

The cyclin-dependent kinase inhibitor KIP-RELATED PROTEIN 6 promotes stem cell death upon DNA damage in *Arabidopsis* roots

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SUMMARY

Maintenance of genome integrity is essential for plant growth and survival. Plants have evolved specialised DNA damage responses that coordinate DNA repair, cell cycle arrest and stem cell-specific cell death. However, the mechanism through which damaged stem cells are selectively eliminated remains unclear. Here, we show that the cyclin-dependent kinase inhibitor *KIP-RELATED PROTEIN 6* (*KRP6*) is induced in *Arabidopsis thaliana* in response to DNA damage. Reporter analyses reveal that *KRP6* induction occurs in the root stem cell niche under genotoxic stress. We further demonstrate that the NAC-type transcription factor, SUPPRESSOR OF GAMMA RESPONSE 1 (*SOG1*), a central regulator of the plant DNA damage response, directly activates *KRP6* expression upon DNA damage. Loss-of-function *krp6* mutant exhibits reduced stem cell death after DNA damage, whereas *KRP6* overexpression enhances cell death. The expression of a dominant-negative *CDKA;1* allele in vascular stem cells promotes DNA damage-induced stem cell death. Together, these findings identify *KRP6* as a key regulator linking genotoxic stress to stem cell death, highlighting the important role of CDK inhibition in safeguarding genome integrity in plants.

Keywords: *Arabidopsis thaliana*, CDK, DNA damage response, KIP-RELATED PROTEIN 6, SUPPRESSOR OF GAMMA RESPONSE 1, stem cell death.

INTRODUCTION

Plants are continuously exposed to a wide range of genotoxic stresses, including ultraviolet radiations, reactive oxygen species (ROS) and chemical agents, all of which can cause DNA lesions (Berdy, 1980; Cooke et al., 2003; Frohn-meyer & Staiger, 2003). Among these lesions, DNA double-strand breaks (DSBs) are particularly deleterious because they directly threaten chromosomal stability. Failure to maintain genome integrity compromises meristematic cell proliferation and stem cell maintenance, leading to defects in organ formation and overall plant growth; therefore, preserving genome stability is essential for plant development. To cope with such DNA damage, plants have evolved sophisticated DNA damage responses, such as the activation of DNA repair pathways, modulation of cell cycle progression and elimination of damaged stem cells. The DNA damage response is initiated by sensor kinases: ATAXIA TELANGIECTASIA MUTATED (*ATM*) and *ATM*

AND RAD3-RELATED (*ATR*), which detect DSBs and single-stranded DNA, respectively (Culligan et al., 2004; Garcia et al., 2003). These kinases subsequently phosphorylate and activate the plant-specific NAC-type transcription factor, SUPPRESSOR OF GAMMA RESPONSE 1 (*SOG1*), which serves as a central regulator of the DNA damage response in plants, analogous to mammalian p53 protein (Sjogren et al., 2015; Yoshiyama et al., 2013). Once activated, *SOG1* binds to the consensus sequence, CTT [N]₇AAG, on target genes to induce their expression (Bourbousse et al., 2018; Ogita et al., 2018). Through this regulatory framework, *SOG1* orchestrates a broad range of downstream responses, including DNA repair, cell cycle arrest and stem cell death (Bourbousse et al., 2018; Ogita et al., 2018). Notably, stem cell-specific cell death constitutes a distinctive plant strategy to safeguard genome integrity. Severely damaged stem cells are selectively eliminated, whereas neighbouring cells compensate for their

loss through regeneration, thereby maintaining meristem function (Fulcher & Sablowski, 2009; Takahashi et al., 2024, 2025).

Cell cycle progression is tightly coupled with DNA damage responses because checkpoint activation allows cells to repair their DNA prior to division. Cyclin-dependent kinase (CDK)/cyclin complexes are the primary drivers of the cell cycle and their activity is tightly regulated through phosphorylation and inhibition by CDK inhibitors (CKIs) (Wang et al., 1997). In Arabidopsis, two CKI families have been characterised: SIAMESE/SIAMESE-RELATED (SIM/SMRs) and Kip-related proteins/interactors of CDK (KRPs/ICKs) (Churchman et al., 2006; De Veylder et al., 2001). Several *SMR* genes, including *SMR4*, *SMR5* and *SMR7*, are rapidly induced by DNA damage and contribute to cell cycle checkpoints (Yi et al., 2014). Importantly, SOG1 directly regulates the transcription of these *SMRs*, thereby linking DNA damage signalling to CDK inhibition and cell cycle arrest (Yi et al., 2014). In contrast, KRPs have been primarily implicated in developmental processes such as leaf morphogenesis, endoreplication and hormonal responses (Sanz et al., 2011; Verkest et al., 2005; Wang et al., 1998, 2000; Weinl et al., 2005). However, whether KRPs also participate in the DNA damage response, and if so, whether they function analogously to SMRs in coupling CDK inhibition with cell cycle arrest, remains unexplored.

SOG1 enforces G2 arrest via additional downstream regulators. In particular, SOG1 directly induces the NAC-type transcription factors, *ANAC044* and *ANAC085*, which stabilise the repressor-type R1R2R3-MYB transcription factors, MYB3R3 and MYB3R5. These repressor-type MYBs (Rep-MYBs) repress a set of G2/M-specific genes, thereby promoting G2 arrest (Chen et al., 2017; Takahashi et al., 2019). Accumulating evidence indicates that DNA damage leads to reduced CDK activity through multiple mechanisms. DNA damage suppresses the expression of mitotic cyclins, resulting in a decrease in the formation of active CDK-cyclin complexes. Simultaneously, SOG1 induces *SMR* genes, leading to a further decrease in CDK activity under genotoxic stress (Yi et al., 2014). Consistent with this, reduced CDK activity leads to decreased phosphorylation and stabilisation of Rep-MYBs, thereby reinforcing G2 arrest (Chen et al., 2017). Intriguingly, mutants of *ANAC044*, *ANAC085*, or Rep-MYBs not only fail to arrest at G2 but also exhibit reduced stem cell death under DNA damage (Chen et al., 2017; Takahashi et al., 2019). These observations support the hypothesis that cell cycle arrest is associated with stem cell death in response to DNA damage. However, the molecular mechanisms through which cell cycle regulation contributes to the induction of stem cell death under genotoxic stress remain largely unknown.

