SCF$^{\text{SKP2B}}$- AND KPC1-DEPENDENT DEGRADATION OF CYCLIN-DEPENDENT KINASE INHIBITOR KRP1 AND CELL CYCLE REGULATION IN *ARABIDOPSIS THALIANA*

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Abstract

In animals and fungi, cyclin-dependent kinase inhibitors play a key role in cell cycle regulation by inhibiting the activities of cyclin-dependent kinase/cyclin complexes. However, little is known about the role of this group of proteins in plant cell cycle regulation. To gain insight into the mechanisms by which the plant cell cycle is regulated, I studied the cyclin-dependent kinase inhibitor KRP1 in *Arabidopsis*. The role of KRP1 in pericycle activation during lateral root initiation and in cell cycle regulation and how ubiquitin-mediated protein degradation regulates KRP1 protein turnover were investigated. My results show that KRP1 plays an important role in the regulation of pericycle activation during lateral root initiation. KRP1 interacts with the CDKA;1/CYCD2;1 complex *in planta* and functions in the G1-S transition of the cell cycle. KRP1 is an unstable protein *in planta* and its degradation depends on the 26S proteasome. Further, an SCF complex composed of CUL1 and SKP2b regulates KRP1 degradation. These results suggest that SCF^{SKP2b} targets KRP1 for degradation by the 26S proteasome to regulate the G1-S transition of the cell cycle. In addition to SCF^{SKP2b}-mediated KRP1 degradation, KRP1 degradation is also regulated by the

RING finger ubiquitin ligase KPC1. To further understand the mechanisms of

KRP1 degradation and identify novel proteins that regulate KRP1

degradation, I performed a genetic screen for mutations that stabilize KRP1

protein. Three mutants called *msk* (mutant stabilizes KRP1) caused by a

recessive mutation were identified. These three *msk* mutants define three

distinct genetic loci. The results of these studies reveal a novel function of

KRP1 in the regulation of pericycle activation during lateral root initiation and

provide new insight into the mechanisms by which plant cell cycle is regulated

by ubiquitin-mediated protein degradation.

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Chapter 1: Cell Cycle Regulation

In eukaryotes, including yeast, animals, and plants, the basic mechanisms of cell cycle regulation are highly conserved. Cell cycle progression is controlled by the activities of cyclin-dependent kinase (CDK)/cyclin complexes. CDK inhibitors (CKIs) function mainly as negative cell cycle regulators that bind the CDK/cyclin complexes and inhibit their activities. A key mechanism in cell cycle progression is the degradation of some important cell cycle regulators by ubiquitin-dependent protein degradation to promote irreversible transitions of the cycle. In this chapter, I will describe the mechanisms of cell cycle regulation in eukaryotes, CDK inhibitors, the ubiquitin-proteasome pathway, and the role of SCF ubiquitin ligases in cell cycle regulation.

Cell cycle regulation

The eukaryotic cell cycle consists of a series of events that ultimately lead to the formation of two daughter cells. The cell cycle is divided into four phases: G1, S, G2, and M (Fig 1-1). In G1 (gap 1) phase, the cell prepares for DNA synthesis. In S phase, DNA synthesis occurs and the genome duplicates. In G2 (gap 2) phase, the cell makes sure that DNA synthesis is complete and prepares for mitosis. In M (mitosis) phase, chromosome segregation and cell division occur to form two daughter cells (Johnson and Walker, 1999). In eukaryotes, the fundamental mechanisms of cell cycle
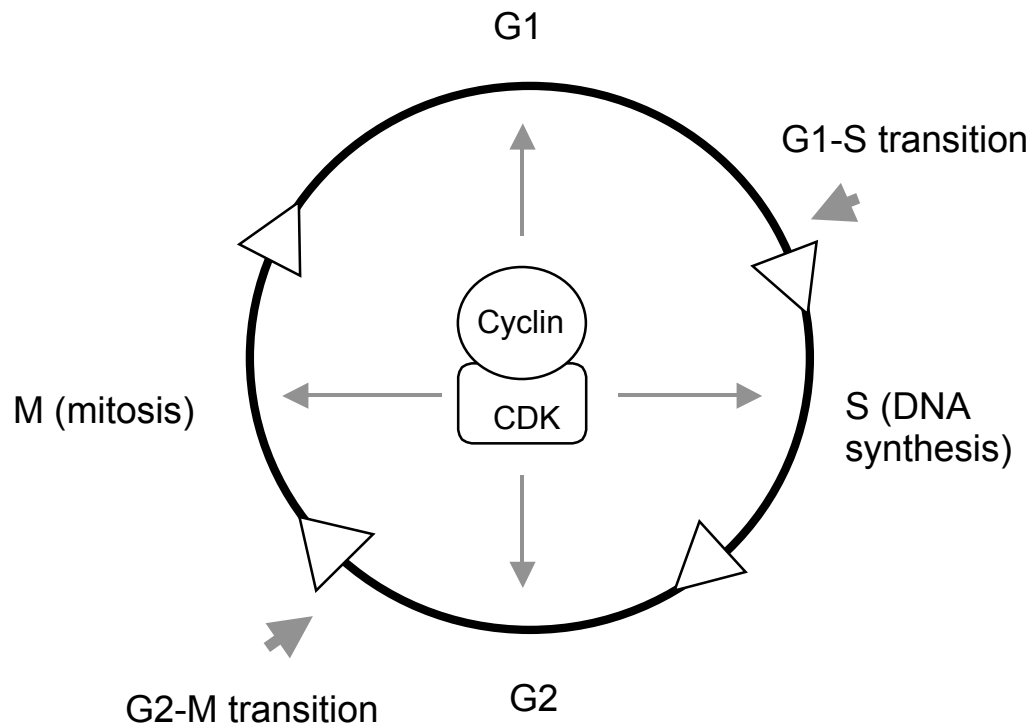
Figure 1-1. Cell cycle regulation in eukaryotes.

Cell cycle is composed of G1, S, G2, and M with two major checkpoints: the G1-S transition and the G2-M transition. The fundamental mechanisms of cell cycle regulation in eukaryotes are highly conserved. The CDK/cyclin complexes drive cell cycle progression from one phase of the cycle to the next (see text for details).

regulation are highly conserved. There are two major checkpoints to control cell cycle progression: the G1-S transition and the G2-M transition (Fig 1-1). Cell cycle progression is controlled by the activities of CDK/cyclin complexes, which consist of a catalytic subunit (CDK) and a regulatory subunit (cyclin). Different combinations of CDKs and cyclins regulate passage from one phase of the cycle to the next (Dewitte and Murray, 2003; De Veylder et al., 2003). Because CDKs are the engine that drives cell cycle progression, cells have evolved numerous mechanisms to modulate CDK activities to tightly and precisely regulate cell cycle transitions. CDK activities are regulated by binding to activating proteins cyclins, binding to inhibitory proteins CKIs, phosphorylation of CDKs by CDK activating kinases (CAKs) and other protein kinases, and dephosphorylation of CDKs by protein phosphatases (Morgan, 1997).

The CDK/cyclin complexes control the cell cycle by phosphorylating a large number of specific protein substrates. For example, in the budding yeast, 181 protein substrates have been identified, including CDK inhibitors Sic1 and Far1, G1 cyclin Cln2, protein kinases Swe1 and Gin4, G1 transcription factor Swi5, and other cell cycle regulators (Ubersax et al., 2003). A group of well-known substrates of the CDK/cyclin complexes are the retinoblastoma (RB) family proteins, which are best known for their role in modulating the activities of E2F/DP (E2F dimerization partner) transcription factors to regulate the G1-S transition of the cell cycle (Fig 1-2). In early G1 phase, RB binds to the E2F/DP heterodimer to repress gene expression
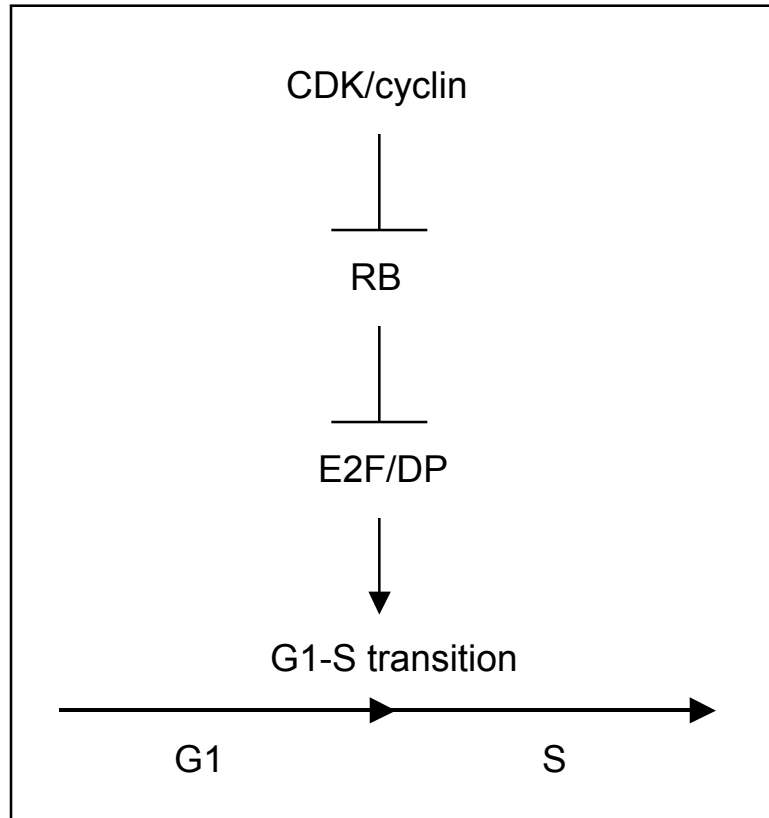
Figure 1-2. The RB-E2F pathway regulates the G1-S transition of the cell cycle (see text for details).

mediated by the E2F/DP transcription factors. In late G1 phase, triggered by certain signals, the G1 CDK/cyclin complexes become active and phosphorylate RB to release the E2F/DP heterodimer. Active E2F/DP transcription factors activate gene expression required for the G1-S transition and S phase progression. The cell passes the G1-S transition and enters S phase to undergo DNA synthesis (Classon and Harlow, 2002; Cobrinik, 2005).

The mammalian genome encodes three RB-related proteins (RB, p107, and p130), seven E2Fs, and two DPs (Cobrinik, 2005; Dimova and Dyson, 2005). RB-related proteins and E2F/DP transcription factors have been identified in plants, indicating the involvement of the RB-E2F pathway in the regulation of the G1-S transition of the plant cell cycle (Rossi and Varotto, 2002; Shen, 2002; Gutierrez et al., 2002; De Veylder et al., 2003; Dewitte and Murray, 2003). The *Arabidopsis* genome encodes a RB-related protein (RBR), three E2Fs, two DPs, and three DP-E2F-like proteins (DELs) (Vandepoele et al., 2002). A previous study showed that the CDKA;1/CYCD2;1 complex phosphorylates a plant RBR protein *in vitro* and the RBR-associated kinase activity peaks at the G1-S transition in *Arabidopsis* (Boniotti and Gutierrez, 2001), supporting an important role of the CDKA;1/CYCD2;1 complex in the regulation of the G1-S transition mediated by the RB-E2F pathway. In contrast to multicellular animals and plants, it has long been thought that the RB-E2F pathway does not exist in the unicellular organism yeast. However, the identification of Whi5 protein, an inhibitor of

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G1-specific gene expression required for the G1-S transition that functions like RB, indicates that a regulatory mechanism similar to the RB-E2F pathway is present in yeast to regulate gene expression required for the G1-S transition and S phase progression (de Bruin et al., 2004; Costanzo et al., 2004).

Cyclin-dependent kinase inhibitors

As described previously, the activities of CDK/cyclin complexes can be regulated by CDK inhibitors (CKIs), which function mainly as negative cell cycle regulators. CKIs play a critical role in cell cycle regulation through inhibiting the activities of CDK/cyclin complexes (Sherr and Roberts, 1999). CKIs have been identified in yeast, mammals, and plants (Table 1-1). In mammals, there are seven CKIs, which are classified into two families: the INK4 family and the Cip/Kip family. The INK4 CKIs have ankyrin repeats and specifically inhibit the activities of CDK4 and CDK6 (Nakayama and Nakayama, 1998; Vidal and Koff, 2000). The INK4 CKIs are composed of p15^{INK4b} (Hannon and Beach, 1994), p16^{INK4a} (Serrano et al., 1993), p18^{INK4c} (Guan et al., 1994; Hirai et al., 1995), and p19^{INK4d} (Chan et al., 1995; Hirai et al., 1995). In contrast to the INK4 CKIs, the Cip/Kip CKIs have different structural properties and have broader CDK inhibitory abilities. The Cip/Kip CKIs have a conserved motif called CDK-binding/inhibitory domain in the N-terminal region and inhibit the activities of CDK4 and CDK6 as well as other CDKs. The Cip/Kip CKIs consist of p21^{Cip1} (Harper et al., 1993; el-Deiry et al.,

Table 1-1. Cyclin-dependent kinase inhibitors in eukaryotes.

Organisms			CDK Inhibitors	References
Yeast	Budding yeast		$p40^{Sic1}$	Nugroho and Mendenhall, 1994; Donovan et al., 1994.
			Far1	Chang and Herskowitz, 1990; Peter and Herskowitz, 1994.
			Pho81	Coche et al., 1990; Schneider et al., 1994.
	Fission yeast		$p25^{Rum1}$	Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995.
Mammals		Cip/Kip family	$p27^{Kip1}$	Polyak et al., 1994; Toyoshima and Hunter, 1994.
			$p21^{Cip1}$	Harper et al., 1993; el-Deiry et al., 1993; Xiong et al., 1993.
			$p57^{Kip2}$	Lee et al., 1995; Matsuoka et al., 1995.
		INK4 family	$p15^{INK4B}$	Hannon and Beach, 1994.
			$p16^{INK4A}$	Serrano et al., 1993.
			$p18^{INK4C}$	Guan et al., 1994; Hirai et al., 1995.
			$p19^{INK4D}$	Hirai et al., 1995; Chan et al., 1995.
Plants	*Arabidopsis*	$p27^{Kip1}$-related proteins	KRP1, 2, 3, 4, 5, 6, 7	Wang et al., 1997; Lui et al., 2000; De Veylder et al., 2001.
	Tobacco		NtKIS1	Jasinski et al., 2002.
	Maize		KRP;1, KRP;2	Coelho et al., 2005

1993; Xiong et al., 1993), p27^{Kip1} (Polyak et al., 1994; Toyoshima and Hunter, 1994), and p57^{Kip2} (Lee et al., 1995; Matsuoka et al., 1995).

Plants do not appear to have INK4-type CKIs, but proteins related to the Cip/Kip family have been identified in *Arabidopsis*, tobacco, and Maize (Wang et al., 1997; De Veylder et al., 2001; Jasinski et al., 2002; Coelho et al., 2005). The *Arabidopsis* genome encodes seven mammalian CKI p27^{Kip1}-related proteins (KRPs) (Fig 1-3), also known as Interactors/Inhibitors of Cdc2 kinase (ICKs) (De Veylder et al., 2001; Zhou et al., 2002a; Vandepoele et al., 2002). The only sequence similarity between KRPs and p27^{Kip1} is in the conserved about 30-amino-acid CDK-binding/inhibitory domain (Wang et al., 1997; De Veylder et al., 2001). The CDK-binding/inhibitory domain is present in the N-terminal region of p27^{Kip1}. In contrast, all KRPs have the conserved CDK-binding/inhibitory domain in the C-terminus (Fig 1-4). Outside the CDK-binding/inhibitory domain, there is no significant sequence identity between KRPs (De Veylder et al., 2001).

Studies on the function and regulation of plant CKIs have largely focused on the effects of ectopic expression on plant growth and development and transcription, respectively. Despite years of efforts by numerous laboratories, the exact role of plant CKIs in cell cycle regulation and in plant growth and development is poorly understood. Ectopic expression studies have confirmed that KRP1, KRP2, and KRP6 are inhibitors of the cell cycle, resulting in dwarfed plants with reduced cell number and organ size (Wang et al., 2000; De Veylder et al., 2001; Zhou et

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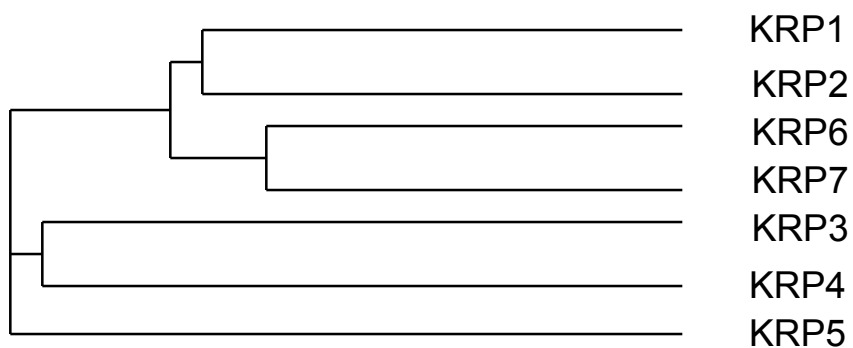
Figure 1-3. Cladogram of KRP protein family in *Arabidopsis*.

Tree was generated using the Clustal W, a multiple sequence alignment program for proteins (http://www.ebi.ac.uk/clustalw/), showing relationship between KRPs in *Arabidopsis*.
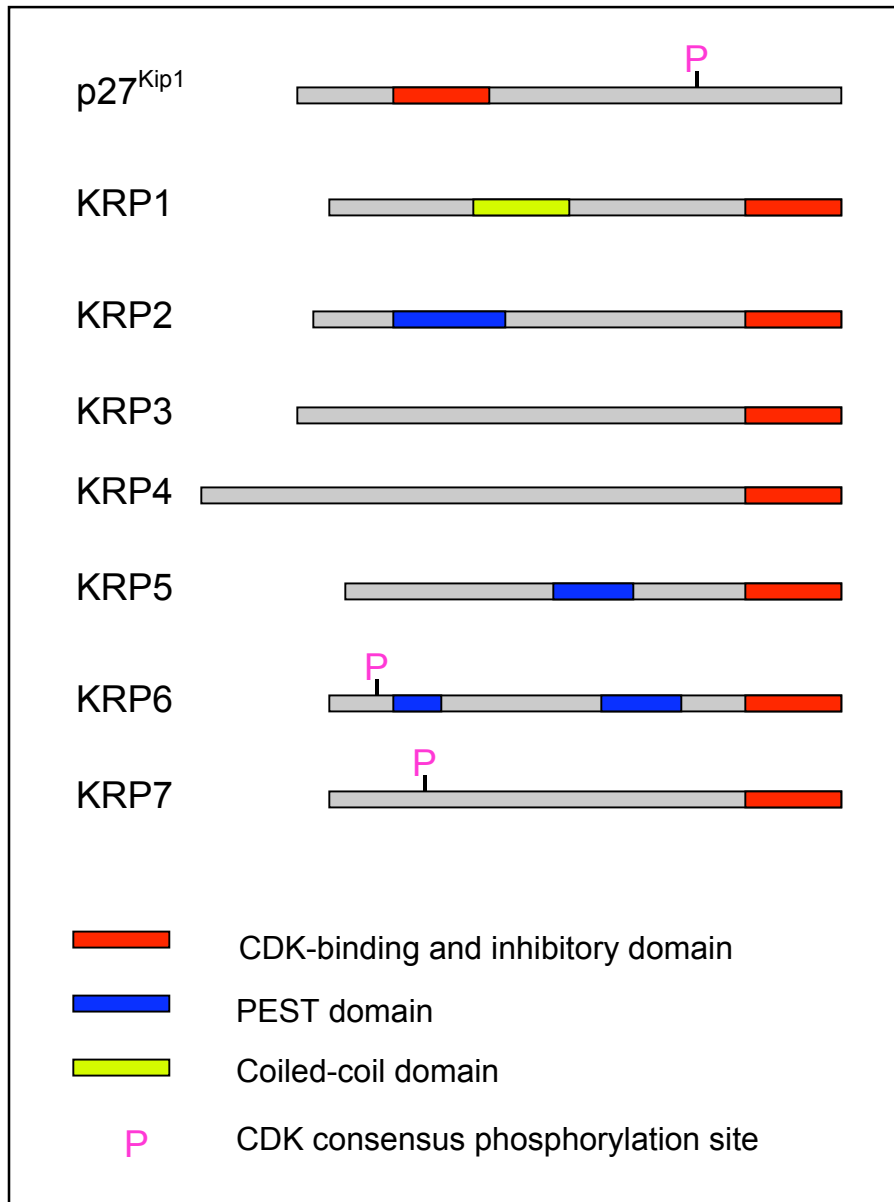
Figure1-4. Protein structure of mammalian p27^Kip1 and _Arabidopsis_ KRPs (not to scale).

