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ORIGINAL RESEARCH

The Protein Arginine Methylase 5 (*PRMT5/SKB1*) Gene Is Required for the Maintenance of Root Stem Cells in Response to DNA Damage

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ABSTRACT

Plant root stem cells and their surrounding microenvironment, namely the stem cell niche, are hypersensitive to DNA damage. However, the molecular mechanisms that help maintain the genome stability of root stem cells remain elusive. Here we show that the root stem cells in the *skb1* (*Shk1 kinase binding protein 1*) mutant undergoes DNA damage-induced cell death, which is enhanced when combined with a lesion of the Ataxia-telangiectasia mutated (ATM) or the ATM/RAD3-related (ATR) genes, suggesting that the *SKB1* plays a synergistically effect with ATM and ATR in DNA damage pathway. We also provide evidence that *SKB1* is required for the maintenance of quiescent center (QC), a root stem cell niche, under DNA damage treatments. Furthermore, we report decreased and ectopic expression of *SHORTROOT* (*SHR*) in response to DNA damage in the *skb1* root tips, while the expression of *SCARECROW* (*SCR*) remains unaffected. Our results uncover a new mechanism of plant root stem cell maintenance under DNA damage conditions that requires SKB1.

KEYWORDS: *SKB1/PRMT5*; DNA damage; Root stem cells; QC maintenance; *Arabidopsis*

INTRODUCTION

Plant root stem cells provide the precursors for the production of all the under-ground tissues throughout the life of a plant, which can last for thousands of years in some cases (Baurle and Laux, 2003). In *Arabidopsis*, a set of specialized stem cells are located in root apical meristems (RAM). These specialized stem cells form a relatively inactive quiescent center (QC) that functions as the organizing center of the RAM. Root stem cells directly surround the QC and give rise to the different cell files, such as the stele, cortex and endodermis, epidermis and the lateral root cap, as well as the columella (Dolan et al., 1993). The highly organized structure of the RAM in *Arabidopsis* is apparently indispensable for its proper growth and development, and also offers an ideal model for the study of stem cells. The *Arabidopsis* QC plays a pivotal role in maintaining root meristem activity and specifies the identity of the surrounding initial cells. It has been reported that only cells that are in direct contact with the QC are maintained as stem cells (Wildwater et al., 2005; Williams and Fletcher, 2005). In the RAM, two GRAS family transcription factors, SHORTROOT (SHR) and SCARECROW (SCR), are involved in maintaining stem cell populations. *SHR* transcription is restricted to the stele, but the protein moves to the adjacent cell layer including the QC, where it activates the expression of the related *SCR* gene (Helariutta et al., 2000; Nakajima and Benfey, 2002). Both the expression level and the nuclear localization of SHR are critical for its function. It has been recently demonstrated that the pattern of cell division within the endodermis is regulated by the SHR protein dosage (Koizumi et al., 2012). SCR itself is required for the nuclear

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localization of SHR. Mutations in either *SCR* or *SHR* result in irregular morphology of the stem cell niche, lack of QC-specific marker expression, and ultimately the collapse of the root meristem (Sabatini et al., 2003; Levesque et al., 2006; Cui et al., 2007). During the life cycle of a plant, its root cells are constantly exposed to adverse environmental hazards, such as high salinity, drought stress, or heavy metal compounds in the soil. All of these hazards can cause DNA damage, and consequently, mutations (Bray and West, 2005; Tuteja et al., 2009). In animals, DNA damage in stem cells usually leads to p53-depended programmed cell death (PCD) in order to prevent cancer and protect the germline. Plants do not have a p53 protein, and their damaged stem cells tend to die to safeguard genome integrity (Fulcher and Sablowski, 2009). In both animals and plants, cellular responses to DNA damage are mediated by the highly conserved Atamia-telangiectasia mutated (ATM) and ATM/RAD3-related (ATR) protein kinases. ATM is activated in response to DNA double-stranded breaks (DSBs), whereas ATR is activated by single-stranded DNA breaks, which are generated during replication stress or by resection of DSBs during DNA repair. The activated ATM and ATR positively regulate downstream events, such as the transcriptional up-regulation of DNA repair genes (Shiloh, 2006; Flynn and Zou, 2010). In plants, the best-characterized response downstream of ATM and ATR is the WEE1 kinase, which is highly expressed in DNA damaged cells and is known to induce G2/M cell cycle arrest (De Schutter et al., 2007). Moreover, it has been demonstrated that plant stem cells and their early descendants selectively undergo ATM/ATR-mediated cell death following mild genotoxic treatments (Fulcher and Sablowski, 2009). However, the mechanisms with which plant stem cells protect themselves against DNA damage remain elusive.

SKB1, also known as protein arginine methyltransferase 5 (PRMT5), is a type II arginine methyltransferase. It has been reported to regulate diverse cellular and biological processes, including cell cycle progression (Gilbreth et al., 1998; Bao et al., 2001), RNA metabolism (Deng et al., 2010; Zhang et al., 2011), transcriptional regulation and apoptosis (Yang et al., 2009; Zhou et al., 2010). Here, we show that the *Arabidopsis SKB1* gene plays a pivotal role in DNA damage-induced stem cell death. In the *skb1* mutant, QC activities vanish following DNA damage treatments. Decreased and ectopic expression of *SHR* was detected in the *skb1* mutant in response to DNA damage, while the expression of *SCR* remained unaffected. Our data uncover a new function of the *SKB1* gene in root stem cell maintenance under DNA damage conditions.

RESULTS

Loss of *SKB1* induces short roots and meristematic cell death in *Arabidopsis*

Previous studies reported that the *skb1* mutant shown late-flowering and dwarfing phenotypes (Pei et al., 2007; Wang et al., 2007). However, although the late-flowering phenotype in the *skb1* could be rescued by *flc* mutation, the dwarfing

