Kip-Related Protein 3 Is Required for Control of Endoreduplication in the Shoot Apical Meristem and Leaves of *Arabidopsis*

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The cell cycle plays an important role in the development and adaptation of multicellular organisms; specifically, it allows them to optimally adjust their architecture in response to environmental changes. Kip-related proteins (KRPs) are important negative regulators of cyclin-dependent kinases (CDKs), which positively control the cell cycle during plant development. The *Arabidopsis* genome possesses seven *KRP* genes with low sequence similarity and distinct expression patterns; however, why *Arabidopsis* needs seven *KRP* genes and how these genes function in cell cycle regulation are unknown. Here, we focused on the characterization of *KRP3*, which was found to have unique functions in the shoot apical meristem (SAM) and leaves. *KRP3* protein was localized to the SAM, including the ground meristem and vascular tissues in the ground part of the SAM and cotyledons. In addition, *KRP3* protein was stabilized when treated with MG132, an inhibitor of the 26S proteasome, indicating that the protein may be regulated by 26S proteasome-mediated protein degradation. *KRP3*-overexpressing (*KRP3* OE) transgenic plants showed reduced organ size, serrated leaves, and reduced fertility. Interestingly, the *KRP3* OE transgenic plants showed a significant reduction in the size of the SAM with alterations in cell arrangement. In addition, compared to the wild type, the *KRP3* OE transgenic plants had a higher DNA ploidy level in the SAM and leaves. Taken together, our data suggest that *KRP3* plays important regulatory roles in the cell cycle and endoreduplication in the SAM and leaves.

INTRODUCTION

Unlike animals, which can sense and respond to environmental changes by actively moving to an optimal habitat, sessile plants adapt to a changing environment by altering their growth and development. Cell division is one of the most important features of environmental adaptation in organisms, particularly in plants. Moreover, plants have the potential to create new organs and to develop new organs post-embryonically from specialized

regions called meristems, where cell division plays a key role both in the developmental processes that create the overall architecture of a plant and in modulating the plant growth rate in response to environmental changes (Barton, 2010; Laufs et al., 1998). The machinery of the cell includes several positive and negative regulators that control the progression and cessation of the cell cycle. Among these regulators, cell cycle phase-specific cyclin (CYC) and cyclin-dependent kinase (CDK) complexes trigger the successive continuation of the cell cycle (Inzé and De Veylder, 2006; Nowack et al., 2012). CDK inhibitors negatively regulate cell division by interacting with CYC/CDK complexes. Plant CDK inhibitors carry a conserved domain found in mammalian CDK inhibitors, known as Kip-related protein (KRPs)/inhibitor of CDK (ICKs). The *Arabidopsis* genome contains seven *KRP* genes (De Veylder et al., 2001; Wang et al., 2006). Previous studies based on the ectopic expression of *KRP1*, *KRP2*, *KRP4*, and *KRP6* confirmed that these proteins function as negative regulators of cell division; reductions in plant size, organ size, and cell number have been observed (De Veylder et al., 2001; Liu et al., 2008; Verkest et al., 2005; Weinl et al., 2005). The reason that *Arabidopsis* needs seven *KRP* genes and how these *KRP* genes function in cell cycle regulation (*i.e.*, collectively or individually) are currently unknown.

Previous reports demonstrated that the seven *KRP* genes in *Arabidopsis* have distinct spatial and temporal expression patterns, as well as distinct functional patterns (De Veylder et al., 2001; Menges and Murray, 2002; Menges et al., 2005; Ormenese et al., 2004; Wang et al., 2006). These findings suggest that the *KRP* family is transcriptionally regulated according to spatial and temporal conditions (*e.g.*, by organ or developmental stage). Based on these previous studies, we propose that the seven *KRP* genes in *Arabidopsis* display distinct substrate specificities (*i.e.*, phase-specific CDKs) and that they affect the spacing and timing of development by regulating the cell cycle.