Motifs were analyzed using the Simple Modular Architecture Research Tool (SMART, http://smart.embl.de/) and the PESTfind Analysis Webtool (PESTfind score > +10) (https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm). PESTfind produces a score ranging from -50 to +50. A score above zero suggests a possible PEST region. Reference: De Veylder et al., 2001.

al., 2002a; Zhou et al., 2002b). Studies of transcriptional regulation have shown that *KRP1* transcription is increased by low temperature and abscisic acid (ABA), while *KRP2* transcription is down-regulated by auxin during lateral root initiation (Wang et al., 1998; Himanen et al., 2002). The expression patterns of the *KRPs* during the cell cycle were characterized using synchronized *Arabidopsis* cultured cells both during re-entry into the cell cycle of cells that have stopped cycling due to sucrose depletion and during cell cycle progression following synchronization at the G1-S boundary using the DNA polymerase inhibitor aphidicolin (Menges and Murray, 2002; Menges et al., 2005). These data show that there are three main patterns of transcriptional regulation of *KRP* genes. *KRP1* is highly expressed in non-dividing cells and is strongly down-regulated during G1 phase in cell cycle re-entry. *KRP1* shows a further clear peak of expression at the G2-M transition, although this is 3-fold lower than the expression in non-dividing cells. *KRP2* is highly expressed in non-dividing cells and is unique in showing a peak of expression only during G1 phase as cells re-enter the cell cycle. In contrast, *KRP3*, *4*, *5*, *6*, and *7* are not highly expressed in non-dividing cells, but are up-regulated or peak during S and early G2 phase. These results implicate *KRP1* and *KRP2* as primary candidates for controlling activation of division by non-dividing cells. However, protein levels of plant CKIs during the cell cycle have not yet been investigated. Among the plant CKIs, only KRP2 is known to be degraded by the 26S proteasome. Interestingly, degradation of KRP2 requires its phosphorylation by the CDKB1;1 complex (Verkest et al., 2005a),

but the detailed mechanism of KRP2 degradation remains to be elucidated.

Ubiquitin-mediated protein degradation

In eukaryotes, the ubiquitin-proteasome pathway is a highly conserved pathway that selectively degrades proteins. The conjugation of ubiquitin (a small protein composed of 76 amino acids) to a substrate requires the sequential action of three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase) (Fig 1-5). First, the carboxy-terminal glycine residue of ubiquitin is linked to the cysteine residue of an E1 via the formation of a thiol-ester bond. This ubiquitin activation reaction is ATP-dependent. Then, ubiquitin is transferred to an E2. Finally, ubiquitin is conjugated to the lysine residue of the substrate with the help of an E3, which provides specificity for the ubiquitin-proteasome pathway. The substrate, conjugated with four or more ubiquitins that are linked through lysine 48, is degraded by the 26S proteasome (Weissman, 2001; Pickart and Eddins, 2004).

The 26S proteasome is a proteolytic machine that degrades ubiquitin-conjugated substrates and is present in both the cytoplasm and nucleus of eukaryotic cells (Wójcik and DeMartino, 2003). The 26S proteasome is composed of two subcomplexes: the 19S regulatory complex and the 20S core complex. The 19S regulatory complex functions to selectively recognize and bind substrates conjugated with a polyubiquitin chain, unfold the substrates, and translocate the unfolded substrates to the 20S core complex.
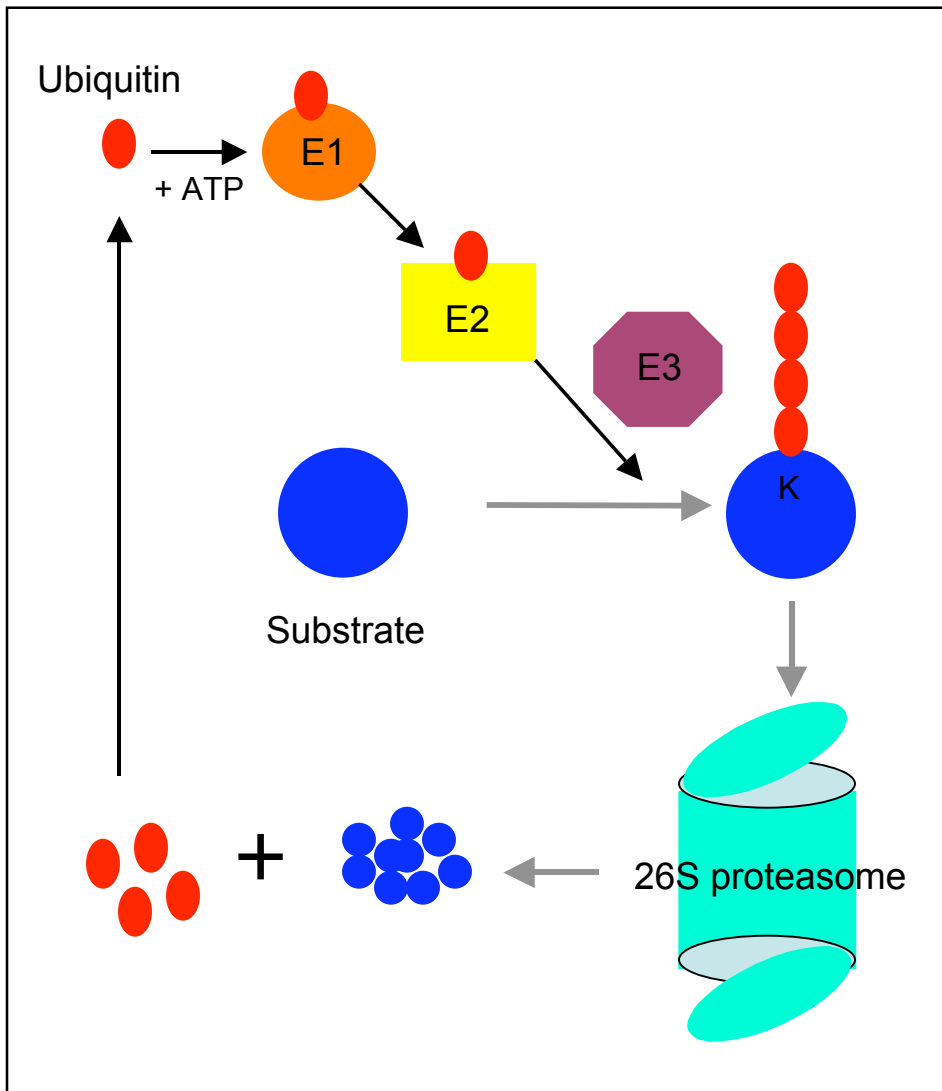
Figure 1-5. The ubiquitin-proteasome pathway (see text for details).

The 20S core complex degrades the substrates into short peptides (Pickart and Cohen, 2004; Wolf and Hilt, 2004).

For yeast and animals, the ubiquitin-proteasome pathway has been extensively studied. Ubiquitin-mediated protein degradation plays a crucial role in diverse cellular processes, including signal transduction, transcription, DNA replication, and cell cycle regulation (Pichart, 2001; Pickart and Eddins, 2004; Petroski and Deshaies, 2005). In the past ten years, important advances have also been made in this field for plants. A large number of the components of the ubiquitin-proteasome pathway have been identified, including E1s, E2s, E3s, and the proteasome subunits (von Arnim, 2001; Vierstra, 2003). Ubiquitin-mediated protein degradation has been implicated in various cellular and developmental processes, including hormone responses, cell cycle regulation, light signaling, circadian rhythms, abiotic stresses, pathogen defense response, self-incompatibility, flower development, trichome morphogenesis, and leaf senescence (Smalle and Vierstra, 2004; Moon et al., 2004).

SCFs and cell cycle regulation

Ubiquitin-mediated protein degradation plays a critical role in the cell cycle by destroying many important cell cycle regulators to promote irreversible transitions of the cycle. Two ubiquitin ligases that play an important role in the cell cycle are the APC/C (anaphase-promoting complex/cyclosome) and SCF (SKP1-Cullin-F-box protein) E3s (Reed, 2003;

Vodermaier, 2004). APC functions mainly in mitosis, regulating sister chromatid separation, spindle-pole separation, and mitosis exit (Peters, 2002; Harper et al., 2002; Castro et al., 2005). APC has been identified in plants, indicating its involvement in the plant cell cycle regulation. However, little is known about the function, regulation, and substrates of APC in the plant cell cycle (Capron et al., 2003; Fulop et al., 2005). The stabilization of CYCA3 and CYCB in the *apc2* and *nomega* mutants deficient in APC functions, respectively, suggests that CYCA3 and CYCB may the substrates of plant APC (Capron et al., 2003; Kwee and Sundaresan, 2003). In contrast to APC, SCFs function mainly in S and G2, regulating the G1-S and G2-M transitions (Yew, 2001; DeSalle and Pagano, 2001; Cardozo and Pagano, 2004; Nakayama and Nakayama, 2005). Here, I focus on SCFs and describe the role of SCFs in cell cycle regulation.

SCFs belong to one of the six types of identified ubiquitin ligases, including SCF E3s, VCB E3s, BTB E3s, APC/C E3s, HECT E3s, and single subunit RING E3s (Schwechheimer and Villalobos, 2004). An SCF complex is composed of four subunits: RBX1, CUL1, SKP1, and F-box protein (Deshaies, 1999; Fig 1-6). The CUL1 subunit is an elongated protein and functions as a scaffold to bind the RBX1 and SKP1-F-box protein subcomplex. The F-box protein subunit provides specificity for SCFs and binds selective substrates (Petroski and Deshaies, 2005). The *Arabidopsis* genome encodes ~700 F-box proteins and 21 ASKs (SKP1-related proteins) (Gagne et al., 2002; Farras et al., 2001; Risseeuw et al., 2003), indicating a

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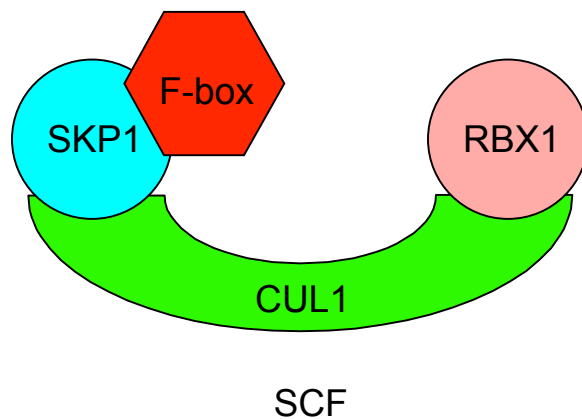
Figure 1-6. The structure of SCF ubiquitin ligases.

An SCF complex is composed of RBX1, CUL1, SKP1, and F-box protein. The RBX1 subunit binds the ubiquitin-conjugating enzyme E2. The CUL1 subunit is an elongated protein that binds the RBX1 and the SKP1-F-box protein subcomplex. The F-box protein recognizes and binds substrates. In *Arabidopsis*, the SKP1-related proteins are ASKs.

potential large number of SCFs in *Arabidopsis*. SCFs have been shown to function in diverse cellular and developmental processes in plants, including hormone response, floral development, self-incompatibility response, light response, leaf senescence, shoot branching, circadian rhythms, and cell cycle regulation (Moon et al., 2004; Smalle and Vierstra, 2004; Thomann et al., 2005). A well-known example is the regulation of AUX/IAA protein degradation by SCF^{TIR1} in auxin signaling (Gray et al., 2001).

In yeast and mammals, the role of SCFs in cell cycle regulation has been extensively investigated. SCFs are responsible for the degradation of cyclins, CKIs, transcription factor E2F-1, and many other cell cycle regulators (Tyers and Jorgensen, 2000; Yew, 2001; DeSalle and Pagano, 2001; Cardozo and Pagano, 2004). A well-known example is the regulation of cell cycle progression by SCF^{SKP2} in mammals (Nakayama and Nakayama, 2005). The best-characterized SCF^{SKP2} substrate is the CKI $p27^{Kip1}$. SCF^{SKP2} targets $p27^{Kip1}$ for degradation to trigger the G1-S transition of the cell cycle. (Tsvetkov et al., 1999; Carrano et al., 1999; Sutterlüty et al., 1999). The SCF^{SKP2}-dependent degradation of $p27^{Kip1}$ requires its phosphorylation at the residue Thr187, which is mediated by the CDK2/cyclin E complex (Sheaff et al., 1997; Vlach et al., 1997; Montagnoli et al., 1999). In addition to $p27^{Kip1}$, SCF^{SKP2} is also involved in the degradation of the following cell cycle regulators: CKIs $p21^{Cip1}$ (Yu et al., 1998; Bornstein et al., 2003) and $p57^{Kip2}$ (Kamura et al., 2003), cyclins D1 (Yu et al., 1998) and E (Nakayama et al., 2000; Yeh et al., 2001), CDK Cdk9 (Kiernan et al., 2001; Barboric et al.,

2005), transcription factor E2F-1 (Marti et al., 1999), pocket protein p130 (Tedesco et al., 2002; Bhattacharya et al., 2003), replication licensing factor Cdt1 (Li et al., 2003; Sugimoto et al., 2004; Kondo et al., 2004), origin recognition complex large subunit Orc1 (Méndez et al., 2002), and transcription factors B-Myb (Charrasse et al., 2000) and c-Myc (Kim et al., 2003; von der Lehr et al., 2003).

In contrast to yeast and mammals, very little is known about the role of SCFs in plant cell cycle regulation (Inzé, 2005; Thomann et al., 2005). The *Arabidopsis* genome encodes two closely related F-box proteins (called SKP2a and SKP2b) that are related to mammalian SKP2 (del Pozo et al., 2002a). SKP2a appears to recruit the phosphorylated form of the transcription factor E2Fc for degradation. Whether SKP2b also regulates E2Fc degradation is unknown. In addition to E2Fc, another cell cycle regulator that may be an SCF substrate is CYCD3;1. This cyclin is unstable and its degradation depends on the 26S proteasome (Planchais et al., 2004). In transgenic plants with reduced levels of *RBX1* and in an *ask1-1 ask2-1* double mutant, CYCD3;1 accumulates, indicating that SCF is involved in its degradation. However, the F-box protein component of this SCF has not been identified (Lechner et al., 2002; Liu et al., 2004).

Chapter 2: SCF^{SKP2b}-dependent Degradation of KRP1 and Cell Cycle Regulation

INTRODUCTION

As described in Chapter one, a group of proteins called cyclin-dependent kinase inhibitors (CKIs) play a key role in cell cycle regulation through inhibiting the activities of CDK/cyclin complexes. In yeast and mammals, the role of CKIs in cell cycle regulation and ubiquitin-dependent degradation of CKIs have been extensively studied and are well understood. In contrast, little is known about the role of these proteins in plant cell cycle regulation and about the post-translational regulation of plant CKIs mediated by ubiquitin-dependent protein degradation. To gain insight into the mechanisms by which plant cell cycle is regulated, I focused on KRP1 in *Arabidopsis*, the first identified plant CKI (Wang et al., 1997). I studied the role of KRP1 in pericycle activation during lateral root initiation and in cell cycle regulation and how an SCF complex regulates KRP1 protein turnover. My results show that KRP1 plays an important role in regulating pericycle activation during lateral root initiation. KRP1 interacts with the CDKA;1/CYCD2;1 complex *in planta* to control the G1-S transition of the cell cycle. KRP1 is an unstable protein *in planta* and an SCF complex composed of CUL1 and SKP2b regulates KRP1 degradation. These results provide new

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insight into the mechanisms by which plant cell cycle is regulated by SCF-dependent protein degradation.

RESULTS

***KRP1* is ubiquitously expressed**

Previous studies by RNA blot and RT-PCR have shown that *KRP1* is expressed in roots, stems, leaves, flowers, inflorescences, and actively dividing cultured cells (Wang et al., 1998; Lui et al., 2000; De Veylder et al., 2001). In addition, *KRP1* expression was examined in leaves and in the shoot apex by *in situ* hybridization (Ormenese et al., 2004). *KRP1* RNA was detected in endoreduplicating tissues of leaves but not in dividing cells of the shoot apical meristem. To further characterize *KRP1* expression, I generated *Arabidopsis* transgenic lines in which the bacterial *β-glucuronidase* reporter gene (*GUS*) was placed adjacent to the *KRP1* promoter. Over 10 independent transgenic lines were analyzed, and all lines exhibited similar *GUS* expression patterns. In young seedlings, GUS staining was observed in the cotyledon, hypocotyl, and root (Fig 2-1A). Within the root, GUS staining was observed in the epidermis, cortex, endodermis, pericycle, and vascular tissues (Fig 2-1B). In older seedlings, strong GUS staining was observed in the rosette leaves (Fig 2-1C). GUS staining was also observed in the lateral root (Fig 2-1D). In the flower, GUS staining was observed in the sepals, anthers, and mature pollens (Fig 2-1E and 2-1F). GUS staining was also

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Figure 2-1. *KRP1* is ubiquitously expressed.
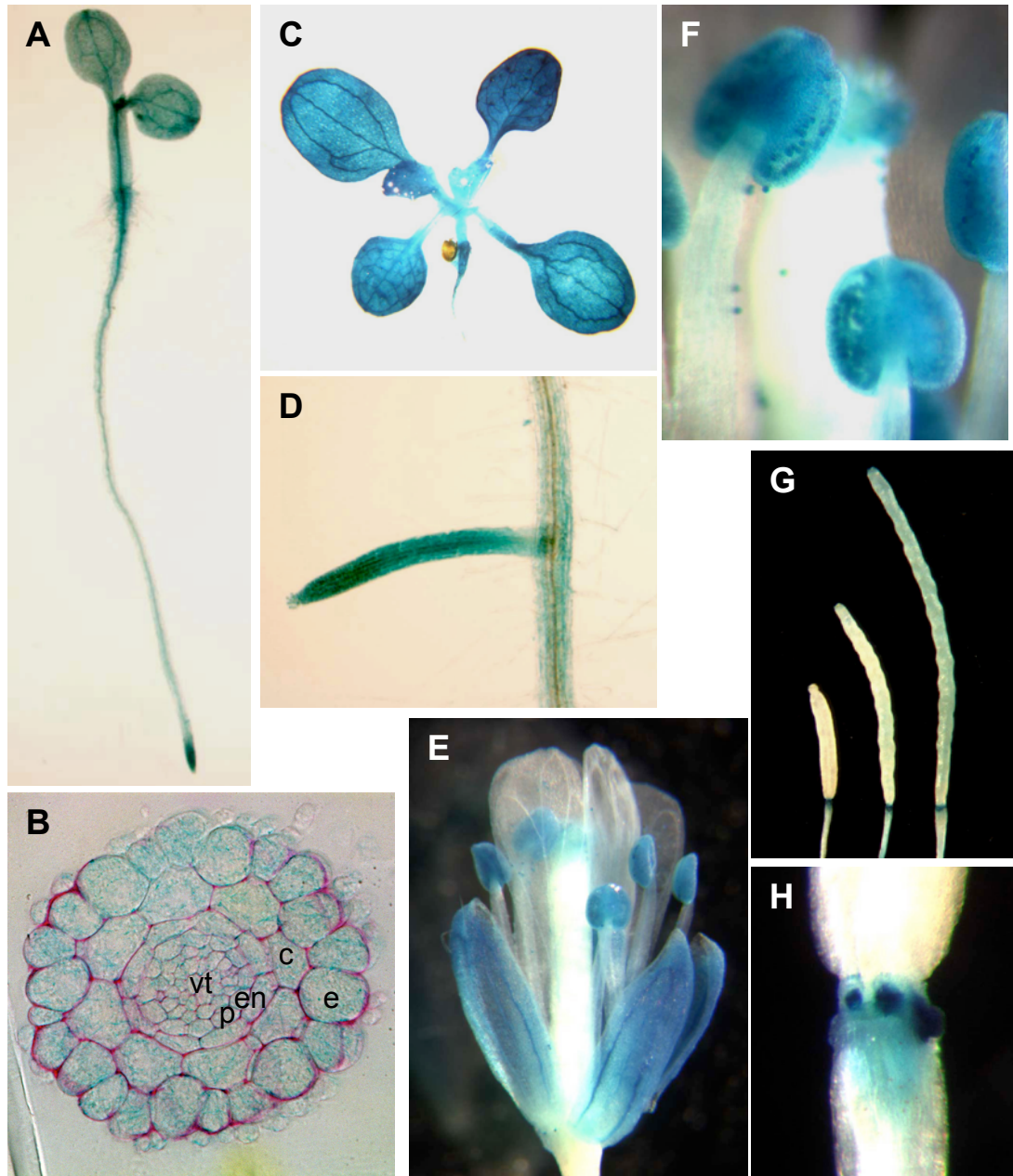
Figure 2-1. *KRP1* is ubiquitously expressed.

A 2062 bp *KRP1* promoter was fused to *GUS*, and *GUS* expression was examined by GUS staining of transgenic plants carrying a *KRP1*::*GUS* transgene. **(A)** Four-day-old light-grown seedling. **(B)** Root transverse section of a 4-day-old light-grown seedling. e, epidermis; c, cortex; en, endodermis; p, pericycle; vt, vascular tissue. **(C)** Rosette leaves of a 10-day-old light-grown seedling. **(D)** Lateral root of a 10-day-old light-grown seedling. **(E)** Mature flower. **(F)** A closer look at the anthers and mature pollens shown in **(E)**. **(G)** Siliques. **(H)** A closer look at the base of siliques shown in **(G)**.

observed in the siliques, especially the base of siliques (Fig 2-1G and 2-1H). These results show that *KRP1* is ubiquitously expressed in various tissues and organs throughout plant development.

