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Analysis of the Spatial Expression Pattern of Seven Kip Related Proteins (KRPs) in the Shoot Apex of *Arabidopsis thaliana*

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• *Background and aims* Kip-related-proteins (KRPs), negative regulators of cell division, have recently been discovered in plants but their *in planta* function is as yet unclear. In this study the spatial expression of all seven *KRP* genes in shoot apices of *Arabidopsis thaliana* were compared.
• *Methods In situ* hybridization analyses were performed on longitudinal sections of shoot apices from 2-month-old Arabidopsis plants.
• *Key Results* The study provides evidence for different expression pattern groups. *KRP1* and *KRP2* expression is restricted to the endoreduplicating tissues. In contrast, *KRP4* and *KRP5* expression is mainly restricted to mitotically dividing cells. *KRP3*, *KRP6* and *KRP7* can be found in both mitotically dividing and endoreduplicating cells.
• *Conclusion* The results suggest differential roles for the distinct *KRP*s. *KRP1* and *KRP2* might specifically be involved in the establishment of polyploidy. In contrast, *KRP4* and *KRP5* might be involved in regulating the progression through the mitotic cell cycle. *KRP3*, *KRP6* and *KRP7* might have a function in both types of cell cycle.

Key words: *Arabidopsis thaliana*, cell cycle, cell differentiation, endoreduplication, KRP expression.

INTRODUCTION

Flowering plants are multicellular organisms where cell division plays a significant role in growth and development. The cell division cycle is regulated in yeast, animals and plants by a molecular machinery where the main drivers are cyclin-dependent kinases (CDKs) (Norbury and Nurse, 1992; Morgan, 1997; Mironov *et al*., 1999). CDK activity is mediated by several mechanisms, more particularly by association with cyclins (reviewed by Pines, 1994) and phosphorylation of the Thr-161 residue (for review, see Dunphy, 1994).

A relatively new mechanism of regulating CDK activity has been found with the identification of CDK inhibitors (CKIs; see reviews by Harper and Elledge, 1996; Pines, 1995; Sherr and Roberts, 1995, 1999). These proteins bind to cyclin/CDK complexes and inhibit CDK activity. In mammals, two CKI families have been recognized, based upon their sequence similarity and mode of action: the INK4 and the Kip/Cip families.

The CKIs of the Kip/Cip family, including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, negatively regulate cell cycle progression and enforce cell cycle arrest when expressed at high levels (Elledge and Harper, 1994; Sherr and Roberts, 1995). The Kip/Cip CKIs are involved in both G1/S and G2/M checkpoint control and the regulation of the cell cycle exit preceding cell differentiation (Zhang *et al*., 1999). The $p21^{Cip1}$ and $p27^{Kip1}$ CKIs have also been found in complexes with active cyclin-CDKs, suggesting that CKIs may also act as positive regulators (LaBaer *et al*., 1997). Indeed the normal up-regulation of CyclinD/CDK4 in mitogen-stimulated fibroblasts depends upon $p21^{Cip1}$ and $p27^{Kip1}$ (Cheng *et al*., 1999).

Proteins of the class that act as inhibitors of CDK were unknown in plants until ICK1 was identified in *Arabidopsis thaliana* (Wang *et al*., 1997) The carboxy-terminal domain of ICK1 shares a conserved 31 amino-acid sequence, including part of the CDK binding domain, with the mammalian $p27^{Kip1}$ kinase inhibitor. ICK1 has been shown to interact directly with both Cdc2a (CDK-a) and CycD3 (a D-type cyclin) by yeast two-hybrid and *in vitro* binding assays (Wang *et al*., 1998). Actually in the *A. thaliana* genome seven CKI-like genes are present, all having a region of approx. 25 amino acids that are highly conserved with the mammalian Kip/Cip proteins, hence their name, KRPs: *K*ip-*r*elated *p*roteins (De Veylder *et al*., 2001; Vandepoele *et al*., 2002). Despite their limited sequence homology with the mammalian counterparts, the KRPs have been shown to be true biochemical homologues of the Kip/Cip proteins, as recombinant KRPs are able to inhibit CDK activity *in vitro* (Wang *et al*., 1998; Lui *et al*., 2000), whereas their overexpression in plants results in a

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decrease of CDK activity *in vivo* (Wang *et al*., 2000; De Veylder *et al*., 2001).