On the other hand, several recent studies in plants reported that *KRP* genes inhibited or induced endoreduplication. The overexpression of *Arabidopsis KRP1*, *KRP2*, and *KRP6* was shown to inhibit endoreduplication, decreasing the ploidy level in leaves and trichomes (De Veylder et al., 2001; Liu et al.,

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2008; Schnitter et al., 2003). In contrast, studies in a variety of organisms have demonstrated a potential positive role for KRPs in endoreduplication. Maize KRPs are reportedly involved in endoreduplication during endosperm development (Coelho et al., 2005). In addition, *KRP2* overexpression was found to trigger the mitosis-to-endocycle transition during leaf development (Verkest et al., 2005).

In this study, we characterized *KRP3* and found that it has unique functions in the shoot apical meristem (SAM) and leaves. GUS expression was identified in the SAM (including the ground meristem and vascular tissues in the ground part of the SAM and cotyledons) in *KRP3*pro:*KRP3*-GUS transgenic plants. In addition, KRP3 protein was stabilized when treated with MG132, an inhibitor of the 26S proteasome, suggesting that KRP3 is regulated by 26S proteasome-mediated protein degradation. *KRP3*-overexpressing (*KRP3* OE) transgenic plants showed well-known *KRP*-overexpression phenotypes, including reduced organ size, serrated leaves, and reduced fertility. Interestingly, the *KRP3* OE transgenic plants showed a reduction in the size of the SAM and alterations in cell arrangement. In addition, compared to the wild type, a higher DNA ploidy level was detected in SAM and leaf cells of the *KRP3* OE transgenic plants. Taken together, our results suggest that *KRP3* is involved in regulating the cell cycle and endoreduplication in the SAM and leaves.

MATERIALS AND METHODS

Plant materials and growth condition

The wild type used in this study was *Arabidopsis thaliana Col-0* (*Col-0*). Transgenic plants with *KRP3*pro:*KRP3*-GUS and transgenic plants with sense *KRP3* constructs driven by the CaMV 35S promoter in *Col-0* background were used for the analyses. Seeds of wild type and transgenic lines were surface sterilized, germinated on Murashige and Skoog (MS) medium and seedlings were transferred to soil after 3-weeks. Plants were grown at 23°C under long-day light condition (50-100 $\mu\text{E}/\text{m}^2\text{s}$, 16 h of light).

DNA constructs and generation of transgenic plants

To construct the *KRP3*pro:*KRP3*-GUS and CaMV35S pro:*KRP3*, we isolated *KRP3* promoter region, full genomic region and *KRP3* protein encoding region from *Col-0* genome and cDNA library, respectively. We amplified DNA fragment from 2.5 kb upstream of transcriptional start codon to stop codon of *KRP3* (AT5G48820, GenBank: BT025290.1) with primers of TOPO-pro*KRP3*-F; 5'-CACCAAGAAGATCTTTTCATGAAAGAGAAAAAAG-3' and *KRP3*-R2; 5'-GGTTTGACTTGCACCAATTCGTAAC-3' and *KRP3* protein encoding region with primers of TOPO *KRP3*-F; 5'-CACCATGGGGAAATACATGAAGAA-3' and *KRP3*-R1; 5'-TCATGGTTTGACTTGCACCCATTCTGT-3'. These DNA fragments were cloned into the pENTR™/D-TOPO® vector (Invitrogen, USA). All clones were confirmed by sequencing. Then, each *KRP3* promoter:*KRP3* fragments and *KRP3* encoding region in the pENTR™/D-TOPO® vector was mobilized into the destination vector, *pGWB3* containing *GUS* gene and *pH2GW7* containing CaMV 35S promoter through LR reaction, a recombination reaction between attL site in pENTR™/D-TOPO® and attR site in destination vectors by LR clonase (Invitrogen, USA). All constructs were introduced into *Arabidopsis thaliana* by the floral-dip method through *Agrobacterium*-mediated transformation. Transgenic plants were isolated by hygromycin as a selection marker.

RT-PCR analysis

To confirm expression level of *KRP3*, semiquantitative RT-PCR was carried out. Total RNA of the wild type and transgenic plants was extracted from aerial parts of 3-weeks seedlings using RNeasy Mini Kit (Quagen, Germany). First-strand cDNA was prepared using the SuperScript first-strand synthesis system (Invitrogen, USA). DNA fragment of *KRP3* was amplified by the following conditions and primer pairs: for *KRP3*, 5'-AGATCATGGTTACACCAGGG-3' and 5'-CATTCGTAACGTCGCTGAG-3'. *TUB4* transcript level was examined as control using the following primer pairs, 5'-CCTCTTCTCTCCTCTCGTAC-3' and 5'-AGAGGTTGA CGA GCAGATGA-3'.

