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Original article

DNA damage inhibits lateral root formation by up-regulating cytokinin biosynthesis genes in *Arabidopsis thaliana*

Short title:

Inhibition of LR formation by DNA damage

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Abstract

Lateral roots (LRs) are an important organ for water and nutrient uptake from soil. Thus, control of LR formation is crucial in the adaptation of plant growth to environmental conditions. However, the underlying mechanism controlling LR formation in response to external factors has remained largely unknown. Here, we found that LR formation was inhibited by DNA damage. Treatment with zeocin, which causes DNA double-strand breaks, up-regulated several DNA repair genes in the LR primordium (LRP) through the signaling pathway mediated by the transcription factor SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1). Cell division was severely inhibited in the LRP of zeocin-treated *sog1-1* mutant, which in turn inhibited LR formation. This result suggests that SOG1-mediated maintenance of genome integrity is crucial for proper cell division during LRP development. Furthermore, zeocin induced several cytokinin biosynthesis genes in a SOG1-dependent manner, thereby activating cytokinin signaling in the LRP. LR formation was less inhibited by zeocin in mutants defective in cytokinin biosynthesis or signaling, suggesting that elevated cytokinin signaling is crucial for the inhibition of LR formation in response to DNA damage. We conclude that SOG1 regulates DNA repair and cytokinin signaling separately and plays a key role in controlling LR formation under genotoxic stress.

Introduction

Plants are inevitably exposed to various stresses throughout their lifetimes. Abiotic stresses, such as high salinity, osmotic stress, drought, strong light illumination, and heavy metals, produce reactive oxygen species (ROSs) in cells, resulting in the breakage of genomic DNA (Mittler 2002; Apel & Hirt 2004). Furthermore, naturally occurring endogenous by-products of cell metabolism and ultraviolet light block replication fork, and ionizing radiation and radiomimetic drugs cause DNA lesions, thereby generating DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) (Britt 1996; Møller *et al*. 2007; Tripathy & Oelmüller 2012).

Because the maintenance of genome integrity is crucial for survival under various environmental conditions, plants have a signaling pathway that senses DNA lesions and transduces the signal to trigger cellular responses to DNA damage (Hu *et al.* 2016). Similar to mammals and yeasts, plants sense DNA damage through the sensor kinases ATAXIA-TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR) (Garcia *et al.* 2003; Culligan *et al.* 2004). ATM is activated by DSBs, whereas ATR responds to SSBs and DNA replication fork blocking. ATM and ATR phosphorylate and activate the plant-specific NAC transcription factor SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) (Yoshiyama *et al.* 2013; Sjogren *et al.* 2015). Phosphorylated SOG1 induces the expression of downstream genes involved in DNA repair and cell cycle regulation (Yoshiyama *et al.* 2013). Some cell cycle-related genes are known to be direct targets of SOG1—for example, SOG1 binds to the promoters of CDK inhibitor genes *SIAMESE-RELATED 5* (*SMR5*) and *7* (*SMR7*)

(Yi *et al*. 2014), which participate in DNA damage-induced cell cycle arrest.

Root development is controlled in a plastic manner to cope with fluctuating environmental conditions. Since lateral roots (LRs) contribute to water and nutrient uptake from soil and account for the majority of plant root mass, control of LR formation is a crucial survival strategy for plants under stressful conditions (Malamy 2005). Under osmotic or salt stress, the growth of newly emerged LRs is promptly inhibited (Van der Weele *et al*. 2000; Deak & Malamy, 2005; Duan *et al*. 2013). However, the molecular mechanism underlying the inhibition of LR formation under environmental stress remains poorly understood.

LRs are formed along the primary root, followed by subsequent ramification (tertiary, quaternary, and further branching) (Nibau *et al*. 2008). LRs are originated from the pericycle, which is located between the endodermis and the central vasculature of primary roots. Local auxin accumulation triggers the specification of pericycle cells into LR founder cells (Dubrovsky *et al*. 2008). During LR initiation, nuclei in a pair of LR founder cells migrate toward the common cell wall, which leads to asymmetric cell division that generates two small daughter cells flanked by two larger cells. Through a series of subsequent periclinal cell divisions, LR primordium (LRP) develops into a more advanced stage forming dome-shaped primordium, and thereafter, LR emerges from the primary root.

Proper auxin accumulation is essential not only for LR initiation but also for LR development and emergence (Benkova *et al*. 2003; Swarup *et al.* 2008). Cytokinins negatively regulate LR development by interfering with the expression of the auxin

efflux carrier *PIN* genes, thereby disrupting the formation of an auxin gradient around LR founder cells (Laplaze *et al.* 2007; Marhavý *et al.* 2011). Therefore, targeted expression of *ISOPENTENYLTRANSFERASE* (*IPT*), a cytokinin biosynthesis gene, in xylem pole pericycle cells disrupts LR initiation and organization (Laplaze *et al.* 2007). However, whether environmental stresses affect LR formation by controlling the activity of hormonal signaling remains unknown.

Cytokinins are a central regulator of plant growth and development, such as embryogenesis, vascular differentiation, root and shoot apical meristem activity, and nodule organogenesis (Hwang *et al.* 2012). In *Arabidopsis*, N^6-(Δ^2-isopentenyl)adenine (iP) and *trans*-zeatin (*tZ*) are known as major bioactive cytokinins (Sakakibara 2006). The initial step of cytokinin biosynthesis is the N^6-prenylation of adenosine 5′-phosphates catalyzed by adenosine phosphate-IPT, which produces iP-riboside 5′-phosphates (iPRPs; Kakimoto 2001; Takei *et al.* 2001). The *trans*-hydroxylation of the prenyl side chain of iPRPs is catalyzed by the cytochrome P450 enzymes CYP735A1 and CYP735A2, and it produces *t*Z-riboside 5′-phosphates (*t*ZRPs). The cytokinin-activating enzyme LONELY GUY (LOG) then converts iP- and *t*Z-riboside 5′-monophosphate to iP and *t*Z, respectively (Kurakawa *et al*. 2007; Kuroha *et al*. 2009). Cytokinins are perceived by the CK receptors *ARABIDOPSIS* HISTIDINE KINASE 2 (AHK2), AHK3, and AHK4/CRE1. The cytokinin signal then phosphorylates and activates type-B *ARABIDOPSIS* RESPONSE REGULATOR (ARR) transcription factors through a two-component phosphorelay pathway, thereby controlling the expression of downstream genes involved in cytokinin response (Hwang & Sheen 2001;

Mason *et al*. 2005).

Here, we found that *sog1* and *atm* mutants are hypersensitive to DSBs in terms of LR development, suggesting that the maintenance of genome integrity is important for proper LR formation. We also revealed that SOG1-dependent DNA damage signaling is involved in the induction of cytokinin biosynthesis genes. LR formation was less inhibited by DSBs in cytokinin biosynthesis and signaling mutants, suggesting that plants actively elevate cytokinin levels to inhibit LR formation in response to DNA damage.

Results

DNA damage inhibits LR formation

To observe LR formation under DNA damage conditions, we treated *Arabidopsis* seedlings with the radiomimetic reagent zeocin, which causes DSBs (Huang *et al.* 1981). When 5-day-old seedlings were transferred onto zeocin-containing medium and grown for seven days, primary root growth was retarded as reported previously (Fig. 1A, B) (Adachi *et al*. 2011), but LR formation was also inhibited compared with that in the untreated control (Fig. 1C). The reduction in LR density (the number of emerged LRs per primary root length) was dependent on zeocin concentration (Fig. 1D). This result indicates that DSBs inhibit LR formation. To examine whether other types of DNA damage also inhibit LR formation, seedlings were treated with bleomycin, methyl methanesulfonate (MMS), cisplatin, mitomycin C (MMC), or hydroxyurea (HU). Bleomycin causes DSBs (Povirk 1996). MMS is an alkylating agent that methylates

guanine and adenine bases, causing base mispairing and replication blocks (Beranek 1990; Llorente *et al.* 2008). MMC generates interstrand cross-links on DNA, whereas cisplatin preferentially forms intrastrand cross-links (Eastman 1985; Rink *et al.* 1996). HU inhibits deoxyribonucleotide production, thereby causing DNA replication stress (Wang & Liu 2006; Saban & Bujak 2009). These DNA damaging agents inhibited primary root growth in a concentration-dependent manner (Fig. S1). Interestingly, bleomycin, MMS, cisplatin, and MMC inhibited LR formation (Fig. 2A–D), whereas HU treatment had no significant effect on LR density although primary root growth was retarded (Fig. 2E, Fig. S1E). These results suggest that DSBs, DNA alkylation, and DNA cross-links, but not DNA replication stress, inhibit LR formation.

Cell division during LR formation is severely inhibited in *sog1* and *atm* mutants

ATM and ATR kinases sense DSBs, and SSBs and DNA replication stress, respectively, and phosphorylate and activate the plant-specific transcription factor SOG1 (Garcia *et al.* 2003; Culligan *et al.* 2004; Yoshiyama *et al.* 2013; Sjogren *et al.* 2015). SOG1 regulates hundreds of genes involved in cell cycle arrest, stem cell death, early onset of endoreplication, and DNA repair (Yoshiyama *et al*. 2009). To reveal whether the inhibition of LR formation in response to DNA damage is an active process involving ATM/ATR-SOG1 pathways, we first observed the response of *sog1-1* mutant carrying a missense mutation in the NAC domain (Yoshiyama *et al.* 2009). Surprisingly, compared with the wild-type, *sog1-1* showed hypersensitivity to zeocin in LR formation (Fig. 3A, B), whereas primary root growth was tolerant to zeocin (Fig. S2) as reported previously

(Adachi *et al*. 2011).

We speculated that even though zeocin-induced inhibition of LR formation is an active response to DNA damage, the *sog1-1* mutation might cause the accumulation of DNA damage, thereby masking suppression of the LR phenotype. To gather hints about this scenario, we observed the distribution of each stage of LR formation. It has been known that LRP is formed through a series of cell divisions according to a well-defined sequence of transversal, periclinal, and anticlinal divisions (Casimiro *et al.* 2003; Péret *et al.* 2009). At the stage I, two pericycle founder cells undergo asymmetric division. The daughter cells undergo further division, and the LRP is made of three and four layers at stage III and IV, respectively. Further cell division in these cell layers eventually results in the emergence of LRs from the primary root (Malamy & Benfey 1997). In wild-type seedlings, zeocin treatment reduced the ratio of emerged LR by half, while that of LRP at the first stage increased more than twofold (Fig. 3C). By contrast, most LRP formation was arrested from stage I to IV in *sog1-1* mutant, and no emerged LR was observed (Fig. 3D). This result suggests that zeocin treatment severely inhibits cell division for LRP formation in *sog1-1*. Indeed, when *sog1-1* was treated with zeocin, periclinal and anticlinal divisions did not occur uniformly during LRP development, generating an irregularly shaped LRP (Fig. 3E).

