, Tsuge T, Sakaguchi N, Luo G, Sun K, Shi D, Aki S, Zheng N, *et al.* 2008. Targeted degradation of the cyclin-dependent kinase inhibitor ICK4/KRP6 by RING-Type E3 ligases is essential for mitotic cell cycle progression during Arabidopsis gametogenesis. *Plant Cell* **20**: 1538–1554. doi: 10.1105/tpc.108.059741.

Lui H, Wang H, DeLong C, Fowke LC, Crosby WL, Fobert PR. **2000**. The Arabidopsis Cdc2a-interacting protein ICK2 is structurally related to ICK1 and is a potent inhibitor of cyclin-dependent kinase activity in vitro. *Plant Journal* **21**: 379–385. doi: 10.1046/j.1365-313x.2000.00688.x.

Massonnet C, Tisne S, Radziejwoski A, Vile D, De Veylder L, Dauzat M, Granier C. **2011**. New insights into the control of endoreduplication: endoreduplication could be driven by organ growth in Arabidopsis leaves. *Plant Physiology* **157**: 2044–2055. doi: 10.1104/pp.111.179382.

Melaragno JE, Mehrotra B, Coleman AW. **1993**. Relationship between endopolyploidy and

763 cell size in epidermal tissue of Arabidopsis. *Plant Cell* **5**: 1661–1668. doi:
764 10.1105/tpc.5.11.1661.

765 **Murashige T, Skoog F**. **1962**. A revised medium for rapid growth and bio assays with tobacco
766 tissue cultures. *Physiologia Plantarum* **15**: 473–497.doi: 10.1111/j.1399-
767 3054.1962.tb08052.x.

768 **Nakai T, Kato K, Shinmyo A, Sekine M**. **2006**. Arabidopsis KRPs have distinct inhibitory
769 activity toward cyclin D2-associated kinases, including plant-specific B-type cyclin-
770 dependent kinase. *FEBS Letters* **580**: 336–340. doi: 10.1016/j.febslet.2005.12.018.

771 **Ohto M-AA, Fischer RL, Goldberg RB, Nakamura K, Harada JJ**. **2005**. Control of seed mass
772 by APETALA2. *Proceedings of the National Academy of Science, USA* **102**: 3123–3128. doi:
773 10.1073/pnas.0409858102.

774 **Pettkó-Szandtner A, Mészáros T, Horváth G V., Bakó L, Csordás-Tóth É, Blastyák A,**
775 **Zhiponova M, Miskolczi P, Dudits D**. **2006**. Activation of an alfalfa cyclin-dependent kinase
776 inhibitor by calmodulin-like domain protein kinase. *Plant Journal* **46**: 111–123. doi:
777 10.1111/j.1365-313X.2006.02677.x.

778 **Ren H, Santner A, Pozo JC Del, Murray JAH, Estelle M**. **2008**. Degradation of the cyclin-
779 dependent kinase inhibitor KRP1 is regulated by two different ubiquitin E3 ligases. *Plant*
780 *Journal* **53**: 705–716. doi: 10.1111/j.1365-313X.2007.03370.x

781 **Schiessl K, Muino JM, Sablowski R**. **2014**. Arabidopsis JAGGED links floral organ patterning
782 to tissue growth by repressing Kip-related cell cycle inhibitors. *Proceedings of the National*
783 *Academy of Science, USA* **111**: 2830–2835. doi: 10.1073/pnas.1320457111.

784 **Schnittger A, Weinl C, Bouyer D, Schöbinger U, Hülskamp M**. **2003**. Misexpression of the
785 cyclin-dependent kinase inhibitor *ICK1/KRP1* in single-celled Arabidopsis trichomes reduces
786 endoreduplication and cell size and induces cell death. *Plant Cell* **15**: 303–315. doi:
787 10.1105/tpc.008342.

788 **Serrano-Mislata A, Schiessl K, Sablowski R**. **2015**. Active control of cell size generates spatial
789 detail during plant organogenesis. *Current Biology* **25**: 2991–2996.doi:

10.1016/j.cub.2015.10.008.

Tsukaya H. **2013**. Does ploidy level directly control cell size? Counterevidence from arabidopsis genetics. *PLoS ONE* **8**. doi: 10.1371/journal.pone.0083729.