Although some data are available on the transcription profiles of KRPs, not much is known about their spatial pattern of expression. Preliminary expression analyses showed that the *KRP1* and *KRP6* genes are expressed ubiquitously in various plant organs (roots, inflorescence stems, flower buds and 3-week-old leaves) and in a 3-day-old, actively dividing suspension culture (De Veylder *et al*., 2001). *KRP4*, *KRP5* and *KRP7* are expressed in the same organs and culture, but mRNA clearly seems to be more abundant in tissues that display high mitotic activity (flowers and suspension cultures), with *KRP4* also being abundantly present in leaves. *KRP2* mRNA seems to be more abundant in flowers, and the level of *KRP3* expression is high in actively dividing suspension cultures but it is not detectable, or is barely so, in intact plant organs (mainly roots and flowers) (De Veylder *et al*., 2001). These transcription profiles suggest that the various KRPs might play distinct roles during plant development.

Here we report the spatial expression pattern of all seven *A. thaliana KRPs* in the shoot apex of plants maintained in vegetative growth for 2 months in short day conditions. This material has proved to be suitable for the characterization of genes involved in the regulation of the mitotic cycle and the endoreduplication cycle (Jacqmard *et al*., 1999). At the cellular level the data confirm the existence of differential expression patterns for the distinct *KRP* genes, and on this basis a classification of the KRPs into different functional groups is suggested.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana (L.) Heynh. (ecotype Col-o) plants were maintained in a vegetative state for 2 months by growth in short days as described in Corbesier *et al*. (1996). Seeds were kindly provided by C. R. Somerville (Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA, USA). Apical buds were then excised for mRNA *in situ* hybridization analysis.

mRNA in situ *hybridization*

Longitudinal sections of shoot apices from 2-month-old *Arabidopsis* plants were hybridized as described by Segers *et al*. (1996). Non-specific binding of the KRPs probes to the adhesive was often observed and was responsible for causing background noise in the results. We have partly succeeded in reducing this non-specic binding of the probes by doubling the duration of the acetylation treatment. Lengthening the acetylation time did not affect the signal with the antisense probe. *KRP*s [^{35}S] UTP-labelled antisense probes were obtained from the linearized transcription vectors by *in vitro* transcription with T7 (*KRP1*, *KRP2*, *KRP3*, *KRP5* and *KRP7*) and SP6 (*KRP4* and *KRP6*) RNA polymerases.

RESULTS

In situ hybridizations were performed on sections of shoot apices of 2-month-old *A. thaliana* plants kept in a vegetative state when grown in short day conditions. This allowed us to characterize the *KRP* genes potentially involved in the regulation of the mitotic cycle and/or the endoreduplication cycle, since discrimination between dividing and endoreduplicating tissues has been established (Jacqmard *et al*., 1999). Without ambiguity, divisions occur in the shoot apical meristem (SAM) in just-emerged leaf primordia up to 70 μm length, in vascular tissues and in the procambium, while endoreduplication is established in cells of the pith and the stipules. The situation is less clear regarding the leaves where, depending on their stage of maturation, both divisions and endoreduplication can be found. Indeed, DNA content in the SAM and young leaf primordia of 30–70 μm of length was mostly 2C with only a small proportion of 4C nuclei, indicating that meristematic cells are euploid (Jacqmard *et al*., 1999). In contrast, in maturing leaves >400 μm in length, endoreduplication is observed in all mesophyll cells while divisions still occur in vascular tissues of these leaves. An intermediate situation is observed in leaf primordia of 300–400 μm of length, where spongy mesophyll cells at the abaxial side are differentiated from the palisade layer at the adaxial side, and where cell divisions occur concomitantly with the establishment of endoreduplication in mesophyll cells.