We also observed the response of *atm-2* and *atr-2* knockout mutants to zeocin. Similar to *sog1-1*, *atm-2* showed zeocin-induced arrest of LRP development at early stages (Fig. S3A). On the contrary, we observed emerged LR in *atr-2* mutant (Fig. S3B), suggesting that cell division was not inhibited as severely as that in *sog1-1* or *atm-2*

mutants. In contrast to the wild-type, zeocin treatment increased the ratio of LRP at stage III to VIII, rather than stage I, in *atr-2* by unknown reasons.

SOG1-mediated control of genome integrity is important for LR formation

The above results indicate that zeocin-induced DSBs severely inhibit cell division during LRP development in *sog1-1* and *atm-2* mutants. Since DSB signals are transmitted through the ATM–SOG1 pathway (Yoshiyama *et al*. 2013), DNA damage may be highly accumulated in *atm-2* and *sog1-1* mutants, thereby inhibiting cell division. To test this hypothesis, expression of a DNA repair gene was monitored in LRP. RAD51 participates in DSBs repair via homologous recombination, and its expression is up-regulated by DNA damage (Osakabe *et al*. 2002; Abe *et al*., 2005). In wild-type seedlings carrying the *pRAD51:GUS* reporter gene, zeocin treatment increased β-D-glucuronidase (GUS) expression in LRP (Fig. 4A). By contrast, a trace level of GUS expression was observed in *sog1-1* even in the presence of zeocin (Fig. 4A). Measurement of *RAD51* transcripts with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) supported the results of the GUS expression experiments (Fig. 4B, C). Similarly, the expression of other DNA repair genes, *BRCA1, RAD17*, and *PARP2*, was induced by DNA damage in wild-type but not significantly in *sog1-1* (Fig. 4B, C). These results suggest that ATM-activated SOG1 is essential for the induction of DNA repair genes, thus maintaining genome integrity during LRP development. Note that primary root growth in *sog1-1* was not inhibited, rather faster than that in the wild-type, in the presence of zeocin (Fig. S2), suggesting that compared with the LRP,

the primary root meristem is more resistant to accumulating DNA damage for unknown reasons.

To examine whether the zeocin-treated LRPs still retain the ability to develop into LRs, 2.5 or 5 μM zeocin-treated seedlings were transferred onto medium without zeocin and measured the number of emerged LR. In wild-type, LR density was gradually increased after transfer to zeocin-free medium; about 20 % increase in LR density was observed within five days after transfer (Fig. 5A). In *sog1-1* mutant, however, LR formation was not recovered within five days (Fig. 5B), suggesting that irregularly formed LRP in *sog1-1* could not restart cell division even in the absence of zeocin. These data indicate that SOG1-mediated maintenance of genome integrity plays a key role in preserving LRPs.

DNA damage up-regulates cytokinin signaling in LRP

It is still unknown whether inhibition of LR formation in zeocin-treated wild-type plants is a consequence of remaining DNA damage that escaped from SOG1-dependent repair, or is an outcome of active response to DNA damage. The phytohormone cytokinin is known to inhibit LR initiation (Laplaze *et al*. 2007), and it down- and up-regulates cell division and cell differentiation, respectively, in the transition zone of roots (Dello Ioio *et al.* 2008; Takahashi *et al.* 2013). When wild-type seedlings were treated with bioactive cytokinins, such as 300 nM kinetin or 40 nM benzyladenine (BA), LR density significantly decreased (Fig. 6A, B), supporting previous observations (Laplaze *et al*. 2007). Detailed analysis of LRP development showed that compared with the untreated

control, cytokinin-treated seedlings showed a greater than twofold increase in the ratio of LRP arrested at stage I (Fig. 6C). Note that this tendency is highly similar to that of zeocin-treated seedlings (Fig. 1, Fig. 3), suggesting that DNA damage inhibits LR formation by modulating cytokinin signaling.

To examine whether the cytokinin signaling is altered in LRP after DNA damage, we observed the expression of the cytokinin signaling marker gene *ARR5*. It has been shown that cytokinin treatment activates the GUS reporter gene expression driven by the *ARR5* promoter (D'Agostino *et al*. 2000). As shown in Figure 6D, *pARR5::GUS* expression was detected in the central cylinder, but not in LRP, in the absence of zeocin. However, zeocin treatment dramatically increased GUS expression in LRP, indicating that the cytokinin signaling is enhanced in LRP in response to DNA damage.

DNA damage actively induces cytokinin biosynthesis genes

Although cytokinin signaling was activated in LRP under DNA damage, previous microarray data showed that the expression of cytokinin signaling genes was not altered by gamma ray irradiation (Table S1). Therefore, we hypothesized that cytokinin biosynthesis is up-regulated in response to DNA damage. We used qRT-PCR to measure the expression levels of cytokinin biosynthesis genes *IPT2*, *IPT3*, *IPT5*, *IPT7*, *IPT9*, *LOG1*, *LOG2*, *LOG3*, *LOG4*, *LOG5*, *LOG6*, *LOG7*, *LOG8*, *CYP735A1*, and *CYP735A2*. The results showed that transcript levels of *IPT2*, *IPT7*, *IPT9*, *LOG2*, *LOG3*, *LOG4*, *LOG5*, *LOG7*, and *LOG8* were increased in roots after treatment with 5 μM or 10 μM

zeocin (Fig. 7A).

To determine whether the SOG1-mediated pathway is required for this induction, we examined the expression of *IPT2*, *IPT7*, *IPT9*, *LOG2*, *LOG3*, and *LOG4* in *sog1-1* mutant. The qRT-PCR results showed that the induction of the expression of these genes by zeocin treatment was suppressed in *sog1-1* (Fig. 7B, C), indicating that the induction of cytokinin biosynthesis genes is a programmed response to DNA damage through the SOG1-mediated pathway.

Elevated cytokinin signaling is crucial for the inhibition of LR formation in response to DNA damage

To determine whether the activation of cytokinin signaling is involved in the inhibition of LR formation under DNA damage conditions, we observed the zeocin response of plants defective in cytokinin biosynthesis or signaling. *ipt3-2;5-1;7-1* has mutations in three major *IPT* genes, and is known to produce a very low amount of cytokinins (Miyawaki *et al*. 2006). We used this mutant because *IPT7* is one of the cytokinin biosynthesis genes induced by zeocin treatment (Fig. 7A). As a cytokinin signaling mutant, *arr1-3;12-1* was used in which type-B response regulators ARR1 and ARR12 are defective, thus signaling through the two-component pathway is weakened (Mason *et al*. 2005). We also used a transgenic line overexpressing cytokinin oxidase-dehydrogenase 1 (*CKX1*) under the cauliflower mosaic virus 35S promoter (*35S::CKX1*), in which endogenous active cytokinins are actively degraded by CKX1 (Werner *et al.* 2003). As shown in Figure 8, reduction of LR density in response to

zeocin treatment was observed in *ipt3-2;5-1;7-1, arr1-3;12-1*, and *35S::CKX1* less frequently than in the wild-type. In *ipt3-2;5-1;7-1* and *35S::CKX1*, LR density was not reduced further at zeocin concentrations higher than 2.5 μM (Fig. 8D, F). However, in *arr1-3;12-1*, it was reduced dependently on zeocin concentration up to 7.5 μM (Fig. 8E). *Arabidopsis* possesses eleven type-B response regulators, and at least several of them have similar activities in cytokinin signaling (Hill *et al.* 2013). Therefore, it is likely that not only ARR1 and ARR12 but also other type-B response regulators are involved in transmitting the cytokinin signal enhanced by DNA damage. As a result, compared with *ipt3-2;5-1;7-1* and *35S::CKX1*, *arr1-3;12-1* might display more sensitive phenotype to zeocin. Overall, our results indicate that an increase in cytokinin signaling is crucial for the inhibition of LR formation in response to DNA damage.

As mentioned above, *sog1-1* was hypersensitive to zeocin, probably due to no induction of DNA repair genes and resultant accumulation of DNA damage. However, cytokinin-related mutants were tolerant to zeocin although cytokinin biosynthesis genes are induced via the SOG1-mediated pathway. To resolve this paradox, we hypothesized that SOG1 differentially regulates genes for DNA repair and cytokinin biosynthesis, and that enhanced cytokinin production inhibits LR formation but not affects DNA repair. We tested this hypothesis by measuring the expression levels of DNA repair genes in *ipt3-2;5-1;7-1*. As shown in Fig. S4, *RAD51*, *BRCA1*, *PARP2*, and *RAD17* were induced by zeocin treatment to a similar extent as that in wild-type. This result suggests that, in cytokinin-related mutants, damaged DNA is properly repaired, thereby exhibiting a tolerant phenotype to zeocin.

Discussion

LRs are an important organ for water uptake and the absorption of nutrients from soil. Thus, precise control of LR formation is crucial for plants to adapt their growth to environmental conditions. In this study, we showed that DNA damage inhibited LR formation. Our data demonstrated that SOG1-mediated DNA damage signaling elevated the expression of genes involved in DNA repair and cytokinin biosynthesis. Since LRP development was severely impaired by DNA damage in the *sog1-1* mutant, SOG1-mediated activation of DNA repair machineries seems crucial for maintaining genome integrity in LRP, thus for enabling LR formation after removal of genotoxic stress (Fig. S5). Furthermore, our results indicated that SOG1-dependent enhancement of cytokinin signaling is required for the inhibition of LR formation under DNA damage conditions. There still remains a possibility that factors associated with cytokinin signaling respond to DNA damage, but our data clearly showed that genes for cytokinin biosynthesis are actively induced by zeocin treatment, suggesting that cytokinin content itself increased in LRP under DNA damage conditions. We found that induction of DNA repair genes was independent from that of cytokinin biosynthesis genes (Fig. S5). Altogether, DNA damage signaling differentially controls DNA repair and cytokinin level, thereby maintaining the ability of plants to produce LR after recovery from genotoxic stress. It is known that various environmental stresses often produce ROS and cause the breakage of genomic DNA (Mittler 2002; Apel & Hirt 2004), suggesting that the above mechanism also underlies programmed response to other stresses in

controlling overall root growth.