The *krp1-1* mutant is a null mutant without an obvious phenotype

To study the role of *KRP1* in plant growth and development, I investigated the effects of *KRP1* loss of function on plant growth and development. I identified three *KRP1* T-DNA insertion mutants in the SALK collection. The *krp1-1*, *krp1-2*, and *krp1-3* mutants have a T-DNA insertion in the third intron, promoter (157 bp before the start codon ATG), and 3'UTR, respectively (Fig 2-2A). To understand the molecular nature of the *krp1-1*, *krp1-2*, and *krp1-3* mutants, I examined *KRP1* expression by RT-PCR in these mutants. The full-length transcripts of *KRP1* were detected at a low level in the *krp1-2* mutant and at a high level in the *krp1-3* mutant, but not in the *krp1-1* mutant (Fig 2-2C). Because the *krp1-1* mutant does not appear to have the full-length *KRP1* transcripts and is possibly a null mutant, I decided to focus on and work with the *krp1-1* mutant. Although the *krp1-1* mutant did not have the full-length *KRP1* transcripts, truncated *KRP1* transcripts were detected (Fig 2-2D). Therefore, truncated KRP1 protein could be produced in the *krp1-1* mutant. If this truncated protein exists, KRP1 will lose the C-terminal 22 amino acids and has an impaired CDK-binding/inhibitory domain (Fig 2-2B). Like the *krp1-1* and *krp1-2* mutants, the *krp1-3* mutant did not exhibit any obvious defects in growth and development (Table 2-1).
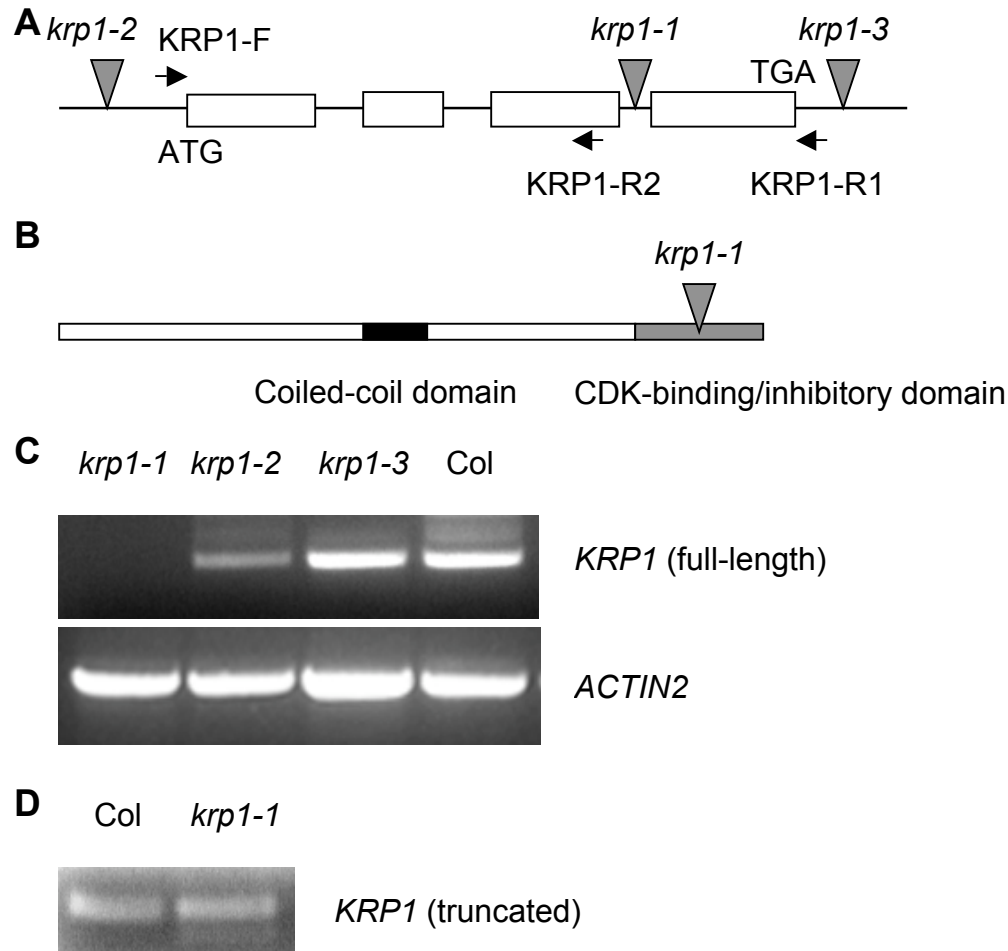
Figure 2-2. RT-PCR analysis of *KRP1* expression in the *KRP1* T-DNA insertion mutants.

(A) Genomic structure of *KRP1* and T-DNA insertion locations (not to scale). Lines represent promoter, introns, and UTRs (untranslated region). Boxes represent exons. Triangles represent T-DNA inserts. Arrows indicate primers that are used for RT-PCR. The *krp1-1*, *krp1-2*, and *krp1-3* mutants have a T-DNA insert in the third intron, promoter (157 bp before the start codon ATG), and 3'UTR, respectively. **(B)** Protein structure of KRP1 (not to scale). Black and gray boxes represent coiled-coil domain and CDK-binding/inhibitory domain, respectively. Triangle represents T-DNA insert. Motifs were analyzed using the Simple Modular Architecture Research Tool (SMART, http://smart.embl.de/). **(C)** and **(D)** RT-PCR analysis of *KRP1* and *ACTIN2* expression in the wild-type (Col), *krp1-1*, *krp1-2*, and *krp1-3* mutants. Total RNAs were extracted from 7-day-old light-grown seedlings. Gene specific primers for amplifying the full-length and truncated transcripts of *KRP1* are shown in **(A)**. PCRs were performed for 35 cycles (*ACTIN2*) and 40 cycles (*KRP1*).

Table 2-1. Phenotypic analysis of the *krp1-1* mutant.

Phenotypic analysis was performed as described by Boyes et al., (2001).
Over twenty plants were analyzed. SD represents standard deviation.

Measurement	Wild-type	*krp1-1*
Primary root length in cm of 7-day-old light-grown seedlings	5.3 ± 0.68 (SD)	5.3 ± 0.41 (SD)
Hypocotyl length in mm of 7-day-old dark-grown seedlings	18.89 ± 1.21(SD)	19.27 ± 1.44 (SD)
Number of lateral roots of 11-day-old light-grown plants	8.72 ± 4.38 (SD)	8.31 ± 3.58 (SD)
Bloting time in days	25.55 ± 2.8 (SD)	24.92 ± 1.92 (SD)
Number of rosette leaves when plants bolt	9.93 ± 1.42 (SD)	9.69 ± 1.54 (SD)
Time in days when the first flower opens	31.18 ± 2.7 (SD)	30.85 ± 2.74 (SD)
Mature plant height in cm	51.13 ± 6.79 (SD)	51.99 ± 6.14 (SD)
Number of stem branches on main bolt >1cm	1.93 ± 0.86 (SD)	1.9 ± 0.68 (SD)
Number of side bolts >1cm	2.5 ± 1.41 (SD)	2.31 ± 1.44 (SD)
The distance in cm between the first silique and the last silique on the main bolt	43.17 ± 6.62 (SD)	45.04 ± 5.32 (SD)
Seeds in mg per plant	199.83 ± 77.38 (SD)	216.8 ± 77.37 (SD)

As an alternative to understand the role of *KRP1* in plant growth and development, I investigated the effects of *KRP1* gain of function on plant growth and development. I generated *Arabidopsis* transgenic plants that express a c-myc epitope tagged KRP1 under the control of the *CaMV 35S* promoter. More than 30 independent lines exhibited similar phenotypes. Plants had serrated rosette leaves, reduced apical dominance, and reduced fertility (Fig 2-3A to 2-3E). This phenotype is very similar to that conferred by overexpression of KRP1 (Wang et al., 2000), indicating that Myc-KRP1 is a functional protein *in planta*. A *35S::Myc-KRP1* line that has weak phenotypes and carries a single T-DNA insertion was chosen for further analysis. Interestingly, KRP1 overexpressors were temperature-sensitive. If grown at 18 ^{0}C, plants were more robust and exhibited increased fertility (Fig 2-3A and 2-3B).

The CDK-binding/inhibitory domain is critical for KRP1 function as a CDK inhibitor. Previous studies showed that KRP1 without or with an impaired CDK-binding/inhibitory domain did not interact or had dramatically reduced interaction with CDKA;1 (Wang et al., 1998; Zhou et al., 2003a). As described above, although the *krp1-1* mutant does not appear to have full-length KRP1 protein, truncated KRP1 protein without the C-terminal 22 amino acids (KRP1-C22) and with an impaired CDK-binding/inhibitory domain might exist in this mutant. To determine whether the *krp1-1* mutant is a null mutant, I examined whether KRP1-C22 is a functional protein *in planta*. I generated *Arabidopsis* transgenic plants that express a c-myc epitope tagged KRP1-
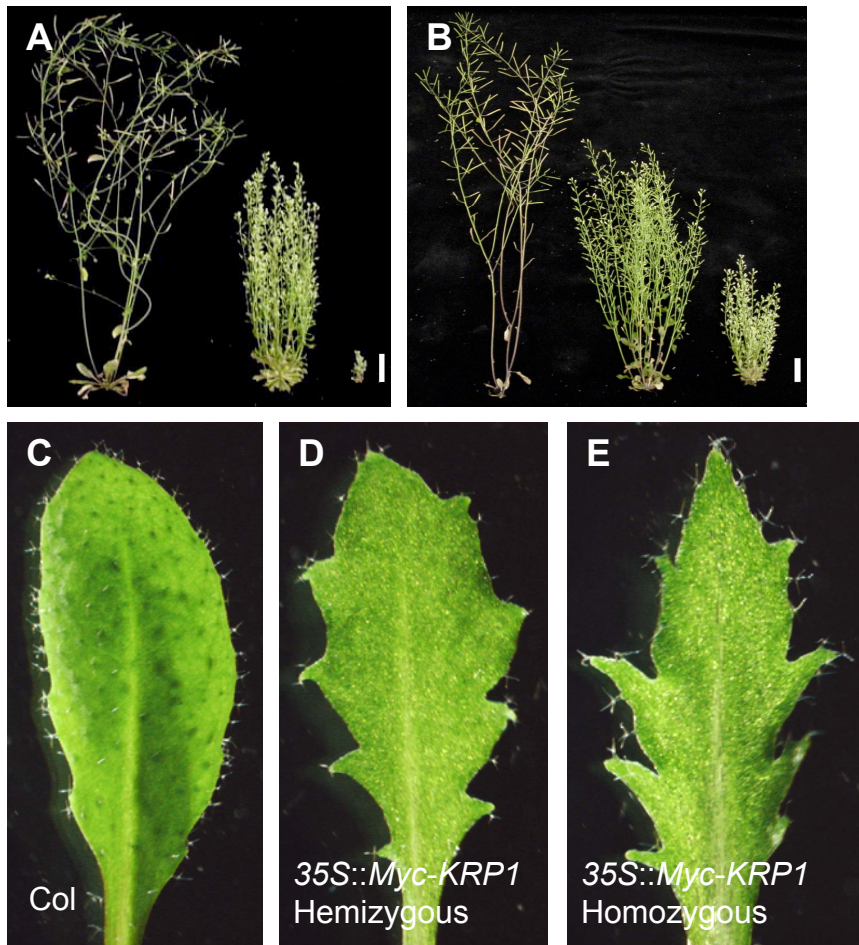
Figure 2-3. KRP1 overexpressors exhibit a pleiotropic phenotype.

(A) Seven-week-old mature plants grown at 22 ^{0}C. (B) Eleven-week-old mature plants grown at 18 ^{0}C. (A) and (B) From left to right, wild-type (Col), *35S*::*Myc-KRP1* (hemizygous), *35S*::*Myc-KRP1* (homozygous). Bar = 2 cm. (C) to (E) Rosette leaves of 47-day-old plants grown at 18 ^{0}C.

C22 under the control of the *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter.

Ten independent *35S*::*Myc-KRP1-C22* lines that show Myc-KRP1-C22

protein expression did not exhibit an obvious phenotype (data not shown).

Here I show results for the line 2. An obvious phenotype of plants that

overexpress KRP1 is serrated rosette leaves. Plants that overexpress Myc-

KRP1-C22 did not exhibit serrated rosette leaves (Fig 2-4A to 2-4C). The

35S::*Myc-KRP1-C22* plants were very similar to wild-type throughout the life

cycle of plants (Fig 2-4A to 2-4F; data not shown). Immunoblot analysis using

an ∝-c-myc antibody showed Myc-KRP1-C22 protein expression in *35S*::*Myc-

KRP1-C22* plants. Interestingly, there are two clear Myc-KRP1-C22 protein

bands (Fig 2-4G). These results indicate that KRP1-C22 probably is not a

functional protein *in planta*, therefore, the *krp1-1* mutant is a null mutant.

KRP1 overexpression inhibits auxin-mediated pericycle cell division

during lateral root initiation

An interesting and previously uncharacterized aspect of the KRP1

overexpression phenotype is a severe defect in lateral root formation (Fig 2-

5A). The *35S::Myc-KRP1* line exhibited only a slight decrease in primary root

growth, but lateral root formation was dramatically inhibited. The number of

lateral roots was reduced 41.22% and 96.19% in the hemizygous and

homozygous *35S::Myc-KRP1* plants, respectively (Fig 2-5B and 2-5C),

suggesting that the effect of KRP1 on lateral root formation was dose-

Figure 2-4. Myc-KRP1-C22 is not a functional protein *in planta*.

A

Col

B

35S::Myc-KRP1

C

35S::Myc-KRP1-C22

D

Col

E

35S::Myc-KRP1

F

35S::Myc-KRP1-C22

G

* Myc-KRP1

Myc-KRP1-C22

Unknown protein

1   2   3
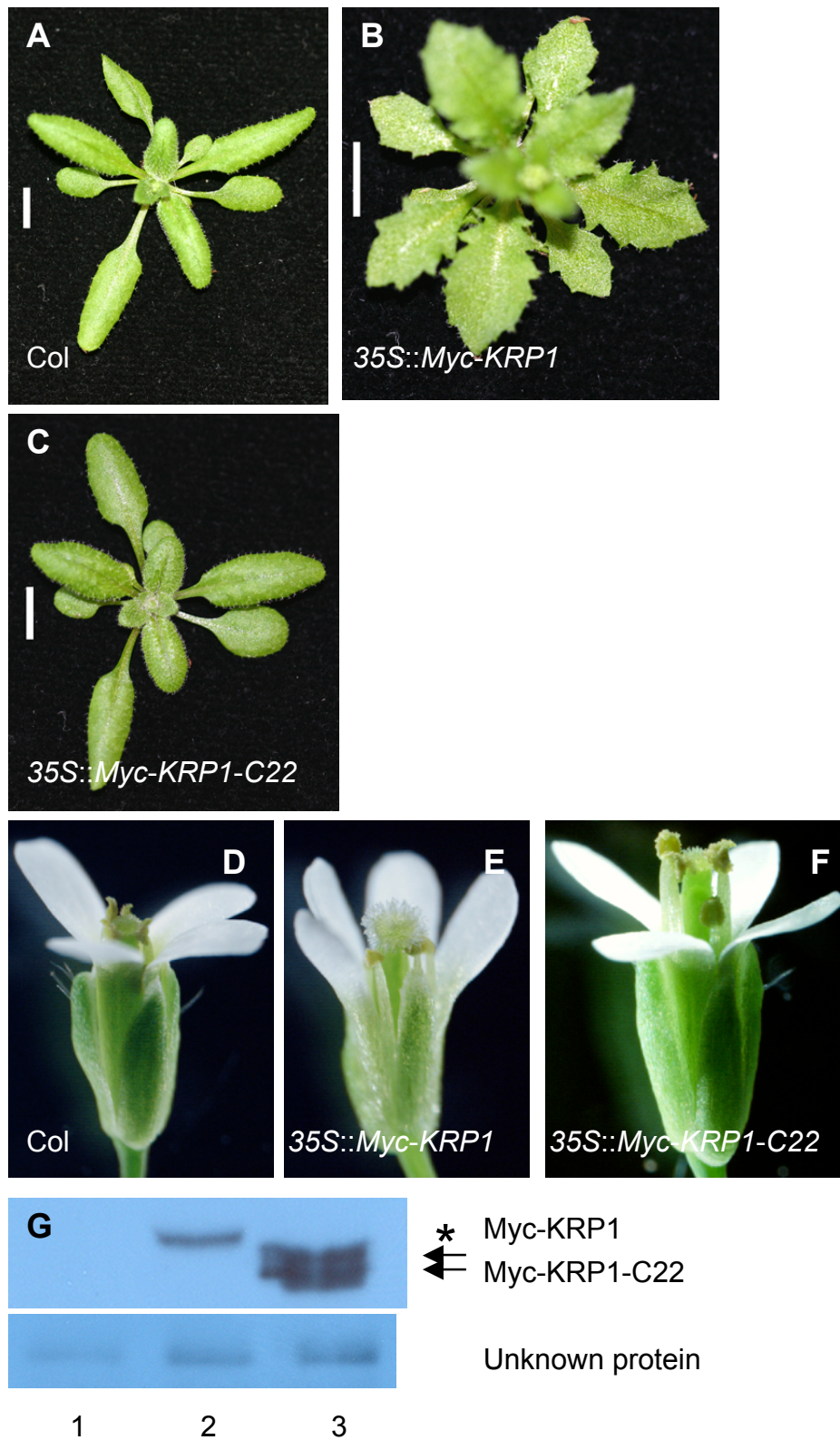
Figure 2-4. Myc-KRP1-C22 is not a functional protein *in planta*.

(A) to (C) Three-week-old plants. Bar = 0.5 cm. **(D) to (F)** Mature flowers. **(A)** and **(D)** Wild-type (Col). **(B)** and **(E)** *35S*::*Myc-KRP1* (hemizygous). **(C)** and **(F)** *35S*::*Myc-KRP1-C22* (hemizygous or homozygous, kanamycin-resistant T2). **(G)** Immunoblot analysis of Myc-KRP1 and Myc-KRP1-C22 with an ∝-c-myc antibody. Protein extracts were prepared from 16-day-old light-grown plants. An unknown protein recognized by the ∝-c-myc antibody was used as a loading control. Lane 1, wild-type (Col); lane 2, *35S*::*Myc-KRP1* (hemizygous); lane 3, *35S*::*Myc-KRP1-C22* (hemizygous, kanamycin-resistant T1).

Figure 2-5. KRP1 overexpression inhibits auxin-mediated pericycle cell division.
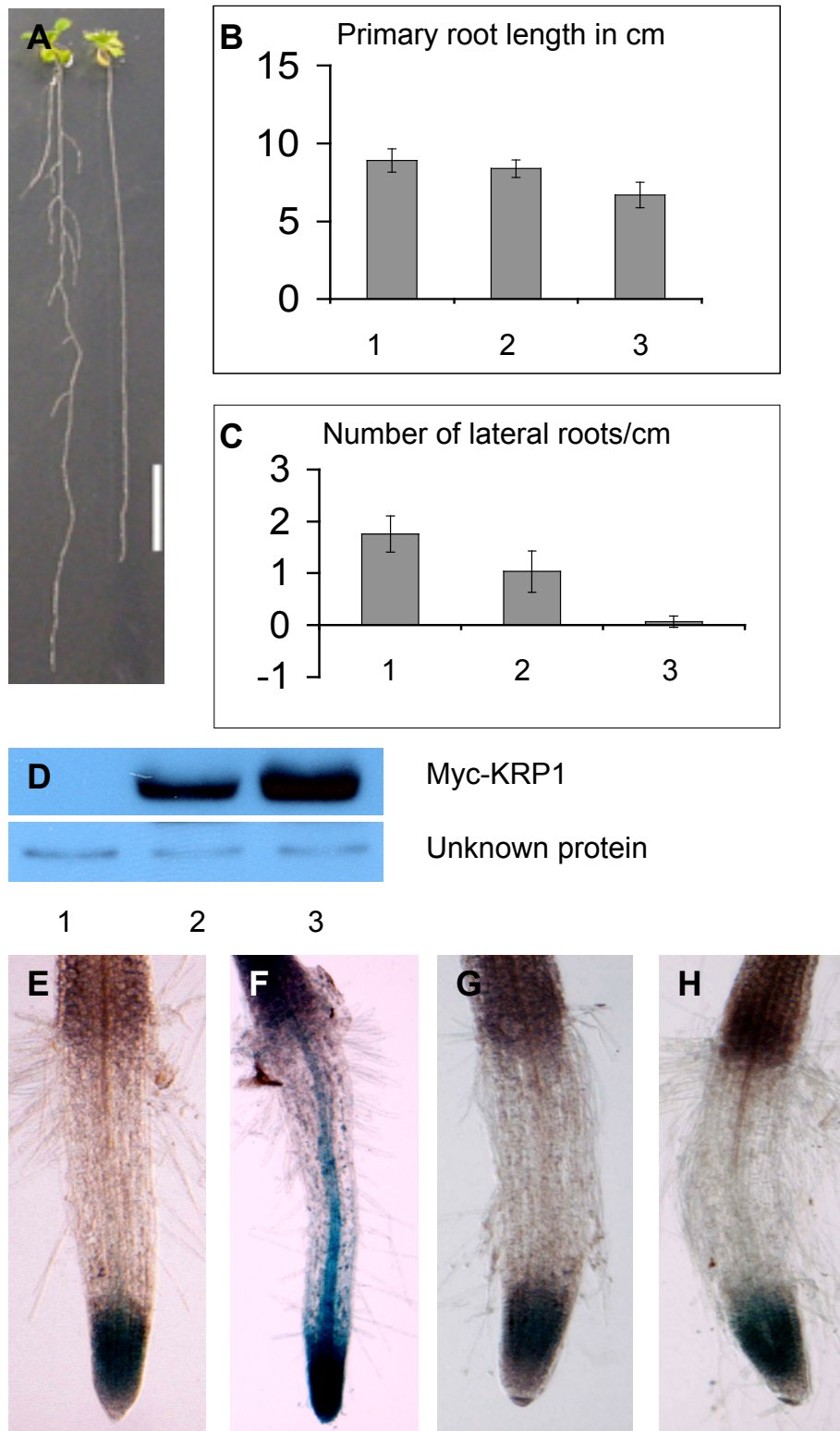
Figure 2-5. KRP1 overexpression inhibits auxin-mediated pericycle cell division.