In the *in situ* hybridization analysis, the *KRP1* and mainly *KRP2* genes were highly expressed in endoreduplicating cells of the pith and in mesophyll cells of maturing leaves (Fig. 1). Expression of both genes was also observed in cells of 300–400 μm long leaf primordia (arrows in Fig. 1, KRP1 and KRP2). *KRP1* and *KRP2* RNA transcripts were barely detected in the SAM, in axillary buds (not shown) and in vascular cells. The distribution of *KRP1* and *KRP2* transcripts in leaves varied depending on the stage of differentiation of the leaf. Transcripts of both genes were distributed in a relatively homogenous pattern in maturing leaf primordia. But in leaf primordia of 300–400 μm length, tissue-specific patterns of expression were observed: *KRP1* transcripts accumulated both in palisade cells of the mesophyll at the adaxial side and in spongy mesophyll cells at the abaxial side (arrows in Fig. 1, KRP1); *KRP2* transcripts accumulated more specifically in spongy mesophyll cells (arrow in Fig. 1, KRP2). Although mitoses were still detected in leaf primordia of that stage, cell differentiation had already started.

In contrast, *KRP4* and *KRP5* were expressed in all tissues where mitotic divisions occur (Fig. 1, KRP4 and Fig. 2, KRP5). The level was high in dividing cells of the SAM and in young leaf primordia of up to 400 μm. While *KRP4* was also slightly expressed in the procambium (Fig. 1, KRP4), *KRP5* hybridization signal was particularly strong in the just-initiated procambial cells and in the peripheral zone of the SAM (Fig. 2, KRP5). For both genes, some expression was also

detected in the vascular bundles of maturing leaves and either a very weak or no signal was observed in the pith and mesophyll cells of maturing leaves.

The *KRP3*, *KRP6* and *KRP7* genes were expressed in both dividing cells of the emerged leaf primordia and endoreduplicating cells of the pith and maturing leaves within the shoot apex (Fig. 1, KRP3 and Fig. 2, KRP6 and 7). *KRP3* transcripts accumulated particularly in the upper cells of the pith just produced by the rib meristem (arrow in Fig. 1, KRP3). The *KRP3* and *KRP7* signals were absent from the SAM while that of *KRP6* was weakly detected. The *KRP3*, *KRP6* and *KRP7* hybridization signals were low in dividing procambial cells of the stem (Fig. 1, KRP3 and Fig. 2, KRP6 and 7). A signal was not observed in control hybridizations with sense probes for six *KRP*s (shown for *KRP2*, *KRP3*, *KRP5*, *KRP6* and *KRP7*, but not shown for KRP4) (Fig. 2A, B, C, E, F and G, respectively). A very low signal was detected in the pith with the sense *KRP1* probe but it is much lower than with the antisense probe (compare Fig. 1, KRP1 and Fig. 2A). Also, a signal was not observed in control hybridizations with antisense probes pretreated with RNase (shown for *KRP3* in Fig. 2D).

DISCUSSION

Analysis of the expression patterns of the seven *KRP*s of *A. thaliana* in the shoot apex by mRNA *in situ* hybridization suggests differential functions for the distinct KRPs in the control of the mitotic division cycle and/or endoreduplication cycle. The expression data presented (summarized in Table 1) provide evidence for different expression pattern groups. The first group comprises KRP1 and KRP2, which are highly expressed in endoreduplicating tissues such as the pith cells and mesophyll cells of maturing leaves, but are not present in the mitotic dividing cells of the SAM or the vascular cells of the shoot apex. Therefore, KRP1 and KRP2 might specifically be involved in the shift of the mitotic cycle to the endoreduplication cycle in the shoot apex, or even perhaps in the control of the endocycle itself. A role for the KRPs in controlling the ploïdy level is evident from transgenic plants overexpressing the *KRP1* and *KRP2* genes. In comparison with control plants, these transgenic plants display a decrease in their ploïdy level (De Veylder *et al*., 2001; Zhou *et al*., 2002). Curiously, whereas *KRP1* mRNA was distributed equally over the whole of the leaf primordia, *KRP2* transcripts accumulated preferentially in the differentiating spongy mesophyll cells. This expression profile is complementary to that reported for *CYCD3;1*, a D-type cyclin shown to be rate-limiting for cell division in dividing calli starved of cytokinin (Riou-Khamlichi *et al*., 1999). Its specific accumulation pattern in the shoot suggests a role for KRP2 in the onset of cell differentiation.