In this study, we found that zeocin treatment severely perturbed LR formation in *sog1-1* mutant, but primary roots grew rather faster in the mutant than in the wild-type after zeocin treatment. It is likely that DNA damage was highly accumulated both in primary roots and LRP; therefore, dividing cells in the primary root meristem are more tolerant to DNA damage as compared to cells constituting LRP. However, factors causing these differences remain unknown. It has been reported that osmotic stress, which is known to cause DNA damage (Balestrazzi *et al.* 2011), also severely inhibits LR formation, while primary root growth is not retarded (Deak & Malamy 2005). This result indicates that stress-induced DNA damage signals are interpreted differentially between primary roots and LRs, allowing changes in root architecture. It is interesting to study the differences in DNA damage signaling between the two root tissues and their physiological roles in survival under changing environmental conditions.

ATM and ATR are activated by DSBs, and SSBs and DNA replication stress, respectively (Garcia *et al*. 2003; Culligan *et al*. 2004). Here, we demonstrated that zeocin-induced DSBs, but not HU-triggered DNA replication stress, inhibit LR formation. Moreover, we showed that compared with the wild type, *atm-2* and *sog1-1*, but not *atr-2*, exhibited hypersensitivity to zeocin in terms of LRP development. These results suggest that DSBs, which can represent fatal damage accompanied by a loss of chromosome arms, have a higher impact on accumulating DNA damage as compared with replication stress, and the ATM–SOG1 pathway, rather than the ATR–SOG1

pathway, plays a major role in provoking DNA repair in LRP. Previous microarray data indicate that the expression of several DNA repair genes is upregulated by gamma ray irradiation in the *atr* mutant, but not in the *atm* or *sog1* mutants, at a level comparable to that in the wild-type (Table S2) (Culligan *et al*. 2006; Yoshiyama *et al.* 2009), supporting the idea that ATM–SOG1 mainly participates in repairing damaged DNA. Recently, it has been shown that high aluminum stress specifically activates the ATR–SOG1 pathway and inhibits root growth (Sjogren *et al.* 2015); therefore, ATM and ATR may respond differentially to external cues and control primary and LR growth.

It has been shown that exogenous cytokinins inhibit the auxin-induced expression of *PIN* genes during LR development, indicating that cytokinins prevent the PIN-dependent establishment of the auxin gradient required for LR initiation (Laplaze *et al*. 2007). In this study, we demonstrated that zeocin treatment increases the expression of cytokinin biosynthesis genes and arrests LRP development at early stages. Therefore, DSBs likely inhibit LR formation by increasing cytokinin levels and down-regulating *PIN* expression. In the transition zone of primary roots, cytokinins induce the expression of *SHY2/IAA3*, one of the Aux/IAA auxin signaling repressors, and inhibit *PIN* expression. This promotes the transition from cell division to cell differentiation, restricting the size of the root meristem (Dello Ioio *et al.* 2008). We previously reported that DSBs promote an early transition from cell division to cell differentiation in the root meristem (Adachi *et al.* 2011); thus, it is possible that cytokinins also participate in DSB-induced cessation of cell division in the primary root. Further studies will reveal whether cytokinin-mediated inhibition of cell division is a

commonly used mechanism for stress response in roots.

Experimental procedures

Plant growth conditions

Arabidopsis thaliana (ecotype Columbia-0) plants were grown vertically under continuous light conditions at 22°C on Murashige and Skoog (MS) plates [0.5 × MS salts, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES), 1% sucrose, and 1.2% phytoagar (pH 6.3)]. For DNA damage and cytokinin experiments, five-day-old seedlings were transferred onto medium containing DNA-damaging agents: zeocin (Invitrogen), bleomycin, MMC, cisplatin, HU (Wako), or MMS (Nacalai Tesque); or cytokinins: kinetin (Sigma) or benzyladenine (Wako).

Plant materials and constructs

sog1-1 (Yoshiyama *et al.* 2009), *atm-2* (Garcia *et al.* 2003), *atr-2* (Culligan *et al.* 2004), *ipt3-2;5-1;7-1* (Miyawaki *et al.* 2006), *arr1-3;12-1* (Mason *et al.* 2005), *35S::CKX1* (Werner *et al.* 2003), and *pARR5::GUS* (D'Agostino *et al.* 2000) have been described previously. The promoter sequence of *RAD51* was amplified from *Arabidopsis* genomic DNA by polymerase chain reaction (PCR) with 5′-AAAAAGCAGGCTTTAGCGTCAAGTAGTTGG-3′ and 5′-AGAAAGCTGGGTTTCTCTCAATCAGAGC-3′ primers and cloned into the pDONR™221 (Invitrogen) entry vector by BP recombination reaction according to the manufacturer's instructions (Invitrogen). To generate the *pRAD51:GUS* construct, the entry clone was mixed with the pGWB3 destination vector (Nakagawa *et al.* 2009) by LR recombination reaction according to the manufacturer's instructions (Invitrogen).

All constructs were transferred into the *Agrobacterium tumefaciens* GV3101 strain harboring plasmid pMP90. The obtained strains were used to generate stably transformed *Arabidopsis* with the floral dip transformation method (Clough and Bent 1998).

Quantitative RT-PCR

Total RNA was extracted from *Arabidopsis* root with a Plant Total RNA Mini Kit (Favorgen Biotech Corp.). First-strand complementary DNA (cDNA) was prepared from total RNA with ReverTra Ace® (Toyobo) according to the manufacturer's instructions. For quantitative PCR, a THUNDERBIRD SYBR qPCR Mix (Toyobo) was used with 100 nM primers and first-strand cDNAs. PCR was performed on a LightCycler 480 Real-Time PCR System (Roche) with the following conditions: 95°C for 5 min; 45 cycles at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 15 sec. *ACTIN2* (At3g18780) was used as a reference gene. Primer sequences are listed in Table S3.

GUS staining

Seedlings were incubated in GUS staining solution (100 mM sodium phosphate, 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 0.5 mM ferricyanide, and 0.5 mM ferrocyanide [pH 7.4]) in the dark at 37°C. 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 (Olympus).

Root growth analysis

For root growth experiments, seedlings were grown vertically on square plates. Root tips were marked on the plates every 24 h. The plates were photographed, and root growth was measured with ImageJ software (http://rsb.info.nih.gov/ij/) by calculating the distance between successive marks along the root axis.

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Figure legends

Figure 1. DNA damage inhibits LR formation. (A) Phenotype of zeocin-treated seedlings. Five-day-old wild-type seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin) and grown for an additional seven days. Black lines indicate the positions of the root tips when the seedlings were transferred onto each medium. Bar = 2 cm. (B) Primary root growth of wild-type plants. Five-day-old seedlings were transferred onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin. Root length was measured. Data are presented as means ± SE (n > 20). (C) Five-day-old wild-type seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin) and grown for an additional seven days. Arrowheads indicate the positions of the LR along the primary roots. Bar = 0.5 cm. (D) LR density after transfer onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin. Data are presented as means ± standard error (SE; n > 20). Significant differences between treated seedlings and the untreated control were determined with the Student's *t*-test: ***, $P < 0.001$.

Figure 2. DNA double-strand breaks, alkylation, and cross-links, but not DNA replication stress, inhibit LR formation. LR density after transfer onto medium containing various DNA-damaging agents. Five-day-old seedlings were transferred onto control medium or medium containing the indicated concentrations of bleomycin (A), methanesulfonate (MMS) (B), cisplatin (C), mitomycin C (MMC) (D), or hydroxyurea (HU) (E) and grown for an additional seven days. Data are presented as means ± SE (n

> 14).

Figure 3. DNA damage severely inhibits cell division to form LRP in *sog1-1* mutant. (A) LR phenotype of zeocin-treated *sog1-1* seedlings. Five-day-old wild-type (WT) and *sog1-1* seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin) and grown for an additional seven days. Arrowheads indicate the positions of the LR along the primary roots. Bar = 0.5 cm. (B) LR density of WT and *sog1-1* after transfer onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin. Data are presented as means ± SE (n > 20). Significant differences between treated seedlings and the WT control were determined with the Student's *t*-test: ***, $P < 0.001$. (C, D) Stage distribution of LRP in the WT and *sog1-1* after transfer onto control medium or medium containing 2.5 μM zeocin (+ zeocin). Data are presented as means ± SE (n > 15). Significant differences between treated seedlings and the untreated control were determined with the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (E) LRPs of zeocin-treated WT and *sog1-1* plants. Five-day-old seedlings were transferred onto medium containing 2.5 μM zeocin (+ zeocin) and grown for an additional seven days. Bar = 25 μm.

Figure 4. SOG1 is essential for the induction of DNA repair genes. (A) LRP of WT and *sog1-1* seedlings harboring *pRAD51:GUS*. Five-day-old seedlings were transferred onto control medium (control) or medium containing 5 μM zeocin (+ zeocin), and GUS staining was conducted after 24 h. Bar = 50 μm. (B, C) Transcript levels of *RAD51*,

BRCA1, *PARP2*, and *RAD17* in roots. Five-day-old wild-type (B) and *sog1-1* (C) seedlings were transferred onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin and grown for seven days. Total RNA was extracted from roots. The mRNA levels were normalized to that of *ACTIN2*, and are indicated as relative values, with that of the control set to 1. Data are presented as means ± SD (n = 3). Significant differences between treated seedlings and the control were determined with the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. The zeocin-treated *sog1-1* has no ability to restart cell division. LR density of zeocin-treated seedlings after transfer onto control medium. Five-day-old seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin or 5.0 μM zeocin for five days, then transferred onto a control medium and grown for a indicated additional days. LR density was normalized to that of the control and is indicated as a relative value, with that of the control set to 1.0. Data are presented as means ± SE (n > 20). Significant differences between values obtained before and after the plants were transferred to the control medium (day 0) were determined with the Student's *t*-test: ***, $P < 0.001$.

Figure 6. Cytokinin signaling is up-regulated in LRP in response to DNA damage. (A) LR phenotype of zeocin- or cytokinin-treated seedlings. Five-day-old seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin), 300 nM kinetin (+ kinetin), or 40 nM benzyladenine (+ BA) and grown for an

additional seven days. Arrowheads indicate the positions of the LR along the primary roots. Bar = 0.5 cm. (B) LR density after transfer onto control medium or medium containing 2.5 μM zeocin (+ zeocin), 300 nM kinetin (+ kinetin), or 40 nM benzyladenine (+ BA). Data are presented as means ± SE (n > 20). Significant differences between treated seedlings and the untreated control were determined with the Student's *t*-test: ***, $P < 0.001$. (C) Stage distribution of LRP after transfer onto control medium or medium containing 2.5 μM zeocin (+ zeocin), 300 nM kinetin (+ kinetin), or 40 nM benzyladenine (+ BA). Data are presented as means ± SE (n > 15). Significant differences between treated seedlings and the non-treated control were determined with the Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$. (D) LRP of wild-type seedlings harboring *pARR5:GUS*. Five-day-old seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin), and GUS staining was conducted after 7 days. Bar = 50 μm.