(A) Two-week-old light-grown plants, wild-type (Col) (left) and *35S*::*Myc-KRP1* (homozygous, right). Bar = 1 cm. **(B)** Primary root length of 2-week-old light-grown plants. **(C)** Lateral root number of 2-week-old light-grown plants. **(D)** Immunoblot analysis of Myc-KRP1 with an ∝-c-myc antibody. Protein extracts were prepared from 2-week-old light-grown plants. An unknown protein recognized by the ∝-c-myc antibody was used as a loading control. **(B)** to **(D)** Lane 1, wild-type (Col); lane 2, *35S*::*Myc-KRP1* (hemizygous); lane 3, *35S*::*Myc-KRP1* (homozygous). **(E)** GUS expression in the root of a *CYCB1;1*::*GUS* seedling grown on an ATS/10 µM NPA plate for 72 hours. **(F)** GUS expression in the root of a *CYCB1;1*::*GUS* seedling grown on an ATS/10 µM NAA plate for 12 hours (after transfer from the ATS/10 µM NPA plate onto the ATS/10 µM NAA plate). **(G)** GUS expression in the root of a *35S*::*Myc-KRP1* (homozygous) seedling carrying a *CYCB1;1*::*GUS* transgene grown on an ATS/10 µM NPA plate for 72 hours. **(H)** GUS expression in the root of a *35S*::*Myc-KRP1* (homozygous) seedling carrying a *CYCB1;1*::*GUS* transgene grown on an ATS/10 µM NAA plate for 12 hours (after transfer from the ATS/10 µM NPA plate onto the ATS/10 µM NAA plate).

dependent. This was confirmed by determining the level of Myc-KRP1 in these lines by protein blot (Figure 2-5D).

To learn more about how KRP1 functions in lateral root formation, I introduced a *CYCB1;1::GUS* transgene into *35S::Myc-KRP1* plants by crossing. *CYCB1;1*, a mitotic cyclin, is expressed in late G2 and M phase and is therefore a marker for cell cycle progression from G2 to M phase. I then used the lateral root induction conditions developed by Himanen et al. (2002) to examine the effect of KRP1 overexpression on auxin-mediated pericycle cell division during lateral root initiation. Seedlings are first treated with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA). This compound prevents pericycle cell division, and all pericycle cells remain in G1 phase. Subsequently, seedlings are treated with the auxin 1-naphthaleneacetic acid (NAA) to activate pericycle cells, causing them to pass the G1-S and G2-M transitions and undergo cell division. After NPA treatment, wild-type and *35S::Myc-KRP1* seedlings did not exhibit GUS staining in the pericycle (Fig 2-5E and 2-5G). As expected, treatment of wild-type seedlings with NAA produced significant GUS staining in the pericycle, showing that these cells have passed though the G2-M transition (Fig 2-5F). In contrast, no staining was observed in the *35S::Myc-KRP1* plants (Fig 2-5H), indicating that KRP1 overexpression inhibits auxin-mediated pericycle cell division during lateral root initiation.

KRP1 interacts with CDKA;1 and CYCD2;1 *in planta*

A crucial step towards understanding the role of KRP1 in cell cycle regulation is to identify its CDK/cyclin complex targets. Analyses using the yeast two-hybrid system have shown that KRP1 interacts with CDKA;1 and D-type cyclins (CYCD1;1, CYCD2;1, and CYCD3;1), but KRP1 does not interact with CDKB1;1, CYCA2;2, and B-type mitotic cyclins (CYCB1;1 and CYCB2;1) (Wang et al., 1998; De Veylder et al., 2001; Zhou et al., 2003b). However, these interactions have not been confirmed in the plant. Because I have transgenic plants overexpressing Myc-KRP1 as well as antibodies to CDKA;1, CDKB1;1, and CYCD2;1, I tested for these interactions by immunoprecipitation. Protein extracts prepared from wild-type and *35S::Myc-KRP1* seedlings were immunoprecipitated with an ∝-c-myc antibody. Immunoblot analyses were performed with ∝-CDKA;1, ∝-CDKB1;1, and ∝-CYCD2;1 antibodies. As shown in Fig 2-6, CDKA;1 and CYCD2;1, but not CDKB1;1, co-immunoprecipitates with Myc-KRP1. These results indicate that KRP1 interacts with CDKA;1 and CYCD2;1 *in planta*.

KRP1 is an unstable protein and its degradation depends on the 26S proteasome

KRP1 is related to mammalian CKI p27^{Kip1}, which is degraded through the action of a ubiquitin ligase called SCF^{SKP2} (Tsvetkov et al., 1999; Carrano et al., 1999; Sutterlüty et al., 1999). Whether KRP1 levels are also regulated by ubiquitin-mediated protein degradation is unknown. To study KRP1 protein
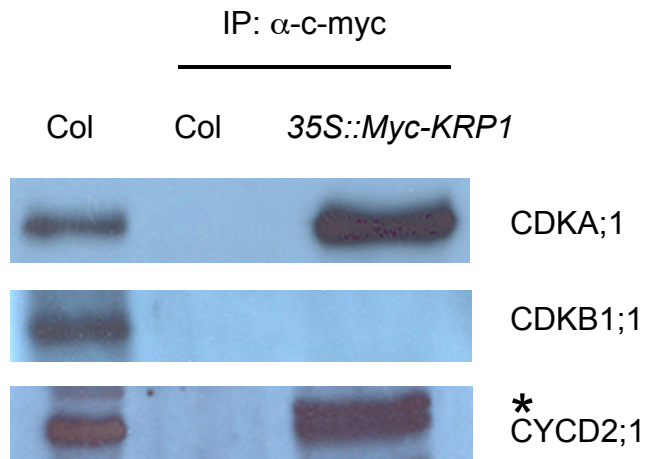
Figure 2-6. KRP1 interacts with CDKA;1 and CYCD2;1 *in planta*.

Protein extracts from wild-type (Col) and *35S::Myc-KRP1* seedlings were immunoprecipitated with an ∝-c-myc antibody. Immunoblot analyses of protein extracts from wild-type (Col) and ∝-c-myc immunoprecipitates were performed with ∝-CDKA;1, ∝-CDKB1;1, and ∝-CYCD2;1 antibodies. Asterisk indicates an unknown protein recognized by the ∝-CYCD2;1 antibody.

stability *in planta*, I generated *Arabidopsis* transgenic plants that express

KRP1-GUS fusion protein under the control of the KRP1 promoter. As shown

in Fig 2-1A and again in Fig 2-7A for comparison, GUS staining was observed

in the cotyledon, hypocotyl, and root of *KRP1:GUS* seedlings. In contrast, no

GUS staining was observed in *KRP1::KRP1-GUS* seedlings (Fig 2-7B). Since

GUS is a stable protein, these results indicate that KRP1 destabilizes GUS,

suggesting that KRP1 is an unstable protein that is quickly degraded *in*

planta.

To determine whether the 26S proteasome is involved in KRP1

degradation, we examined the effect of MG132, a proteasome inhibitor, on

KRP1-GUS and Myc-KRP1 protein stability. Unlike the DMSO-treated

seedlings (Fig 2-7C and 2-7D), GUS staining was observed in the cotyledon

and root of MG132-treated *KRP1::KRP1-GUS* seedlings (Fig 2-7E and 2-7F).

Inside the root, GUS staining was observed in the epidermis, cortex,

endodermis, pericycle, and vascular tissues (Fig 2-7G). The KRP1-GUS

localization in roots is consistent with that of *KRP1* expression (Figure 2-1B).

Similarly, MG132 treatment resulted in increased Myc-KRP1 levels in

35S::Myc-KRP1 seedlings (Fig 2-7H). CDKA;1, a stable protein, was used as

a loading control. MG132 did not affect CDKA;1 protein stability. The

stabilization of both KRP1-GUS and Myc-KRP1 by MG132 indicates that

KRP1 degradation depends on the 26S proteasome.
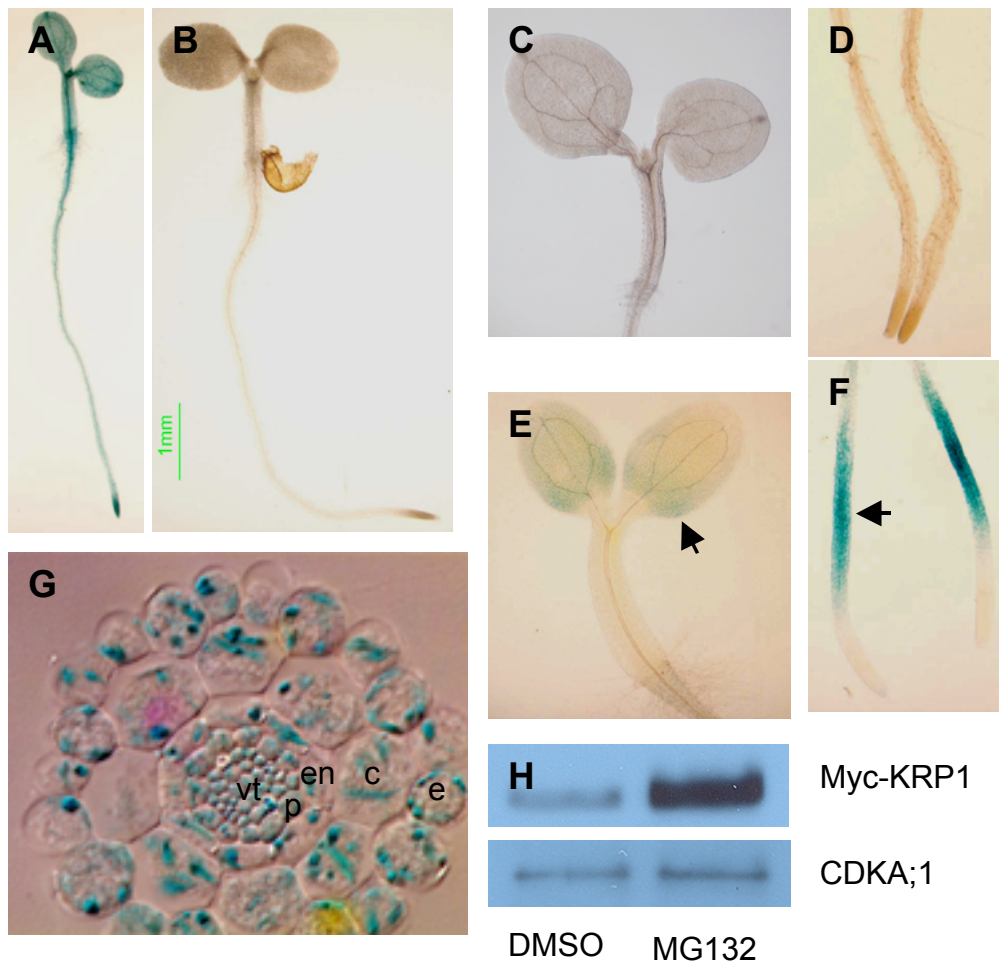
Figure 2-7. KRP1 degradation depends on the 26S proteasome.

(A) GUS staining of a 4-day-old light-grown *KRP1*::*GUS* seedling. **(B)** GUS
staining of a 4-day-old light-grown *KRP1*::*KRP1-GUS* seedling. **(C)** and **(D)**
GUS staining of 4-day-old light-grown *KRP1*::*KRP1-GUS* seedlings treated
with DMSO for 8 hours. **(E)** and **(F)** GUS staining of 4-day-old light-grown
KRP1::*KRP1-GUS* seedlings treated with 50 µM MG132 for 8 hours. MG132
is a 26S proteasome inhibitor. DMSO was used as a control. **(G)** Root
transverse section of a GUS-stained 4-day-old light-grown *KRP1*::*KRP1-GUS*
seedling treated with 50 µM MG132 for 12 hours. e, epidermis; c, cortex; en,
endodermis; p, pericycle; vt, vascular tissue. **(H)** Immunoblot analysis of
protein extracts from *35S*::*Myc-KRP1* (homozygous) seedlings treated with
DMSO and 50 µM MG132 for 12 hours, respectively, with ∝-c-myc and ∝-
CDKA;1 antibodies. CDKA;1, a stable protein, was used as a loading control.
Arrows indicate GUS staining.

The AXR1-dependent RUB conjugation pathway regulates KRP1 degradation

Like ubiquitin, RUB1 (related to ubiquitin-1, also called NEDD8) is a post-translational modifier that regulates diverse cellular processes. At present, the only known RUB1 targets are the cullin family proteins, including the CUL1 subunit of SCFs (Lammer et al., 1998; Osaka et al., 1998; Wada et al., 1999; Liakopoulos et al., 1999; Hori et al., 1999; del Pozo et al., 1999). In *Arabidopsis*, RUB1 conjugation to CUL1 requires the AXR1-ECR1 heterodimer (RUB-activating enzyme E1), RCE1 (RUB-conjugating enzyme E2), and RBX1 (RUB-protein ligase E3). RUB conjugation modulates the activity of many, perhaps all CUL1-based SCFs (Parry and Estelle, 2004). Our previous data demonstrated that the ubiquitin-proteasome pathway regulates KRP1 degradation. To determine if the RUB conjugation pathway is required for this degradation, I examined KRP1-GUS and Myc-KRP1 protein stability in the *axr1-3* mutant, which has an impaired RUB conjugation pathway (Lincoln et al., 1990; Leyser et al., 1993; del Pozo et al., 1998).

I introduced the *KRP1::KRP1-GUS* transgene into *axr1-3* plants by crossing. The results shown in Fig 2-8A and 2-8B indicate that the *axr1-3* mutation acts to stabilize KRP1-GUS in both the cotyledon and hypocotyl. However, no GUS staining was observed in the *axr1-3* roots (data not shown). Homozygous *KRP1::KRP1-GUS* plants are very similar to wild-type in appearance, while *axr1-3* plants exhibit a pleiotropic phenotype that includes reduced stature and decreased apical dominance (Fig 2-8C and 2-

Figure 2-8. The AXR1-dependent RUB conjugation pathway regulates KRP1 degradation.
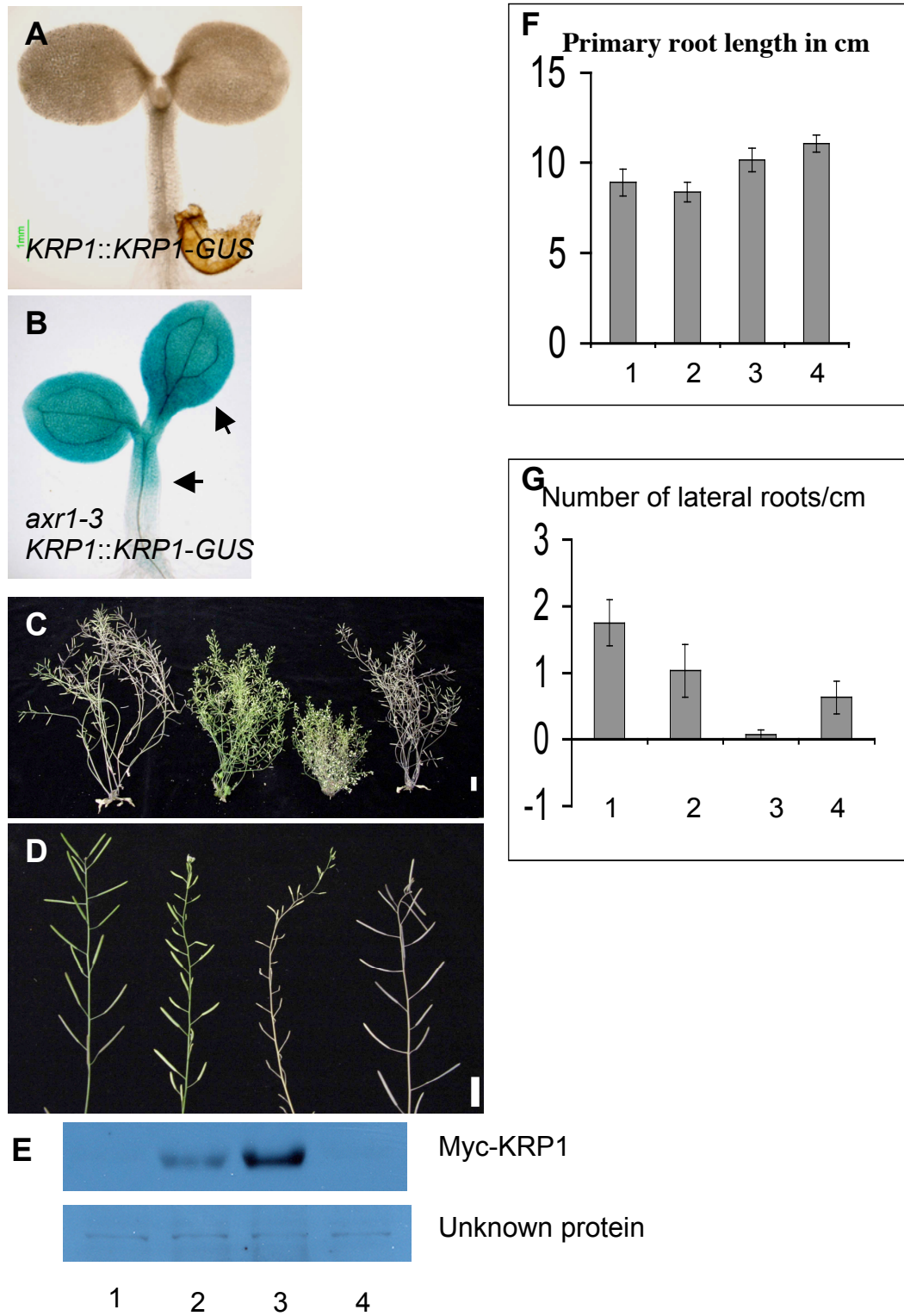
A
KRP1::KRP1-GUS

B
axr1-3
KRP1::KRP1-GUS

C

D

E
Myc-KRP1

Unknown protein

1   2   3   4

F
Primary root length in cm

G
Number of lateral roots/cm

Figure 2-8. The AXR1-dependent RUB conjugation pathway regulates KRP1 degradation.

(A) GUS staining of a 4-day-old light-grown *KRP1*::*KRP1-GUS* seedling. **(B)** GUS staining of a 4-day-old light-grown *axr1-3* seedling carrying a *KRP1*::*KRP1-GUS* transgene. **(C)** Seven-week-old mature plants. From left to right, wild-type (Col), *axr1-3*, *axr1-3 KRP1*::*KRP1-GUS*, and *KRP1*::*KRP1-GUS*. Bar = 2 cm. **(D)** Inflorescences of 7-week-old mature plants shown in **(C)**. Bar = 1 cm. **(E)** Immunoblot analysis of Myc-KRP1 with an $\propto$-c-myc antibody. Protein extracts were prepared from 2-week-old light-grown plants. An unknown protein recognized by the $\propto$-c-myc antibody was used as a loading control. **(F)** Primary root length of 2-week-old light-grown plants. **(G)** Lateral root number of 2-week-old light-grown plants. **(E)** to **(G)** Lane 1, wild-type (Col); lane 2, *35S*::*Myc-KRP1* (hemizygous); lane 3, *axr1-3 35S*::*Myc-KRP1* (hemizygous); lane 4 *axr1-3*. Arrows indicate GUS staining.

8D). Interestingly, the introduction of the *KRP1::KRP1-GUS* transgene into the *axr1-3* background enhances this mutant phenotype. The effects of the transgene were variable, but approximately 34% (29/85) had a severe phenotype as illustrated in Fig 2-8C and 2-8D.