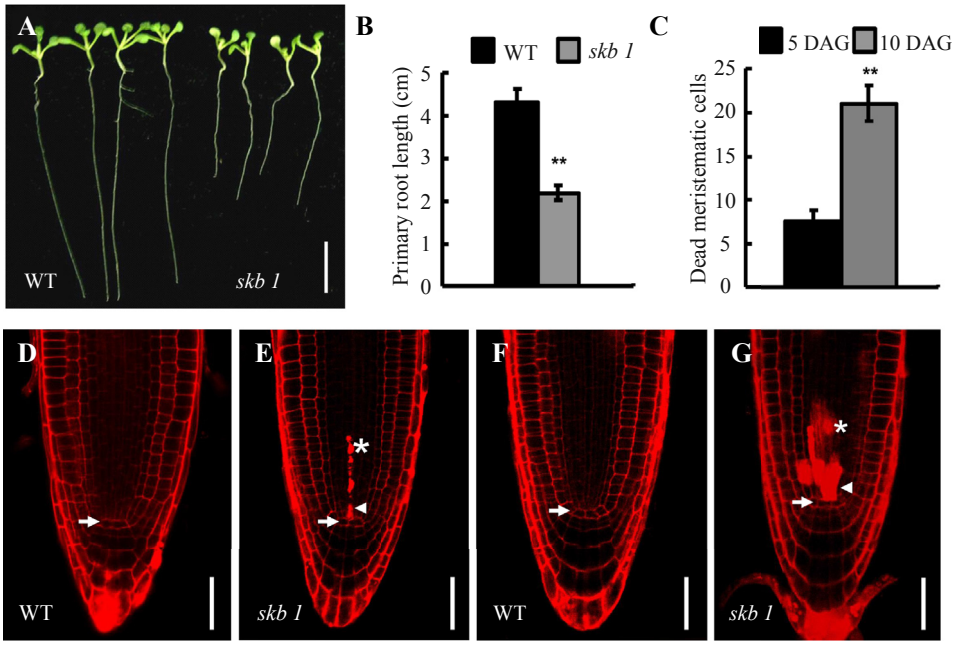


Fig. 1. Cell death in the *skb1* meristem.

A: The primary root length of 10-day-old wild type and *skb1* mutant seedlings. Scale bar: 1 cm. **B:** Quantitative result of the primary root length. At least 20 plants were scored. Error bars indicate mean standard error. $^{**}P < 0.01$. **C:** Quantitative analysis of dead meristematic cells in 5-day-old and 10-day-old *skb1* root tips. The number of dead cells was scored in 15−20 roots. DAG, days after germination. Error bars indicate mean standard error. $^{**}P < 0.01$. **D−G:** Representative confocal images of 5-day-old (**D** and **E**) and 10-day-old (**F** and **G**) root tips stained with PI (propidium iodide). The arrows indicate the QC, arrowheads point to dead stem cells in the stem cell niche, and asterisks indicate the dead cells in non-stem cell niche area. Scale bars: 50 μm.

phenotype was not affected. This dwarfing phenotype is aggravated when plants were grown in soil (Zhang et al., 2011). These findings prompted us to examine whether the root development is affected in the *skb1* mutant plants. As shown in Fig. 1, the *skb1* mutant plants exhibited a short-root phenotype. The primary root lengths of the *skb1* mutant were reduced by about 50% compared to those of wild type in 10-day-old plants (Fig. 1A and B). We further examined whether the root cells are affected in the *skb1* mutant plants. The seedlings of 5-day-old wild type and *skb1* mutant were stained with propidium iodide (PI), a dye that stains the cell walls of living cells but is also used to detect dead cells that have lost their membrane integrity. PI-stained root tips were then visualized by confocal microscopy. We observed some dead cells in the *skb1* mutant root meristem, mostly in stele initials and their early descendants (Fig. 1E). Dead cells were not seen in wild type plants (Fig. 1D). Staining of 10-day-old root tips revealed a similar, but enhanced meristematic cell death

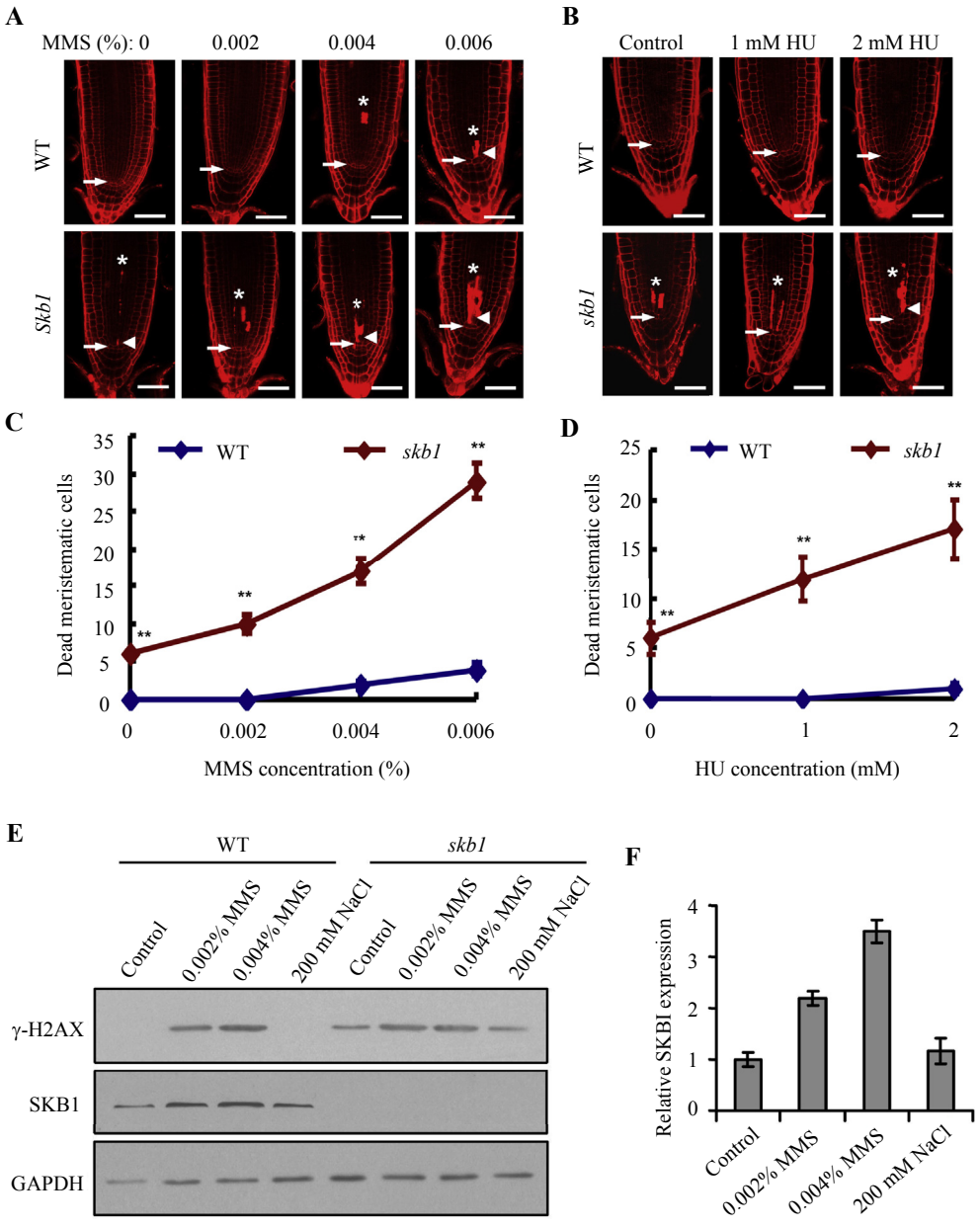


Fig. 2. The root meristematic cells of the *skb1* mutant were hypersensitive to DNA damage-inducing reagents.