Figure 7. DNA damage induces cytokinin biosynthesis genes. (A) Transcript levels of cytokinin biosynthesis genes in roots. Five-day-old seedlings were transferred onto control medium or medium containing 5 or 10 μM zeocin for seven days. (B, C) Transcript levels of *IPT2*, *IPT7*, *IPT9*, *LOG3*, and *LOG4* in roots. Five-day-old wild-type (B) and *sog1-1* (C) seedlings were transferred onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin for seven days. Total RNA was extracted from roots. The mRNA levels were normalized to that of *ACTIN2*, and are indicated as relative values, with that of the control set to 1. Data are presented as means ± SE (n =

3). Significant differences between treated seedlings and the control were determined with the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 8. Cytokinin signaling is important for inhibition of LR formation in response to DNA damage. (A–C) LR phenotype of cytokinin mutant seedlings. Five-day-old wild-type (WT), *ipt3-2;5-1;7-1* (A), *arr1-3;12-1* (B), and *35S::CKX1* (C) seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin), and grown for an additional seven days. Arrowheads indicate the positions of the LR along the primary roots. Bar = 0.5 cm. (D–F) LR density of WT, *ipt3-2;5-1;7-1* (D), *arr1-3;12-1* (E), and *35S::CKX1* (F) after transfer onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin. Data are presented as means ± SE ($n > 20$). Significant differences between treated seedlings and the WT control were determined with the Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$.

Figure 1

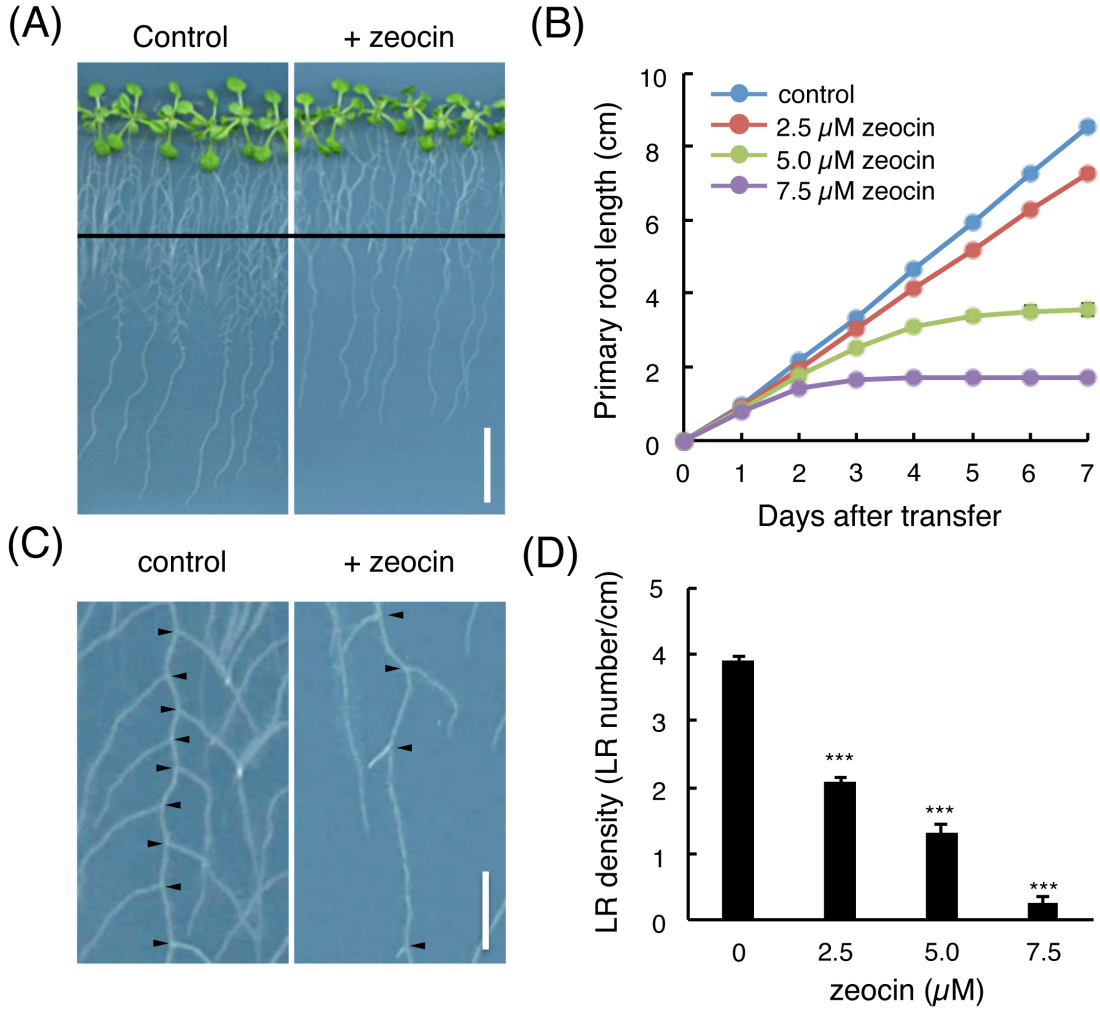

(A)
Control
+ zeocin
(B)
control
2.5 μM zeocin
5.0 μM zeocin
7.5 μM zeocin
Primary root length (cm)
Days after transfer
(C)
control
+ zeocin
(D)
LR density (LR number/cm)
zeocin (μM)
***

Figure 2

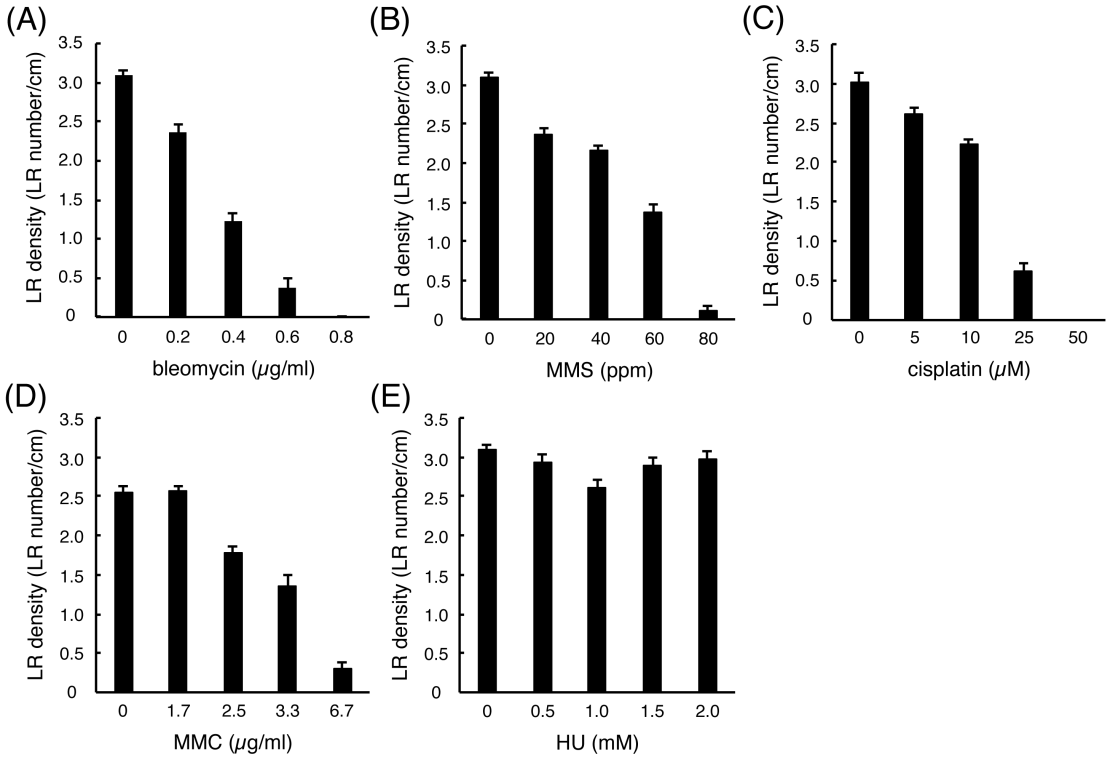

(A)
LR density (LR number/cm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
0.2
0.4
0.6
0.8
bleomycin (μg/ml)
(B)
LR density (LR number/cm)
0
20
40
60
80
MMS (ppm)
(C)
LR density (LR number/cm)
0
5
10
25
50
cisplatin (μM)
(D)
LR density (LR number/cm)
0
1.7
2.5
3.3
6.7
MMC (μg/ml)
(E)
LR density (LR number/cm)
0
0.5
1.0
1.5
2.0
HU (mM)