In addition, SOG1 integrates multiple hormonal signalling pathways into the DNA damage response. In

Arabidopsis, DNA damage stimulates cytokinin biosynthesis, which in turn activates type-B ARABIDOPSIS RESPONSE REGULATOR (ARRs) transcription factors (Takahashi et al., 2021). ARR2 induces *CELL CYCLE SWITCH 52A1* (*CCS52A1*), which encodes an activator of the anaphase-promoting complex/cyclosome (APC/C), a conserved E3 ubiquitin ligase that controls cell cycle progression by targeting key cell cycle regulators for proteasomal degradation (Takahashi et al., 2013). Activation of APC/C promotes the degradation of mitotic cyclins, thereby facilitating their exit from the mitotic cell cycle and entry into endoreplication, a modified cell cycle characterised by repeated rounds of DNA replication without cell division. Concurrently, the type-B ARRs ARR1 and ARR12 induce SHY2/IAA3, an AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) family member that represses *PIN-FORMED* (*PIN*) genes encoding auxin efflux carriers. This repression reduces auxin transport downward towards the root tip, resulting in a local reduction in auxin level in the root apex. This decrease in auxin level contributes to both G2 arrest and stem cell death in the root meristem. Moreover, a recent study revealed that SOG1 directly induces additional Aux/IAA repressors, *IAA5* and *IAA29*, which specifically suppress auxin signalling in the stem cell niche in roots, thereby promoting cell death (Takahashi et al., 2025). Nevertheless, the link between decreased auxin signalling and stem cell death remains poorly understood.

In this study, we investigated the role of KRP6/ICK4, a member of the Arabidopsis KRP/ICK family, in DNA damage response. We showed that *KRP6* expression was induced by DNA damage in a SOG1-dependent manner and was preferentially activated in the root stem cell niche. Genetic analyses suggested that KRP6 promotes DNA damage-induced stem cell death. These findings suggest that KRP6 is involved in linking DNA damage signalling to stem cell death in roots.

RESULTS

***KRP6* is induced in a tissue-specific manner in response to DNA damage**

To assess the early transcriptional responses of cell cycle regulators to DNA damage, we reanalysed published transcriptomic data from 2-week-old Arabidopsis seedlings treated for 2 h with the DSB-inducing agent zeocin (Ogita et al., 2018). At this time point, the transcript levels of core CDKs, G1/S regulators (RBR and E2F/DP) and G2/M-related genes, including MYB3R transcription factors and APC/C regulators, remained largely unchanged (Figure S1a–d). Consistent with this, the repression of mitotic cyclins reported at later stages of the DNA damage response (Adachi et al., 2011) was not yet evident, except for the induction of *CYCB1;1* (Figure S1e) (Weimer et al., 2016). In contrast, several CDK inhibitor genes associated with DNA

damage response, including *SMR4*, *SMR5* and *SMR7*, were induced (Figure S1f) (Weimer et al., 2016; Yi et al., 2014). Notably, among the seven KRP/ICK family members, *KRP6*/*ICK4* showed a pronounced increase in transcript levels after zeocin treatment (Figure S1g). This induction was also observed in roots treated with bleomycin, another DSB-inducing agent (Figure S2). Together, these analyses indicate that *KRP6* is transcriptionally upregulated at an early stage of the DNA damage response.

To investigate the tissue specificity of *KRP6* induction after DNA damage, we generated transgenic plants harbouring a *pKRP6:GFP* reporter construct, in which *GFP* expression was driven by a 2-kb upstream promoter region of *KRP6*. To obtain an overview of the spatial pattern of *KRP6* induction across tissues, 5-day-old seedlings were transferred to medium supplemented with 10 μ M zeocin, and GFP fluorescence was analysed after 24 h. Under control conditions, GFP signals were undetectable in aerial tissues and were only weakly detectable in the root vasculature (Figure 1A–C). In contrast, zeocin treatment resulted in a pronounced induction of GFP fluorescence in the root tips, accompanied by enhanced signal intensity in the root vascular tissue (Figure 1A–C). To further examine the spatial and temporal dynamics of *KRP6* induction, we examined GFP expression in the root tips at earlier time points after zeocin treatment. At 0 h, the weak GFP fluorescence was largely restricted to the outermost layer of the root cap. By 10 h, GFP signals became detectable in the stem cell niche as well as in vascular initials and their daughter cells. With prolonged zeocin treatment, both the intensity of the GFP signal and the number of GFP-positive cells increased (Figure 1D). A comparable time-dependent increase in GFP signal was also observed in the root cap (Figure 1D).

We next examined the spatial distribution of KRP6 protein accumulation in root tips. We transformed the *krrp6-1* mutant with a *pKRP6:KRP6-GFP* reporter construct. In this construct, *GFP* was fused in-frame to the C-terminus of the *KRP6* coding sequence, and its expression was driven by the 2-kb promoter. As described below, this construct functionally complemented the *krrp6-1* mutant phenotype by suppressing DNA damage-induced stem cell death. In the untreated roots, KRP6-GFP fluorescence was detected in the outermost layer of the root cap. Upon zeocin treatment, GFP signals were markedly enriched in the stem cell niche as well as in vascular initials and their daughter cells (Figure 1E), indicating that the KRP6 protein accumulates in this region in response to DNA damage. To assess the temporal relationship between KRP6 accumulation and stem cell death, we monitored KRP6-GFP fluorescence at 10 h after zeocin treatment, when cell death was not yet detectable, and at 12 h, when stem cell death first became apparent. Notably, KRP6-GFP signals were evident in the stem cell niche prior to the onset of cell death