A and **B:** Increased root stem cell death in the *skb1* mutant following MMS (**A**) and HU (**B**) treatments. The arrows indicate the QC, arrowheads point to dead stem cells in the stem cell niche, and asterisks indicate the dead cells in non-stem cell niche area. Scale bars: 50 μm. **C** and **D:** Quantitative results of dead meristematic cells after MMS (**C**) and HU (**D**) treatments. At least 20 roots were scored. Error bars indicate mean standard error. $^{**}P < 0.01$. **E:** SKB1 is specific for DNA damage. Five-day-old root tips under different concentration of MMS or 200 mmol/L NaCl were collected, and Western blotting was performed using the indicated antibodies. **F:** Quantitative result of SKB1 expression in wild type. GAPDH was used as an internal control for each condition, and SKB1 expression in control medium was set as 1. Results are from three independent experiments.

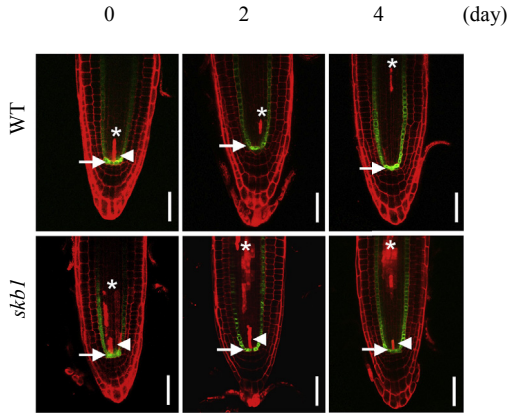


Fig. 3. Stem cell death in the *skb1* mutant.
Representative confocal images of *pSCR::GFP* root tips treated with 0.006% MMS for 24 h and then imaged at indicated times after being moved back to MS medium. The arrows indicate the QC, arrowheads point to dead stem cells in the stem cell niche, asterisks indicate the dead cells in non-stem cell niche area. Scale bars: 50 μm.

pattern in the *skb1* roots (Fig. 1C and E−F), further illustrating the cell death phenotype in the *skb1* mutant plants. We further introduced a *35S::SKB1* construct that expressed *SKB1* cDNA constitutively into *skb1* mutant plants (Wang et al., 2007). Expression of the *35S::SKB1* could rescue the short-root and stem cell death phenotypes in the *skb1* mutant, resulting in transgenic plants with a wild type phenotype (Fig. S1).

SKB1 is functionally involved in DNA damage-induced root stem cell death

It has been demonstrated that the cells in plant stem cell niches are hypersensitive to DNA damage, leading to programmed cell death (Fulcher and Sablowski, 2009). We therefore wondered whether the root stem cell death in the *skb1* mutant might be induced by DNA damage. To directly examine this, 5-day-old seedlings were treated with various concentrations of methyl methanesulfonate (MMS), a radiomimetic compound that produces DNA DSBs in cells (Lee et al., 2007), for 24 h prior to PI staining. As shown in Fig. 2A and C, the numbers of dead stem cells in the *skb1* mutant were significantly elevated by MMS treatment in a dosage-dependent manner. This indicated that SKB1 is required for stem cell maintenance in response to MMS stress. Additionally, we examined the sensitivity of the *skb1* mutant to hydroxyurea (HU), a drug that stalls DNA replication through inhibition of ribonucleotide reductase (Sakano et al., 2001; Koc et al., 2004). For this analysis, 5-day-old wild type and *skb1* seedlings were grown for an additional 24 h in the absence or presence of different concentrations of HU, after which root tips were stained with PI. We obtained similar results as with the MMS treatment: a higher dosage of HU induced much more dead meristematic cells in the *skb1* mutant than in the wild type (Fig. 2B and D). These results suggest that the *SKB1*-deficient root stem cells are more sensitive to DNA damage-causing agents such as MMS and HU.

To detect whether there is a spontaneous DNA damage in the *skb1* mutant and if the SKB1 is specific for DNA damage, γ-H2AX and SKB1 expression levels were detected by Western blotting under different treatment conditions. We found that γ-H2AX was expressed in the *skb1* mutant under non-treated condition, indicates there is a spontaneous DNA damage in the *skb1* (Fig. 2E). The γ-H2AX and SKB1 expression levels were increased under MMS treatment in a dosage-dependent manner, but were not induced by 200 mmol/L NaCl (Fig. 2E and F), suggesting that the SKB1 is specific for DNA damage response instead of a generalized response to all environmental stresses.