Tsukaya H. **2014**. Comparative leaf development in angiosperms. *Current Opinion in Plant Biology* **17**: 103–109. doi: 10.1016/j.pbi.2013.11.012.

Vandepoele K, Vlieghe K, Florquin K, Hennig L, Beemster GTS, Gruissem W, Van de Peer Y, Inzé D, De Veylder L. 2005. Genome-Wide Identification of Potential Plant E2F Target Genes. *Plant Physiol.* **139**: 316 - 328. doi: 10.1104/pp.105.066290.

Vanhaeren H, Gonzalez N, Coppens F, De Milde L, Van Daele T, Vermeersch M, Eloy NB, Storme V, Inzé D. **2014**. Combining growth-promoting genes leads to positive epistasis in *Arabidopsis thaliana. eLife* **2014**: 1–19. doi: 10.7554/eLife.02252.

Van Leene J, Hollunder J, Eeckhout D, Persiau G, Van De Slijke E, Stals H, Van Isterdael G, Verkest A, Neirynck S, Buffel Y, *et al.* 2010. Targeted interactomics reveals a complex core cell cycle machinery in *Arabidopsis thaliana*. *Molecular Systems Biology* **6**. doi: 10.1038/msb.2010.53.

Verkest A, de O. Manes C-L-, Vercruysse S, Maes S, van der Schueren E, Beeckman T, Genschik P, Kuiper M, Inze D, De Veylder L. **2005a**. 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. *Plant Cell* **17**: 1723–1736. doi: 10.1105/tpc.105.032383.

Verkest A, Weinl C, Inzé D, De Veylder L, Schnittger A. **2005b**. Switching the cell cycle. Kip-related proteins in plant cell cycle control. *Plant Physiology* **139**: 1099–1106. doi: 10.1104/pp.105.069906.

Wang H, Fowke LC, Crosby WL. **1997**. A plant cyclin-dependent kinase inhibitor gene. *Nature* **386**: 451–452. doi: 10.1038/386451a0.

Weber H, Borisjuk L, Wobus U. **1996**. Controlling seed development and seed size in *Vicia faba*: A role for seed coat-associated invertases and carbohydrate state. *Plant Journal* **10**:

823–834. doi: 10.1046/j.1365-313X.1996.10050823.x.

Weinl C, Marquardt S, Kuijt SJH, Nowack MK, Jakoby MJ, Hülskamp M, Schnittger A. **2005**. Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. *Plant Cell* **17**: 1704–1722. doi: 10.1105/tpc.104.030486.

Wuyts N, Palauqui J, Conejero G, Verdeil J-L, Granier C, Massonnet C. **2010**. High-contrast three-dimensional imaging of the Arabidopsis leaf enables the analysis of cell dimensions in the epidermis and mesophyll. *Pant Methods* **6**: 17. doi: 10.1186/1746-4811-6-17.

Zamariola L, De Storme N, Tiang CL, Armstrong SJ, Franklin FCH, Geelen D. **2013**. SGO1 but not SGO2 is required for maintenance of centromere cohesion in *Arabidopsis thaliana* meiosis. *Plant Reproduction* **26**: 197–208. doi: 10.1007/s00497-013-0231-x.

Zamariola L, De Storme N, Vannerum K, Vandepoele K, Armstrong SJ, Franklin FCH, Geelen D. **2014**. SHUGOSHINs and PATRONUS protect meiotic centromere cohesion in *Arabidopsis thaliana*. *Plant Journal* **77**: 782–794. doi: 10.1111/tpj.12432.

Zhao X, Bramsiepe J, Van Durme M, Komaki S, Prusicki MA, Maruyama D, Forner J, Medzihradszky A, Wijnker E, Harashima H, *et al.* 2017. RETINOBLASTOMA RELATED1 mediates germline entry in *Arabidopsis*. *Science* **356:** 6336. doi: 10.1126/science.aaf6532.

Zhou Y, Fowke L, Wang H. **2002**. Plant CDK inhibitors: Studies of interactions with cell cycle regulators in the yeast two-hybrid system and functional comparisons in transgenic *Arabidopsis* plants. *Plant Cell Reports* **20**: 967–975. doi: 10.1007/s00299-001-0434-8.