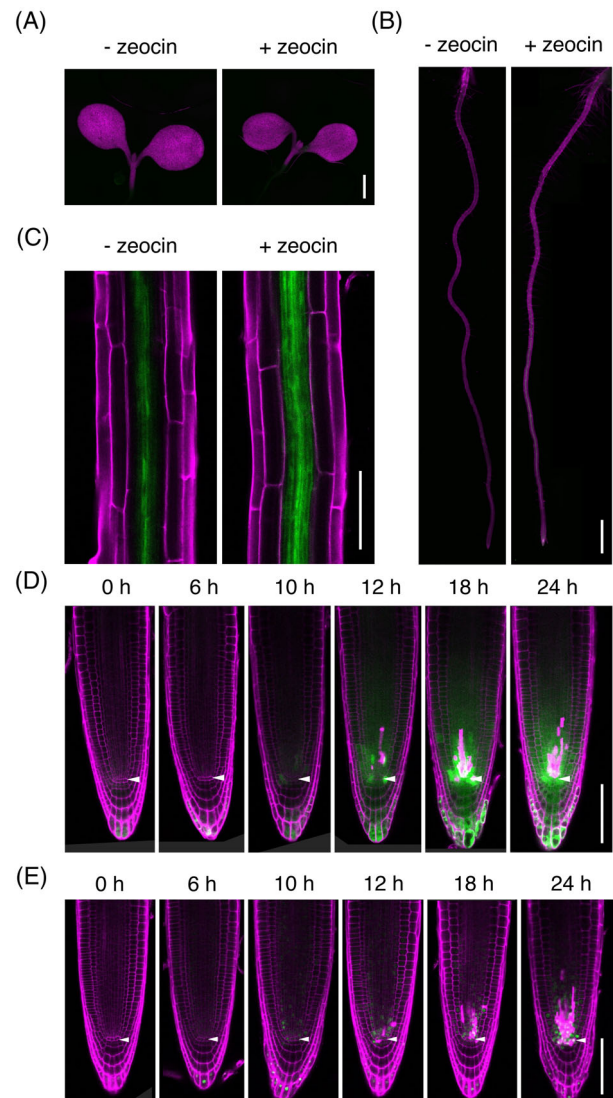


Figure 1. DNA damage induces *KRP6* expression in roots.

(A–C) Five-day-old *pKRP6:GFP* seedlings were transferred onto MS plates supplemented with (+ zeocin) or without (– zeocin) 10 μ M zeocin and grown for 24 h. GFP fluorescence in cotyledons (A), whole roots (B) and root vasculatures (C) was observed after counterstaining with PI. Bars = 1 mm (A, B) and 100 μ m (C).

(D, E) Five-day-old *pKRP6:GFP* (D) and *pKRP6:KRP6-GFP* (E) seedlings were treated with 10 μ M zeocin for the indicated times. GFP fluorescence in root tips was detected after PI staining. Arrowheads indicate the QC cells. Bars = 100 μ m.

(Figure 1E). These results indicate that KRP6 accumulation is not a consequence of cell death but rather occurs before cell death is initiated.

SOG1 directly induces *KRP6* expression in response to DNA damage

The NAC-type transcription factor SOG1 is a central regulator of the plant DNA damage response (Yoshiyama et al., 2009). To examine whether SOG1 is required for

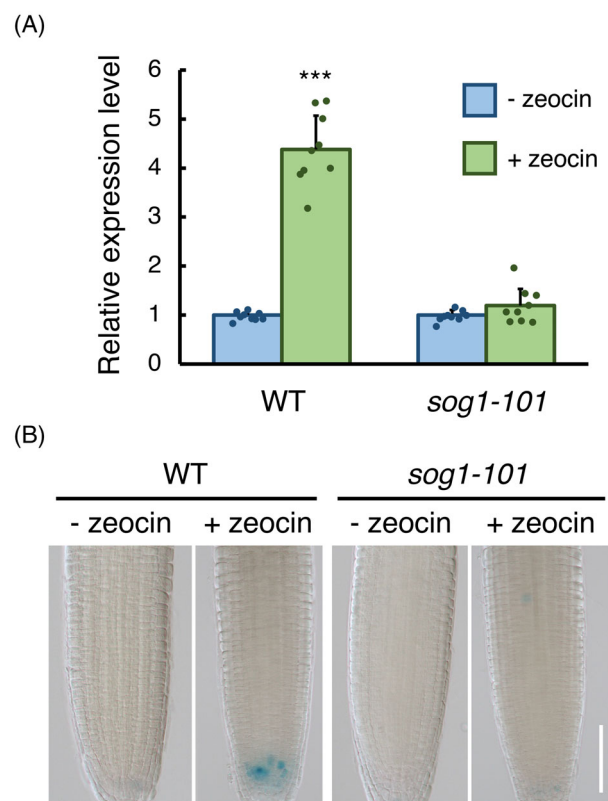


Figure 2. SOG1 induces *KRP6* expression upon DNA damage.

(A) Transcript levels of *KRP6* in the presence of zeocin. Five-day-old WT and *sog1-101* seedlings were transferred onto MS plates supplemented with (+ zeocin) or without (– zeocin) 10 μ M zeocin and grown for 24 h. Total RNA was extracted from roots and subjected to qRT-PCR. Transcript level of *KRP6* was normalised to that of *ACTIN2*, and are indicated as relative values, with that of the control set to 1. Data are presented as mean $\pm$ standard deviation (SD) calculated from three biological replicates. Significant differences from the control were determined by Student's *t*-test: ****P* < 0.001.

(B) Five-day-old WT and *sog1-101* seedlings harbouring *pKRP6:GUS* were transferred to MS plates supplemented with (+ zeocin) or without (– zeocin) 10 μ M zeocin, and grown for 24 h, followed by GUS staining. Bar = 100 μ m.

DNA damage-induced *KRP6* expression, 5-day-old WT and *sog1-101* seedlings, a T-DNA insertion null mutant of *SOG1*, were transferred to medium containing 10 μ M zeocin and total RNA was isolated from roots after 24-h treatment. Quantitative RT-PCR (qRT-PCR) analysis revealed that *KRP6* transcript levels increased approximately four-fold in WT seedlings after zeocin treatment compared to the untreated controls. However, this induction was not observed in the *sog1-101* mutant (Figure 2A). Consistent with the qRT-PCR results, analysis of *pKRP6:GUS* transcriptional reporter, in which *GUS* expression was driven by the 2-kb *KRP6* promoter, revealed strong GUS activity in WT root tips upon zeocin treatment. In contrast, no detectable GUS staining was observed in *sog1-101* roots, even under DNA damage conditions (Figure 2B). These findings

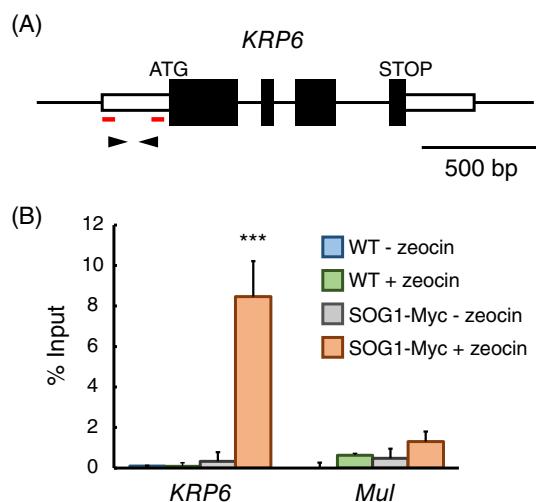


Figure 3. SOG1 directly binds the *KRP6* locus under zeocin treatment.