Figure 3

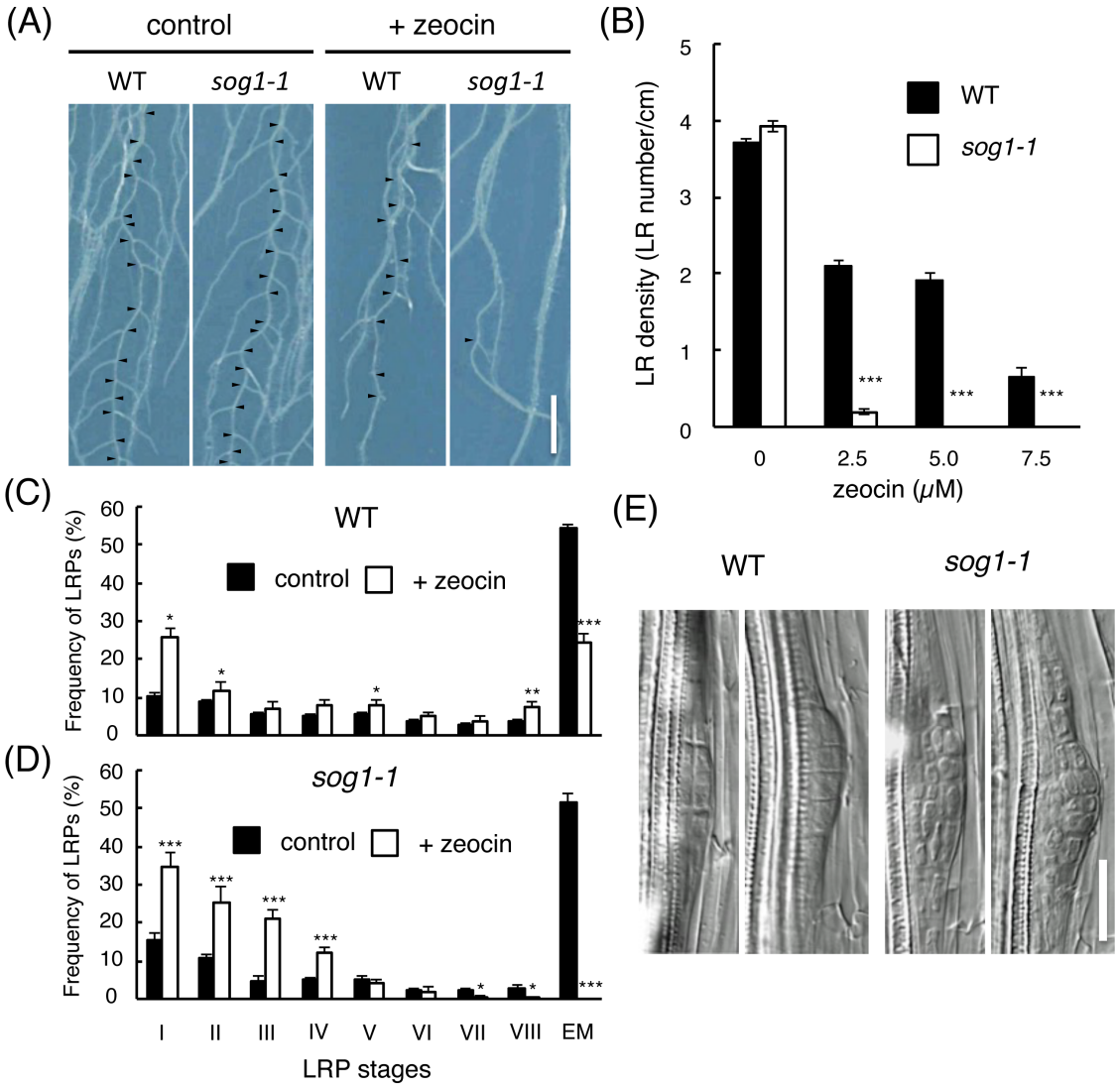

(A)
control
+ zeocin
WT
sog1-1
WT
sog1-1
(B)
LR density (LR number/cm)
WT
sog1-1
zeocin (μM)
0
2.5
5.0
7.5
(C)
WT
control
+ zeocin
Frequency of LRPs (%)
(D)
sog1-1
control
+ zeocin
Frequency of LRPs (%)
I
II
III
IV
V
VI
VII
VIII
EM
LRP stages
(E)
WT
sog1-1

Figure 4

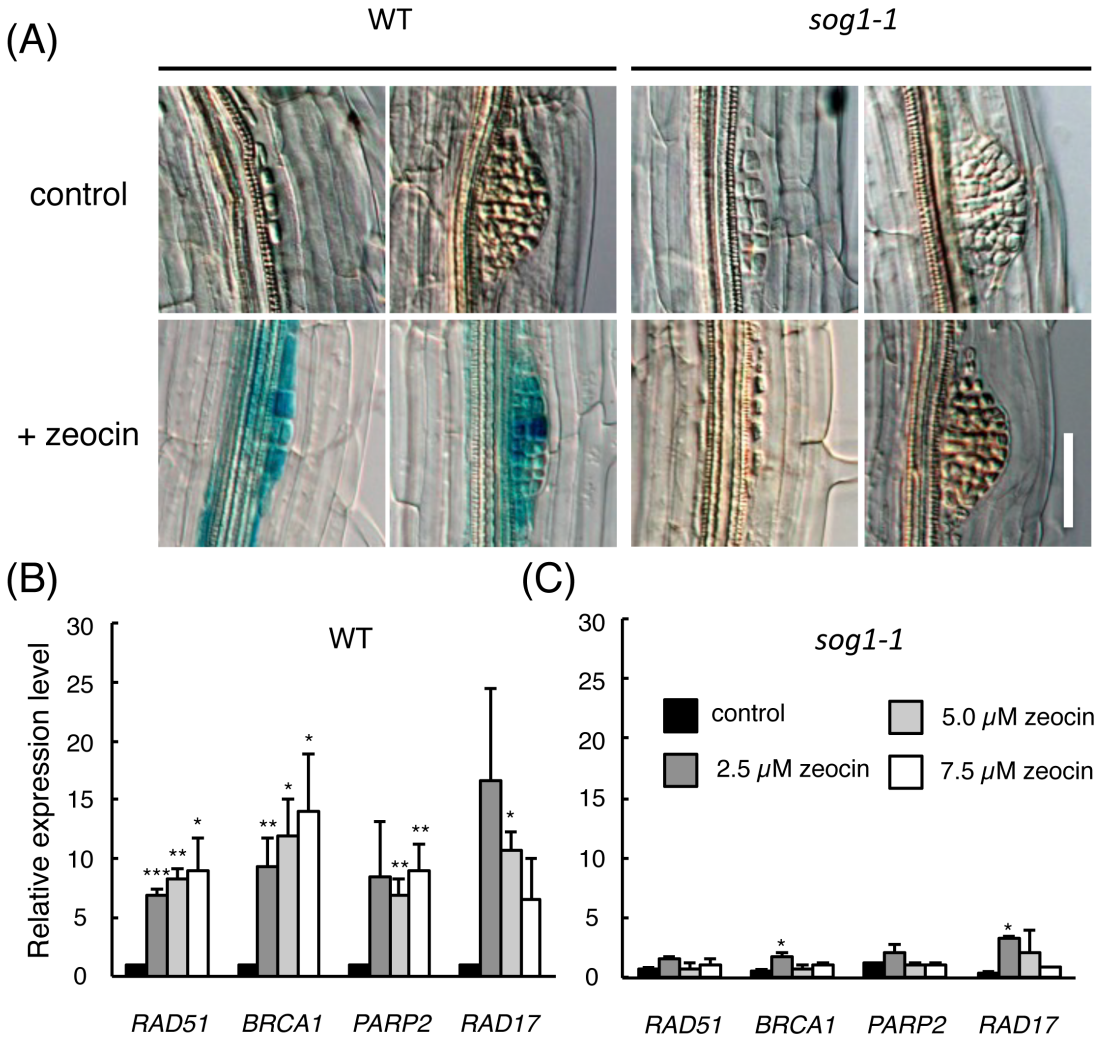

(A)
WT
sog1-1
control
+ zeocin
(B)
WT
Relative expression level
30
25
20
15
10
5
0
RAD51
BRCA1
PARP2
RAD17
(C)
sog1-1
control
2.5 μM zeocin
5.0 μM zeocin
7.5 μM zeocin
RAD51
BRCA1
PARP2
RAD17

Figure 5

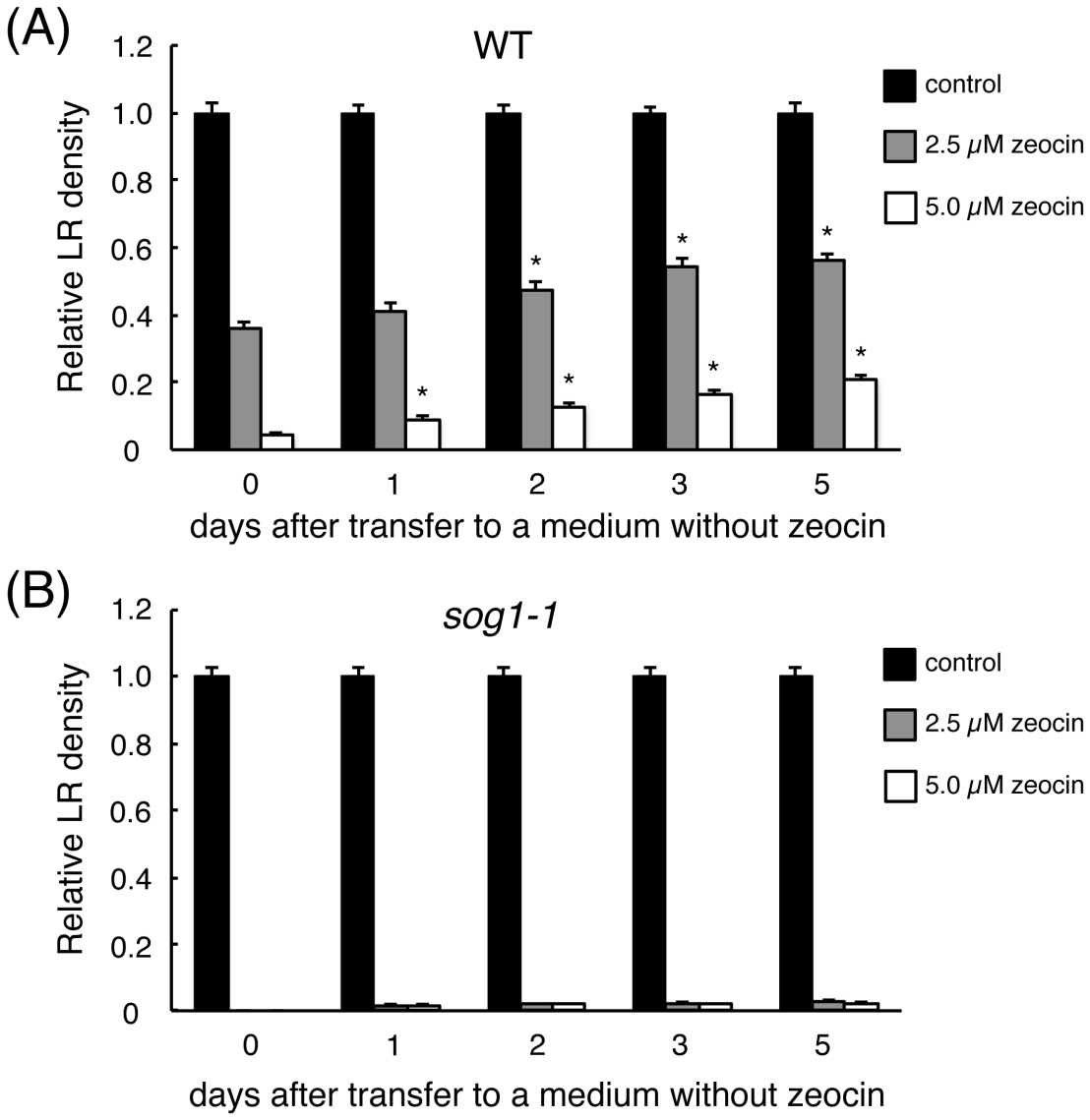

(A)
WT
Relative LR density
1.2
1.0
0.8
0.6
0.4
0.2
0
control
2.5 μM zeocin
5.0 μM zeocin
*
0
1
2
3
5
days after transfer to a medium without zeocin
(B)
sog1-1
Relative LR density
1.2
1.0
0.8
0.6
0.4
0.2
0
control
2.5 μM zeocin
5.0 μM zeocin
0
1
2
3
5
days after transfer to a medium without zeocin