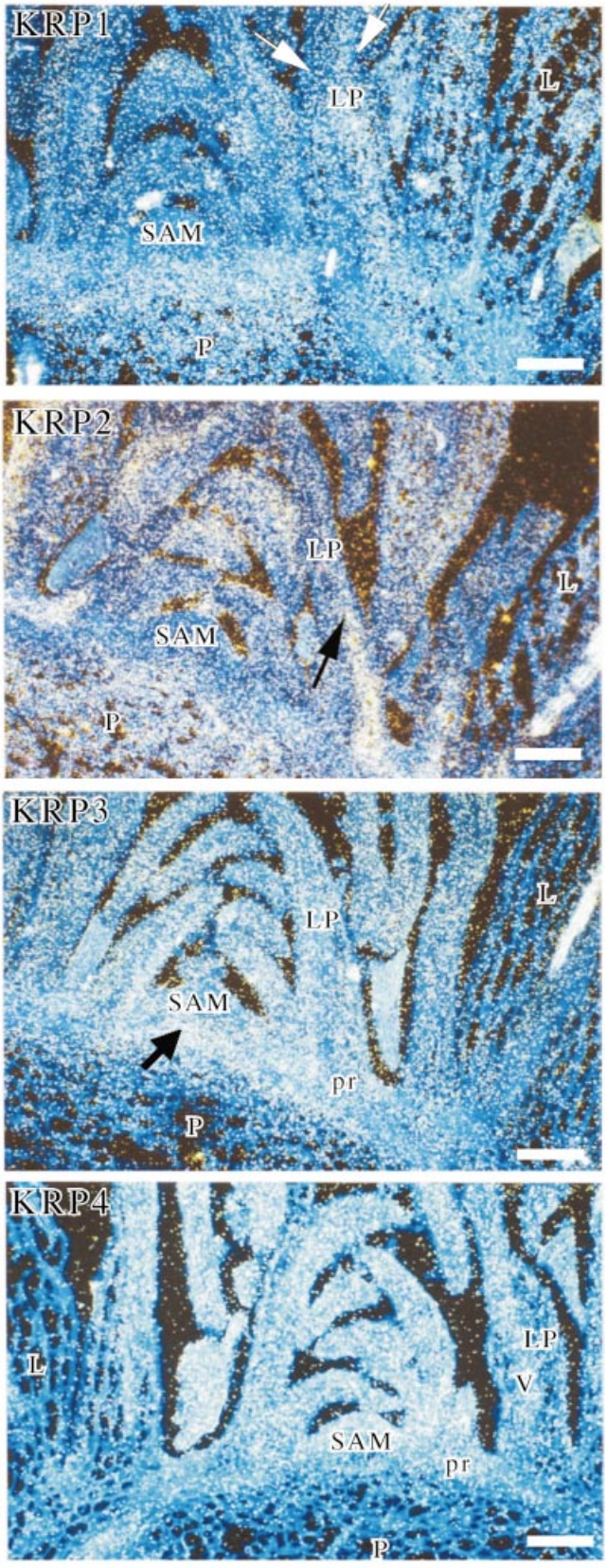

FIG. 1. mRNA *in situ* localization of *KRP*s in the shoot apex. Longitudinal sections of shoot apices of 2-month-old *A. thaliana* plants (ecotype Col-0) hybridized with ^{35}S-labelled antisense riboprobes of *KRP1*, *2*, *3* and *4*. The autoradiographic signal was visualized in dark-field illumination. L, maturing leaf of >400 µm length; LP, 300–400 µm-long leaf primordium; P, pith; pr, procambium; SAM, shoot apical meristem; V, vascular tissue. Scale bar = 100 µm. Arrows indicate transcripts accumulation.