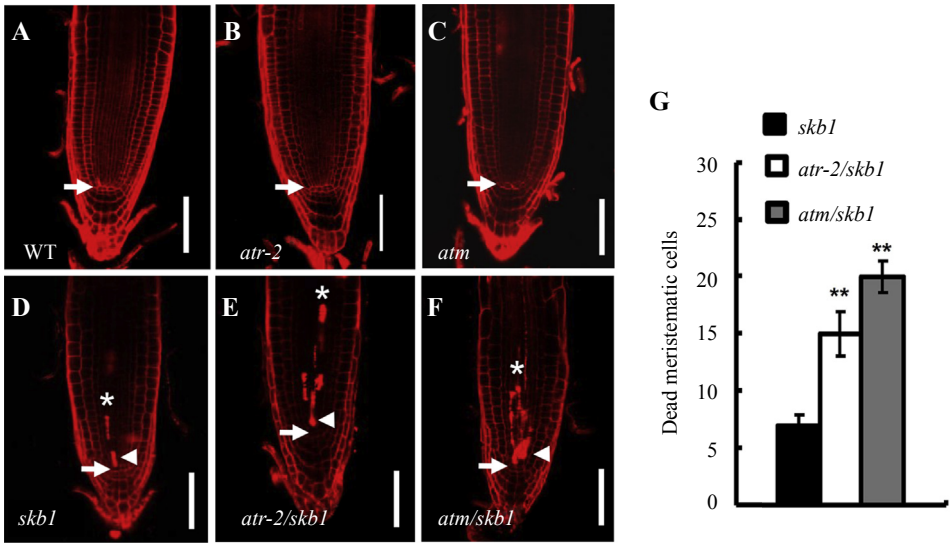
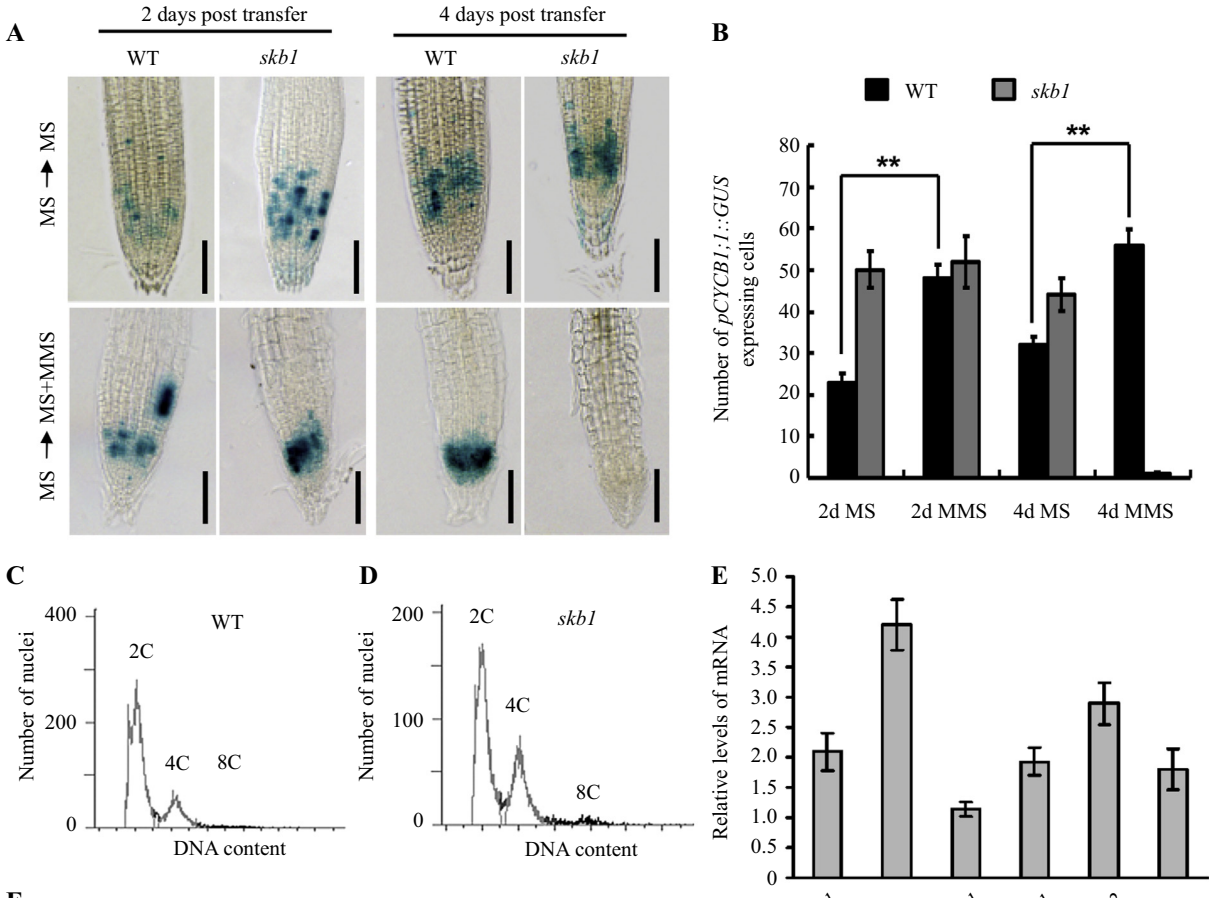


Fig. 4. Enhanced root stem cell death phenotype in *atr-2/skb1* and *atm/skb1* double mutants.
A−F: 5-day-old root tips with the indicated genotypes were stained with PI and photographed. The arrows indicate the QC, arrowheads point to dead stem cells in the stem cell niche, and asterisks indicate the dead cells in non-stem cell niche area. Scale bars: 50 μm. **G:** Quantitative results of dead meristematic cells for the indicated genotypes. Fifteen to twenty roots were scored for each line. Error bars indicate mean standard error. $^{**}P < 0.01$.

To investigate the effects of *SKB1* on root cell death in more detail, *pSCR::erGFP* was introgressed into the *skb1* mutant by genetic crosses. The reporter line *pSCR::erGFP* marks the endodermis as well as the QC in root tips. The wild type and *skb1* mutant plants carrying the *pSCR::erGFP* were treated with 0.006% MMS for 24 h, and subsequently transferred back to MS medium. We found that the surviving initials expanded to replace the dead cells, which appeared to be located away from the QC in both the wild type and *skb1* mutant plants (Fig. 3). Remarkably, new dead cells surrounding the QC were observed in the *skb1* mutant, while no such additional cell death was seen in the wild type (Fig. 3). These observations support the idea that these dead meristematic cells were indeed stem cells in the *skb1* mutant, which provides spatially-resolved evidence that these dead cells originated from damaged stem cells.

To examine whether or not root cells outside of the stem cell niche were also affected by DNA damage, 5-day-old seedlings grown on MS medium were transferred to a medium supplemented with MMS or HU, and incubated for 10 additional days. Some enlarged and highly vacuolated root tip cells were observed in the *skb1* mutant (Fig. S2), resembling



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	WT (%)	*skb1* (%)
G1	74.94	61.48
G2/M	19.43	30.05
Endoreduplicate	1.62	3.73

Fig. 5. SKB1 regulates the G2/M checkpoint in response to DNA damage.

A: Five-day-old wild type and *skb1* plants carrying *pCYCB1;1::GUS* were transferred to MS or MS plus 0.008% MMS plates for the indicate periods of time. *CYCB1;1* expression was detected using a histochemical *GUS* assay. At least 20 individual plants were stained for each treatment and time point. A representative example is shown for each treatment. Scale bars: 50 μm. **B:** Quantitative result of *pCYCB1;1::GUS* expressing cells. Twenty roots were scored. Error bars indicate mean standard error. $^{**}P < 0.01$. **C** and **D:** Flow cytometric analyses of the wild type (**C**) and *skb1* mutant (**D**) seedlings. 2C denotes the normal diploid DNA content of cells in G1 phase; 4C denotes cells that have passed S phase and in G2/M phase; 8C denotes endoreduplicated cells. **E**: qRT-PCR analysis of levels of mRNAs for cell cycle-regulated genes in the wild type and *skb1*. The values are expressed as ratios to the values in wild type. **F:** The percentages of cells in different cell cycle phase in the wild type and *skb1* mutant.

differentiated cells' responses to DNA damage treatments. By contrast, no obvious defects were found in the wild type roots under the same treatment conditions (Fig. S2). Thus, root cells of the *skb1* mutant display a general increase in sensitivity to MMS and HU.

SKB1 functions synergistically with ATM and ATR in DNA damage pathway

Given that the ATM and ATR protein kinases are key regulators in DNA damage responses (Shiloh, 2006; Flynn and Zou, 2010), and in order to elucidate the link between DNA damage response and root stem cell death in the *skb1* mutant, we generated *atm/skb1* and *atr-2/skb1* double mutants and analyzed their root stem death phenotype following PI staining. Although no dead root cells were observed in either *atm* or *atr-2* root tips (Fig. 4B and C), a significantly higher number of PI-stained cells was detected in the *atm/skb1* and *atr-2/skb1* double mutants as compared to the *skb1* single mutant (Fig. 4E−G). We further examined whether the *atm* or *atr-2* mutation enhances the sensitivity of *skb1*-deficient root cells to DNA damage-inducing agents. As expected, compared to wild type, the *atr-2* root tips showed hypersensitivity to DNA replication stress induced by HU treatment, while the *atm* mutant root cells were more sensitive to DNA DSBs caused by MMS (Fig. S3). The sensitivity of root cells to DNA damage-inducing agents was apparently enhanced in both the *atm/skb1* and *atr-2/skb1* double mutants (Fig. S3). Taken together, these results indicate that the *atm* or *atr-2* mutation exerts a synergistic effect on the observed *skb1* root stem cell death phenotype.