Figure 6

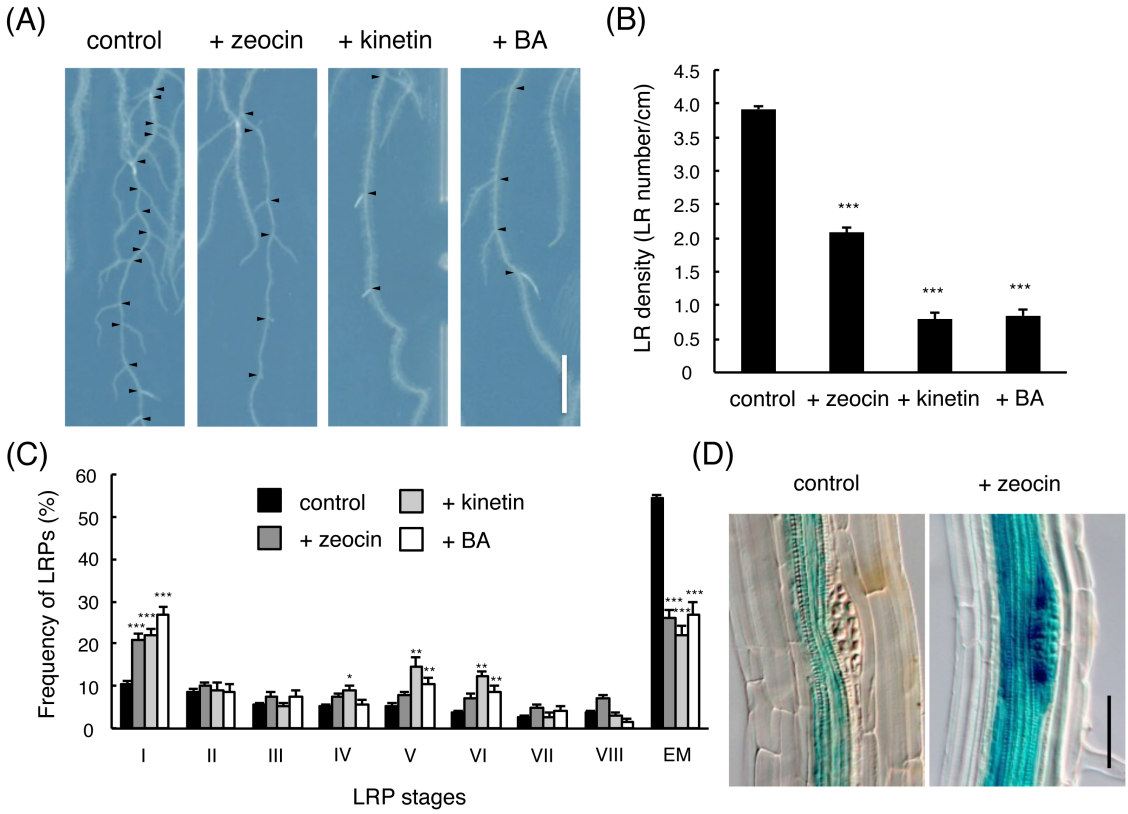

(A)
control
+ zeocin
+ kinetin
+ BA
(B)
LR density (LR number/cm)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
***
***
***
control
+ zeocin
+ kinetin
+ BA
(C)
Frequency of LRPs (%)
60
50
40
30
20
10
0
control
+ zeocin
+ kinetin
+ BA
I
II
III
IV
V
VI
VII
VIII
EM
LRP stages
(D)
control
+ zeocin

Figure 7

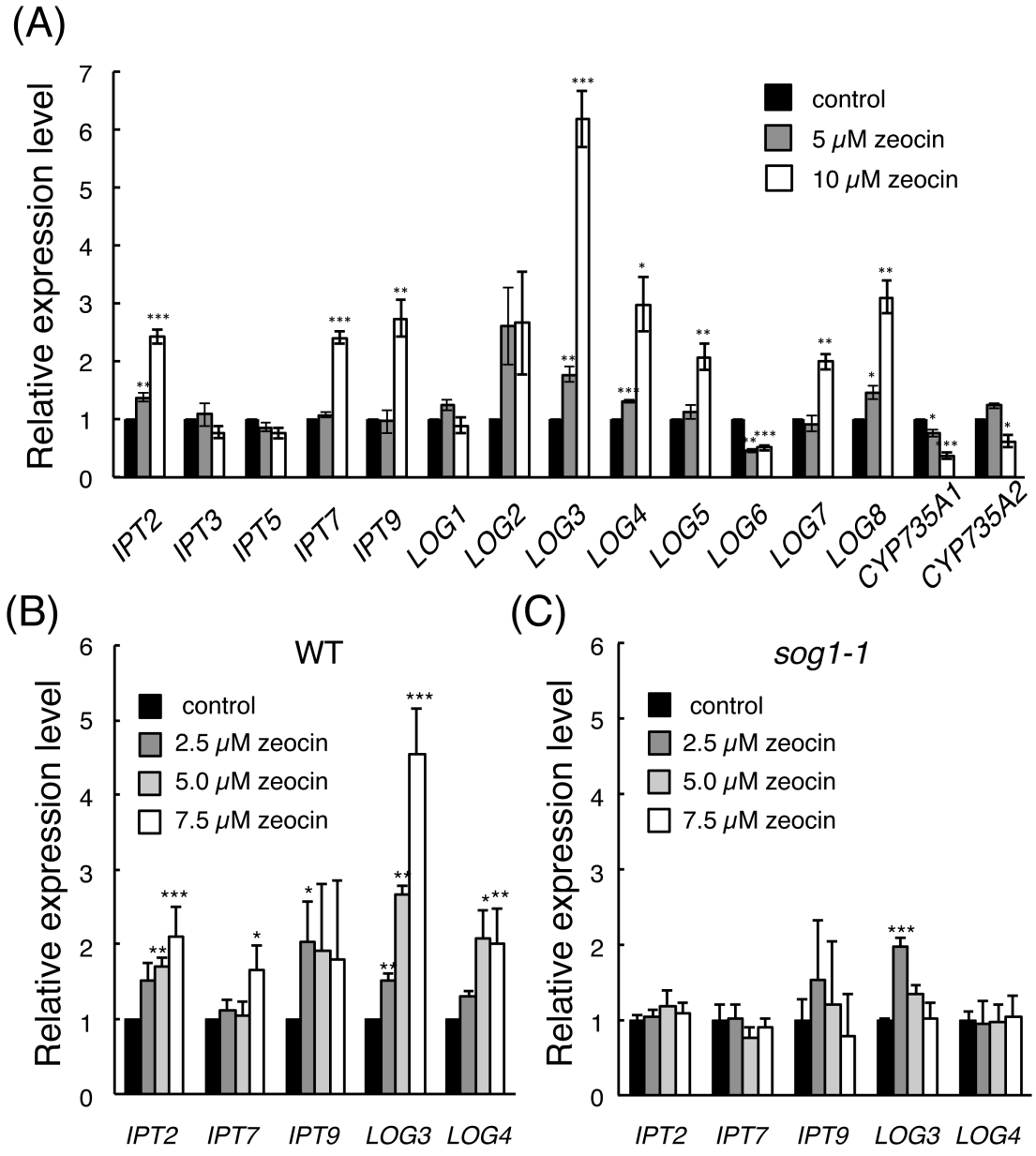

(A)
Relative expression level
control
5 μM zeocin
10 μM zeocin
IPT2
IPT3
IPT5
IPT7
IPT9
LOG1
LOG2
LOG3
LOG4
LOG5
LOG6
LOG7
LOG8
CYP735A1
CYP735A2
(B)
WT
control
2.5 μM zeocin
5.0 μM zeocin
7.5 μM zeocin
IPT2
IPT7
IPT9
LOG3
LOG4
(C)
sog1-1