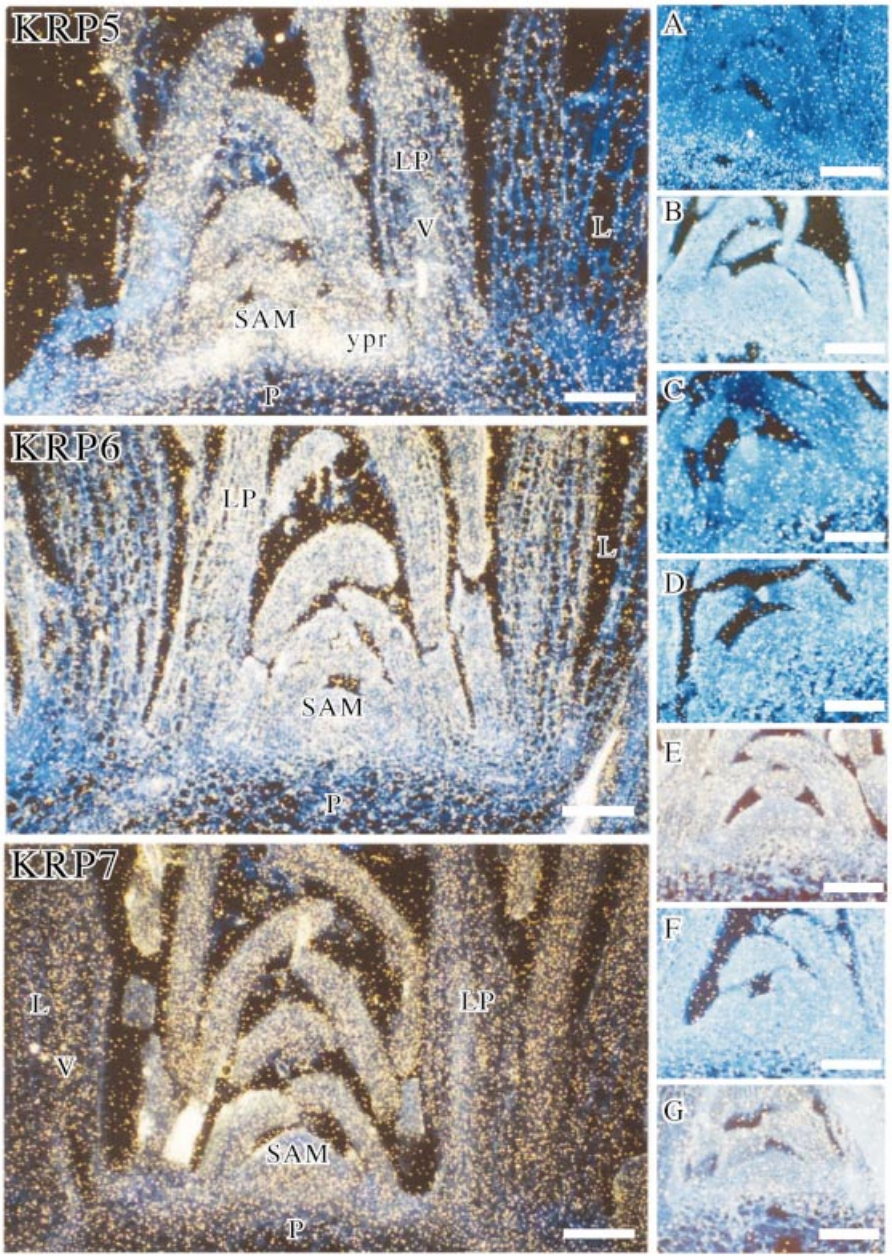

FIG. 2. mRNA *in situ* localization of *KRP*s in the shoot apex. Longitudinal sections of shoot apices of 2-month-old *A. thaliana* plants (ecotype Col-0) hybridized with ^{35}S-labelled antisense riboprobes of *KRP5*, *6* and *7*, or sense riboprobes of (A) *KRP1*, (B) *KRP2*, (C) *KRP3*, (E) *KRP5*, (F) *KRP6* and (G) *KRP7*. (D) Section pretreated with RNase and hybridized with ^{35}S-labelled antisense riboprobes of *KRP3*. The autoradiographic signal was visualized in dark-field illumination. L, maturing leaf of >400 μm length; LP, 300–400 μm-long leaf primordium; P, pith; SAM, shoot apical meristem; V, vascular tissue; ypr, young procambium. Scale bar = 100 μm.

The second class of *KRP*s comprises *KRP4* and *KRP5*, which are highly expressed in the dividing cells of the SAM, young leaf primordia, procambium and vascular tissue of the shoot apex. Since the mitotic cyclin *CYCB1;1* is also expressed in these cells (Segers *et al*., 1996), we hypothesize that KRP4 and KRP5 might be involved in regulating the progression through the mitotic cell cycle. There is an apparent discrepancy between our data for KRP4 and those previously published by De Veylder *et al*. (2001), who have observed from transcription profiles a *KRP4* expression in 3-day-old leaves where most cells are elongating and are probably endoreduplicating. Since divisions occur in the vascular cells of these leaves, we cannot exclude the possibility that KRP4 might be part of regulating the progression through the mitotic cell cycle. Curiously, the *KRP5* hybridization signal is particular high in just-initiated procambial cells and in the peripheral zone of the SAM, suggesting a more specific role for KRP5 in the

TABLE 1. *Expression levels of seven* KRP*s of* A. thaliana *in the shoot apex*