Zhou Y, Li G, Brandizzi F, Fowke LC, Wang H. **2003**. The plant cyclin-dependent kinase inhibitor ICK1 has distinct functional domains for *in vivo* kinase inhibition, protein instability and nuclear localization. *Plant Journal* **35**: 476–489. doi: 10.1046/j.1365-313X.2003.01821.x.

Figures:

Figure 1. The effect of *KRP* mutations on leaf size of *Arabidopsis thaliana*: Change of (a) leaf blade area, (b) cell size, (c) cell number, and (d) stomatal index of leaf 1&2 of single (*krp4, krp6, krp7*), double (*krp4/6, krp4/7, krp6/7*) and triple mutants (*krp4/6/7*) relative to the WT (Col-0). For leaf blade area measurements n = 20-100 mature leaves, whereas for cellular measurements n = 3-9 leaves (120-220 abaxial epidermal cells per leaf were measured). Error bars represent standard error. Asterisks indicate a significant difference at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively.

Figure 2. Kinematic growth analysis of the first leaf pair of *Arabidopsis thaliana* Col-0 and *krp4/6/7* plants: (a) leaf blade area, (b) relative leaf expansion rate, (c) epidermal cell number on the abaxial side of the leaf, (d) cell division rate (CDR), (e) epidermal cell area, and (f) stomatal index. Insets show the same data on a linear scale to show absolute differences. Error bars represent ± standard error; n = 8-22 for leaf blade area and 3-6 leaves for cellular analysis (50-190 abaxial epidermal cells per leaf were measured).

Figure 3. Ploidy distribution of *Arabidopsis thaliana* Col-0 and *krp4/6/7*: (a) Relative endoreplication index of first leaf pair of Col-0 (WT), single, double and triple mutants on D26, (b) endoreduplication index and ploidy distribution, (c)-(f) percentage of 2C, 4C, 8C and 16C nuclei. Error bars represent ± standard error; (n = 4 biological replicates each containing 3-20 leaves for which 13 - 16 000 cells per replicate were measured). Asterisks indicate a significant difference at * $P < 0.05$ and *** $P < 0.001$.

Figure 4. Transcriptome analysis of *Arabidopsis thaliana* Col-0 and *krp4/6/7*: Overlap between genes differentially expressed on 9 and 15 DAS in *krp4/6/7*. Numbers represented by up and down arrows show up and down regulation of genes, respectively. Transcripts were considered differentially expressed if $\log_2$ of fold change > 0.75 and FDR corrected $P < 0.05$ (n = 2 on 9D and 3 on 15D biological replicates).

Figure 5. Seed and embryo size of *Arabidopsis thaliana* Col-0 and *krp4/6/7*: (a) seed size as projected area of dry seeds (n = 6 plants for which 110-130 seeds per plant measured), (b) embryo size measured as projected area of dissected embryos (n = 30-40). Error bars represent ± standard error. Asterisks indicate a significant difference at *** $P < 0.001$.

Figure 6. Effect of seed size on leaf growth of *Arabidopsis thaliana* Col-0 and *krp4/6/7*: (a) seed size distribution of Col-0 and *krp4/6/7,* the dotted oval represents the median size of each line (n = 3 plants for which 320-480 seeds per plant were measured), (b) leaf blade area of Col-0 and *krp4/6/7* seedlings grown from seed sizes of 80, 100, 120, 130 and 140 μm^2 (C = Col-0 and k = *krp4/6/7*). Error bars represent ± standard error (n = 15-35 leaves). Asterisks indicates a significant difference at *** $P < 0.001$.

Figure 7. Cellular analysis of embryo size of *Arabidopsis thaliana* Col-0 and *krp4/6/7*: (a) cotyledon area (n = 50), (b) cotyledon cell number and (c) cotyledon cell area (n = 15 for which 20-30 cells per cotyledons ware measured). Error bars represent ± standard error.