(A) Schematic representation of the *KRP6* genomic region. Black and white boxes indicate the coding region and untranslated regions (UTRs), respectively. Red lines and triangles represent the locations of the SOG1-binding motifs and regions amplified by chromatin immunoprecipitation quantitative PCR (ChIP-qPCR), respectively.

(B) ChIP-qPCR. Two-week-old WT and *pSOG1:SOG1-Myc* (SOG1-Myc) seedlings grown in liquid MS medium were further cultured in a medium with (+ zeocin) or without (– zeocin) 20 μ M zeocin for 2 h. Genomic DNA was extracted from whole seedlings, and chromatin bound to SOG1-Myc was immunoprecipitated. qPCR was conducted to amplify the *KRP6* genomic regions shown in (A). *Mutator-like transposon* (*Mul*) was used as a negative control. The enrichments of DNA fragments by ChIP-qPCR is normalised against the corresponding input DNA. Data are represented as mean $\pm$ SD (*n* = 3) calculated from three biological replicates. Significant differences from the control were determined by Student's *t*-test: ****P* < 0.001.

indicate that SOG1 is required for DNA damage-dependent transcriptional activation of *KRP6*.

A previous chromatin immunoprecipitation (ChIP) sequence analysis indicated significant SOG1-binding peaks within the *KRP6* genomic region (Bourbousse et al., 2018). We therefore searched for the SOG1-binding consensus motif, CTT(N)₇AAG, within the *KRP6* locus and identified two putative motifs (CTTTGTTAAGAAG and CTTTGAAAGGAAG) located in the 5'-untranslated region (UTR) (Figure 3A). To determine whether SOG1 directly binds to this region *in vivo*, we performed ChIP assays using transgenic plants expressing a functional SOG1-Myc fusion protein under the control of its native promoter (*pSOG1:SOG1-Myc*) (Yoshiyama et al., 2013). The immunoprecipitated DNA was analysed by qPCR using primers targeting the 5'-UTR of *KRP6* (Figure 3A). Under normal growth conditions, no significant enrichment of SOG1 binding was detected at the *KRP6* 5'-UTR (Figure 3B). In contrast, following zeocin-induced DNA damage, SOG1-bound DNA fragments were significantly enriched in this region, which contained consensus-binding motifs (Figure 3B). SOG1 is constitutively expressed, particularly in proliferative tissues such as the root meristem, and its

function is primarily regulated at the level of protein activity rather than expression (Yoshiyama et al., 2013). Upon DNA damage, SOG1 is activated through ATM/ATR-dependent phosphorylation, which enables its association with target promoters and the subsequent transcriptional activation of downstream genes (Ogita et al., 2018; Yoshiyama et al., 2013, 2017). Taken together, these results demonstrate that SOG1 directly associates with the *KRP6* promoter in a DNA damage-dependent manner.

KRP6 is involved in DNA damage-induced stem cell death

To investigate the physiological role of KRP6 in DNA damage response, we analysed the phenotype of the *krp6-1* mutant (Cheng et al., 2013). qRT-PCR analysis detected transcripts corresponding to the N-terminal region of *KRP6* but not to the C-terminal region (Figure S3), indicating that *krp6-1* is unable to produce a full-length KRP6 protein. The KRP family proteins are small CDK inhibitors whose C-terminal domain is required for interaction with CDKs and for their inhibitory activity (Schnittger et al., 2003; Zhou et al., 2003). Therefore, although truncated transcripts were present, *krp6-1* was expected to lack functional KRP6 activity and could be regarded as a functional loss-of-function mutant. We first examined whether the loss of *KRP6* affected overall root growth under DNA damage conditions. Five-day-old WT and *krp6-1* seedlings grown on MS medium were transferred to medium supplemented with 0, 4, 6 or 10 μM zeocin, and grown for an additional 6 days. Under both control and zeocin-treated conditions, the root growth of the *krp6-1* mutant was indistinguishable from that of the WT (Figure S4a–d), suggesting that the loss of *KRP6* does not alter overall root growth or sensitivity to DNA damage. To assess meristem activity in more detail, we measured the root meristem size by counting the number of cortical cells between the QC and the first elongated cell. In the WT roots, zeocin treatment for 24 h reduced the meristem cell number by 27%, reflecting the suppression of cell proliferation in response to DNA damage. A comparable reduction was observed in *krp6-1* mutant (Figure S4e,f), indicating that KRP6 is dispensable for the regulation of meristem cell proliferation and elongation under DNA-damaging conditions.

Next, we examined DNA damage-induced stem cell death. Roots were treated with 10 μM zeocin for 24 h and stained with propidium iodide (PI) to visualise dead cells. Note that although PI staining provides a simple and reproducible estimate of cell death in the root tip, it does not clearly distinguish individual cell types or different stages of cell death within the affected region. In WT roots, cell death was predominantly detected in vascular initial cells and their daughters. Although a similar spatial pattern was observed in the *krp6-1* mutant, the extent of stem cell death was significantly reduced to approximately 50% of that observed in the WT (Figure 4). The extent of stem cell

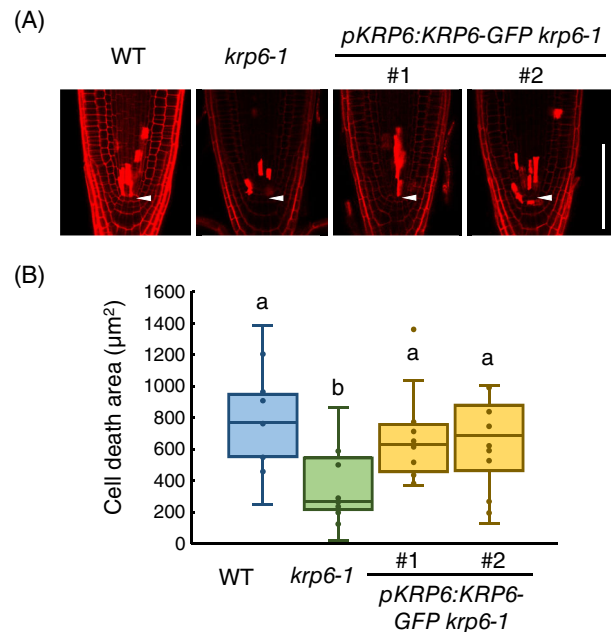


Figure 4. DNA damage-induced stem cell death is suppressed in *krp6* mutant.

(A) Root tips of *krp6* mutant after zeocin treatment. Five-day-old WT, *krp6-1* and two independent *pKRP6:KRP6-GFP krp6-1* (#1 and #2) seedlings were treated with 10 μM zeocin for 24 h and subjected to PI staining. Arrowheads indicate the QC. Bar = 100 μm .