Type of *KRP*s	Shoot apical mersitem	Vascular cells	Pith	Cortex	Young leaf primordia		Mature leaf primordia
					Palisade mesophyll	Spongy mesophyll	
KRP1	O	O	XX	X	XX	XX	XX
KRP2	O	O	XXX	X	XX	XXX	XX
KRP3	X	X	XX	X	XX	XX	X
KRP4	XX	X	O	O	XX	XX	O
KRP5	XXX	XX	X	X	XX	XX	X
KRP6	XX	X	X	X	XX	XX	XX
KRP7	X	XX	XX	XX	XX	XX	XX

Abbreviations: O = not expressed; X = slightly expressed; XX = expressed; XXX = highly expressed.

divisions that occur in the zone where leaf primordia initiate and in the zone where the periphery of the stem is constructed.

The third group of *KRP*s comprises *KRP3*, *KRP6* and *KRP7*, for which transcripts can be detected in both mitotically dividing and endoreduplicating cells. The *KRP3* transcripts accumulate particularly in the upper cells of the pith, just below the L3 layer. It would be of interest to determine if *KRP3* interacts with either gene involved in the maintenance of the SAM state (Sharma and Fletcher, 2002).

At this time it is still unclear why *A. thaliana* has so many different KRPs that are expressed in the same tissues. It is probable that KRPs might participate in regulating CDK activity in response to different developmental or environmental signals. This hypothesis is supported by the fact that *KRP1* expression is induced by abscisic acid (Wang *et al*., 1998). Moreover, the *KRP4* expression pattern in the shoot apex is highly similar to that of *CYCD3;1* (Riou-Khamlichi *et al*., 1999). Since *CYCD3* is specifically induced by cytokinin, *KRP4* might also be under the control of this mitogen. In order to further understand the functions of the *KRP* genes it would be interesting to identify the distinct mitogenic and environmental factors regulating their expression.

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