Figure 8

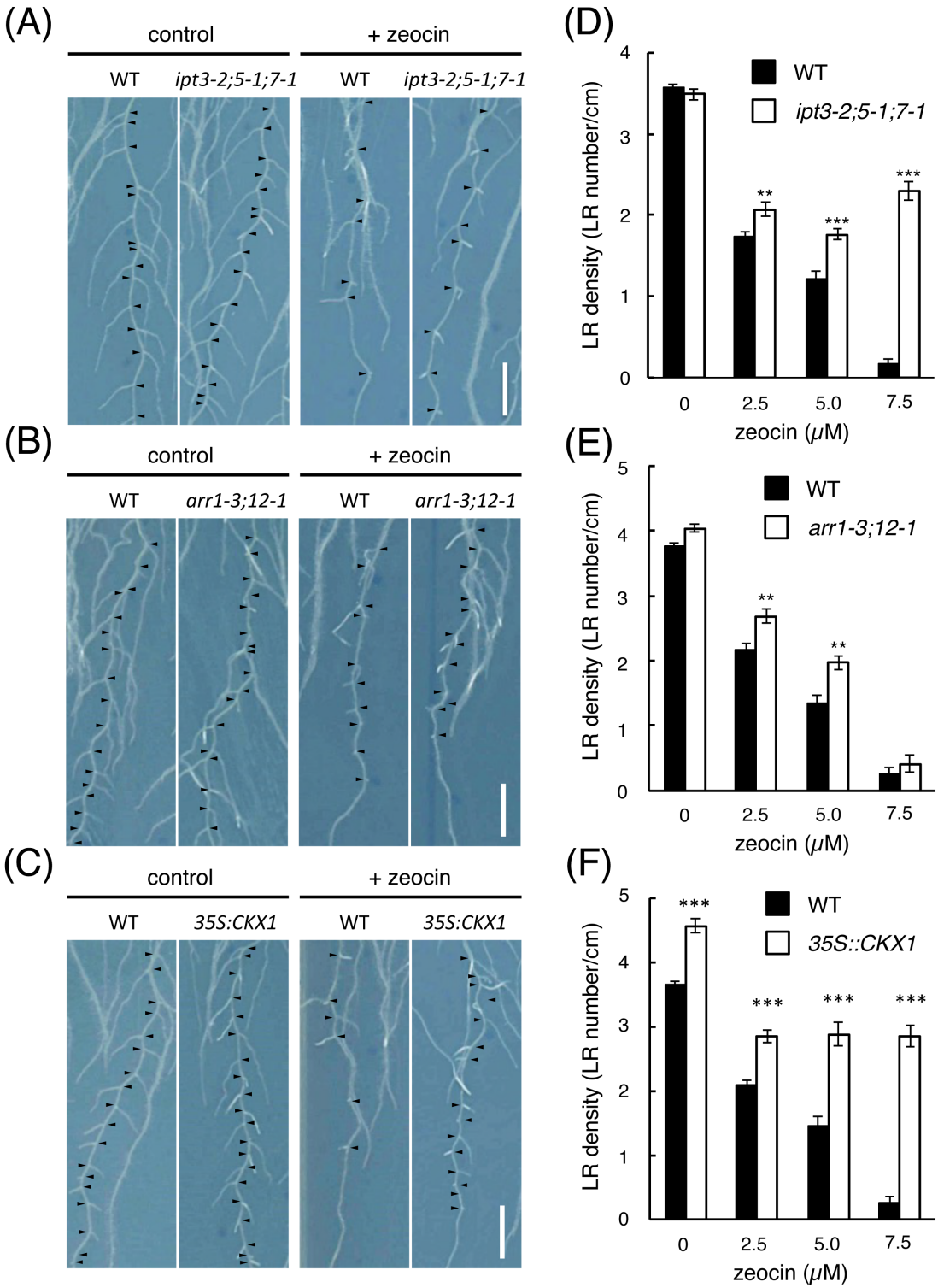

(A)
control
+ zeocin
WT
ipt3-2;5-1;7-1
WT
ipt3-2;5-1;7-1
(B)
control
+ zeocin
WT
arr1-3;12-1
WT
arr1-3;12-1
(C)
control
+ zeocin
WT
35S:CKX1
WT
35S:CKX1
(D)
WT
ipt3-2;5-1;7-1
LR density (LR number/cm)
zeocin (μM)
0
2.5
5.0
7.5
(E)
WT
arr1-3;12-1
LR density (LR number/cm)
zeocin (μM)
(F)
WT
35S::CKX1
LR density (LR number/cm)
zeocin (μM)

Figure S1. Primary root growth of wild-type seedlings treated with various DNA damaging agents. Five-day-old wild-type seedlings were transferred onto control medium or medium containing the indicated concentrations of bleomycin (A), methanesulfonate (MMS) (B), cisplatin (C), mitomycin C (MMC) (D), or hydroxyurea (HU) (E), and primary root length was measured. Data are presented as means ± SE (n > 14).

Figure S2. Primary root growth of zeocin-treated *sog1-1* seedlings. (A) Five-day-old wild-type (WT) and *sog1-1* seedlings were transferred onto control medium (control) or medium containing 2.5 μM zeocin (+ zeocin) and grown for an additional seven days. Black lines indicate the positions of the root tips when the seedlings were transferred onto each medium. Bar = 2 cm. (B, C) Primary root growth of wild-type and *sog1-1*. Root length of wild-type (A) and *sog1-1* (B) seedlings. Five-day-old seedlings were transferred onto control medium or medium containing 2.5, 5.0, or 7.5 μM zeocin. Root length was measured. Data are presented as means ± SE (n > 20).

Figure S3. Stage distribution of LRP in *atm-2* and *atr-2*. Five-day-old *atm-2* (A) and *atr-2* (B) seedlings were transferred onto control medium or medium containing 2.5 μM zeocin, and grown for an additional seven days. Data are presented as means ± SE (n > 10). Significant differences between treated seedlings and the untreated control were determined with the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure S4. Transcript levels of DNA repair-related genes in *ipt3-2;5-1;7-1*. Five-day-old wild-type (A) and *ipt3-2;5-1;7-1* (B) seedlings were transferred onto control medium or medium containing 2.5 or 5.0 μM zeocin and grown for seven days. Total RNA was extracted from roots. The mRNA levels were normalized to that of *ACTIN2*, and are indicated as relative values, with that of the control set to 1. Data are presented as means ± SD (n = 3). Significant differences between treated seedlings and the control were determined with the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure S5. Model for ATM/SOG1-mediated maintenance of genome integrity in LRP. DSBs activate the ATM sensor kinase, which then phosphorylates and activates SOG1. Activated SOG1 differentially induces the expression of DNA repair genes and cytokinin biosynthesis genes, thereby repairing damaged DNA and inhibiting cell division in LRPs. DSBs, DNA double-strand breaks; LRPs, lateral root primordium.

Table S1. Transcript levels of cytokinin signaling genes after gamma ray irradiation

AGI	Description	Culligan *et al.* (2006)	Yoshiyama *et al.* (2009)
AT1G27320	*AHK3*	0.79	0.84
AT3G29350	*AHP2*	1.16	1.03
AT5G39340	*AHP3*	1.11	1.14
AT3G16360	*AHP4*	1.01	1.06
AT1G03430	*AHP5*	1.72	1.31
AT3G16857	*ARR1*	0.83	0.61
AT4G16110	*ARR2*	0.94	0.88
AT4G31920	*ARR10*	0.79	0.91
AT1G67710	*ARR11*	0.98	0.63
AT2G25180	*ARR12*	0.92	0.76
AT2G01760	*ARR14*	0.88	0.94

Five-day-old wild-type seedlings were irradiated at 100 Gy and harvested 1.5 h after the end of the irradiation period. The numbers shown are normalized fold changes with respect to unirradiated plants. Data were obtained from Culligan *et al.* (2006) and Yoshiyama *et al.* (2009).

Table S2. Overview of the transcriptionally induced core DNA repair genes in wild-type, *atm-2, atr-2,* and *sog1-1* after gamma ray irradiation

		Culligan et al. (2006)			Yoshiyama et al. (2009)	
AGI	Description	WT	*atr-2*	*atm-2*	WT	*sog1-1*
AT4G21070	*AtBRCA1*	250.79	226.01	3.16	57.79	0.97
AT5G20850	*AtRAD51*	58.10	54.70	1.57	31.20	1.38
AT3G19210	*AtRAD54*	2.12	2.29	0.95	2.36	1.20
AT5G40840	*SYN2*	56.90	62.41	1.63	27.47	1.06
AT5G24280	*GMI1*	58.51	47.33	1.18	42.83	1.18
AT2G31320	*AtPARP1*	24.60	22.69	1.16	9.97	1.07
AT4G02390	*AtPARP2*	70.35	55.26	1.44	59.32	1.52

Five-day-old seedlings were irradiated at 100 Gy and harvested 1.5 h after the end of the irradiation period. The numbers shown are normalized fold changes with respect to unirradiated plants. Data were obtained from Culligan *et al.* (2006) and Yoshiyama *et al.* (2009).

Table S3. Primers used for qRT-PCR

Genes	Primer sequences
ACT2	5’- CTGGATCGGTGGTTCCATTC -3’
	5’- CCTGGACCTGCCTCATCATAC -3’
IPT2	5’- AGGCTCCTTCGTCGTCAA -3'
	5’- CCATGATTCTTCAGATTTGCTTAATA -3'
IPT3	5’- CGGGTTCGTGTCTGAGAGAG -3’
	5’- CTGACTTCCTCAACCATTCCA -3’
IPT5	5’- AGTTACAGCGATGACCACCA -3’
	5’- GGCAGAGATCTCCGGTAGG -3’
IPT7	5’- ACTCCTTTGTCTCAAAACGTGTC -3’
	5’- TGAACACTTCTCTTACTTCTTCGAGT -3’
IPT9	5’- TGGATTGTATCTGCGATGGTT -3’
	5’- TGGGCCTCAGCGATAACTT -3’
LOG1	5’- GAACTCGGAACCGAACTGG -3’
	5’- TCAAACCCATTAAACCAATGC -3’
LOG2	5’- TTTGAAGAGTTGTTGGAAGTCATC -3’
	5’- TCCATCCACGTTCAATAGTCC -3’
LOG3	5’- TGATGCTTTTATTGCCTTACCA -3’
	5’- CCACCGGCTTGTCATGTAT -3’

LOG4	5’- GTTTGATGGGTTTGGTTTCG -3’ 5’- CACCGGTCAACTCTCTAGGC -3’
LOG5	5’- ATGGGTTTGGTCTCACAAGC -3’ 5’- CTCCGGTTATCTCTTTGTCCA -3’
LOG6	5’- CAATGGGAACAAAGCTAGTTATCAA -3’ 5’- AAGATCAATCTTCCTCATCATCACCA -3’
LOG7	5’- CATGTTCTAGGGGTCATTCCA -3’ 5’- CTCCGATGGTCTCACCAGTT -3’
LOG8	5’- ATTGCACTCCCTGGAGGTTA -3’ 5’- CCCATCAACATTCAATAGACCA -3’
CYP735A1	5’- GGCCTTCCCTCAGTCGAT -3’ 5’- TTCAAATGCCATCCTTGGTAG -3’
CYP735A2	5’- GAACAGCTCTCAAGTCTTACTTCGT -3’ 5’- TCAAATGCCATTCTTGGTAAAA -3’
RAD17	5’- CTAGTGCGACTCAAGAAGAC -3’ 5’- GCCTGTATTTGTCAACCCAC -3’
RAD51	5’- GATCACGGGAGCTCGATAAA -3’ 5’- GCGGAACTCACCATATAACTCTG -3’
BRCA1	5’- TCTTGCTCAGGGCTCACAGTTGAAG -3’ 5’- TTTCCCCTCCAAGATTGCCATCATG -3’
PARP2	5’- AGCCTGAAGGCCCGGGTAACA -3’

5’- GCTGTCTCAGTTTTGGCTGCCG -3’