Microscopic observation

Samples of seedlings and leaves were fixed in Fixation solution [5% (v/v) acetic acid, 5% (v/v) formaldehyde, and 45% (v/v) ethanol in water] under a vacuum. To analyze the number and size of the palisade cell, fixed leaves were transparently rendered by incubation in solution of chloral hydrate [200 g chloral hydrate and 20 g glycerol in 50 ml dH₂O] (Kim et al., 1999). Palisade cells between the midvein and the leaf margin in the center of the leaf blade were analyzed. The number and size of palisade cells per unit area of this region was determined. For histological analysis, fixed seedlings were dehydrated by sequential 30-min incubation in 50%, 60%, 70%, 80%, 90%, 95%, and 99.5% (v/v) ethanol, and 1 h incubation in 100% (v/v) ethanol twice. The dehydrated seedlings were set in Technovit 7100 resin (Heraeus Kulzer, Wehrheim/Ts., Germany) at room temperature, once in 50% (v/v) resin and twice in 100% resin. Seedlings were embedded in molds and harden for 24 h. Longitudinal section of seedling resin block were performed with a rotary microtome (4- μm thick) (MICROM International, Germany), and then slices were stained with a 0.1% (w/v) solution of toluidine blue in 0.1 M sodium phosphate buffer (pH 2.0) for 2 min. Whole leaves, leaf cells, and sectional slices were observed by stereoscopic microscopy (Leica MZ12.5, Leica, Germany) and a light microscopy (Axioskop2, Carl Zeiss, Germany).

GUS staining of *Arabidopsis* seedling

Plant seedlings were incubated in 90% (v/v) acetone for 1 h at 20°C, and washed with 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM K₃Fe(CN₆)III, 3 mM EDTA, and 0.05% (v/v) Triton X-100. For GUS staining, washed seedlings were immersed in above mentioned 50 mM sodium phosphate buffer containing 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-glucuronide at 37°C for overnight. To terminate staining, seedlings were mounted in 70% (v/v) ethanol.

DNA ploidy analysis

To visualize DNA contents of cells in SAM, 4',6-diamidino-2-phenylindole (DAPI) staining was utilized. Slice of SAM in 14-day-old old seedlings was prepared from histological analysis method and then stained with 10 mg/ml DAPI for 30 min. DAPI stained nuclei in SAM cells was observed by fluorescent microscopy (Axioskop2, Zeiss, Germany). To measure the amount of DNA contents in leaves, DNA ploidy analyzer was used. Frozen leaves were chopped with a razor blade in nuclei extraction solution and supplemented with nuclei staining solution (<http://www.partech.com>). The homogenate was filtered over a 30- μm mesh. The nuclei were analyzed with the Cystain UV Precise P 05-5002 (<http://www.partech.com>).

Fig. 1. Spatial expression pattern of KRP3 protein. (A) GUS stained young leaf (left), root tip (middle) and root (right) of 14-day-old seedlings of *KRP3pro:KRP3-GUS* transgenic plants treated dimethyl sulfoxide (DMSO) or 100 μM MG132 dissolved in DMSO for 6 h. (B) GUS stained shoot apex (left) and its longitudinal sections (right) of 6 DAS seedlings treated DMSO or 100 μM MG132 dissolved in DMSO for 6 h. Scale bars, 1 mm (left) and 50 μm (right).

Fig. 2. Phenotypes of *KRP3* overexpressing transgenic plants. (A-D) Phenotypes of *KRP3* OE transgenic plants in aerial part. From the left, wild type (*Col-0*), *KRP3* OE-30 (moderate phenotype), and *KRP3* OE-45 (strong phenotype). (A) Leaf phenotype of at the age of 21 days. (B) The phenotype of the mature plants. Scale bars, 1 cm. (C, D) The phenotype of floral organs in side view (C) and upper view (D). (E) Root growth curve of *Col-0* and *KRP3* OE transgenic plants. (F) The expression level of *KRP3* in *Col-0* and *KRP3* OE transgenic plants.