I also introduced the *35S::Myc-KRP1* transgene into *axr1-3* plants by crossing. As shown in Figure 2-8E, the *axr1-3* mutation also stabilized Myc-KRP1. The accumulation of Myc-KRP1 was associated with severe growth defects of *axr1-3* plants, especially lateral root formation. Two-week-old plants had very few lateral roots (Fig 2-8F and 2-8G). These data indicate that the AXR1-dependent RUB conjugation pathway regulates KRP1 degradation.

KRP1 degradation is dependent on SCF[SKP2b]

The RUB conjugation pathway is probably required for function of all cullin-based E3 ubiquitin ligases, including SCF and CUL3-BTB E3s (Pintard et al., 2003; Parry and Estelle, 2004; Pan et al., 2004). To determine if an SCF might be involved in KRP1 degradation, I introduced the *KRP1::KRP1-GUS* and *35S::Myc-KRP1* transgenes into the *axr6-3* mutant by crossing. The *axr6-3* mutant contains a recessive and temperature-sensitive mutation of *CUL1* that has been shown to stabilize SCF substrates (Quint et al., 2005). As shown in Figures 2-9A, 2-9B, and 2-9D, the *axr6-3* mutant stabilized both KRP1-GUS and Myc-KRP1, exhibiting GUS staining in the cotyledon and an increased Myc-KRP1 protein level. Interestingly, overexpression of either KRP1-GUS or Myc-KRP1 enhances the *axr6-3* growth defects. Plants

41
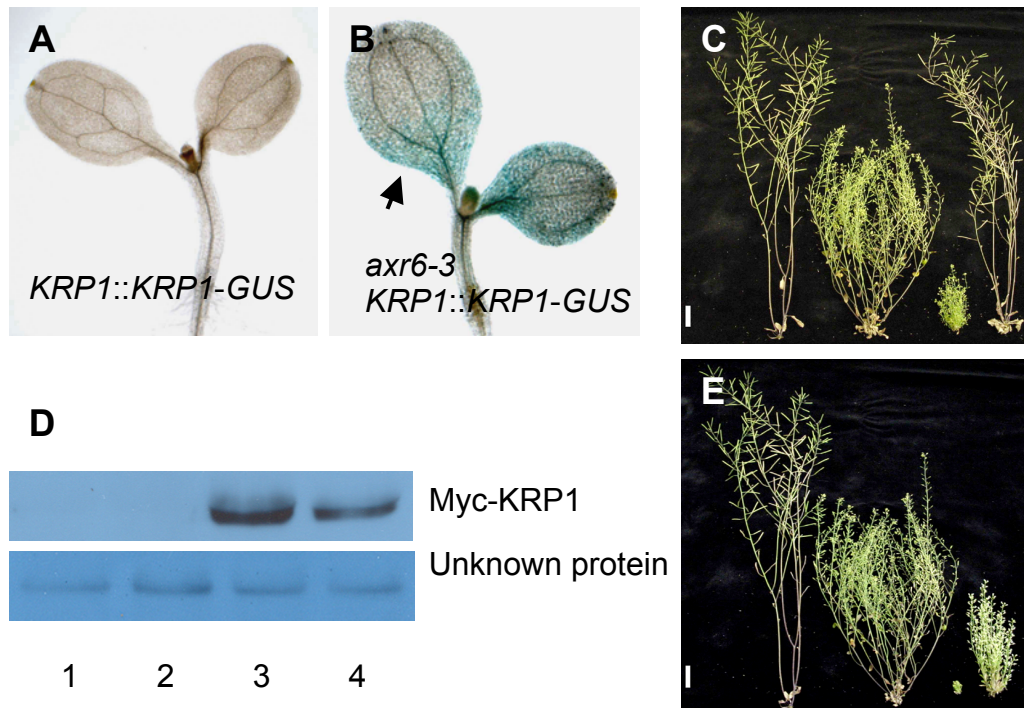
Figure 2-9. CUL1, a core component of SCFs, is required for KRP1 degradation.

(A) GUS staining of a 6-day-old light-grown *KRP1*::*KRP1-GUS* seedling. **(B)** GUS staining of a 6-day-old light-grown *axr6-3* seedling carrying a *KRP1*::*KRP1-GUS* transgene. **(C)** Eleven-week-old mature plants grown at 18 ^{0}C. From left to right, wild-type (Col), *axr6-3*, *axr6-3 KRP1*::*KRP1-GUS*, and *KRP1*::*KRP1-GUS*. Bar = 2 cm. **(D)** Immunoblot analysis of Myc-KRP1 with an ∝-c-myc antibody. Protein extracts were prepared from 40-day-old plants grown at 18 ^{0}C. An unknown protein recognized by the ∝-c-myc antibody was used as a loading control. Lane 1, wild-type (Col); lane 2, *axr6-3*; lane 3, *axr6-3 35S*::*Myc-KRP1* (hemizygous); lane 4 *35S*::*Myc-KRP1* (hemizygous). **(E)** Eleven-week-old mature plants grown at 18 ^{0}C. From left to right, wild-type (Col), *axr6-3*, *axr6-3 35S*::*Myc-KRP1* (homozygous), and *35S*::*Myc-KRP1* (homozygous). Bar = 2 cm. Arrow indicates GUS staining.

exhibited a dramatically decreased height and were sterile (Fig 2-9C and 2-9E). As for *axr1-3*, the *axr6-3* mutation did not appear to stabilize KRP1 in the root (data not shown). These results indicate that CUL1 is required for KRP1 degradation.

The involvement of CUL1 in KRP1 degradation reveals that an SCF mediates KRP1 protein turnover. Among the SCF subunits, the F-box protein recognizes and binds substrates. In mammals, the F-box protein SKP2 binds CKI p27^{Kip1} (Tsvetkov et al., 1999; Carrano et al., 1999; Sutterluty et al., 1999) and the transcription factor E2F-1 (Marti et al., 1999) as well as other cell cycle regulators (Nakayama and Nakayama, 2005), targeting them for degradation. *Arabidopsis* has two SKP2-related F-box proteins, SKP2a and SKP2b, which are 83% identical at the amino acid sequence level. *SKP2a* and *SKP2b* are located in a duplicated region of chromosome 1, suggesting that they are probably duplicated genes with redundant functions. It has been shown that SKP2a binds the transcription factor E2Fc and appears to mediate its degradation (del Pozo et al., 2002a). Because of the sequence and functional relationship between KRP1 and p27^{Kip1}, I decided to investigate the possibility that *Arabidopsis* SKP2 is involved in KRP1 degradation.

To test this possibility, I examined KRP1-GUS protein stability in the *SKP2a* and *SKP2b* T-DNA insertion mutants. I identified *SKP2a* and *SKP2b* T-DNA insertion mutants in the GABI-KAT and SALK collection (Fig 2-10A). Neither *skp2a-1* and *skp2b-1* nor a *skp2a-1 skp2b-1* double mutant exhibited an obvious phenotype. In addition, none of these mutants stabilized KRP1-

Figure 2-10. RT-PCR analysis of *SKP2a* and *SKP2b* expression in the *SKP2a* and *SKP2b* T-DNA insertion mutants.
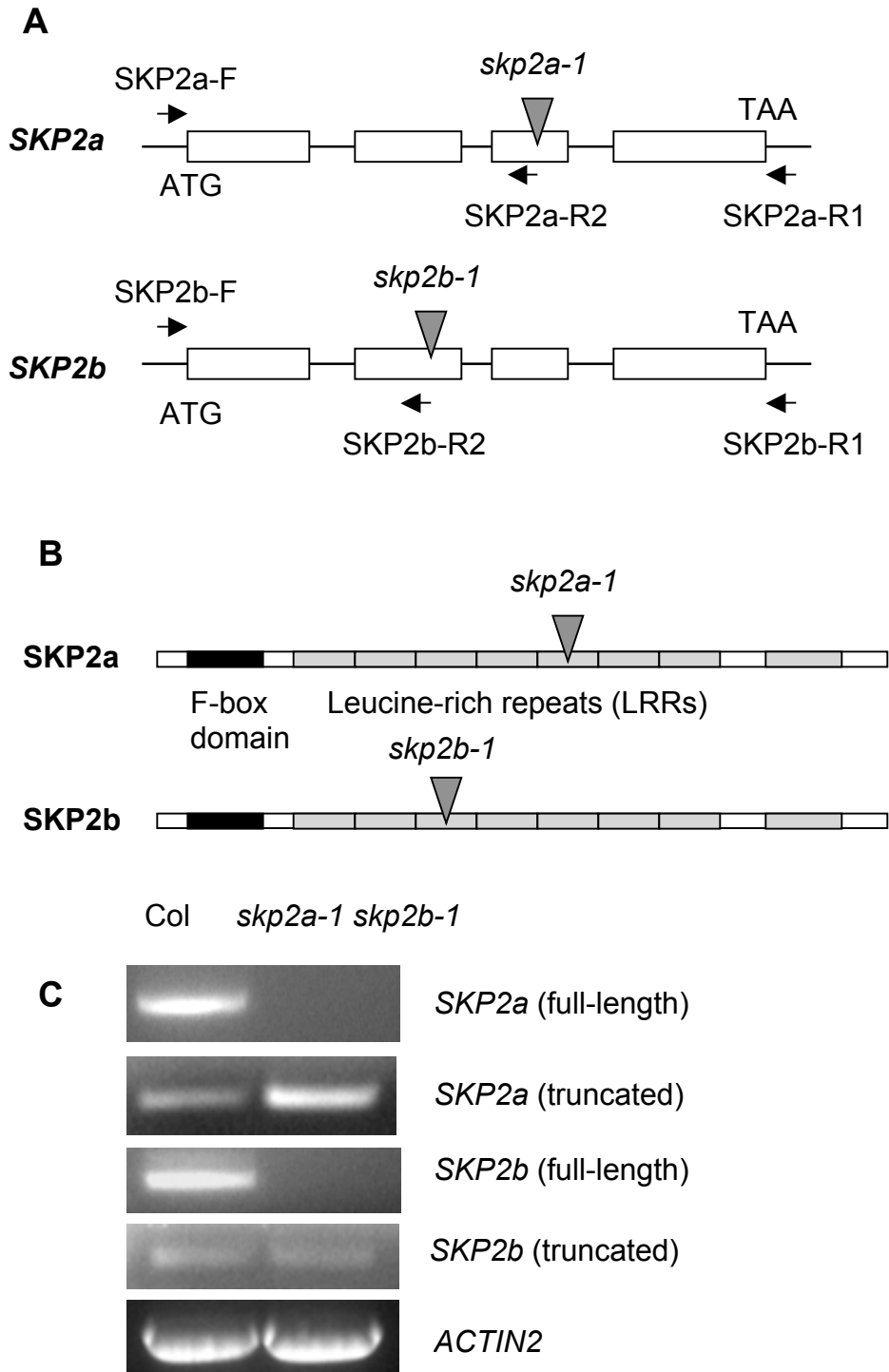
Figure 2-10. RT-PCR analysis of *SKP2a* and *SKP2b* expression in the *SKP2a* and *SKP2b* T-DNA insertion mutants.

(A) Genomic structure of *SKP2a* and *SKP2b* and T-DNA insertion locations (not to scale). Lines and boxes represent introns and exons, respectively. Triangles represent T-DNA inserts. Arrows indicate primers that are used for RT-PCR. The *skp2a-1* and *skp2b-1* mutants have a T-DNA insert in exon 3 and exon 2, respectively. **(B)** Protein structure of SKP2a and SKP2b (not to scale). Black and gray boxes represent F-box domain and leucine-rich repeat (LRR), respectively. Triangles represent T-DNA inserts. Both SKP2a and SKP2b have an F-box domain and eight LRRs. Motifs were analyzed using the Simple Modular Architecture Research Tool (SMART, http://smart.embl.de/). **(C)** RT-PCR analysis of *SKP2a*, *SKP2b*, and *ACTIN2* expression in the wild-type (Col) and a *SKP2a-1 skp2b-1* double mutant. Total RNAs were extracted from 7-day-old light-grown seedlings. Gene specific primers for amplifying the full-length and truncated transcripts of *SKP2a* and *SKP2b* are shown in **(A)**. PCRs were performed for 35 cycles (*ACTIN2*) and 40 cycles (*SKP2a* and *SKP2b*).

GUS (data not shown). To understand the molecular nature of the *skp2a-1* and *skp2b-1* mutants, I examined *SKP2a* and *SKP2b* expression by RT-PCR in the *skp2a-1 skp2b-1* double mutant. The full-length transcripts of *SKP2a* and *SKP2b* could not be detected. However, truncated transcripts were detected at a high level for *SKP2a* and at a low level for *SKP2b* (Fig 2-10C). Therefore, truncated SKP2a and SKP2b proteins could be produced. If these truncated proteins exist, SKP2a and SKP2b will have the F-box domain as well as four and two leucine-rich repeats (LRRs), respectively (Fig 2-10B). Thus it is possible that truncated SKP2a and SKP2b could form a functional SCF complex that targets substrates for degradation.

As an alternative approach, I examined KRP1-GUS protein stability in *SKP2* RNA interference (RNAi) transgenic plants with reduced levels of both *SKP2a* and *SKP2b*. I worked with two independent lines and obtained similar results. *SKP2-RNAi* transgenic lines did not exhibit any obvious defects in growth and development (data not shown). Here we show results for the line RNAi-29. Compared with WT, the expression of both *SKP2a* and *SKP2b* was strongly decreased in this line (Fig 2-11A). The *KRP1::KRP1-GUS* transgene was introduced into *SKP2-RNAi* transgenic plants by crossing, and F1 plants were examined for GUS expression. GUS staining was observed in the cotyledon of RNAi-29 seedlings, but not the hypocotyl and root (Fig 2-11B and 2-11C; data not shown). Therefore, *SKP2-RNAi* transgenic plants stabilize KRP1-GUS, indicating that SKP2a and SKP2b are involved in KRP1 degradation.

Figure 2-11. The F-box protein SKP2b is involved in KRP1 degradation.

A

SKP2a

SKP2b

ACTIN2

Col   RNAi-29

B

C

D

E   Myc-KRP1

Unknown protein

F   Myc-KRP1

SKP2b-TAP

ACTIN2

1   2   3   4

G

H

47
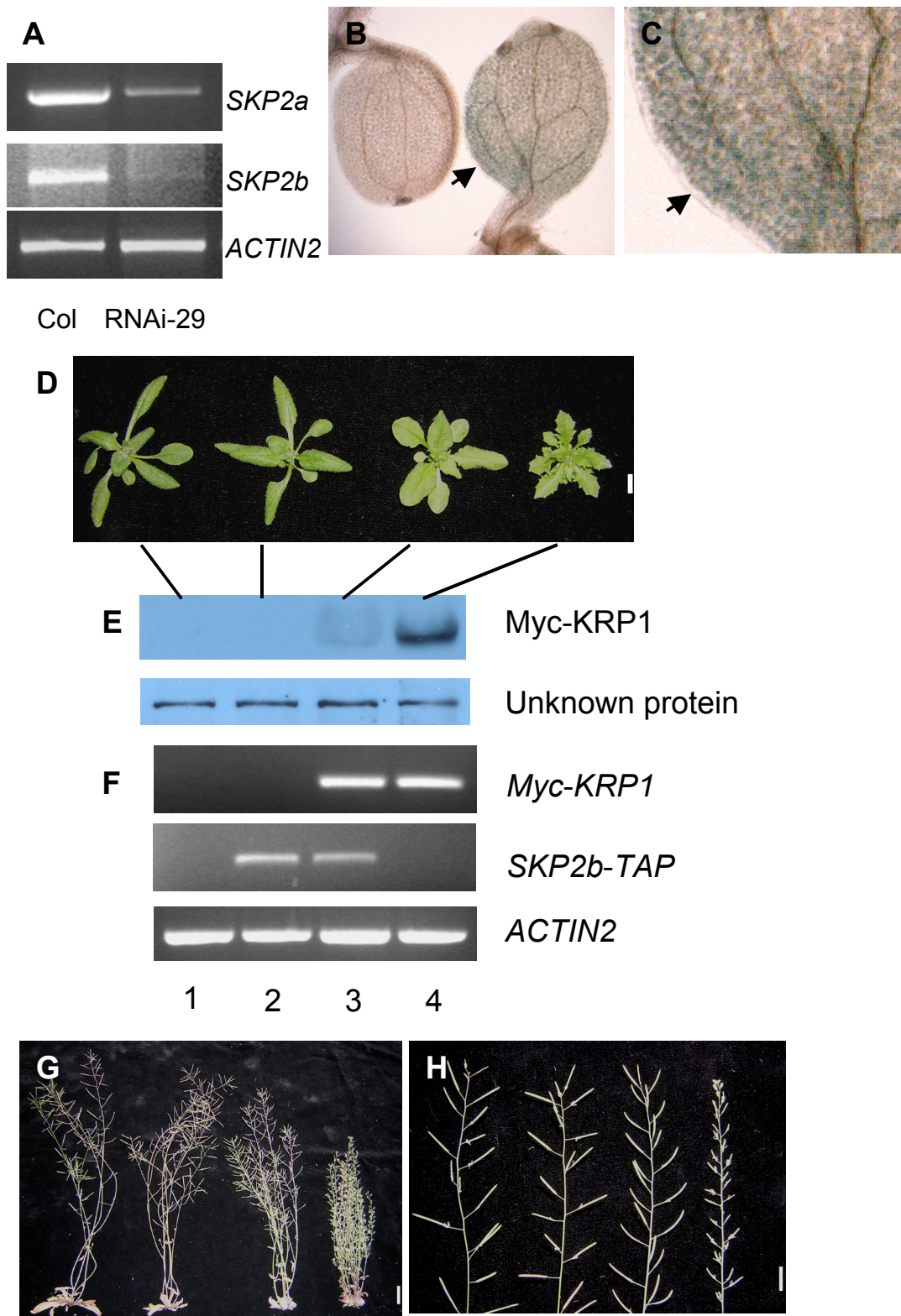
Figure 2-11. The F-box protein SKP2b is involved in KRP1 degradation.

(A) RT-PCR analysis of *SKP2a*, *SKP2b*, and *ACTIN2* expression in the wild-type (Col) and *SKP2-RNAi* line (RNAi-29). Total RNAs were extracted from 7-day-old light-grown seedlings. PCRs were performed with gene specific primers for 25 cycles (*ACTIN2*) and 40 cycles (*SKP2a* and *SKP2b*). **(B)** GUS staining of 5-day-old light-grown *KRP1::KRP1-GUS* seedlings in the wild-type (Col) (left) and *SKP2-RNAi* line (RNAi-29) (right) background. **(C)** A closer look at the GUS-stained cotyledon of *SKP2-RNAi* line (RNAi-29) shown in **(G)**. **(D)** Three-week-old plants. Bar = 0.5 cm. **(E)** Immunoblot analysis of Myc-KRP1 with an ∝-c-myc antibody. Protein extracts were prepared from 3-week-old plants. An unknown protein recognized by the ∝-c-myc antibody was used as a loading control. **(F)** RT-PCR analysis of *Myc-KRP1*, *SKP2b-TAP*, and *ACTIN2* expression. Total RNAs were extracted from 3-week-old plants. The c-myc epitope and TAP tag transcripts were amplified to show *Myc-KRP1* and *SKP2b-TAP* expression, respectively. PCRs were perfomed for 25 cycles. **(D)** to **(F)** Lane 1, wild-type (Col); lane 2, *35S*::*SKP2b-TAP*; lane 3, *35S*::*SKP2b-TAP 35S*::*Myc-KRP1* (hemizygous); lane 4, *35S*::*Myc-KRP1* (hemizygous). **(G)** Seven-week-old mature plants. Bar = 2 cm. **(H)** Inflorescences of 7-week-old mature plants. Bar = 0.5 cm. **(G)** and **(H)** From left to right: wild-type (Col); *35S*::*SKP2b-TAP*; *35S*::*SKP2b-TAP 35S*::*Myc-KRP1* (hemizygous); *35S*::*Myc-KRP1* (hemizygous). Arrows indicate GUS staining.

To gain further evidence for a role for *Arabidopsis* SKP2 in KRP1 degradation, I examined the effect of SKP2 overexpression on KRP1 degradation *in planta*. I worked with *Arabidopsis* transgenic plants that express a TAP (tandem affinity purification) tagged SKP2a or SKP2b under the control of the *CaMV 35S* promoter. I introduced the *35S::SKP2a-TAP* and *35S::SKP2b-TAP* transgenes into *35S::Myc-KRP1* plants by crossing. A *35S::SKP2a-TAP* line did not appear to alter the effects of Myc-KRP1overexpression (data not shown). Interestingly, two independent *35S::SKP2b-TAP* lines suppressed the effects of Myc-KRP1 overexpression. Here I show results for the line 5. An obvious phenotype of KRP1 overexpressors is serrated rosette leaves. Plants that overexpress both SKP2b-TAP and Myc-KRP1 did not exhibit serrated rosette leaves (Fig 2-11D). In addition to the loss of serrated rosette phenotype, mature plants that overexpress both SKP2b-TAP and Myc-KRP1 exhibited increased height and fertility (Fig 2-11 G and 2-11H). The loss of serrated leaf phenotype and increased height and fertility were associated with a decreased Myc-KRP1 protein level. Three-week-old *35S::SKP2b-TAP 35S::Myc-KRP1* plants had much less Myc-KRP1 than *35S::Myc-KRP1* plants (Fig 2-11E). I also examined *Myc-KRP1* transcript levels in both lines and found them to be similar (Fig 2-11F), confirming that decreased Myc-KRP1 protein levels are due to increased degradation. Taken together, my data indicate that KRP1 degradation is dependent on an SCF complex that consists of CUL1 and SKP2b.