(B) The area of PI-stained dead cells in roots grown in the presence of zeocin was measured ($n > 11$). In the box plots, centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range; and circles indicate each value in the data set. Different letters indicate significant differences ($P < 0.05$; Student's *t*-test).

death was quantified as the area of root tip tissue containing cells showing intracellular PI staining, which is indicative of the loss of plasma membrane integrity. A comparable reduction was also observed after bleomycin treatment as well as after exposure to 100 Gy of gamma rays, which is high-energy electromagnetic radiation known to induce DNA damage (Figure S5). To confirm that the reduced cell death phenotype was specifically attributable to the loss of KRP6 function, we analysed *krp6-1* plants expressing *pKRP6:KRP6-GFP*. In these lines, the area of zeocin-induced stem cell death was restored to the WT levels (Figure 4). Taken together, these results demonstrate that KRP6 is required for DNA damage-induced stem cell death.

Expression of a dominant-negative *CDKA;1* allele promotes DNA damage-induced stem cell death

To assess whether KRP6 contributes to the promotion of stem cell death, we analysed transgenic plants overexpressing *KRP6*. Two independent overexpression lines were selected in which *KRP6* transcript levels increased by approximately 69-fold (35S:*KRP6* #1) and 286-fold (35S:*KRP6* #2) relative to those in the WT, respectively

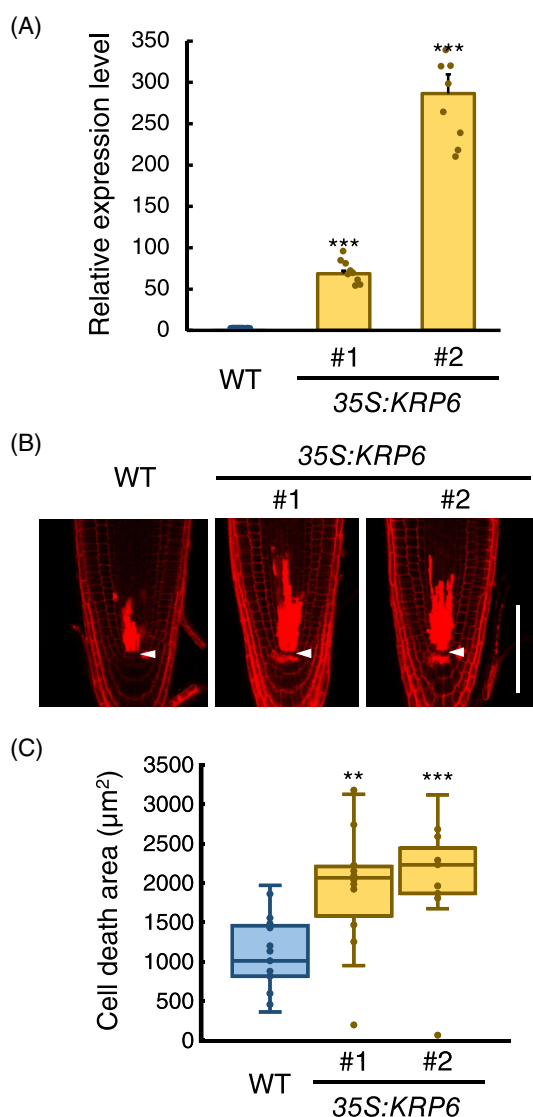


Figure 5. KRP6 promotes stem cell death under DNA damage. (A) Transcript levels of *KRP6*. Total RNA was extracted from 5-day-old WT and two independent *35S:KRP6* (#1 and #2) roots and subjected to qRT-PCR. Transcript levels of *KRP6* were normalised to that of *ACTIN2*, and are indicated as relative values, with that for WT set to 1. Data are presented as mean $\pm$ SD calculated from three biological replicates. Significant differences from WT were determined by Student's *t*-test: ****P* < 0.001. (B) Root tips of *KRP6*-overexpressing plants under zeocin treatment. Five-day-old WT and *35S:KRP6* (#1 and #2) seedlings were treated with 10 μ M zeocin for 24 h and subjected to PI staining. Arrowheads indicate the QC. Bar = 100 μ m. (C) The area of dead cells was measured (*n* > 14). In the box plots, centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range; and circles indicate each value in the data set. Significant differences from WT were determined by Student's *t*-test: ***P* < 0.01; ****P* < 0.001.

(Figure 5A). Under normal growth conditions, both overexpression lines exhibited reduced root growth and a decreased number of meristematic cells compared to the WT, likely due to reduced CDK activity caused by elevated

KRP6 levels (Figure S6). Despite these growth defects, neither line displayed detectable stem cell death under non-stress conditions (Figure S6b), indicating that increased *KRP6* expression alone was insufficient to trigger cell death. We next examined the response of these overexpression lines to DNA damage. Upon zeocin treatment, the area of cell death in the root tips was significantly increased in both *KRP6* overexpression lines compared to that in the WT (Figure 5B,C). Notably, while cell death occurred predominantly in the proximal stem cell region of WT roots, *KRP6* overexpression led to additional cell death in columella stem cells. We note, however, that these lines represent strong overexpression conditions and may not fully reflect the physiological level or spatial pattern of endogenous *KRP6* induction. Thus, these results should be interpreted as gain-of-function evidence supporting a role for *KRP6* in promoting DNA damage-induced stem cell death.

KRP6 interacts with CDK-cyclin complexes, particularly those containing *CDKA;1*, to suppress CDK activity (De Veylder et al., 2001; Nakai et al., 2006). Given that *KRP6* is transcriptionally induced in the stem cell niche after DNA damage (Figure 1E), we reasoned that *KRP6* induction might be associated with a local reduction in CDK activity within the stem cell region, which could contribute to the enhanced sensitivity of stem cells to DNA damage-induced cell death. To test this hypothesis, we expressed the dominant-negative form of *CDKA;1*, *CDKA;1.N146*, in which the conserved aspartate residue at position 146 is substituted with asparagine, which has been shown to suppress cell division and promote cell cycle arrest in shoot and stomatal lineages (Borowska-Wykręć et al., 2013; De Veylder et al., 2000; Yang et al., 2014). Its expression is driven by the *WOODENLEG* (*WOL*) promoter, which is active in vascular stem cells and steles (Petricka et al., 2012). Two independent transgenic lines expressing *pWOL:CDKA;1.N146* were selected for phenotypic analysis (Figure 6A). Under control conditions, in the absence of zeocin, neither line exhibited detectable stem cell death, indicating that expression of *CDKA;1.N146* alone is insufficient to trigger cell death (Figure 6B, – zeocin). In contrast, upon zeocin treatment, both lines displayed a significant increase in the area of stem cell death compared to the WT roots (Figure 6B,C). As a control, we generated transgenic lines expressing wild-type *CDKA;1* under control of the *WOL* promoter (Figure S7a). These lines did not show enhanced stem cell death in response to zeocin treatment (Figure S7b,c), demonstrating that the observed phenotype was specific to the dominant-negative *CDKA;1* allele rather than to ectopic *CDKA;1* expression. Taken together, these results suggest that reduced CDK activity in vascular stem cells and the stele is positively correlated with the extent of DNA damage-induced stem cell death.