SKB1 regulates the G2/M checkpoint response to MMS treatment

In plants, the activation of ATM/ATR in response to DNA damage usually leads to a G2/M cell cycle arrest in order to prevent damaged cells from entering mitosis (M-phase) (Culligan et al., 2004; Ricaud et al., 2007). To investigate whether *SKB1* regulates the G2/M checkpoint in root tips, a *CYCB1;1::GUS* reporter was introgressed into the *skb1* mutant background. We then compared the *pCYCB1;1::GUS* expression between the wild type and *skb1* mutant following MMS treatment. The wild type root tips displayed typical patterns of *pCYCB1;1::GUS* expression on control plates, representing the fraction of G2/M cells that are present during normal growth (Fig. 5A). Compared to the wild type, the *skb1* mutant displayed a substantial increase in the number of *pCYCB1;1::GUS* expressing cells, and in the intensity of their staining (Fig. 5A and B). Treatment of wild type seedlings with MMS resulted in an increased number of *CYCB1;1::GUS* expressing cells both at day 2 and at day 4 following treatment (Fig. 5B). In contrast, less MMS-induced *CYCB1;1::GUS* accumulation was observed in the *skb1* mutant at day 2, and no detectable *GUS* activity was seen in *skb1* root tips at day 4 after MMS treatment (Fig. 5A and B). To further analyze the defect of cell cycle progression in *skb1* mutant, cell cytometry assay was performed. The result indicated a strong accumulation of G2/M phase cells with a 4C DNA content in *skb1* mutant as compared with the wild type (30.05% in the *skb1* mutant *versus* 19.43% in the wild type), concomitantly with a decrease in the number of 2C cells (61.48% in the *skb1* mutant *versus* 74.94% in the wild type) (Fig. 5 C, D and F). This

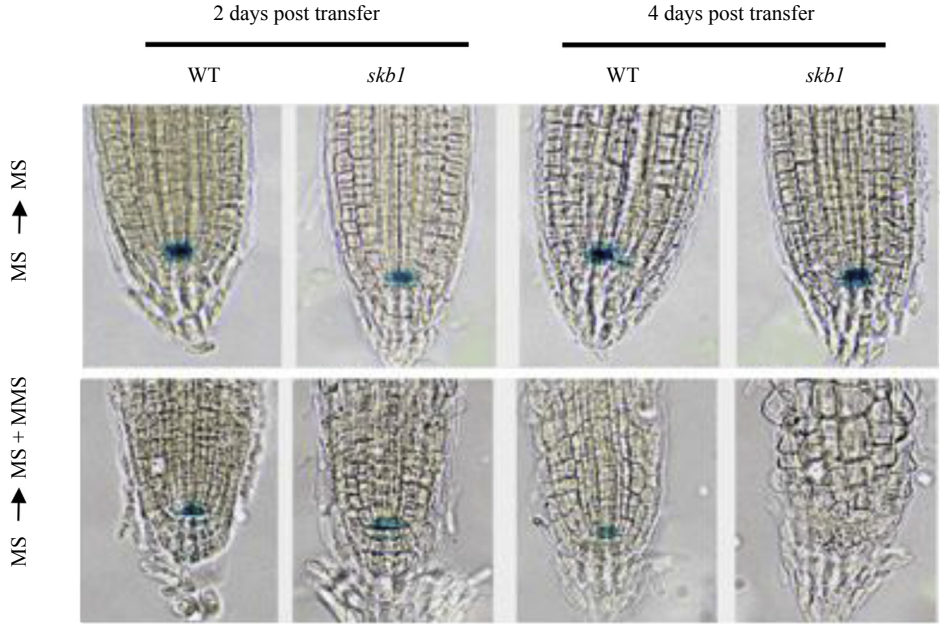

Fig. 6. MMS treatment results in loss of the QC in the *skb1* mutant root tips.
Five-day-old wild type and *skb1* plants carrying the *pQC25::GUS* reporter were transferred to MS or MS plus 0.008% MMS plates for the indicated periods of time. QC activity was measured using a histochemical *GUS* assay. Blue represents *QC25*-expressing cells.