DISCUSSION

In recent years, SCFs have been implicated in many aspects of cellular regulation and developmental processes in plants, especially in hormone signaling. Although SCF^{SKP2a} appears to regulate the transcription factor E2Fc degradation, a critical role for SCFs in plant cell cycle regulation has not yet been clearly established (Moon et al., 2004; Thomann et al., 2005). The mammalian CKI p27^{Kip1}-related proteins have been identified in plants, however, very little is known about the posttranslational regulation of plant CKIs (Verkest et al., 2005b). Here, I provide clear evidence for the important role of an SCF in plant cell cycle regulation. I show that SCF^{SKP2b} mediates CKI KRP1 degradation to regulate the G1-S transition of the cell cycle. In addition, ubiquitin-mediated protein degradation might regulate pericycle activation to initiate lateral root formation through the degradation of KRP1.

KRP1 is expressed in dividing and differentiated cells

To understand the role of *KRP1* in plant growth and development, I have determined the pattern of *KRP1* expression using *GUS* as a reporter under the control of the *KRP1* promoter. Consistent with previous northern blot and RT-PCR data, *GUS* expression reveals that *KRP1* is broadly expressed in various tissues and organs throughout plant development. In roots, *KRP1* is expressed in the root meristem as well as the elongation and differentiation regions. Unlike the closely related *KRP2* gene, which is only

expressed in pericycle cells (Himanen et al., 2002), *KRP1* is expressed in all

cell types of the mature root. *KRP1* is highly expressed in the rosette leaves

of 10-day-old plants. This is consistent with previous *in situ* hybridization data,

showing high *KRP1* expression in endoreduplicating tissues of leaves

(Ormenese et al., 2004). High *KRP1* expression in leaves indicates a possible

role of *KRP1* in leaf development. This hypothesis is supported by a report by

Wang et al. (2000), showing that overexpression of *KRP1* results in serrated

rosette leaves. In flowers, *KRP1* is expressed in anthers and mature pollens.

Interestingly, *KRP1* is expressed in the floral organ abscission zone at the

base of siliques. Whether *KRP1* plays a role in floral organ abscission is

unknown, but its expression pattern suggests this possibility. In conclusion,

KRP1 is expressed in both dividing cells and differentiated cells throughout

plant development. In addition to its role in cell division, KRP1 might also play

a role in regulating cell differentiation and endoreduplication. The role of

KRP1 in endoreduplication was reported by a recent study by Weinl et al.

(2005). Using *Arabidopsis* trichomes as a system, the authors demonstrated

that KRP1 inhibits mitosis entry but allows DNA synthesis to cause

endoreduplication.

KRP1 interacts with the CDKA;1/CYCD2;1 complex to control the G1-S
transition of the cell cycle

In eukaryotes, cell cycle progression is controlled by the activities of

CDK/cyclin complexes. In *Arabidopsis*, there are 5 CDKs with known direct

roles in the cell cycle and at least 31 cyclins of the three main classes of A-, B-, and D-types (Vandepoele et al., 2002; Menges et al., 2005). The combinations of various CDKs and cyclins regulate cell cycle progression. CDKA;1 is the orthologue of yeast Cdc2/Cdc28 and mammalian CDK1 and functions in both the G1-S and G2-M transitions. The CDKBs are plant-specific CDKs that are cell cycle regulated and show a peak of expression in G2 (*CDKB1;1* and *CDKB1;2*) or G2-M (*CDKB2;1* and *CDKB2;2*). There are two genes encoding each of the two CDKB subclasses in *Arabidopsis* (de Jager et al., 2005; Menges et al., 2005). D-type cyclins are thought to control the G1-S transition. Among the 10 D-type cyclins in *Arabidopsis*, only CYCD2;1 and CYCD3;1 are extensively studied. Both have a LxCxE motif near their N terminus, which mediates the interaction between D-type cyclins and retinoblastoma protein (Huntley et al., 1998; Oakenfull et al., 2002; de Jager et al., 2005). We have shown that KRP1 forms a complex with CDKA;1 and CYCD2;1 *in vivo*, but not with CDKB1;1. These results are consistent with previous genetic findings. Overexpression of CDKA;1 and CYCD2;1 in *Arabidopsis* whole plants and trichomes, respectively, suppress the effects of KRP1 overexpression (Zhou et al., 2003b; Schnittger et al., 2003).

CYCD2;1 is a stable protein that is present in both dividing and non-dividing *Arabidopsis* cultured cells (Healy et al., 2001; Planchais et al., 2004). A previous study by immunoprecipitation reported that CYCD2;1 interacts with CDKA;1 *in vivo*, but not CDKB1;1 (Healy et al., 2001). Further, the purified CDKA;1/CYCD2;1 complex from growing *Arabidopsis* cultured cells

phosphorylates a plant retinoblastoma-related protein (RBR) *in vitro* (Boniotti

and Gutierrez, 2001). These data support a critical role of the

CDKA;1/CYCD2;1 complex in regulating the G1-S transition in cells that

reactivate division. The *in vivo* interactions between KRP1 and

CDKA;1/CYCD2;1 strongly suggest that KRP1 functions to regulate the G1-S

transition during cell cycle reactivation. The role of KRP1 in the G1-S

transition control is supported by previous transgenic studies. KRP1

overexpression inhibits cell division and endoreduplication. Plants have a

reduced number of cells and a decreased ploidy level in leaves (Wang et al.,

2000; Zhou et al., 2002a).

In addition to the CDKA;1/CYCD2;1 complex, KRP1 may have other

targets. A recent study reported that besides its role in the G1-S transition,

KRP1 also functions in the G2-M transition to regulate mitosis entry (Weinl et

al., 2005). However, the CDK/cyclin complex target of KRP1 at the G2-M

transition is unknown. It will be of importance to identify other CDK/cyclin

complex targets of KRP1 in the future to further define the role of KRP1 in cell

cycle regulation.

KRPs have redundant functions

The *Arabidopsis* genome encodes seven KRPs. All KRPs have the

conserved CDK-binding/inhibitory domain in the C-terminus. Outside this

region, there is no significant sequence identity (De Veylder et al., 2001; Zhou

et al., 2002a; Vandepoele et al., 2002). At present, whether KRPs have

redundant or distinct functions is unknown. The distinct expression patterns of *KRPs* in the cell cycle and in various plant organs suggest that *KRPs* might play different roles in cell cycle regulation and in plant growth and development (De Veylder et al., 2001; Menges et al., 2005). Analysis using the yeast two-hybrid system demonstrated that all KRPs except KRP5 interacts with CDKA;1 (De Veylder et al., 2001), indicating that KRP5 might bind to an unknown CDK and plays a distinct role in cell cycle regulation. Studies on the functions of plant CKIs have relied on the gain of function approach by overexpressing *KRPs* under the control of the *CaMV* 35S promoter or other tissue-specific promoters (Wang et al., 2000; De Veylder et al., 2001; Zhou et al., 2002a; Zhou et al., 2002b; Schnittger et al., 2003; Weinl et al., 2005; Verkest et al., 2005). However, the effects of loss of *KRPs* on plant growth and development have not yet been reported.

To reveal the role of *KRP1* in plant growth and development by a loss of function approach, I have identified a *krp1-1* mutant with a T-DNA insertion in the third intron, which prevents formation of full-length KRP1 protein. However, truncated KRP1 protein without the C-terminal 22 amino acids (KRP1-C22) and with an impaired CDK-binding/inhibitory domain might be produced in the *krp1-1* mutant. In previous studies, deletion analysis demonstrated that KRP1 with deletion of the C-terminal 16 or 29 amino acids (KRP1-16 or KRP1-29) showed almost no interaction with CDKA;1 in the yeast two-hybrid system (Wang et al., 1998). Consistent with the yeast two-hybrid results, KRP1-15 and KRP1-29 proteins do not appear to bind the CDK

complex *in vivo* (Zhou et al., 2003a). In addition, recombinant KRP1-15 and KRP1-29 proteins lose the CDK kinase inhibition ability (Zhou et al., 2003a). Further, transgenic studies showed that deletion of the C-terminal 15 and 29 amino acids strongly weaken and completely abolish the effects of KRP1 on transgenic plants, respectively (Zhou et al., 2003a). Plants that overexpress KRP1-15 and KRP1-29 exhibited only a weak flower phenotype and no obvious phenotype, respectively. Like the KRP1-29 overexpressors, transgenic plants overexpressing Myc-KRP1-C22 do not show any obvious defects in plant growth and development, indicating that Myc-KRP1-C22 is probably not a functional protein *in planta*. As described previously, KRP1 forms a complex with CDKA;1 and CYCD2;1 *in vivo*. To gain further evidence to support that KRP1-C22 is not a functional protein, it will be interest to know whether Myc-KRP1-C22 forms a complex with CDKA;1 and CYCD2;1 *in vivo*. In conclusion, these data suggest that the *krp1-1* mutant is a null mutant. The absence of an obvious phenotype of the *krp1-1* mutant suggests that in the absence of KRP1, other KRPs compensate the role of KRP1. Therefore, other KRPs may have redundant functions with KRP1. In the future, identification of other *KRPs* T-DNA insertion mutants and construction of higher levels of mutations are required for revealing the phenotype of plants that loss KRPs.

KRP1 regulates pericycle activation during lateral root initiation

Lateral root formation is an example of postembryonic *de novo* organogenesis. In *Arabidopsis*, lateral roots are derived from pericycle cells adjacent to the xylem poles. The process of lateral root formation can be divided into two major steps: pericycle activation and meristem formation (Himanen et al., 2002; Casimiro et al., 2003). Using a gain of function approach, I have demonstrated that KRP1 plays an important role in regulating pericycle activation during lateral root initiation. KRP1 overexpression causes a dramatic decrease in a number of lateral roots in a dose-dependent manner. Two-week-old homozygous *35S::Myc-KRP1* plants have almost no lateral roots. Further, KRP1 overexpression inhibits auxin-mediated pericycle cell division. Although treated with NAA for 12 h, *GUS* expression driven by the *CYCB1;1* promoter was not induced in the pericycle of *35S::Myc-KRP1* seedlings. Therefore, KRP1 overexpression inhibits the first pericycle cell division required for lateral root initiation. The role of KRP1 in lateral root formation is supported by its gene expression and protein localization in the pericycle.

In addition to KRP1, another CKI KRP2 also plays a similar role in lateral root formation. A previous study reported that KRP2 overexpression causes a decreased number of lateral roots and inhibits auxin-mediated pericycle cell division. It was proposed that KRP2 controls the G1-S transition of pericycle cells to regulate pericycle activation and auxin regulates pericycle activation through down-regulating *KRP2* expression (Himanen et al., 2002).

Unlike *KRP2*, *KRP1* expression does not seem to be regulated by auxin in *Arabidopsis* cultured cells (Richard et al, 2002). Whether auxin might affect KRP1 activity at the posttranslational level is unknown, and this awaits further investigation.

Ubiquitin-mediated protein degradation has been implicated in regulating pericycle activation during lateral root initiation. Mutations in *TIR1*, an F-box protein, and *AXR1*, an E1 component of the RUB conjugation pathway, cause a decreased number of lateral roots and inhibit pericycle cell division. Both *TIR1* and *AXR1* are expressed at the lateral root initiation sites and are required for the first pericycle cell division (Gray et al., 1999; del Pozo et al., 2002b). A previous report demonstrated that auxin regulates the G1-S transition of pericycle cells during pericycle cell activation (Himanen et al., 2002). Although it was proposed that auxin possibly promotes pericycle cell division through degrading one or more cell cycle regulators by SCF^{TIR1} (Gray et al., 1999), these cell cycle regulators have not yet been identified.

I have shown that AXR1 regulates KRP1 degradation. Interestingly, KRP1 overexpression enhances the *axr1-3* lateral root defect, which is associated with an increased Myc-KRP1 protein level. One possible explanation for these results is that KRP1 is a target for AXR1-mediated protein degradation during pericycle activation. As discussed later, the ubiquitin-proteasome pathway regulates KRP1 degradation. Whether KRP1 is a substrate of SCF^{TIR1} during pericycle activation is unknown, but it is an interesting area for future investigation. Taken together, I propose that KRP1

functions in the G1-S transition of pericycle cells to regulate pericycle activation. Unlike the regulation of KRP2 at the transcriptional level, KRP1 is regulated at the posttranslational level. Ubiquitin-mediated protein degradation might therefore regulate KRP1 degradation to control pericycle activation during lateral root initiation.

SCF^{SKP2b} mediates KRP1 degradation

Although KRP1 was the first plant CKI to be identified (Wang et al., 1997), nothing is known about its regulation at the posttranslational level. My results indicate that KRP1 is an unstable protein that is degraded by the 26S proteasome. Further, I have demonstrated that the AXR1-dependent RUB conjugation pathway regulates KRP1 degradation. Because the only known substrates for RUB conjugation are the cullin proteins, these results imply that KRP1 degradation requires a cullin-based E3 such as an SCF. In *Arabidopsis*, an SCF complex is composed of four subunits: RBX1, CUL1, ASK (SKP1-related proteins), and F-box protein. I have shown that CUL1 is required for KRP1 degradation. The involvement of CUL1 in KRP1 degradation strongly suggests that an SCF regulates KRP1 degradation.

To identify the F-box component of the SCF, I worked with two mammalian SKP2-like F-box proteins SKP2a and SKP2b in *Arabidopsis*. I have demonstrated that *SKP2-RNAi* lines with strongly decreased levels of both *SKP2a* and *SKP2b* stabilize KRP1-GUS. Surprisingly, *SKP2-RNAi* lines do not exhibit an obvious phenotype. The presence of residual *SKP2a*

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transcript might provide a possible explanation. Further, I have shown that SKP2b overexpression promotes KRP1 degradation *in planta*. Transgenic plants that overexpress both SKP2b-TAP and Myc-KRP1 lose the serrated rosette leaf phenotype and exhibit increased height and fertility conferred by KRP1 overexpression, which is associated with a strongly decreased Myc-KRP1 protein level. A *35S::SKP2a-TAP* line does not appear to suppress the effects of Myc-KRP1 overexpression. The reason for this is unknown. The above results suggest that SKP2b targets KRP1 for degradation, but I could not exclude the possibility that SKP2a is also involved in KRP1 degradation. It will be of importance to examine the biochemical interactions between KRP1 and SKP2b in the future. Taken together, my data clearly indicate that an SCF complex that is composed of CUL1 and SKP2b mediates KRP1 degradation.

In addition to the CUL1 and F-box protein SKP2b, the ASK component of the SCF^{SKP2b} complex is unknown. The *Arabidopsis* genome encodes 21 SKP1-related proteins called ASKs (Farras et al., 2001; Risseeuw et al., 2003). Among the 21 ASKs, a previous study showed that ASK1 and ASK2 have partially redundant functions. The cell division defects and stabilization of CYCD3;1 in plants that loss both *ASK1* and *ASK2* suggest that ASK1 and ASK2 function in cell cycle regulation (Liu et al., 2004). It will be of importance to determine whether ASK1 and ASK2 are involved in KRP1 degradation in the future.

KRP1 degradation is regulated by both SCF-dependent and SCF-independent mechanisms

As described above, an SCF regulates KRP1 degradation. In addition to SCF-dependent degradation of KRP1, several lines of evidence imply that an SCF-independent pathway also exists to regulate KRP1 protein turnover. In the WT background, *KRP1::KRP1-GUS* seedlings do not exhibit any GUS staining. The *axr6-3* mutant and *SKP2-RNAi* transgenic plants exhibit GUS staining at a low level in the cotyledon, while the *axr1-3* mutant exhibit GUS staining at a high level in both the cotyledon and hypocotyl. However, surprisingly, no GUS staining is observed in the root of all these mutants deficient in SCF functions. In contrast, *KRP1::KRP1-GUS* seedlings treated with the proteasome inhibitor MG132 for 8 h strongly accumulate KRP1-GUS in the root, while only a weak GUS staining is observed in the cotyledon. These different GUS staining patterns in the mutants deficient in SCF functions and in the MG132-treated seedlings suggest that in young seedlings, KRP1 degradation is regulated by two distinct mechanisms: both SCF-dependent and SCF-independent pathways. The regulation of KRP1 degradation by these two pathways depends on the 26S proteasome.

Based on my data presented in this paper, I propose a model to explain how SCF^{SKP2b} mediates KRP1 degradation to regulate the G1-S transition of the cell cycle (Fig 2-12). The G1-S transition is controlled by a CDKA;1/CYCD2;1 complex, whose activity is regulated by a negative cell cycle regulator KRP1. The binding of KRP1 to the CDKA;1/CYCD2;1 complex

inhibits the complex activity and blocks the G1-S transition. In late G1 phase, triggered by certain signals, SCF^{SKP2b} targets KRP1 for degradation by the 26S proteasome. The degradation of KRP1 releases the inhibition to the CDKA;1/CYCD2;1 complex. An active CDKA;1/CYCD2;1 complex phosphorylates the retinoblastoma-related protein (RBR) to allow E2F transcription factors activate gene expression required for the G1-S transition and S phase progression. The cell passes the G1-S transition and enters S phase to undergo DNA synthesis. Whether SCF^{SKP2b}-mediated degradation of KRP1 is dependent on the phosphorylation of KRP1 by the CDKA;1/CYCD2;1 complex is unknown.
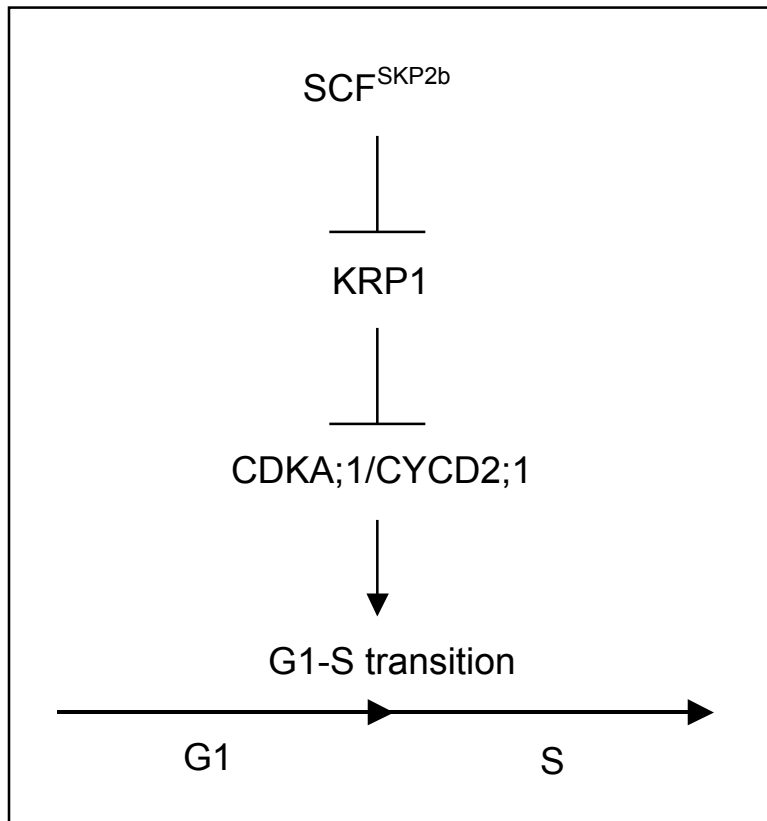
Figure 2-12. Model for SCF^{SKP2b}-mediated degradation of KRP1 and regulation of the G1-S transition of the cell cycle.

KRP1 interacts with the CDKA;1/CYCD2;1 complex and functions to regulate the G1-S transition of the cell cycle. SCF^{SKP2b}-mediated degradation of KRP1 is required for triggering the G1-S transition (see text for details).