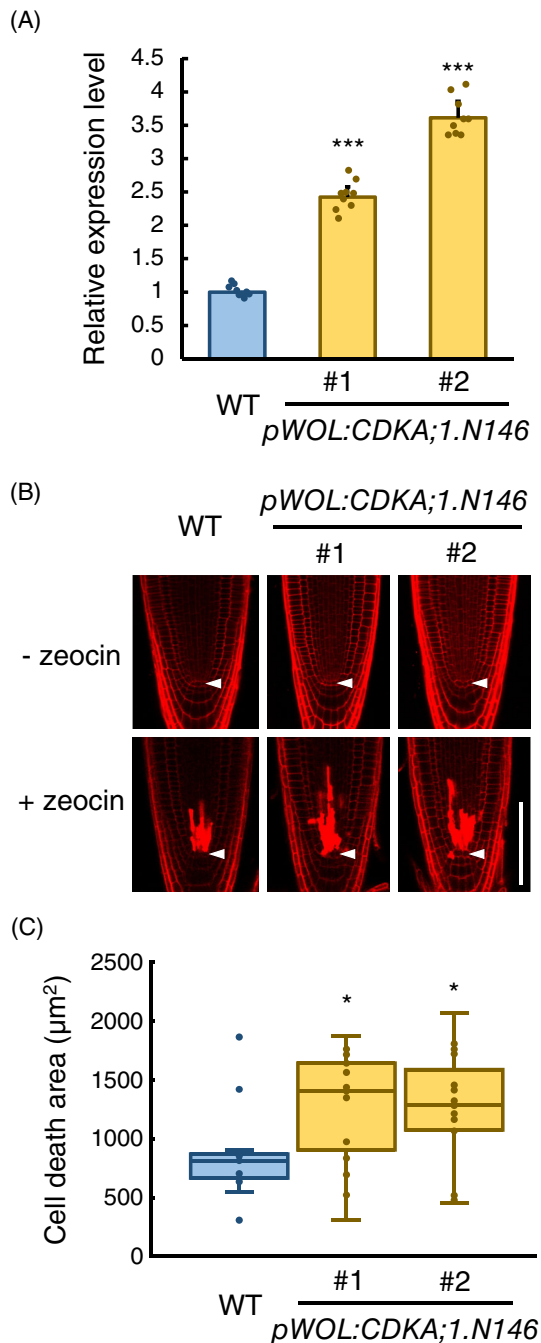


Figure 6. DNA damage-induced stem cell death is enhanced in *pWOL:CDKA;1.N146* plants.

(A) Transcript levels of *CDKA;1.N146*. Total RNA was extracted from roots of 5-day-old WT and two independent *pWOL:CDKA;1.N146* lines (#1 and #2). Transcript levels of *CDKA;1.N146* were quantified by qRT-PCR using *CDKA;1* primers and normalised to that of *ACTIN2*, and are indicated as relative values, with that of *CDKA;1* in WT set to 1. Data are presented as mean $\pm$ SD calculated from three biological replicates. Significant differences from WT were determined by Student's *t*-test: ****P* < 0.001.

(B) Root tips of *pWOL:CDKA;1.N146* lines. Five-day-old WT and *pWOL:CDKA;1.N146* (#1 and #2) seedlings were treated with (+ zeocin) or without (– zeocin) 10 µM zeocin for 24 h and subjected to PI staining. Arrowheads indicate the QC. Bar = 100 µm.

(C) The area of PI-stained dead cells in roots grown in the presence of zeocin was measured (*n* > 13). In the box plots, centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range; and circles indicate each value in the data set. Significant differences from WT were determined by Student's *t*-test: **P* < 0.05.

associated with subsequent stem cell replenishment and restoration of meristem homeostasis (Fulcher & Sablowski, 2009; Takahashi et al., 2024, 2025). However, the molecular mechanisms underlying the predisposition of stem cells to death under genotoxic stress remain poorly understood. In this study, we identified the CDK inhibitor KRP6 as a factor involved in DNA damage-induced stem cell death. *KRP6* is transcriptionally induced by SOG1 and positively contributes to stem cell death under DNA damage conditions.

In plants, SOG1 functions as a central transcription factor integrating multiple DNA damage responses (Bourbousse et al., 2018; Ogita et al., 2018). Within this regulatory network, the suppression of CDK activity represents a key downstream event. Previous studies have shown that members of the SMR family of CDK inhibitors, including *SMR4*, *SMR5* and *SMR7*, are broadly induced in meristematic tissues in an SOG1-dependent manner upon DNA damage, and are crucial for checkpoint-mediated cell cycle arrest (Yi et al., 2014). In this study, we show that another CDK inhibitor, *KRP6*, is also induced downstream of SOG1, but displays a spatially restricted expression pattern, with preferential induction in the root stem cell niche in response to DNA damage. This spatial specificity suggests that the differential expression of SOG1-regulated CDK inhibitors may contribute to distinct DNA damage responses in different cellular contexts. While SMRs broadly induced across the meristem likely suppress CDK activity and promote cell cycle arrest to facilitate DNA repair, the additional induction of *KRP6* in stem cell regions may further reinforce CDK inhibition, specifically in these cells, thereby predisposing stem cells to death under severe genotoxic stress. Thus, the coordinated activation of broadly expressed SMRs and stem cell-specific *KRP6* may underlie the selective elimination of damaged stem cells, allowing SOG1 to balance genome maintenance with tissue integrity by promoting cell cycle arrest in the meristem while triggering cell death in damaged stem cells.