RESULTS

KRP3 protein is expressed in the SAM, root meristem, and leaf primordia

Due to the low expression level, it was difficult to detect mRNA expression in our *KRP3pro:KRP3-GUS* analysis. Several reports have demonstrated the expression pattern of mRNAs in various tissues by *in situ* hybridization, microarray, and RT-PCR (Menges and Murray, 2002; Ormenese et al., 2004); however, the precise expression pattern of *KRP3* mRNA is controversial.

To assess the KRP3 protein expression pattern, we generated transgenic plants harboring *KRP3pro:KRP3-GUS* fusion proteins. GUS expression was observed in the SAM, cotyledons, and leaf primordia, and in the root meristem and dividing cells (Fig. 1A). Longitudinal sections of *KRP3pro:KRP3-GUS* transgenic seedlings showed GUS expression in the SAM region, including in the ground meristem and vascular tissues in the ground part of the SAM and cotyledons (Fig. 1B). These results suggest that cell cycle inhibition by KRP3 in the SAM, root meristem, and leaf primordia is important for proper plant

development.

KRP3 protein is regulated by 26S proteasome-mediated degradation

A recent paper suggested that KRPs are regulated by 26S proteasome-mediated protein degradation (Liu et al., 2008; Ren et al., 2008).

To investigate KRP3 protein degradation and its spatial expression pattern, we analyzed the GUS expression pattern in *KRP3pro:KRP3-GUS* transgenic plants treated with or without MG132, a 26S proteasome inhibitor. MG132 treatment enhanced GUS expression in the SAM and leaf primordia (Fig. 1). Marked GUS expression was observed in the dividing zone of the root tip after treatment with MG132 (Fig. 1A). In addition, a sectional analysis of 7-day-old MG132-treated seedlings indicated that KRP3 was stabilized in the shoot apex and vascular tissue (Fig. 1B). These results suggest that not only the spatial expression but also the temporal degradation of KRP3 is crucial in the regulation of cell division in the shoot apex and root tips.

Fig. 3. Effect of MG132 treatment on wild type and *KRP3* OE transgenic plants. (A, B) From the left, wild type (*Col-0*), *KRP3* OE-30, and *KRP3* OE-45 plants of 21-day-old seedling germinated in MS media after treatment with DMSO (A) or 100 μ M MG132 dissolved in DMSO (B). Scale bars, 1 cm.

***KRP3* overexpression caused size reductions in the floral organs, serrated leaves, and reduced root growth**

To investigate the function of *KRP3*, T-DNA insertion mutants (Supplementary Fig. 2) were characterized. However, the mutants showed no alternation in phenotype compared to the wild type under our growth conditions (data not shown). Despite their low level of sequence similarity, the seven KRPs in *Arabidopsis* have conserved motifs, including a nuclear localization signal domain and CYC/CDK-binding domain (Supplementary Fig. 1). KRP family proteins might have redundant functions in binding CYC/CDKA and CYC/CDKA complexes as cell cycle inhibitors (Verkest et al., 2005). However, their expression patterns and inhibitory effect on kinase activity are probably distinct.

In this study, we characterized *KRP3* function using transgenic plants overexpressing *KRP3* under control of the cauliflower mosaic 35S promoter. Homozygous lines showing moderate expression (*KRP3* OE-30) and a strong phenotype (*KRP3* OE-45) were selected and characterized. Both types of transgenic plants exhibited varying degrees of dwarfism, reductions in size, and serrated leaves and floral organs (e.g., sepals and petals) (Figs. 2A-2D). The extent of *KRP3* expression in selected transformants was detected by RT-PCR (Fig. 2F). *KRP3* expression in the transgenic plants was not correlated with phenotype severity. The expression level of *KRP3* in line OE-45, which showed a strong phenotype, was lower than that in line OE-30, which showed a moderate phenotype (Figs. 2A and 2F), indicating that the post-transcriptional regulation of *KRP3* might be more important than transcriptional regulation. In addition, the reduction in size and serration of the leaves in the *KRP3* OE transgenic plants were more severe in the presence of MG132 (Fig. 3 and Supplementary Table 1), suggesting that the temporal degradation of *KRP3* is crucial in the regulation of cell division during leaf development.