Chapter 3. Role of the RING Finger Ubiquitin Ligase KPC1 in KRP1 Degradation

INTRODUCTION

In Chapter two, I described SCF^{SKP2b}-dependent degradation of KRP1. However, the mechanism of SCF-independent degradation of KRP1 is unknown. In G1 phase, a non-SCF complex called KPC (Kip1 ubiquitination-promoting complex) regulates mammalian CKI p27^{Kip1} degradation. KPC consists of two subunits: KPC1 (a RING finger E3) and KPC2 (a protein containing a ubiquitin-like domain and two ubiquitin-associated domains). Different from the phosphorylation-dependent p27^{Kip1} degradation by SCF^{SKP2} in the nucleus, KPC-mediated p27^{Kip1} degradation is phosphorylation-independent and occurs in the cytoplasm (Kamura et al., 2004; Kotoshiba et al., 2005). Because KRP1 is homologous to p27^{Kip1}, a similar KPC-dependent protein degradation mechanism might exist in *Arabidopsis* to regulate KRP1 protein turnover. The *Arabidopsis* genome encodes 469 RING finger proteins. Interestingly, there is a KPC1-related RING finger protein called At2g22010 in *Arabidopsis* (Kosarev et al., 2002; Stone et al., 2005). Like KPC1, At2g22010 has a RING finger domain in the C-terminus and a SPRY domain with an unknown function near the N-terminus (Fig 3-1A). To understand the mechanism of SCF-independent degradation of KRP1, I worked with

Arabidopsis At2g22010 RING finger E3 and investigated whether this ubiquitin ligase regulates KRP1 degradation.

RESULTS

The *kpc1-1* and *kpc1-2* mutants stabilize KRP1-GUS fusion protein

To determine whether the *Arabidopsis* RING finger ubiquitin ligase KPC1 is involved in KRP1 degradation, I examined KRP1-GUS protein stability in *KPC1* T-DNA insertion mutants. I identified *KPC1* T-DNA insertion mutants in the SAIL and Wisconsin collection (Fig 3-1B). The *kpc1-1* and *kpc1-2* mutants have T-DNA insertions in exon 2 and exon 7, respectively. Neither mutant exhibited an obvious phenotype (data not shown). I introduced the *KRP1*::*KRP1-GUS* transgene into the *kpc1-1* and *kpc1-2* mutants by crossing. As shown in Fig 3-1C to 3-1F, the *kpc1-1* and *kpc1-2* mutants stabilized KRP1-GUS, exhibiting GUS staining in the cotyledon, but not in the hypocotyl and root (data not shown). These results indicate that KPC1 is involved in KRP1 degradation.

Overexpression of HA-KPC1 causes increased Myc-KRP1 degradation
in planta

To gain further evidence for a role of KPC1 in KRP1 degradation, I examined the effect of KPC1 overexpression on KRP1 degradation *in planta*. I generated *Arabidopsis* transgenic plants that express a HA epitope tagged

Figure 3-1. The RING finger ubiquitin ligase KPC1 regulates KRP1 degradation.

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Col   35S::HA-KPC1   35S::Myc-KRP1   35S::HA-KPC1
                                              35S::Myc-KRP1

G

H   Myc-KRP1

                                              Unknown protein
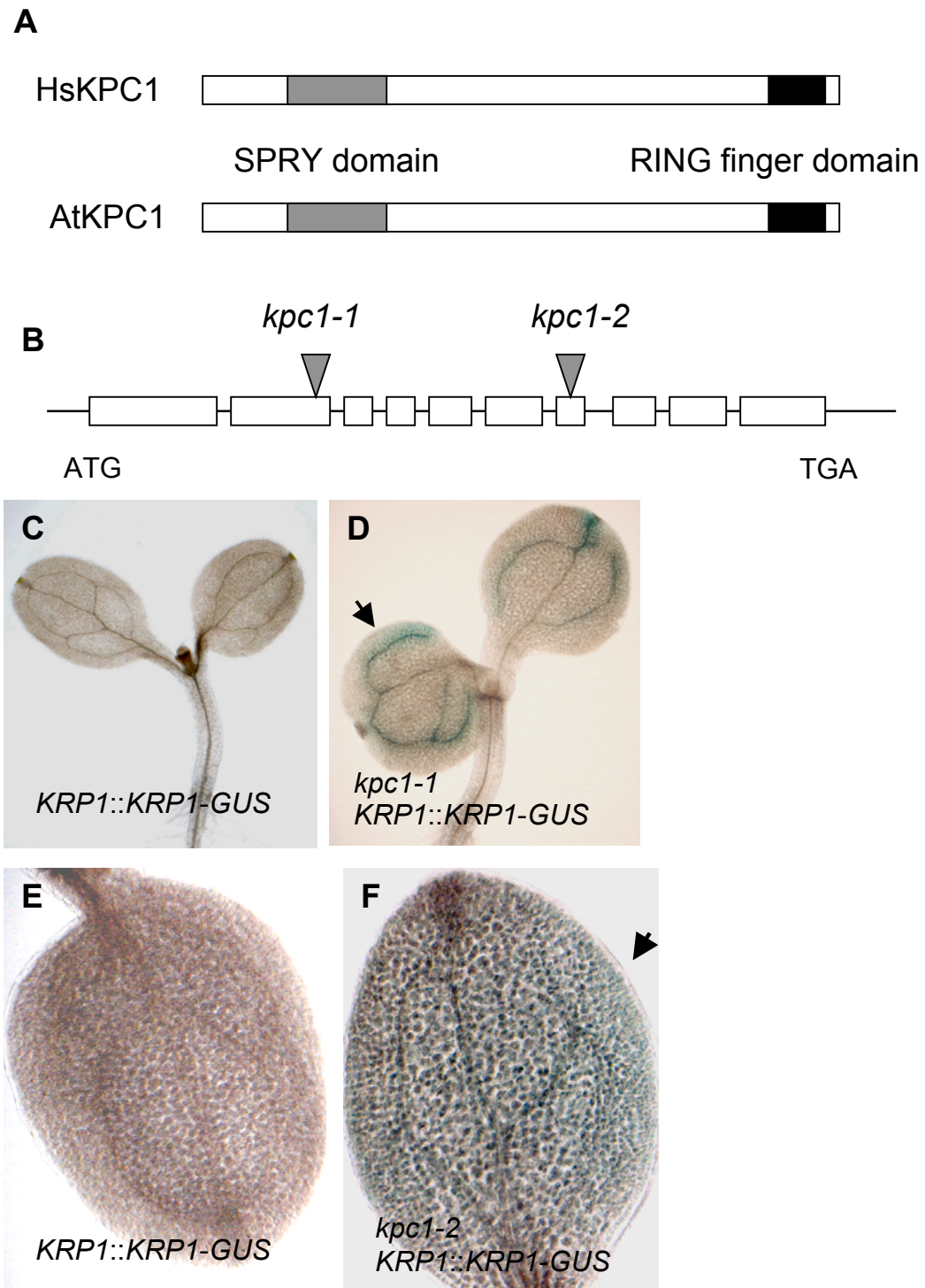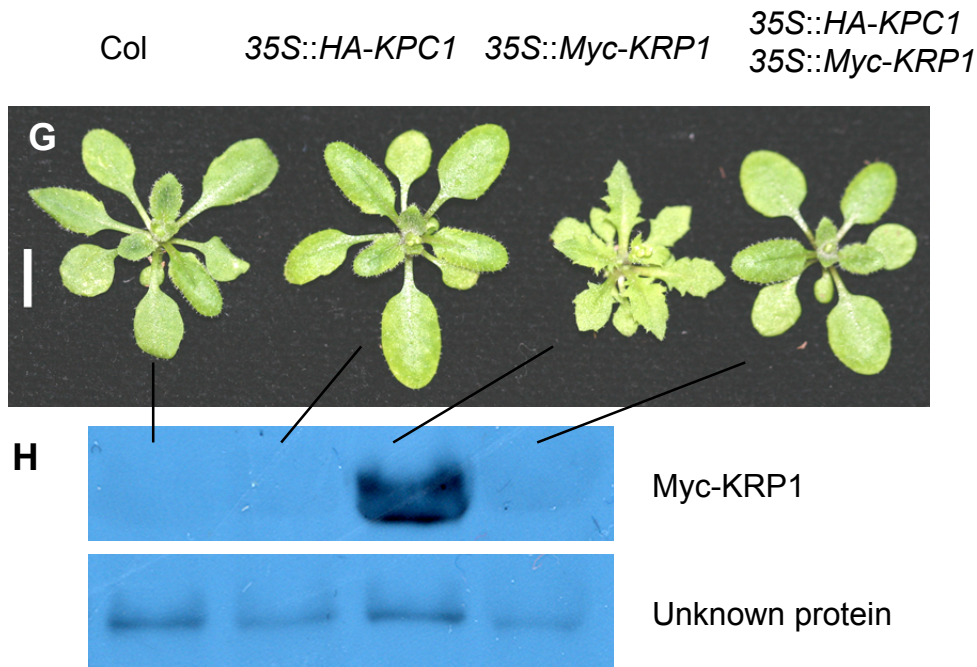
Figure 3-1. The RING finger ubiquitin ligase KPC1 regulates KRP1 degradation.

(**A**) Protein structure of human KPC1 (HsKPC1) and *Arabidopsis* KPC1 (AtKPC1) (not to scale). Gray and black boxes represent SPRY domain and RING finger domain, respectively. The function of the SPRY domain (domain in SPla and RYanodine receptor) is unknown (Ponting et al., 1997). The RING finger domain is a protein interaction domain that can bind the ubiquitin-conjugating enzyme E2 (Freemont, 2000). Motifs were analyzed using the Simple Modular Architecture Research Tool (SMART, http://smart.embl.de/). (**B**) Genomic structure of KPC1 and T-DNA insertion locations (not to scale). Line and boxes represent introns and exons, respectively. Triangles represent T-DNA inserts. The *kpc1-1* and *kpc1-2* mutants have T-DNA insertions in exon 2 and exon 7, respectively. (**C**) GUS staining of a 6-day-old light-grown *KRP1*::*KRP1-GUS* seedling. (**D**) GUS staining of a 6-day-old light-grown *kpc1-1* seedling that carries a *KRP1*::*KRP1-GUS* transgene. (**E**) GUS staining of a 4-day-old light-grown *KRP1*::*KRP1-GUS* seedling. (**F**) GUS staining of a 4-day-old light-grown *kpc1-2* seedling that carries a *KRP1*::*KRP1-GUS* transgene. (**G**) Three-week-old plants. (**H**) Immunoblot analysis of Myc-KRP1 with α–c-myc antibody. Protein extracts were prepared from 4-week-old plants. An unknown protein recognized by the α–c-myc antibody was used as a loading control. (**G**) and (**H**) Wild-type (Col); *35S*::*HA-KPC1* (T2); *35S*::*Myc-KRP1* (hemizygous); *35S*::*HA-KPC1 35S*::*Myc-KRP1* (hemizygous, T2). Arrows indicate GUS staining.

KPC1 under the control of the *CaMV 35S* promoter. I introduced the *35S*::*HA-KPC1* transgene into *35S*::*Myc-KRP1* plants by transformation. An obvious phenotype of plants that overexpress KRP1 is serrated rosette leaves (Fig 2-4C to 2-4E). Interestingly, in the T1 generation, 92% (34/37) *35S*::*Myc-KRP1* plants did not exhibit serrated rosette leaves. I examined Myc-KRP1 protein levels in seven independent lines (two lines showing serrated rosette leaves and five lines without serrated leaves) by immunoblot analysis using an α-c-myc antibody. The five lines that did not exhibit serrated rosette leaves had much less Myc-KRP1 protein levels that the two lines showing serrated rosette leaves (data not shown). Here, I show results for line 6. As shown in Fig 3-1G, plants that overexpress both HA-KPC1 and Myc-KRP1 did not exhibit serrated rosette leaves. The loss of serrated leaf phenotype was associated with a decreased Myc-KRP1 protein level. Four-week-old *35S*::*HA-KPC1 35S*::*Myc-KRP1* plants had much less Myc-KRP1 than *35S*::*Myc-KRP1* plants (Fig 3-1H). Although the transcript levels of *Myc-KRP1* in *35S*::*HA-KPC1 35S*::*Myc-KRP1* plants have not been examined and the possibility that *Myc-KRP1* is silenced can not be ruled out, these results indicate that possibly KPC1 overexpression causes increased degradation of KRP1 *in planta*, supporting the role of KPC1 in KRP1 degradation.

DISCUSSION

Although there are 469 RING finger proteins in *Arabidopsis*, very little is known about their functions and protein substrates. At present, only a small

number of these proteins have been shown to be involved in specific cellular and developmental processes, including COP1, CIP8, and TED3 in photomorphogenesis (Deng et al., 1991; Osterlund et al., 2000; Holm et al., 2002; Hardtke et al., 2002; Hu et al., 2002), RMA1 in secretory pathway (Matsuda and Nakano, 1998; Matsuda et al., 2001), SINAT5 in auxin signaling (Xie et al., 2002), BRH1 in brassinosteroid response (Molnar et al., 2002), RIE1 in seed development (Xu and Li, 2003), ATL2 in defense response (Serrano and Guzman, 2004), and XBAT32 in lateral root development (Nodzon et al., 2004). Here, I present evidence for a role of the RING finger E3 KPC1 in KRP1 degradation, indicating a role of KPC1 in cell cycle regulation. I have shown that two independent *KPC1* T-DNA insertion mutants stabilize KRP1-GUS fusion protein, exhibiting GUS staining in the cotyledon. In addition, KPC1 overexpression suppresses the effects of KRP1 overexpression, causing increased KRP1 degradation *in planta*. Transgenic plants that overexpress both HA-KPC1 and Myc-KRP1 lose the serrated leaf phenotype conferred by KRP1 overexpression, which is associated with a strongly decreased Myc-KRP1 protein level. To rule out the possibility that the decreased Myc-KRP1 protein levels are caused by the silencing of *Myc-KRP1* transgene, RT-PCR or an RNA blot needs to be performed to examine *Myc-KRP1* transcripts in *35S*::*HA-KPC1 35S*::*Myc-KRP1* plants. To understand the molecular nature of the *kpc1-1* and *kpc1-2* mutants, RT-PCR or an RNA blot needs to be performed to examine *KPC1* transcripts in these mutants. It will be important to examine the biochemical interaction between KRP1 and

KPC1 in the future. Taken together, my data indicate that the RING finger

ubiquitin ligase KPC1 regulates KRP1 degradation.

Chapter 4: Isolation of Mutants that Stabilize KRP1

INTRODUCTION

As described in Chapters two and three, KRP1 is an unstable protein
in planta that is degraded by the 26S proteasome. The AXR1-dependent RUB
conjugation pathway regulates KRP1 degradation. KRP1 protein turnover is
further regulated by an SCF complex, which is composed of CUL1 and the F-
box protein SKP2b. The ASK subunit of SCF^{SKP2b} is unknown. In addition to
SCF^{SKP2b}-dependent degradation, KRP1 degradation is also regulated by the
RING finger ubiquitin ligase KPC1. In mammals, the SCF^{SKP2} complex targets
the CKI p21^{Cip1} for degradation by the 26S proteasome (Bornstein et al.,
2003). In addition to ubiquitin-mediated degradation, p21^{Cip1} is also degraded
by the 26S proteasome by an unknown mechanism that does not require
p21^{Cip1} ubiquitination (Sheaff et al., 2000; Chen et al., 2004). Whether a
ubiquitination-independent mechanism exists to regulate KRP1 degradation is
unknown. Although some proteins that are involved in KRP1 degradation
have been identified, many players remain to be identified. To identify novel
proteins that regulate KRP1 protein turnover, I have pursued a forward
genetics approach to screen for mutations that stabilize KRP1. This screen
might identify the ASK subunit of SCF^{SKP2b}, the components of the 26S
proteasome, as well as other proteins that are involved in KRP1 degradation.

RESULTS

Isolation of mutants that stabilize KRP1-GUS fusion protein

In the WT background, young seedlings that express KRP1-GUS fusion protein under the control of the KRP1 promoter do not exhibit any GUS staining in the cotyledon, hypocotyl, and root (Fig 4-1D). In contrast, the 26S proteasome inhibitor MG132-treated *KRP1*::*KRP1-GUS* seedlings accumulate KRP1-GUS in the cotyledon and root (Fig 2-7E and 2-7F). The *axr1-3* and *axr6-3* mutants deficient in SCF functions accumulate KRP1-GUS in the cotyledon and hypocotyl (Fig 2-8B and 2-9B). Based on these findings, I decided to use a single cotyledon for GUS staining to identify mutants that stabilize KRP1-GUS. I mutagenized homozygous *KRP1*::*KRP1-GUS* plants with EMS (ethyl methane sulfonate) and screened for mutants that were deficient in the degradation of KRP1-GUS.

The procedure that was used for the screening is shown in Fig 4-1. For the first-round screening, I removed one cotyledon from each of 20 6-day-old EMS-mutagenized *KRP1*::*KRP1-GUS* M2 seedlings and pooled these cotyledons in a tube for GUS staining. As described above, in the WT background, no GUS staining is observed in the cotyledon. The desired mutant that stabilizes KRP1-GUS will exhibit GUS staining in the cotyledon, as indicated by an arrowhead in Fig 4-1B. Seedlings corresponding to the positive pool were transferred to soil and were allowed to set seeds. In the
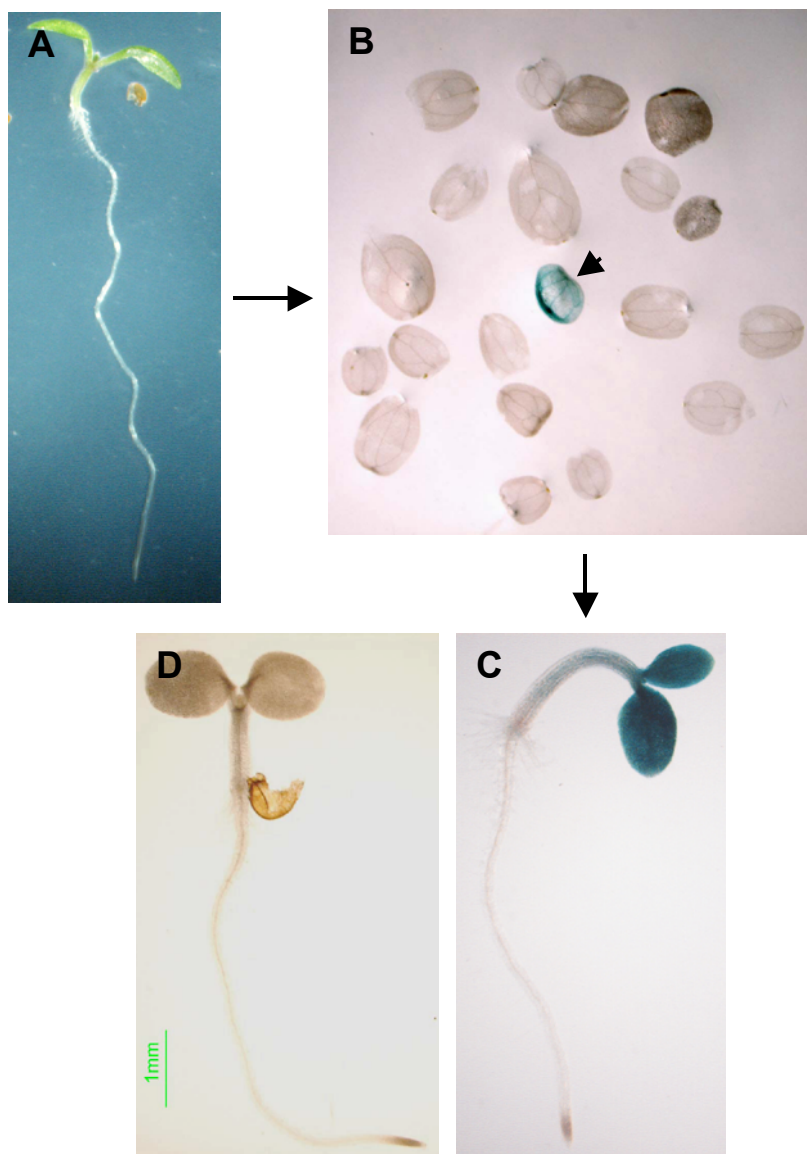
Figure 4-1. Screen for mutants that stabilize KRP1-GUS fusion protein.

(A) Six-day-old light-grown EMS-mutagenized M2 *KRP1::KRP1-GUS* seedling. **(B)** GUS staining of cotyledons from 6-day-old light-grown EMS-mutagenized M2 *KRP1::KRP1-GUS* seedlings. Arrowhead indicates a blue cotyledon. **(C)** GUS staining of a 4-day-old light-grown mutant that stabilizes KRP1-GUS in the cotyledon and hypocotyl. **(D)** GUS staining of a 4-day-old light-grown *KRP1::KRP1-GUS* seedling.

next generation, I performed the second-round screening using 4-day-old

seedlings to identify mutants that exhibits GUS staining in the cotyledon. I

screened ~20,000 M2 plants (from ~5,000 parents) and identified three

mutants that stabilize KRP1-GUS. These mutants are named as *msk* (mutant

stabilizes KRP1). The *msk* mutants did not exhibit an obvious phenotype

(data not shown). As shown in Fig 4-2, the *msk1-1* and *msk3-1* mutants

accumulated KRP1-GUS in the cotyledon, while the *msk2-1* mutant

accumulated KRP1-GUS in the cotyledon and hypocotyl. None of these

mutants exhibited GUS staining in the root (data not shown).