DISCUSSION

In plants, DNA damage activates a coordinated response that includes cell cycle arrest, DNA repair and cell death. In Arabidopsis, severe genotoxic stress induces selective death of stem cells within the stem cell niche, thereby preventing the propagation of cells carrying irreparable genomic damage. Accumulating evidence suggests that stem cell death is not merely a passive outcome of genotoxic stress, but is an actively controlled process closely

The KRP family proteins inhibit CDKA;1-cyclin complexes in Arabidopsis (De Veylder et al., 2001; Nakai et al., 2006). Previously, overexpression of *KRP1/ICK1* was shown to induce cell death in Arabidopsis trichomes (Schnittger et al., 2003), raising the possibility that KRP influences cell death by modulating CDK activity. Consistent with this idea, the expression of the dominant-negative *CDKA;1* allele, specifically in vascular stem cells, enhanced DNA damage-induced stem cell death (Figure 6), indicating that a substantial reduction in CDK activity is sufficient to promote stem cell death under genotoxic stress. In addition to *CDKA;1*, plants possess plant-specific B-type CDKs (CDKBs), which are essential for G2/M progression (Boudolf et al., 2004). Notably, *cdkb1* mutants exhibit hypersensitivity to DNA damage (Weimer et al., 2016), suggesting that CDKB activity also contributes to genome stability. Although it remains unclear whether KRP6 directly interacts with CDKBs, simultaneous suppression of both CDKA and CDKB activities by KRP6 could reduce total CDK activity below a critical threshold, thereby promoting stem cell death. In animal systems, CDK activity plays a dual role; transient CDK inhibition supports checkpoint-mediated cell cycle arrest, whereas sustained or severe suppression of CDK activity triggers apoptosis (Shapiro & Harper, 1999; Wohlbold & Fisher, 2009). For example, inhibition of CDKs by roscovitine, which acts as a competitive inhibitor of CDK by binding to the ATP-binding site, thereby preventing substrate phosphorylation, induces apoptosis by downregulating the anti-apoptotic factor Mcl-1 (Rossi et al., 2006). These findings highlight that CDKs function not only as drivers of cell cycle progression but also as regulators of cell survival. However, the mechanistic link between CDK activity and cell death remains poorly understood in plants. One plausible scenario is that CDKs normally restrain the activity of pro-death factors such as vacuolar-processing enzymes or metacaspases, and that a strong reduction in CDK activity leads to the de-repression of these factors, culminating in vacuolar collapse, a hallmark of plant programmed cell death (Kabbage et al., 2017). While speculative, this model provides a conceptual framework for understanding how CDK suppression predisposes stem cells to death under DNA damage.

However, CDK activity in the stem cell niche after DNA damage has not been directly examined in this study. Therefore, it remains unclear whether a reduction in CDK activity is directly responsible for the induction of stem cell death. Direct measurement of CDK activity would help determine whether DNA damage reduces CDK activity in the root stem cell niche and whether such reduction correlates with stem cell death. It is also possible that KRP6 promotes stem cell death not only through suppression of CDK activity but also via additional, potentially CDK-independent mechanisms. In animal systems, CDK

inhibitors such as p21/Cip1 and p27/Kip1 interact with a wide range of non-CDK targets, including transcription factors, cytoskeletal regulators and components of apoptotic pathways, thereby regulating diverse cellular processes beyond cell cycle control (Starostina & Kipreos, 2012). Similarly, plant KRPs may have evolved additional functions through their interactions with non-CDK proteins. Identifying KRP6-interacting partners and determining whether such interactions contribute to stem cell death are essential for fully elucidating the molecular functions of KRP6 in maintaining genome integrity.

An alternative, non-mutually exclusive possibility is that KRP6 influences stem cell fate decisions rather than directly activating cell death pathways. Reduced CDK activity caused by the altered function of the CDK-activating kinase CDKF;1/Cak1At has been shown to promote the vascular differentiation of stem cells in Arabidopsis roots (Umeda et al., 2000). Importantly, differentiation toward the vascular lineage particularly xylem differentiation is intrinsically coupled with programmed cell death as a terminal developmental event. From this perspective, enhanced stem cell death observed in *KRP6* overexpression lines or in plants expressing dominant-negative *CDKA;1* under genotoxic stress may reflect an accelerated commitment to a vascular differentiation program that ultimately culminates in cell death. Determining whether KRP6 functions as a regulator of cell fate transitions or as a direct activator of cell death requires future studies that combine lineage tracing with markers of vascular identity and cell death.

Previous studies have shown that DNA damage reduces auxin levels in the root meristem, thereby contributing to the induction of stem cell death (Takahashi et al., 2021). Moreover, a recent study revealed that SOG1 directly induces the Aux/IAA transcriptional repressors *IAA5* and *IAA29*, which specifically suppress auxin signalling in the root stem cell niche, thereby promoting stem cell death (Takahashi et al., 2025). These findings establish that reduction of auxin signalling is a key upstream event in DNA damage-induced stem cell death. Previous studies have shown that auxin stimulates the expression of cyclins and CDKs. In Arabidopsis, auxin upregulates the expression of *CDKA;1* (Hemerly et al., 1993), while genes encoding A2-type cyclins (*CYCA2s*) are induced by exogenous auxin treatment (Himanen et al., 2002). Conversely, treatment with the auxin antagonist α -(phenyl ethyl-2-one)-IAA (PEO-IAA) rapidly reduces the transcript levels of *CDKB1;1* and *CYCA2;3* (Ishida et al., 2010), supporting a close link between auxin signalling and CDK activity in the root meristem. Therefore, the reduction of auxin signalling in the stem cell niche after DNA damage is likely to result in decreased CDK activity. In the present study, we identified KRP6 as an additional SOG1-induced factor that promotes DNA damage-induced stem cell death. Importantly, the

iaa5-1 iaa29-1 krp6-1 triple mutant did not exhibit a further reduction in stem cell death compared with either the *iaa5-1 iaa29-1* double mutant or the *krp6-1* single mutant (Figure S8). This genetic relationship suggests that the Aux/IAA-dependent pathway and KRP6 do not act as fully independent pathways. Instead, these pathways may act cooperatively within a closely connected SOG1-dependent regulatory network that converges on the reduction of CDK activity and promotion of stem cell death. One possible interpretation is that reduced auxin signalling lowers the expression of CDKs and cyclins, while KRP6 directly inhibits residual CDK activity at the protein level. The combined action of these transcriptional and post-translational mechanisms may reduce total CDK activity below a critical threshold required for stem cell death, thereby facilitating the selective elimination of damaged stem cells under genotoxic stress.

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis (ecotype Columbia-0) seeds were sown on Murashige and Skoog (MS) plates [0.5 × MS salts, 0.5 g L⁻¹ 2-(N-morpholino)ethanesulfonic acid (MES), 1% sucrose and 1.2% phytoagar (pH 6.3)]. After incubation at 4°C for 2 days, the plates were placed vertically under continuous light at 23°C.