To evaluate the effects of *KRP3* overexpression on root growth, we measured the root length in *KRP3* OE transgenic plants for 14 days after sowing (DAS). Striking defects in root growth were observed (Fig. 2E). The reduced root length in the transgenic plants was caused by an overall reduction in growth rate throughout development. This suggests that *KRP3* controls the cell cycle by lowering the frequency of cell division through

Fig. 4. Anatomical analysis of shoot apical meristem (SAM) and palisade cells of leaves. (A, B) Longitudinal section of the SAM in *Col-0* (A) and *KRP3* OE-45 transgenic plant (B) on 7 DAS. (C, D) Lined image of the boundary of each cells of the SAM in A panel (C) and B panel (D). (E, F) Paradermal images of palisade cells of the first leaf in *Col-0* (E) and *KRP3* OE-45 transgenic plant (F) on 14 DAS. Scale bars, 50 μ m.

a decrease in the cell cycle rate.

Alterations in size and structure in the SAM of the *KRP3* OE transgenic plants

An anatomical analysis of the SAM in the transgenic plants was carried out to identify the function of *KRP3*. The *KRP3* OE transgenic plants showed reductions in the size of the SAM (Figs. 4A and 4B). Longitudinal sections of the SAM from *KRP3* OE-45 transgenic plants showed good organization but enlargement of cells in layers L1 and L2 (Figs. 4B and 4D). However, the cells in layer L3 in the transgenic plants were altered and irregularly sized compared to the wild type (Figs. 4A-4D). The *KRP3* OE transgenic plants showed a reduced cell number but increased cell size in SAM (Fig. 4B and Table 1). The number of cells in the SAM was significantly reduced (to 54% of that in the wild type), while the cell size was increased (to 150% of that in the wild type; Table 1). In spite of decreased cell number, interestingly, total size of SAM in *KRP3* OE transgenic plants was not significantly reduced, because of enlarged cell size (Figs. 4A-4D). This result may due to increase of DNA ploidy by enhanced endoreduplication. Together, these findings indicate that *KRP3* plays important roles in the maintenance of stem cell proliferation and in control of cell expansion in the SAM.

Table 1. Statistical analysis of cell size and number in SAM and of palisade cells in the first leaf pair in *Col-0* and *KRP3* OE transgenic plants

SAM	Actual			Relative		
	Cell number	Total area (μm^2)	One cell area (μm^2)	Cell number	Total area	One cell area
<i>Col-0</i>	81.5 ± 2.1	3466.6 ± 386.5	42.49 ± 3.6	100	100	100
<i>KRP3</i> OE-45	44.3 ± 4.5	2802.4 ± 480.9	63.51 ± 9.7	54.3	80.8	149.5

Leaf	Actual		Relative	
	Cell number in direction of leaf width	One cell area (μm^2)	Cell number in direction of leaf width	One cell area
<i>Col-0</i>	184.17 ± 35.9	993.42 ± 214.8	100	100
<i>KRP3</i> OE-45	47.01 ± 7.8	2191.74 ± 678.3	25.5	220.6

Data are means $\pm$ standard error which is analyzed from the SAM of 7 DAS and 1st leaf of 14 DAS of 5 plants.

Fig. 5. Anatomical analysis of DNA contents in SAM and leaf primordia. (A, B) DAPI stained nuclei of cells in SAM and leaf primordial of *Col-0* (A) and *KRP3* OE-45 transgenic plant (B). (C, D) Close-up image of *Col-0* (C) and *KRP3* OE-45 transgenic plant (D).

Reduced cell number and increased cell size in the leaves of the *KRP3* OE transgenic plants

To analyze the effects of *KRP3* overexpression on leaves, we performed an anatomical analysis of the first leaf pair in wild-type and transgenic plants 14 DAS. The cell numbers in the leaves of the *KRP3* OE-45 transgenic plants was reduced significantly (Figs. 4E and 4F) (to 26% of that in the wild type; Table 1). However, cell size in the plants was increased (to 221% of that in the wild type; Table 1). Therefore, the reduced organ size caused by *KRP3* overexpression was due to a reduction in cell number resulting from the inhibition of mitosis. Compensation was also noted in the altered leaves of the transgenic plants. Compared to the alteration in cell number and cell size in the SAM, the alteration in the leaves was relatively severe. Together, these results suggest that *KRP3* plays important roles in the regulation of cell division and cell enlargement during morphogenesis.