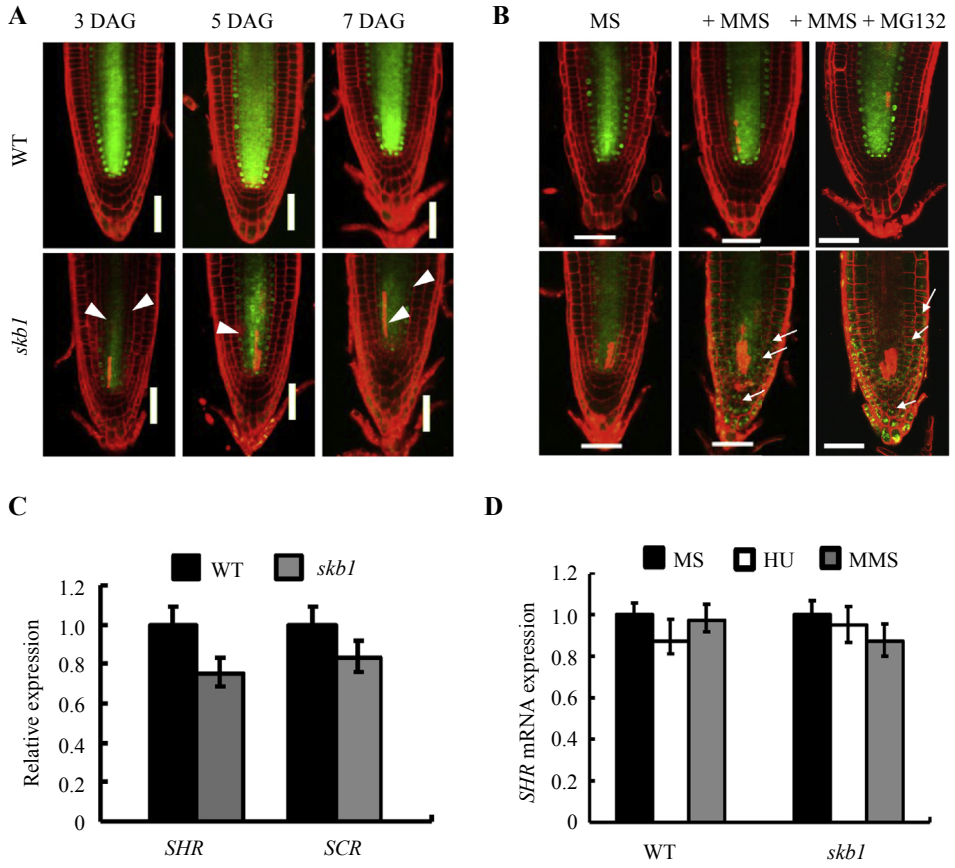

Fig. 7. SHR-GFP protein expression and localization were altered in the *skb1* root tips.
A: The *pSHR::SHR-GFP* expression pattern in the root tips of the wild type and *skb1* mutant plants at different developmental stages. The arrowheads indicate cell files showing reduced expression of *pSHR::SHR-GFP*. DAG, days after germination. Scale bars: 50 μm. **B:** The *pSHR::SHR-GFP* expression pattern in the root tips after two days of MMS and MMS plus MG132 treatment. The arrows indicate the ectopic expression of *pSHR::SHR-GFP*. Scale bars: 50 μm. **C:** Relative *SHR* and *SCR* expression levels in the root tips of 7-day-old wild type and *skb1* mutant, error bars indicate mean standard error. **D:** Relative *SHR* expression in the *skb1* mutant after 2 days of MMS or HU treatment. Error bars indicate mean standard error.

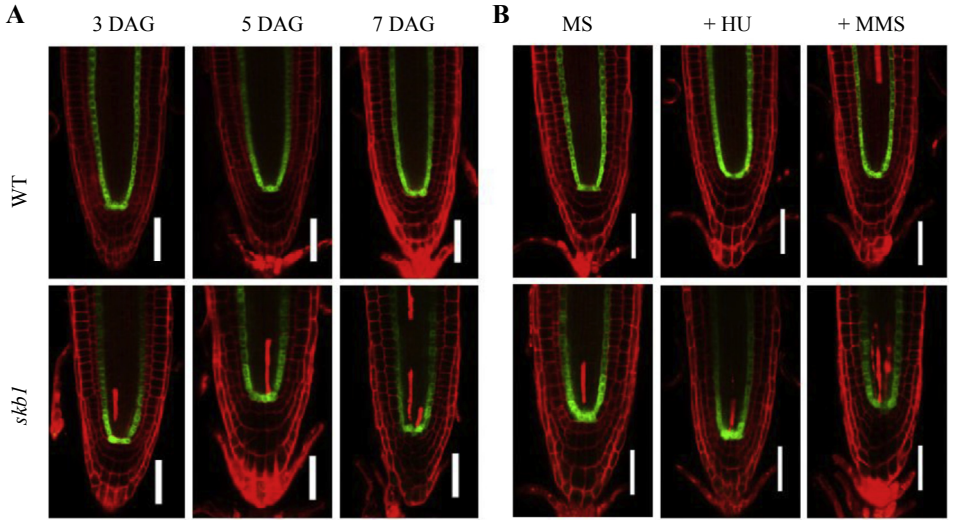

Fig. 8. The *pSCR::erGFP* expression pattern in the root tips of wild type and *skb1* mutant.
A: The *pSCR::erGFP* expression in the root tips of wild type and *skb1* mutant plants at different developmental stages. Scale bars: 50 μm. DAG, days after germination. **B:** The *pSCR::erGFP* expression in the root tips after two days of HU or MMS treatment. Scale bars: 50 μm.

indicates that the G2/M checkpoint in response to DNA damage is defective in the *skb1* root tips.

We also performed qPT-PCR to examine the expression of *CYCB1;1* and other cell cycle-regulated genes in root tips of wild type and *skb1* mutant. Consistent with the observations in *pCYCB1;1::GUS* transgenic plants and cell cytometry assay results, we found that the level of the *CYCB1;1* transcript was significantly higher in *skb1* than in wild type (Fig. 5E). The level of transcript for *WEE1*, a previously reported G2/M cell cycle regulator gene (De Schutter et al., 2007), was increased about 4.2-fold in the *skb1* mutant (Fig. 5E). We also examined the expression of the plant-specific *CDKB*, whose expression is strictly regulated during the cell cycle and is activated between S- and M-phases. There were increases in the expression levels of *CDKB2;1*, *CKKB1;2* and *KRP4* expression in *skb1*. However, the level of *CDKB1;1* did not differ between wild type and *skb1* (Fig. 5E), probably because *CDKB1;1* is expressed from S- to early M-phase, and other genes were all expressed in the more restrict phase of G2/M (Umeda et al., 1999; Menges et al., 2002). These results support the idea that the G2/M progression of the cell cycle is defective in the *skb1* root tips.

SKB1 is required for QC maintenance upon MMS treatment

We further examined the integrity of the root stem cell niche by monitoring the expression of the *QC25* gene that encodes a marker of the quiescent center. A *GUS*-based *QC25* (*pQC25::GUS*) was introgressed into the *skb1* background. Seedlings of the wild type and *skb1* mutant, each carrying *QC25*, were treated with 0.008% MMS for different durations, after which roots were stained for *GUS* activity. In the absence of MMS, both the wild type and *skb1* roots displayed clear staining at the location expected for the QC (Fig. 6, upper panel). Normal QC staining persisted in both the wild type and *skb1* mutant for two days of MMS treatment. However, after four days of MMS treatment, *skb1* roots showed no detectable QC staining, while wild type roots still stained positive for GUS (Fig. 6, lower panel). These results suggest that the QC maintenance response to MMS treatment requires SKB1.

To explore the possibility that the *skb1* roots were dead or too sick to express *CYCB1;1* and *QC25* after four days of MMS treatment, *skb1* mutant plants carrying the *pCYCB1;1::GUS* and *pQC25::GUS* constructs were transferred back to MS medium. Both *QC25* and *CYCB1;1* started to show expression after two days, and showed a normal expression pattern after four days back on the MS medium (Fig. S4), demonstrating that the *skb1* root tips were in fact alive after four days of MMS treatment.

Decreased and ectopic SHR expression in the *skb1* mutant

Given that *SHR* and *SCR* genes are required for QC and stem cell patterning, we further examined whether the expression of these genes was affected in the *skb1* mutant. To test this, *pSHR::SHR-GFP* was introgressed into the *skb1* mutant by genetic crossing. In the *skb1* mutant, *SHR-GFP* expression was significantly reduced in the stele cells and endodermis cell layers, while it retained typical expression and nuclear localization in the wild type root tips (Fig. 7A). We also observed ectopic and cytoplasmic *SHR-GFP* expression in the cortex and epidermal cells of 7-day-old *skb1* seedlings (Fig. 7A). This ectopic expression was strikingly enhanced after HU or MMS treatment (Fig. 7B). Treatment of seedlings with MMS plus MG132, a 26S proteasome inhibitor, did not affect the MMS-induced SHR-GFP localization in the *skb1* mutant (Fig. 7B). Consistent with previously studies, we saw no obviously effects on the level of *SHR-GFP* expression in wild type plants (Cruz-Ramirez et al., 2012; Koizumi and Gallagher, 2013; Wu and Gallagher, 2013). Interestingly, and in contrast to the aberrant *SHR* expression, *SCR* expression was not affected in the *skb1* mutant at the same developmental stages, even following HU or MMS treatment (Fig. 8A and B). These observations indicate that SKB1 regulates the expression and localization of SHR, but not SCR, in response to DNA damage.