Genetic analysis of the *msk* mutants

To rule out the possibility that the *msk* mutants might contain mutations

in the *KRP1* transgene, which caused the increased stability of KRP1-GUS, I

sequenced the *KRP1* transgene of all three *msk* mutants. No mutations were

found (data not shown). These results suggest that increased KRP1-GUS

protein stability is caused by mutations outside the *KRP1* transgene. To

understand the genetic basis of the *msk* phenotype, each *msk* mutant was

backcrossed twice to *KRP1*::*KRP1-GUS* plants and the F1 and F2 progenies

from the second backcross were examined for GUS expression. In the F1

generation, none of the F1 plants exhibited GUS staining in the cotyledon,

indicating that the *msk* mutants are caused by recessive mutations (Table 4-

1). In the F2 generation, ~25% of the F2 seedlings exhibited GUS staining in

the cotyledon, indicating that the *msk* mutants are caused by a single
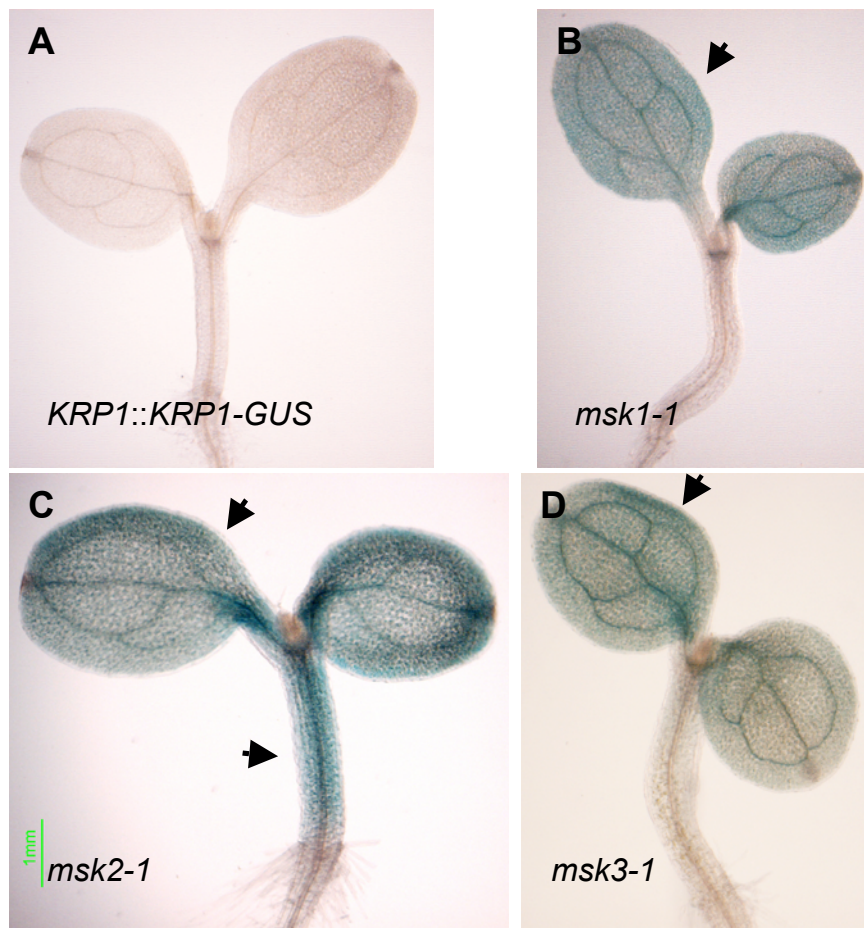
Figure 4-2. The *msk* mutants stabilize KRP1-GUS fusion protein.

(A) GUS staining of a 5-day-old light-grown *KRP1*::*KRP1-GUS* seedling. **(B)** GUS staining of a 5-day-old light-grown *msk1-1* seedling that carries a *KRP1*::*KRP1-GUS* transgene. **(C)** GUS staining of a 5-day-old light-grown *msk2-1* seedling that carries a *KRP1*::*KRP1-GUS* transgene. **(D)** GUS staining of a 5-day-old light-grown *msk3-1* seedling that carries a *KRP1*::*KRP1-GUS* transgene. Arrows indicate GUS staining.

recessive mutation (Table 4-1). To determine the number of genetic loci that the *msk* mutations define, complementation tests were performed. The results shown in Table 4-2 indicate that the three *msk* mutants define three distinct genetic loci.

As described in chapter two, the *axr1-3* and *axr6-3* mutations stabilize KRP1. Both mutants are auxin-resistant mutants that are involved in auxin signaling (Lincoln et al., 1990; Quint et al., 2005). To determine whether the *msk* mutants define genes that are involved in auxin signaling, I tested the *msk* mutants for auxin-resistance using 85 nM 2, 4-D (a synthetic auxin) by a root elongation assay. Like WT, all three *msk* mutants were sensitive to auxin (data not shown). These results indicate that the *msk* mutations do not affect auxin signaling and probably define genes other than *AXR1* and *CUL1*. The absence of an obvious phenotype of the *msk* mutants provides further evidence to support that the *msk* mutants probably do not contain mutations in the *AXR1* and *CUL1* genes.

DISCUSSION

To further understand the mechanisms of KRP1 degradation and to identify novel proteins that regulate KRP1 degradation, I have pursued a forward genetics approach and identified three *msk* mutants that stabilize KRP1. These three mutants define three distinct genetic loci. Among the

Table 4-1. Genetic analysis of the *msk* mutants.

A single cotyledon was removed from individual 5-day-old F1 seedlings for GUS staining. Five-day-old F2 seedlings were used for GUS staining. Chi-square values are given.

Mutants	GUS staining				X^2
	F1	F2			
		Total seedlings	Seedlings with blue cotyledons	Seedlings with white cotyledons	
msk1-1 × *KRP1*::*KRP1-GUS*	White cotyledons	77	17	60	0.35
msk2-1 × *KRP1*::*KRP1-GUS*	White cotyledons	120	26	94	0.71
msk3-1 × *KRP1*::*KRP1-GUS*	White cotyledons	139	29	110	1.27

Table 4-2. Complementation analysis of the *msk* mutants.

A single cotyledon was removed from individual 5-day-old F1 seedlings for GUS staining. Only one blue cotyledon was observed in the F1 plants of *msk1-1* × *msk3-1* and *msk2-1* × *msk3-1*.

Cross	GUS staining		
	Total seedlings	Blue cotyledons	White cotyledons
msk1-1 × *msk2-1*	20	0	20
msk1-1 × *msk3-1*	20	1	19
msk2-1 × *msk3-1*	20	1	19

three *msk* mutants, the *msk2-1* mutant might be the most interesting one.

Like the *axr1-3* mutant, the *msk2-1* mutant strongly accumulates KRP1-GUS

in the cotyledon and hypocotyl, but not in the root. Different from the *axr1-3*

mutant, the *msk2-1* mutant is not resistant to auxin and does not exhibit an

obvious phenotype. Therefore, the *msk2-1* mutant possibly defines a novel

gene that plays an important role in KRP1 degradation. It will be of

importance to clone the affected gene of the *msk2-1* mutant by a map-based

cloning approach in the future in order to understand the role of MSK2 in

KRP1 degradation.

Although I have identified three *msk* mutants, only a single allele is

isolated for each mutant, indicating that the screen is not yet saturated.

Additional screening needs to be performed to identify more mutations that

stabilize KRP1. One disadvantage of the screen described previously is that

mutations causing an embryo-lethality or a seedling-lethality will not be

identified. As described in Chapter two, the 26S proteasome inhibitor MG132

strongly stabilizes KRP1-GUS in the root. Further screening for mutants that

are seedling-lethal and/or accumulate KRP1-GUS in roots will be of great

importance to further understand the KRP1 degradation mechanisms.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana plants were grown under 24 hour light conditions at 22 ^{0}C or 18 ^{0}C when necessary. All mutants and transgenic lines were in the Columbia ecotype. The *krp1-1* (SALK_100189), *krp1-2* (SALK_057417), and *krp1-3* (SALK_078320) mutants, T-DNA insertions in At2g23430, were acquired from the *Arabidopsis* Biological Research Center (ABRC). The *skp2a-1* mutant (GABI-Kat 293D12), a T-DNA insertion in At1g21410, was acquired from GABI-KAT at the Max-Planck Institute for Plant Breeding Research (Cologne, Germany). The *skp2b-1* mutant (SALK_028396), a T-DNA insertion in At1g77000, was acquired from ABRC. The *kpc1-1* (SAIL_3_E3) and *kpc1-2* (WiscDsLox466C1) mutants, T-DNA insertions in At2g22010, were acquired from ABRC. All these T-DNA insertion mutants were confirmed by PCR and sequencing. T-DNA left border primers SALK-LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'), GABI-Kat-LB (5'-CCCATTTGGACGTGAATGTAGACAC-3'), Wisc-p745 (5'-AACGTCCGCAATGTGTTATTAAGTTGTC-3'), and SAIL-LB1 (5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3') were used for genotyping.

Seeds were surface sterilized in a 30% bleach and 0.04% triton X-100 solution for 15 minutes and were washed three times in sterile water. Seeds were cold treated for 2-3 days at 4 ^{0}C to synchronize germination and were

grown on ATS plates supplemented with 1% sucrose and 0.8% agar or in ATS liquid medium supplemented with 1% sucrose (Lincoln et al., 1990). A 1× ATS nutrient solution is composed of 5 mM KNO_3, 2.5 mM KPO_4, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 µM Fe-EDTA, and micronutrients. The components of a 1000× micronutrient solution are 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM Na_2MoO_4, 10 mM NaCl, and 0.01 mM $CoCl_2$. Soil was watered with Adept (1 OZ per 200 liters of water, Crompton Uniroyal Chemical) to control fungus gnat larvae. Plants were fertilized with All Purpose Plant Food (1 teaspoon per gallon of water, Miracle-Gro). N-1-naphthylphthalamic acid (10 µM NPA), 1-Naphthaleneacetic acid (10 µM NAA), herbicide basta (0.01%, AgroEvo), and antibiotics were added to autoclaved ATS medium when necessary.

Transgenic Lines

A 2062 bp *KRP1* (At2g23430) promoter was amplified from genomic DNA with primers KRP1-PF (5'-GTTCAAGCGAGTGACACATCTC-3') and KRP1-PR (5'-CTTCGATTTAGGTTACGTGTGCG-3'). A 602 bp *KRP1* full-length cDNA was amplified from a yeast two-hybrid cDNA library (Gray et al. 1999) with primers KRP1-F (5'-ACGCACACGTCACCTAAATC-3') and KRP1-R (5'-CTTCACTCTAACTTTACCCATTCG-3'). The amplified DNA fragments were cloned to pCR2.1 using the TA cloning kit (Invitrogen) and were sequenced. The *KRP1*::*GUS* (transcriptional fusion) plasmid was constructed by cloning the *KRP1* promoter to the Spe I and Sma I sites of pCB308

containing the *E. coli β-glucuronidase* gene (*GUS*) (Xiang et al., 1999). The

KRP1::*KRP1-GUS* (translational fusion) plasmid was constructed by cloning

both the *KRP1* promoter and the *KRP1* full-length cDNA to the Spe I and Sma

I sites of pCB308. The stop codon TGA of *KRP1* was mutated to TGCA using

the Quikchange site-directed mutagenesis kit (Stratagene) with primers

KRP1-TGA-F (5'-GGGTAAAGTTAGAGTGCAAGAAGCCGAATTCG-3') and

KRP1-TGA-R (5'-CGAATTCGGCTTCTTGCACTCTAACTTTACCC-3').

To make a *35S*::*Myc-KRP1* construct, a *KRP1* full-length cDNA was

cloned to the Sma I site of pGEM7Z and was fused to the C-terminus of 6 × c-

myc epitopes. The start codon ATG of *KRP1* was mutated to ATA using the

Quikchange site-directed mutagenesis kit with primers KRP1-ATG-F (5'-

CGCACACGTCACCTAAATCGAAGATAGTGAGAAAATATAG-3') and KRP1-

ATG-R (5'-CTATATTTTCTCACTATCTTCGATTTAGGTGACGTGTGCG-3').

The *Myc-KRP1* insert was then cloned to the Sma I and Sac I sites of pROK2.

A 3861 bp *KPC1* full-length cDNA was amplified from a yeast two-

hybrid cDNA library (Gray et al. 1999) with primers KPC1-F (5'-

ATATGGCTGAAGACAGCCTACGGG -3') and KPC1-R (5'-

GCAACTAACCCGAGCTTCATGTGC -3'). The amplified DNA fragment was

cloned to pCR-Blunt II-Topo using the Zero Blunt TOPO PCR cloning kit

(Invitrogen) and was sequenced. The full-length *KPC1* cDNA without the start

codon ATG was amplified with primers pENTR-KPC1-F (5'-

CACCTTGGCTGAAGACAGCCTACGG-3') and KPC1-R (5'-

GCAACTAACCCGAGCTTCATGTGC -3') and was cloned to pENTR/D-

TOPO using the pENTR directional TOPO cloning kit (Invitrogen). The *KPC1* insert in pENTR/D-TOPO was sequenced and was then cloned to pGWB15 containing the *CaMV 35S* promoter and 3 × HA epitopes to make a *35S::HA-KPC1* construct.

All above constructs in the binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101. Plants were transformed by the vacuum infiltration method (Bechtold and Pelletier, 1998). Transgenic plants were selected on ATS plates supplemented with necessary antibiotics or herbicide. The antibiotics or herbicide-resistant T1 plants were transferred to soil to allow plants to set seeds.

Plant DNA Isolation

Plant DNAs were prepared using the CTAB extraction method (Lukowitz et al. 2000). A single rosette leaf was ground in 300 µl 2× CTAB buffer (2% cetyl-trimethyl-ammonium bromide [CTAB], 1.4 M NaCl, 100 mM Tris HCl [pH 8.0], and 20 mM EDTA) in a 1.5 ml eppendorf tube using a plastic pestle. The sample was incubated at 65 ^{0}C for at least 10 minutes and was then cooled to room temperature. An equal volume of chloroform was added, and the mixture was vortexed thoroughly and was centrifuged for 5 minutes. The supernatant was transferred to a fresh 1.5 ml eppendorf tube. Three volume of ethanol was added, and the mixture was vortexed thoroughly and was placed at -20 ^{0}C for at least 30 minutes to allow DNA to precipitate. The sample was centrifuged for 20 minutes at 4 ^{0}C, and DNA pellet was

washed in 1 ml 70% ethanol. DNA pellet was dried at room temperature and was then resuspended in 100 µl TE buffer.

Lateral Root Counts

Sterilized seeds were cold treated for 3 days at 4 ^{0}C to synchronize germination and were sown on ATS plates supplemented with 1% sucrose and 0.8% agar. Plates were placed vertically to allow roots to grow along the agar surface under 24 hour light conditions at 22 ^{0}C in a plant growth chamber. Four-day-old seedlings with a similar primary root length were transferred onto fresh ATS plates supplemented with 1% sucrose and 0.8% agar. Lateral roots were counted on the tenth day after transfer under a Nikon SMZ1500 dissecting microscope.

GUS Assays

To examine GUS expression, seedlings were incubated in a GUS staining solution (100 mM $NaPO_4$ [pH 7.0], 10 mM EDTA, 0.5 mM $K_4Fe[CN]_6$, 0.5 mM $K_3Fe[CN]_6$, 0.1% Triton X-100, and 1mM 5-bromo-4-chloro-3-indodyl-β-D-glucuronic acid [X-Gluc]) at 37 ^{0}C (Oono et al., 1998). GUS-stained seedlings were incubated in 70% ethanol to remove chlorophyll. GUS staining patterns were examined under a Nikon SMZ1500 dissecting microscope.

For histochemical localization of GUS, GUS-stained seedlings were fixed in a 4% formaldehyde and 0.02% triton X-100 fixative (in 1 × PBS, pH 7.0) at 4 ^{0}C overnight. Seedlings were dehydrated in a series of 50%, 70%,

85%, 95%, and 100% ethanol. Dehydrated seedlings were embedded in the

Technovit 7100 resin (Kulzer Histo-Technique) using an embedding mold

(Sigma). Root transverse sections of 5 µm were cut using a glass knife with a

microtome. Sections were counterstained for cell wall in 0.05% ruthenium red

for 30 second and were dried at room temperature. Finally, sections were

mounted in 50% glycerol with cover slips and nail polish for analysis under a

Nikon E800/metamorph microscope.

RT-PCR Analysis

Total RNAs were extracted using the TRI reagent (Sigma). The first-

strand cDNAs were synthesized from 5 µg total RNAs using Oligo(dT)$_{20}$

primer and SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). PCRs

were performed with the following gene specific primers in 25 µl reactions:

KRP1-F, 5'-ACGCACACGTAACCTAAATC-3'; KRP1-R1, 5'-

CTTCACTCTAACTTTACCCATTCG-3'; KRP1-R2, 5'-

CTCCCGCTACAACAACAATC-3'; SKP2a-F, 5'-

CCGCTTCATTTTAGTCATTAAAC-3'; SKP2a-R1, 5'-

GGCCGTTTATATATACAACATAAC-3'; SKP2a-R2, 5'-

TGATTGCAGTTATTCCCAATAG-3'; SKP2b-F, 5'-

CATATTTACTTTTGATCTCGTGG-3'; SKP2b-R1, 5'-

CATACTAGAGAGTAGTAGACC-3'; SKP2a-R2, 5'-

CGAGTTTAGTCAGGTTAGTA-3'; Myc-F, 5'-

GACTCTAGAGGATCCCCAAAGC-3'; Myc-R, 5'-

AGCCGAATTCGATGGGGTACCG-3'; TAP-F, 5´-

TAGCCGTCTCAGCAGCCAACC-3´; TAP-R, 5´-

CTTCCCCGCGGAATTCGCGTC-3´; ACTIN2-F, 5'

GGCTGAGGCTGATGATATTC-3'; ACTIN2-R, 5'-

TCTGTGAACGATTCCTGGAC-3'.

Immunoblot Analysis and Immunoprecipitation

Protein extracts were prepared from seedlings grown in sterile ATS

liquid medium plus 1% sucrose or on ATS plates plus 1% sucrose. Seedlings

were homogenized in ice-cold protein extraction buffer C (50 mM Tris·HCl,

150 mM NaCl, 0.5% NP-40, 1 mM PMSF, and protease inhibitor cocktail

[Roche] at pH 7.5) (Gray et al. 1999). After 20 minutes on ice, extracts were

spun at a maximal speed for 15 minutes in a microcentrifuge at 4 ^{0}C. The

supernatants were used for further analysis.

For immunoblot analysis, 50 µg protein extracts were mixed with SDS-

PAGE sample buffer and were boiled for 5 minutes. Denatured proteins were

separated on a 10% acrylamide SDS gel and were transferred to a

nitrocellulose membrane. The membrane was immersed in Tris-buffered

saline (pH 7.6) containing 5% nonfat dry milk and 0.1% Tween 20 to block

non-specific binding sites. The α-c-myc 9E 10 antibody (Covance Research

Products) was used at a 1:1000 dilution. The horseradish peroxidase-

conjugated goat α-mouse secondary antibody (Sigma) was used at a 1:3000

dilution. Proteins were detected with the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

For immunoprecipitation, 5 µl α-c-myc 9E 10 antibody was added to 3 mg protein extracts and was incubated for 1-3 hours at 4 ^{0}C. To collect immune complexes, 30 µl protein A agarose beads (Roche) were added and were incubated for 3 hours to overnight. Immune complexes were washed three times in 1 ml protein extraction buffer C. Finally, agarose beads were resuspended in SDS-PAGE sample buffer. Immunoblot analysis was carried out as described above. The α-CDKA;1 antibody was used at a 1:5000 dilution. The α-CDKB1;1 and α-CYCD2;1 antibodies (Healy et al., 2001) were used at a 1:3000 dilution. The horseradish peroxidase-conjugated goat α-rabbit secondary antibody (Chemicon International) was used at a 1:2500 dilution.

EMS Mutagenesis

Dry homozygous *KRP1*::*KRP1-GUS* seeds were placed in 40 ml 0.3% EMS (Ethyl methane sulfonate, Sigma) in a 50 ml tube. About 23550 seeds were mutagenized in two 50 ml tubes. Seeds were mixed on a shaker at room temperature for 16 hours. Seeds were washed in water for 8 times (15 minutes for each washing) and were then transferred to a fresh 50 ml tube. Seeds were washed in water for additional 7 times (15 minutes for each washing). After washing, the mutagenized seeds (M1 generation) were resuspended in 0.1% agar and were sown at about 3 seeds per square

centimeter. Seeds were cold treated for 3 days at 4 ^{0}C to synchronize germination. Plants were grown until they die naturally and were dried completely. M2 seeds were collected and were used in mutant screens.

Molecular Biology Techniques

Standard techniques, including LB medium preparation, bacterial culture, gene cloning, bacterial transformation, PCR, DNA agarose gel electrophoresis, etc, were done following the procedures of Sambrook (2001). Plasmids were prepared using the QIAprep Spin Miniprep kit (Qiagen). DNAs in agarose gels were purified using the QIAquick gel extraction kit (Qiagen).

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Curriculum Vitae

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Publications

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