KRP3 overexpression triggered endoreduplication in the SAM and leaves

KRP3 overexpression triggered a reduction in the size of the

Fig. 6. Analysis of DNA ploidy level in 14-day-old first leaves of wild type and *KRP3* OE-45 transgenic plants.

SAM and leaves due to a reduction in cell number. This phenomenon was probably caused by an increase in endoreduplication (Verkest et al., 2005), which can increase the nuclear DNA content in cells. To determine the effect of *KRP3* overexpression on DNA content, we observed the nuclei of cells in SAM using DAPI staining and the distribution of DNA in 3-week-old first leaves by flow cytometry (Figs. 5 and 6). Brightness of DAPI-stained nuclei was enhanced in the cells of SAM and leaf primordia of the *KRP3* OE transgenic plants compared to the wild type (Fig. 5). The leaves of the *KRP3* OE transgenic plants had a higher DNA ploidy level (16C and 32C) compared to the wild type (Fig. 6). Verkest et al. (2005) reported that the dosage of *KRP* regulates the onset of endoreduplication and arrest of the cell cycle. These results indicate that *KRP3* overexpression inhibited mitosis in the SAM and leaves.

DISCUSSION

KRP3 is involved in regulation of the SAM and leaf architecture

Despite low levels of similarity in DNA sequence and conserved motifs between *KRP* gene family members, *KRP* overexpression inhibits the cell cycle, causing reductions in plant organ size and serrated leaves (De Veylder et al., 2001; Wang et al., 2006). However, studies have shown that the seven *KRP* genes in *Arabidopsis* have distinct spatial and temporal expression patterns according to the developmental phase of the plant (Ormenese et al., 2004) and phase of the cell cycle (Memges et al., 2005; Menges and Murray, 2002). In addition, *KRP1* and *KRP2* are reported to be degraded by the 26S proteasome (Jakoby et al., 2006; Ren et al., 2008; Weini et al., 2005; Zhou et al., 2003). *KRP6* is targeted for degradation by specific E3 ligases (Liu et al., 2008). Thus, despite redundant functions in the inhibition of CDK activity, the specific expression and degradation patterns of proteins with spatial and temporal regulation play important roles in plant development.

In this study, we analyzed the expression pattern of *KRP3* using *KRP3pro:KRP3-GUS* transgenic plants. GUS was detected in the SAM, leaf primordia, and root tips in dividing cells (Fig. 1A). *In situ* hybridization and RT-PCR analyses of *KRP* expression support our data showing marked *KRP3* expression in the SAM and leaf primordia (Menges et al., 2005; Ormenese et al., 2004). In addition, *KRP3* protein was also regulated by 26S proteasome-mediated protein degradation (Fig. 1). The GUS signals in the SAM and root tips and leaf phenotype of the *KRP3* OE plants were strongly enhanced by treatment with MG132 (Fig. 1). These results indicate that inhibition of the cell cycle in the SAM, leaf primordia, and root tips by *KRP3* is extremely important for proper plant development.

The morphology and arrangement of cells in the SAM affects the direction of cell division, positioning of the leaf primordia, and cell fate specification (Barton, 2010; Laufs et al., 1998). Borghi et al. (2010) reported that the negative cell cycle regulator *RETINOBLASTOMA-RELATED* is required not only for regulation of the cell cycle but also for maintenance of stem cell identity and promotion of differentiation. In addition, *CDKA;1* and *CDKB2* play a role in meristem identification and organization as well as cell cycle regulation (Andersen et al., 2008; Gaamouche et al., 2010). Cooperation among the cell cycle machinery and meristem management through cell cycle regulators control the organization of the meristem and cell fate determination. Our *KRP3* OE transgenic plants showed an alteration in SAM architecture, including a reduced dome size and slightly altered cell morphology below the L3 layer (Figs. 4A

and 4B). Although these alterations did not cause changes in phyllotaxy, leaf morphology was affected (Fig. 2A). Could the alignment of cells in the SAM affect leaf morphology (e.g., shape and size) as well as phyllotaxy? The regulation of leaf morphology in the SAM is poorly understood. Therefore, it is important to identify the regulatory mechanisms that control meristem organization and cell fate determination through the interaction of SAM-specific genes and the cell cycle machinery. Our data suggest that tissue-specific patterns in mRNA expression and protein degradation underlie the different functional roles of *KRP* genes in plant development and growth.