We further performed qRT-PCR to test *SHR* and *SCR* mRNA expression levels in 7-day-old root tips, and found that there were no significant changes in expression in the *skb1* mutant compared with the wild type (Fig. 7C). *SHR* expression was not changed following two days of either HU or MMS treatment (Fig. 7D). These results suggest that the decreased and ectopic SHR expression in the *skb1* is not due to transcriptional changes of *SHR*.

DISCUSSION

We demonstrated that SKB1 is critical for root stem cell maintenance and the G2/M checkpoint in response to DNA damage. We observed that cells in the root stem cell niche died preferentially in the *skb1* root tips, and that the number of these dead stem cells increased at later developmental stages (Fig. 1C−G; more dead cells were detected in 10-day-old seedlings than that of 5-day-old). We therefore surmise that cells in the *skb1* mutant are constitutively exposed to DNA damage-inducing stresses. This notion was further corroborated by treating plants with two DNA damage-inducing compounds, MMS and HU, which cause DSBs and replication stresses, respectively (Sakano et al., 2001; Lee et al., 2007). We found an agent dosage-dependent and enhanced stem cell death phenotype in the *skb1* mutant (Fig. 2). In agreement with the idea that the *skb1* mutant cells are constitutively exposed to DNA damage stresses, accumulation of *CYCB1;1*-positive cells was found in the *skb1* mutant even when no external genotoxic treatment was applied (Fig. 5). In *Arabidopsis*, *CYCB1;1* is expressed upon entry into the G2 phase, and *GUS* staining marks cells in the G2 and early M phase (Colon-Carmona et al., 1999). Normal progression through the cell cycle should result in few cells being in the G2/M stage at any time, whereas treatments that inhibit the cell cycle would be expected to cause a detectable increase in the number of cells trapped in the G2/M stage, thus resulting

in an increased accumulation of *GUS* activity (Culligan et al., 2006). In addition, *skb1* plants carrying the *pCYCB1;1::GUS* reporter showed the capability, albeit somewhat reduced, to accumulate *GUS* after two days of MMS treatment, but not after four days of MMS treatment, whereas treatment of wild type seedlings resulted in the expected increased numbers of *CYCB1;1*-positive cells in the root tips following both two and four days of MMS treatment (Fig. 5). One explanation for these data is that the mutant cells are constitutively exposed to DNA damage-inducing stresses, so the *skb1* mutant could not further respond to external genotoxic treatment. Another possible explanation is that the *skb1* mutant is responding more rapidly to the DNA damage, as the 2-day-MMS-treated *skb1* plants look very similar to the 4-day-MMS-treated wild type plants.

The ectopic formation of SHR-expressing cell files in the *skb1* mutant (Fig. 7) is intriguing. This said, the expression pattern of SCR, which was reported to form a SHR/SCR complex to regulate stem cell patterning and QC activity (Sabatini et al., 2003; Cui et al., 2007), was not altered in the *skb1* plants, even at later developmental stages (7-day-old seedlings) and under DNA damage-inducing conditions (Fig. 8). Thus, the ectopic SHR expression might indicate a specific involvement of SHR in stem cell death induced by DNA damage. The result of our QC activity analysis, namely the correct expression of *QC25* in the *skb1* mutant following two days of MMS treatment (Fig. 6), suggests that the QC in *skb1* plants remains intact at that time point. Taken together, these observations imply that the stem cell death and the vanished QC activity following 4 days of MMS treatment are possibly due to down-regulated SHR expression and its ectopic localization. It remains unclear whether SKB1 directly regulates the SHR protein under DNA damage-inducing treatments. Further studies need to be performed to elucidate the mechanism by which SKB1 regulates SHR and QC activities in response to DNA damage.

SKB1/PRMT5, is a type II argnine methyltransferase that catalyzes argnine symmetric dimethylation (Bao, et al., 2001). It methylates a wide spectrum of substrates, including histone and non-histone proteins, to regulate gene transcription, RNA elongation, pre-mRNA splicing, protein interaction, and protein stability (Gilbreth et al., 1998; Bao et al., 2001; Yang et al., 2009; Deng et al., 2010; Zhou et al., 2010). We report here that mutation of *SKB1* results in hypersensitivity to DNA damage agents. However, whether the methylase activity of SKB1/PRMT5 is required for the DNA damage response, as reported in other biological processes, remains elusive. It was reported that arginine methylation of Rad9 by PRMT5 plays a critical role in S/M and G2/M cell cycle checkpoints in response to DNA damage in *Caenorhabditis elegans* (He et al., 2011). It is likely that the SHR protein is a SKB1/PRMT5 substrate during DNA damage response in root meristem maintenance. It is also possible that histone methylation and other unknown substrates catalyzed by SKB1/PRMT5 regulates this biological process. Further studies in this aspect are needed to uncover the mechanism of SKB1 regulated DNA damage response.

SKB1/PRMT5 is an evolutionally highly conserved protein (Fig. 3) (Wang et al., 2007), and is reported to regulate diverse cellular and biological processes across species (Gilbreth et al., 1998; Bao et al., 2001; Yang et al., 2009; Deng et al., 2010; Zhou et al., 2010). In *Arabidopsis*, SKB1 has been reported to regulate the transition from vegetative phase to reproductive phase as well as a salt stress response through symmetrically dimethylating H4R3 (Wang et al., 2007; Deng et al., 2010; Zhang et al., 2011; Yue et al., 2013). In yeast, SKB1 has been identified as a G2/M cell cycle regulator under DNA stress conditions (Gilbreth et al., 1998; Pal et al., 2004; Scoumanne et al., 2009). In *C. elegans*, the SKB1 homolog PRMT5 has been reported to negatively regulate DNA damage-induced apoptosis (Yang et al., 2009). Several studies in mammalian cells have also demonstrated that SKB1 regulates cell proliferation and cell cycle progression (Pal et al., 2004; Scoumanne et al., 2009). *In vivo*, SKB1 is required for embryonic stem cell maintenance in early mouse development. Our results in this study reveal a new function of SKB1 in plants, namely regulating DNA damage response. Thus, a role for SKB1 in the DNA damage pathway, cell cycle regulation, and stem cell maintenance might be evolutionarily conserved and span both the animal and plant kingdoms.