Figure 8. Silique and seed development in *Arabidopsis thaliana* Col-0, *krp4/6/7* and *sgo1*: Mature siliques of (a) Col-0, (b) *krp4/6/7* and (c) *sgo1.* Scale bar = 5 mm

Figure 9. Contribution of seed size on the leaf phenotype of *Arabidopsis thaliana sgo1*: Differences in (a) leaf blade area, (b) cell number and (c) cell area of *sgo1* relative to the WT, (d) leaf 1&2 blade area of *sgo1* and Col-0 seedlings grown from equal seed size ($150\mu m^2$). Error bars represent ± standard error (n = 10 for leaf blade area, and 5 for which 110-190 abaxial epidermal cells per leaf were measured). An asterisk indicates a significant difference at * $P < 0.05$.

Figure 10. Contribution of seed size to the phenotype of enhanced leaf size mutants and transgenic lines of *Arabidopsis thaliana*. (a) leaf blade area, (b) seed size, (c) leaf blade area

of seedlings grown from equal seed size (160µm²), (d) Estimated % of seed size contribution to increased leaf blade area. Error bars represent ± standard error (n = 20-30 for leaf blade area and 3-5 plants for which 100-150 seeds per plant were measured). An asterisk indicates a significant difference at * $P < 0.05$.

Tables:

Table 1. Estimated rate constants for conversion of leaf ploidy fractions during leaf development (9 to 26 days DAS) of wild type and triple mutant of *Arabidopsis thaliana.* Rate constants (expressed in days^{-1}) are indexed according to the reaction step with '1' indicating the 2C to 4C conversion, '2' for 4C to 8C, '3' for 8C to 16C, and the 'f' or 'r' referring to forward or reverse steps (4 > 2C in mitotic cells).

	k_{1f} [d^{-1}]	k_{1r} [d^{-1}]	k_2 [d^{-1}]	k_3 [d^{-1}]
Col-0	0.24 (SE=0.019)	0.11 (SE=0.013)	0.076 (SE=0.0057)	0.0092 (SE=0.0036)
Krp4/krp6/krp7	0.48 (SE=0.041)	0.25 (SE=0.027)	0.10 (SE=0.0080)	0.024 (SE=0.0049)

Table 2. The effect of *krp4/6/7 and sgo1 on* seed production and size in *Arabidopsis thaliana*. Plants were grown side by side under identical conditions (n = 5 inflorescences for each line). Values represent averages ± standard error. Asterisks indicate significant differences at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Silique	**Col-0**	***krp4/6/7***	***sgo1***
Total no. of siliques	29 ± 2	27 ± 2	32 ± 3
Siliques length (mm)	11 ± 1	9 ± 1	7 ± 1
Fertility			
Total no. of normal seeds	1285 ± 94	714 ± 106	343 ± 32***
Total no. of aborted seeds	148 ± 7	314 ± 45*	404 ± 17***
% of abnormal seeds	10 ± 1	31 ± 3***	54 ± 1***

Seed yield			
Seed size (mm²)	0.12 ± 0.01	0.16 ± 0.01***	0.18 ± 0.01***
Total seed weight (mg)	0.17 ± 0.02	0.12 ± 0.01*	0.13 ± 0.02*
Total no. of seed/ plant	10549 ± 776	4978 ± 618*	4673 ± 284*

916

917

918 **Supplemental Figures:**

919 **Fig. S1** Mathematical model for time dependent evolution of ploidy level (fraction of nuclei).

920 **Fig. S2** Gene enrichment analysis of differentially expressed genes.

921 **Fig. S3** NGS reads mapping for *KRP4, KRP6* and *KRP7*.

922 **Fig. S4** Genotyping and RT-PCR of *krp4, krp6 and krp7.*

923 **Fig. S5** Gene expression of *KRPs on 9 and 15 DAS.*

924 **Fig. S6** Relative gene expression of *ami-KRP1-3-5* constructs.

925 **Fig. S7** Leaf blade area of *ami-KRP1-3-5* transgenic constructs.

926 **Fig. S8** Correlation of seed size and rosette area.

927 **Fig. S9** Seed size of single, double and triple mutants.

928 **Table S1** Primers for genotyping of T-DNA insertion lines and artificial miRNA cloning.

929 **Table S2** List of significantly expressed cell cycle genes in *krp4/6/7* on 9 and 15 DAS.

930 **Methods S1** Construction of transgenic plants.

931 **Methods S2** Transcriptome analysis.

932 **Methods S3** Model construction and analysis