KRP3 is involved in the regulation of endoreduplication during SAM and leaf development

Several recent studies in plants have shown that *KRPs* regulate endoreduplication. In this study, the *KRP3* OE transgenic plants had increased DNA content in the nuclei of the SAM and leaf primordia compared to the wild type (Fig. 5). Additionally, the leaves of the *KRP3* OE transgenic plants had a higher DNA ploidy level (16C and 32C) compared to the wild type (Fig. 6). *CDKA;1* transcripts were detected in both dividing and endoreduplicating cells at the shoot apex and leaves, suggesting that *CDKA;1* plays important roles in mitosis and endoreduplication (Verkest et al., 2005). Thus, our data indicate that the inhibition of *CDKA;1* activity by *KRP3* in mitotically dividing cells in the SAM and leaves results in entry into endoreduplication.

Overall, cell growth, and therefore organ size, is the result of cell proliferation and expansion. In addition, DNA content is a deciding factor in organ size. In particular, the relationship between DNA content and cell expansion is regarded as a key to understanding the mechanism of cell expansion (Breuer et al., 2010). Our study indicates that *KRP3* overexpression reduced the number of cells but increased their size in leaves with a high DNA content (Figs. 4B and 6; Table 1). Cell enlargement by endoreduplication has been reported in several genetic studies. For example, *HIGH PLOIDY2* and *ROOT HAIRLESS*, both of which control entry into the endocycle, showed DNA ploidy-dependent cell expansion (Ishida et al., 2009; Sugimoto-Shirasu et al., 2005). In contrast, Ferjani et al. (2007) suggested that DNA ploidy-independent cell expansion can compensate for the inhibition of cell proliferation to protect against changes in organ size. However, these hypotheses are unable to explain all features of the control of cell and organ size. Further study of the relationship between cell size and DNA content will help explain the role of cell elongation in organ development. In addition, our *KRP3* expression analysis will further our understanding of the evolution and development of this gene family in plants.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

- Andersen, S., Buechel, S., Zhao, Z., Ljung, K., Novák, O., Busch, W., Schuster, C., and Lohmann, J. (2008). Requirement of B2-type cyclin-dependent kinases for meristem integrity in *Arabidopsis thaliana*. *Plant Cell* 20, 88-100.
- Barton, M. (2010). Twenty years on: the inner workings of the