MATERIALS AND METHODS

Plant materials and growth conditions

All *Arabidopsis thaliana* lines used in this study were of the Columbia-0 (Col-0) ecotype. Some of the plant materials have been described previously: *skb1* mutant (Wang et al., 2007), *pCYCB1;1::GUS* (Li et al., 2008), *pQC25::GUS* (Sabatini et al., 2003), as well as *pSHR::SHR-GFP* and *pSCR::erGFP* (Nakajima et al., 2001). The *atm* (Salk_006953) and *atr-2* (Salk_032841) mutants were obtained from the SALK T-DNA collection. Homozygous lines were isolated by using three primers (LBb1 + LP + RP) strategy following the protocol for SALK T-DNA primer design and mutant confirmation (http://signal.salk.edu/tdnaprimers.2.html). The following primers were used for SALK_006953, LBb1: 5′-GCGTGGACCGCTTGCTGCAACT-3′, LP: 5′-ATCCATGTGGTTCAGTCTTGC and RP: TTGGTATCCTGCAGAGGAAAG-3′. And LBb1: 5′-GCGTGGACCGCTTGCTGCAACT-3′, LP: 5′-GCAGCAAAAATTTCTTGGTTG-3′ and RP: 5′-ACTTCAAGGGTTCCGATGTTC-3′ were used for SALK_032841 line. *Arabidopsis* seeds were surface sterilized for 15 min in 10% bleach, washed four times with sterile water, and plated on plates containing Murashige and Skoog (MS) salts, 3% (w/v) sucrose, 0.8% agar (sigma, USA) with a pH of 5.85 and were grown under long-day conditions (16 h of light, 8 h of darkness) at a temperature of 22°C.

PI staining and measurement of dead meristematic cells

To examine stem cell death, root tips were stained with a 5 μg/mL PI solution and observed with a confocal laser scanning microscope (Zeiss, Germany) with an argon laser. For each

treatment, the total number of dead meristematic cells was scored in each of 15−20 unique roots.

Drug treatment

For testing the sensitivity of roots to DNA damage-inducing reagents, 5-day-old seedlings grown on MS medium were transferred to MS medium containing 1 mmol/L hydroxyurea (HU) or different concentrations of methyl methanesulfonate (MMS) and grown for another 10 days prior to taking pictures. For observation the *pSHR::SHR-GFP* expression after MMS or MG132 treatment, the seedlings of 5-day-old wild type and *skb1* mutant were transferred to 0.006% MMS with or without 10 μmol/L MG132 as indicated. Each experiment was repeated independently at least three times. The representative results from one experiment are presented.

Histology

For histochemical *GUS* assay, seedlings were fixed with 90% acetone for 30 min, washed with 0.1 mol/L sodium phosphate buffer (pH 7.2), and soaked overnight in X-Gluc solution (0.1 mol/L) sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM ethylenediaminetetraacetic acid, 0.5 mmol/L potassium ferrocyanide, 0.5 mmol/L potassium ferricyanide and 1 mmol/L 5-bromo-4-chloro-3-indoxyl-β-d-glucuronic acid cyclohexylammonium salt. Seedlings were then washed in 70% ethanol overnight to clear the tissue, and *GUS* expression was observed and photographed using a metallurgical microscope (BX51M, OLYMPUS, Japan).

RNA extraction and qRT-PCR analysis

Total RNA was extracted from 7-day-old root tips using Trizol reagent (Invitrogen, USA). The complementary DNA (cDNA) was synthesized from 2 μg RNA using Super script III reverse transcriptase (Invitrogen). After RT reaction, the cDNA template was subjected to qRT-PCR using SYBR Green (Real Master Mix; Tiangen). The primer sequences were as follows: 5′-GCACTCAATGGCCGACTCTT-3′ and 5′-GATGCGACG CCGTTTGAT-3′ for *SHR*; 5′-GGGACTTCAATGGCC TGGTT-3′ and 5′-TTTCCCTGTAGCCTGAAGAGCTT-3′ for *SCR*; 5′-TAAGCAGATTCAGTTCCGGTCAAC-3′ and 5′-GGGAGCTTTACGAAAGAAATACTCC-3′ for *CYCB1;1*; 5′-TTGGACAAAAGCTTACCAGTAGAAG-3′ and 5′-AGAGA AGATATCGACTTTATCAAGG-3′ for *WEE1*; 5′-GAAGTT-CATTGCTGTATCTGTTGTC-3′ and 5′-CCAAACATAAGA-CACTAATGTGTCG-3′ for *CDKB1;1*; 5v-AATCTTCAG TTAGTATCTTTCCAAG-3′ and 5′-GCTAAAGAAAGGATG ATTCATAGAGG-3′ for *CDKB2;1*. 5′-TGGATG-CATCTTTGCCGAGA-3′ and 5′-TCTCTCACTCTCGCTG AACAA-3′ for *CDKB1;2*; 5′-TCCGATTTTTCTCACACCTC CCAATC-3′ and 5′-TTACTAGTTTCAGCACCCGA-GAAAAACT-3′ for *KRP4*. qRT-PCR was performed using three replicates and the *ACTIN2* (At3g18780) was used as a reference gene. Primers 5′-GGTAACATTGTGCTCAGTGG TGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′ were used for *ACTIN2*. Data presented are means from three biological replicates with standard error. The statistical significance was evaluated by Student's *t*-test.

Western blotting analysis

Proteins were separated by 12% SDS-PAGE and transferred to Nitrocellulose Transfer Membrane (Whatman GmbH, USA). Primary anti-SKB1 and anti-GAPDH polyclonal antibodies (Wang et al., 2007) were diluted 1:5000 and a secondary anti-rabbit antibody (Pierce Company, USA) was diluted 1:5000 respectively. ECL™ Western Blotting Detection Reagents (GE Healthcare, USA) was used to detect the HRP-conjugated secondary antibodies. Image-J software was used to quantify of the SKB1 protein expression.

Flow cytometric analyses

Seven-day-old wild type and *skb1* mutant root tips were chopped with a razor blade in 300 μL of chopping buffer (45 mmol/L $MgCl_2$, 30 mmol/L sodium citrate, 20 mmol/L 3-[*N*-morpholino]propanesulfonic acid, pH 7, and 1% Triton X-100). To the supernatants, 20 μL of 4′,6-diamidino-2-phenylindole from a stock of 1 mg/mL was added, which was filtered over a 30-μm mesh. The nuclei were analyzed with the flow cytometer from beckman counter (Cell Lab Quanta™, USA).

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SUPPLEMENTARY DATA

Fig. S1. Root phenotypes of wild type, *skb1* mutant and *35S::SKB1/skb1* seedlings.
Fig. S2. Root phenotypes of the *skb1* mutant in response to HU and MMS treatments.
Fig. S3. Root phenotypes of the *atm/skb1* and *atr-2/skb1* double mutants in response to HU and MMS treatment.
Fig. S4. The *pQC25::GUS* and *pCYCB1;1::GUS* expression were rescued in the *skb1* mutant.
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