DNA damage treatment

For zeocin treatment, seedlings were transferred to MS medium supplemented with 10 µM zeocin (Thermo Fisher Scientific) and grown under continuous light conditions. For the gamma-ray treatment, 5-day-old seedlings were exposed to 50 Gy h⁻¹ of ⁶⁰Co gamma radiation for 2 h at the Cobalt-60 irradiation facility, Takasaki Institute for Advanced Quantum Science, QST, and subsequently grown under the same conditions.

Plant materials and constructs

sog1-101 (Ogita et al., 2018), *ProSOG1:SOG1-Myc* (Yoshiyama et al., 2013), *krp6-1* (Cheng et al., 2013) and *iaa5-1 iaa29-1* (Takahashi et al., 2025) have been described previously. *pKRP6:GUS sog1-101* and *krp6-1 iaa5-1 iaa29-1* were generated by crossing. WT and *krp6-1* used for analyses were obtained from the same segregating population. To generate the *pKRP6:GFP* and *pKRP6:GUS* fusion constructs, the 2-kb promoter region upstream of *KRP6* start codons was PCR-amplified from Arabidopsis genomic DNA using the primer set listed in Table S1 and cloned into the pDONR221 entry vector (Thermo Fisher Scientific) by a BP recombination reaction according to the manufacturer's instructions. Subsequently, LR reactions were conducted using the destination vectors pGWB605 (Nakamura et al., 2010) or pGWB3 (Nakagawa et al., 2007) to fuse with *GFP* or *GUS*, respectively. To generate the *pKRP6:KRP6-GFP* construct, the genomic fragment containing the 2-kb promoter and coding regions of *KRP6* was amplified with the primer set listed in Table S1 and cloned into the pDONR221 entry vector (Thermo Fisher Scientific) by a BP recombination reaction. The LR reactions were conducted using the destination vector pGWB605 (Nakamura et al., 2010). To generate the overexpression construct, the full-length open reading frame of *KRP6* was amplified from Arabidopsis cDNA by PCR using the primer

sets listed in Table S1 and cloned into the pDONR221 entry vector (Thermo Fisher Scientific) by a BP recombination reaction. The LR reaction was performed using the destination vector pGWB602 (Nakamura et al., 2010). To generate *pWOL:CDKA;1* and *pWOL:CDKA-DN*, the *WOL* promoter and *CDKA;1* gene were amplified by PCR using the primer sets listed in Table S1 and cloned into entry vectors pDONR P4-P1R and pDONR221 (Thermo Fisher Scientific), respectively, by a BP reaction. To introduce a mutation into *CDKA;1* (*CDKA;1.N146*), PCR was performed using the primer sets listed in Table S1. LR reactions were performed using the destination vector R4pGWB601 (Nakamura et al., 2010). All constructs were introduced into the *Agrobacterium tumefaciens* GV3101 strain and stably transformed Arabidopsis plants were generated using the floral dip transformation method (Clough & Bent, 1998).

Microscopic observation

Roots were stained with 10 µM propidium iodide (PI) solution for 1 min at room temperature. Root tips were observed using a confocal laser scanning microscope (LSM880; Zeiss). The area of cell death was measured using the Fiji image analysis software (<http://fiji.sc>) by defining the field in which the PI infiltrated the cells.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the roots using the Plant Total RNA Mini Kit (Favorgen). First-strand cDNA was synthesised from total RNA using the ReverTra Ace qPCR RT Master Mix (Toyobo) according to the manufacturer's instructions. qRT-PCR was performed using the KAPA SYBR Fast qPCR Kit (Kapa Biosystems) with 100 nm primers and first-strand cDNAs. The primer sequences are listed in Table S1. PCR reactions were conducted with the PikoReal Real-Time PCR system (Thermo Fisher Scientific) according to the following conditions: 95°C for 5 min; 50 cycles at 95°C for 10 sec, 60°C for 15 sec and 72°C for 30 sec. Gene expression was analysed using the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001) and normalised using *ACTIN2* as a reference gene.

Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR)

pSOG1:SOG1-MYC transgenic seeds were germinated in liquid MS medium and cultured under continuous light at 23°C with gentle shaking at 50 rpm. After 2 weeks, whole seedlings were treated with or without 20 µM zeocin for 2 h and subsequently fixed with 1% formaldehyde for 15 min. The seedlings were ground and chromatin DNA was extracted. Chromatin DNA was sheared into fragments of 150–500 base pairs (bp) by sonication. After sonication, chromatin bound to the SOG1-Myc fusion protein was immunoprecipitated using an anti-Myc antibody (Millipore) (Gendrel et al., 2005). The specific primers listed in Table S1 were used for qRT-PCR to quantify genomic DNA bound to the precipitated chromatin. PCR was conducted using the PikoReal Real-Time PCR system (Thermo Fisher Scientific) according to the following conditions: 95°C for 5 min; 70 cycles at 95°C for 10 sec, 60°C for 15 sec and 72°C for 30 sec. Relative gene expression was analysed using the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001).

GUS staining

Five-day-old seedlings were incubated in GUS staining solution [100 mM sodium phosphate, 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 0.5 mM ferricyanide and 0.5 mM ferrocyanide (pH 7.4)] at 37°C in the dark. The samples were cleared with a

transparent solution (chloral hydrate, glycerol and water [8 g:1 ml:1 ml]) and observed under a light microscope.

Root growth analysis

Seedlings were grown vertically, and root tips were marked every 24 h. The seedlings were photographed, and the distance between successive marks along the root axis was measured using Fiji software (<https://fiji.sc/>) to determine root growth.

AUTHOR CONTRIBUTIONS

TW and NT designed the experiments. TW, ANS, MU and NT performed the experiments. TW performed the data analysis. TW and NT prepared the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its [Supporting Information](#).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Transcriptional response of core cell cycle genes under zeocin treatment.

Figure S2. Bleomycin treatment increases KRP6 expression.

Figure S3. Characterization of *krp6-1* mutant.

Figure S4. Phenotype of *krp6* mutant upon DNA damage.

Figure S5. Cell death in *krp6* mutant after bleomycin treatment or gamma.

Figure S6. Root growth in KRP6-overexpressing plants.

Figure S7. Cell death in *pWOL:CDKA;1* lines under zeocin treatment.

Figure S8. Cell death in *krp6 iaa5 iaa29* under zeocin treatment.

Table S1. Primers used for cloning, qRT-PCR and ChIP-qPCR.

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