- shoot apical meristem, a developmental dynamo. *Dev. Biol.* **341**, 95-113.
- Borghi, L., Gutzat, R., Fütterer, J., Laizet, Y., Hennig, L., and Grissem, W. (2010). *Arabidopsis* RETINOBLASTOMA-RELATED is required for stem cell maintenance, cell differentiation, and lateral organ production. *Plant Cell* **22**, 1792-1811.
- Breuer, C., Ishida, T., and Sugimoto, K. (2010). Developmental control of endocycles and cell growth in plants. *Curr. Opin. Plant Biol.* **13**, 654-660.
- Coelho, C., Dante, R., Sabelli, P., Sun, Y., Dilkes, B., Gordon-Kamm, W., and Larkins, B. (2005). Cyclin-dependent kinase inhibitors in maize endosperm and their potential role in endoreduplication. *Plant Physiol.* **138**, 2323-2336.
- De Veylder, L., Beeckman, T., Beemster, G.T.S., Krols, L., Terras, F., Landrieu, I., Van Der Schueren, E., Maes, S., Naudts, M., and Inzé, D. (2001). Functional analysis of cyclin-dependent kinase inhibitors of *Arabidopsis*. *Plant Cell* **13**, 1653-1667.
- Ferjani, A., Horiguchi, G., Yano, S., and Tsukaya, H. (2007). Analysis of leaf development in fugu mutants of *Arabidopsis* reveals three compensation modes that modulate cell expansion in determinate organs. *Plant Physiol.* **144**, 988-999.
- Gaamouche, T., Manes, C.L., Kwiatkowska, D., Berckmans, B., Koumproglou, R., Maes, S., Beeckman, T., Vernoux, T., Doonan, J.H., Traas, J., et al. (2010). Cyclin-dependent kinase activity maintains the shoot apical meristem cells in an undifferentiated state. *Plant J.* **64**, 26-37.
- Inzé, D., and De Veylder, L. (2006). Cell cycle regulation in plant development. *Annu. Rev. Genet.* **40**, 77-105.
- Ishida, T., Fujiwara, S., Miura, K., Stacey, N., Yoshimura, M., Schneider, K., Adachi, S., Minamisawa, K., Umeda, M., and Sugimoto, K. (2009). SUMO E3 ligase HIGH PLOIDY2 regulates endocycle onset and meristem maintenance in *Arabidopsis*. *Plant Cell* **21**, 2284-2297.
- Jakoby, M.J., Weini, C., Pusch, S., Kuijt, S.J., Merkle, T., Dissmeyer, N., and Schnittger, A. (2006). Analysis of the subcellular localization, function, and proteolytic control of the *Arabidopsis* cyclin-dependent kinase inhibitor ICK1/KRP1. *Plant Physiol.* **141**, 1293-12305.
- Kim, G.-T., Tsukaya, H., and Uchimiya, H. (1999). The ROTUNDIFOLIA3 gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev.* **12**, 2381-2391.
- Laufs, P., Grandjean, O., Jonak, C., Kiéuc, K., and Traas, J. (1998). Cellular parameters of shoot apical meristem in *Arabidopsis*. *Plant Cell* **10**, 1375-1389.
- 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.
- Menges, M., and Murray, J.M. (2002). Synchronous *Arabidopsis* suspension cultures for analysis of cell-cycle gene activity. *Plant J.* **30**, 203-212.
- Menges, M., de Jager, S.M., Grissem, W., and Murray, J.A.H. (2005). Global analysis of the core cell cycle regulators of *Arabidopsis* identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. *Plant J.* **41**, 546-566.
- Nowack, M., Harashima, H., Dissmeyer, N., Zhao, X., Bouyer, D., Weimer, A., De Winter, F., Yang, F., and Schnittger, A. (2012). Genetic framework of cyclin-dependent kinase function in *Arabidopsis*. *Dev. Cell* **22**, 1030-1040.
- Ormenese, S., de Almeida Engler, J., De Groodt, R., De Veylder, L., Inzé, D., and Jacqmard, A. (2004). Analysis of the spatial expression pattern of seven kip-related proteins (KRPs) in the shoot apex of *Arabidopsis thaliana*. *Ann. Bot.* **93**, 575-580.
- Ren, H., Santner, A., de Pozo, J.C., Murray, J.A.H., and Estelle, M. (2008). Degradation of the cyclin-dependent kinase inhibitor KRP1 is regulated by two different ubiquitin E3 ligase. *Plant J.* **53**, 705-716.
- Schnittger, A., Weini, C., Bouyer, D., Schöbinger, U., and Hülskamp, M. (2003). Misexpression of the cyclin-dependent kinase inhibitor ICK1/KRP1 in single-celled *Arabidopsis* trichomes reduces endoreduplication and cell size and induces cell death. *Plant Cell* **15**, 303-315.
- Sugimoto-Shirasu, K., Roberts, G.R., Stacey, N.J., McCann, M.C., Maxwell, A., and Roberts, K. (2005). RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. *Proc. Natl. Acad. Sci. USA* **102**, 18736-18741.
- Verkest, A., Manes, C.L., Vercruysse, S., Maes, S., Van Der Schueren, E., Beeckman, T., Genschik, P., Kuiper, M., Inzé, D., and De Veylder, L. (2005). 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.
- Wang, H., Zhou, Y., and Fowke, L. (2006). The emerging importance of cyclin-dependent kinase inhibitors in the regulation of the plant cell cycle and related processes. *Can. J. Bot.* **84**, 640-650.
- Weini, C., Marquardt, S., Kuijt, S., Nowack, M., Jakoby, M., Hülskamp, M., and 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.
- Zhou, Y., Li, G., Brandizzi, F., Fowke, L.C., and 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 J.* **35**, 476-489.