Figure S1

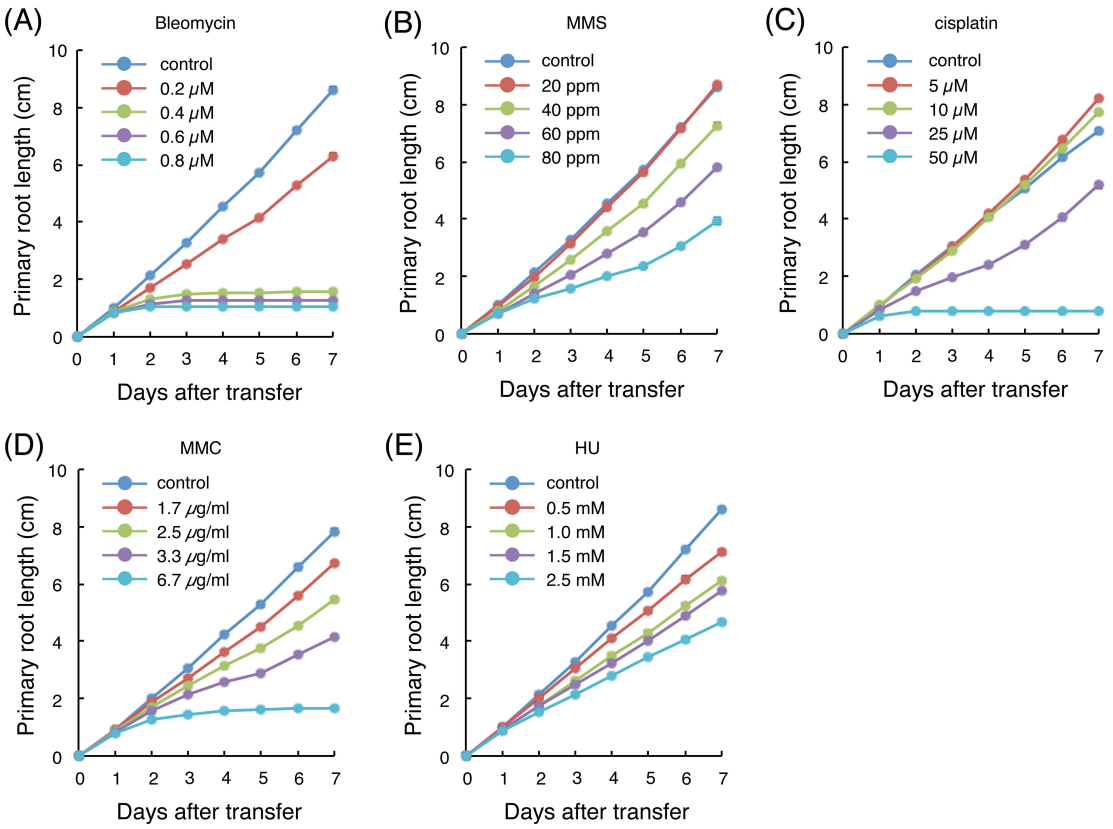

(A)
Bleomycin
control
0.2 μM
0.4 μM
0.6 μM
0.8 μM
Primary root length (cm)
Days after transfer
(B)
MMS
control
20 ppm
40 ppm
60 ppm
80 ppm
Primary root length (cm)
Days after transfer
(C)
cisplatin
control
5 μM
10 μM
25 μM
50 μM
Primary root length (cm)
Days after transfer
(D)
MMC
control
1.7 μg/ml
2.5 μg/ml
3.3 μg/ml
6.7 μg/ml
Primary root length (cm)
Days after transfer
(E)
HU
control
0.5 mM
1.0 mM
1.5 mM
2.5 mM
Primary root length (cm)
Days after transfer

Figure S2

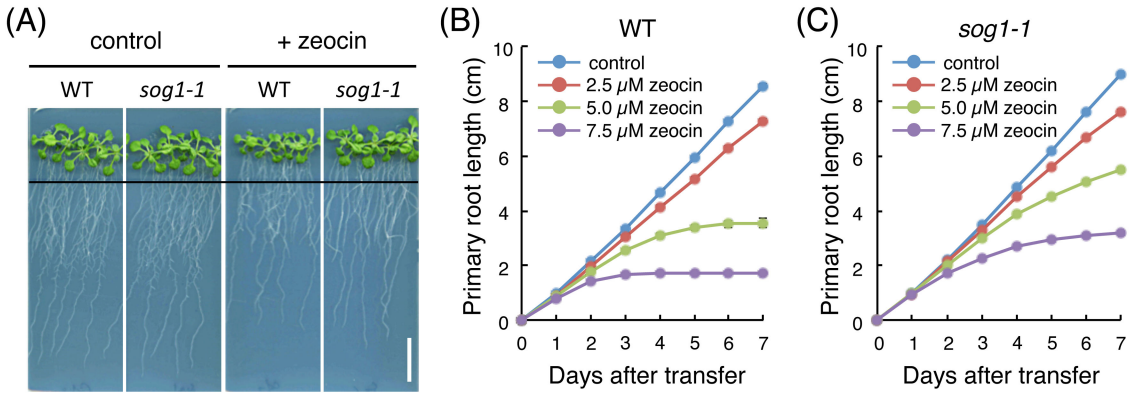

(A)
control
+ zeocin
WT
sog1-1
WT
sog1-1
(B)
WT
Primary root length (cm)
control
2.5 μM zeocin
5.0 μM zeocin
7.5 μM zeocin
Days after transfer
(C)
sog1-1
Primary root length (cm)
control
2.5 μM zeocin
5.0 μM zeocin
7.5 μM zeocin
Days after transfer

Figure S3

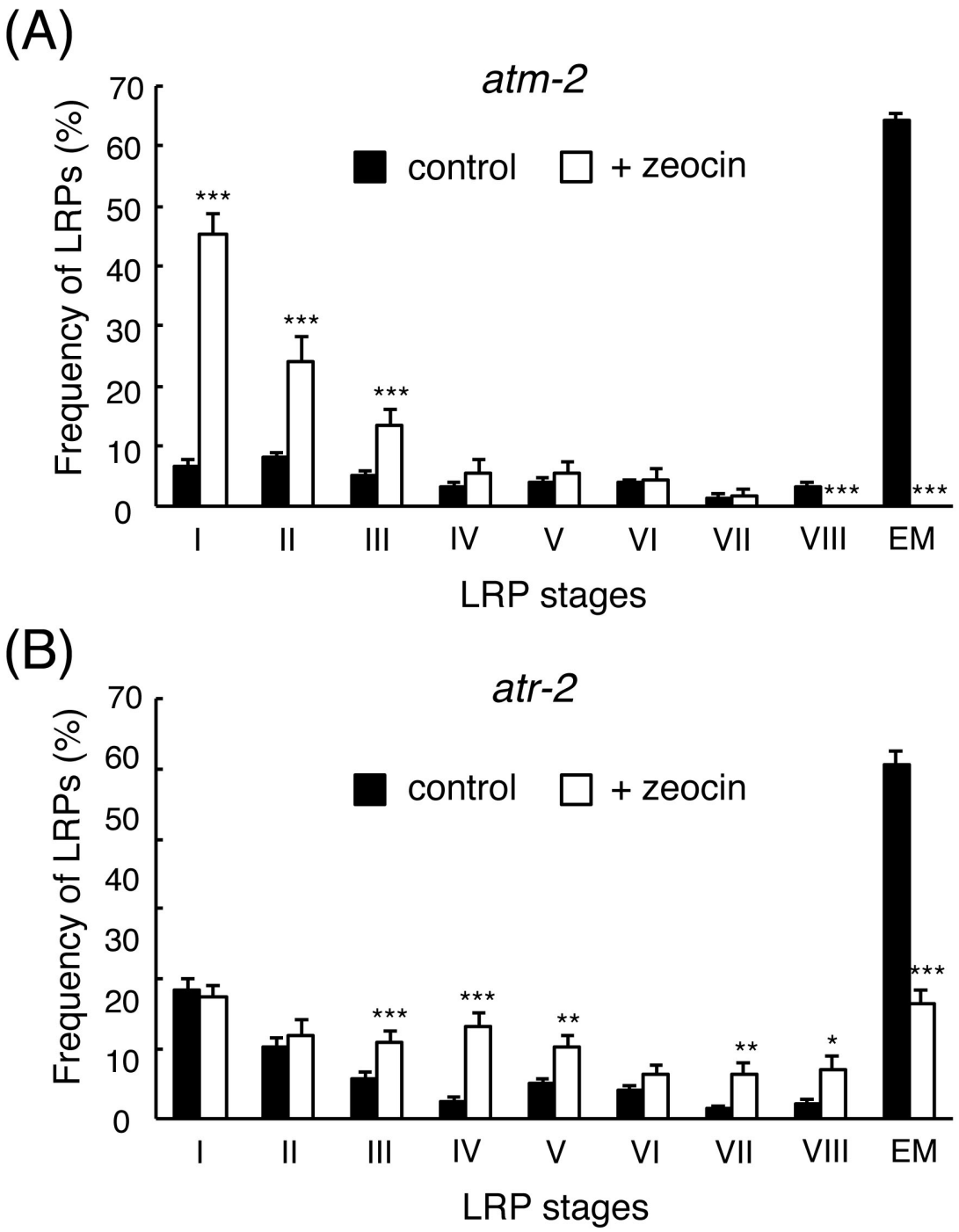

(A)
atm-2
control
+ zeocin
Frequency of LRPs (%)
70
60
50
40
30
20
10
0
***
***
***
***
***
I
II
III
IV
V
VI
VII
VIII
EM
LRP stages
(B)
atr-2
control
+ zeocin
Frequency of LRPs (%)
70
60
50
40
30
20
10
0
***
***
**
**
*
***
I
II
III
IV
V
VI
VII
VIII
EM
LRP stages

Figure S4

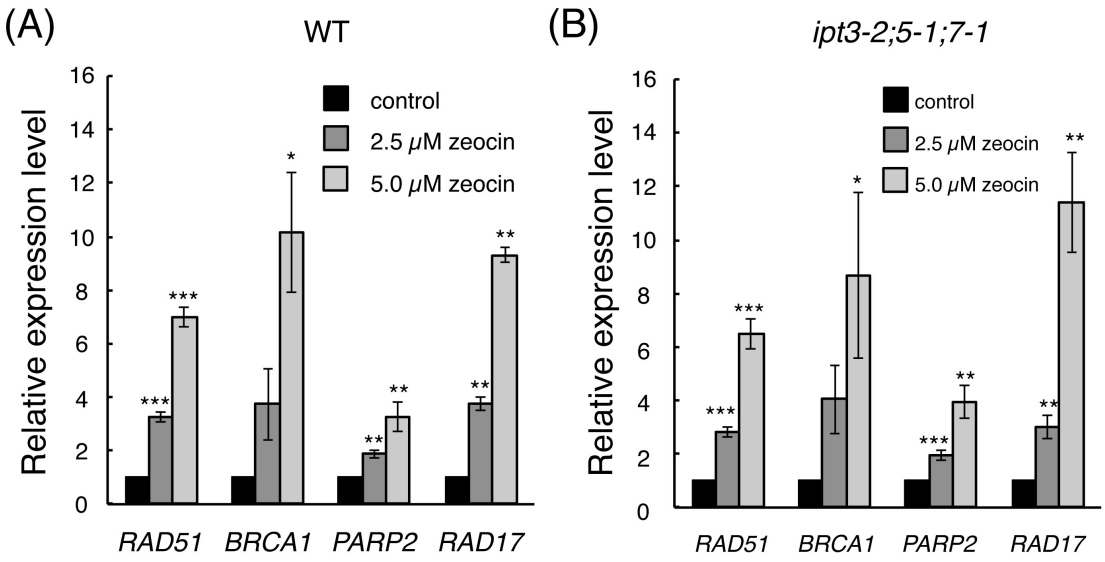
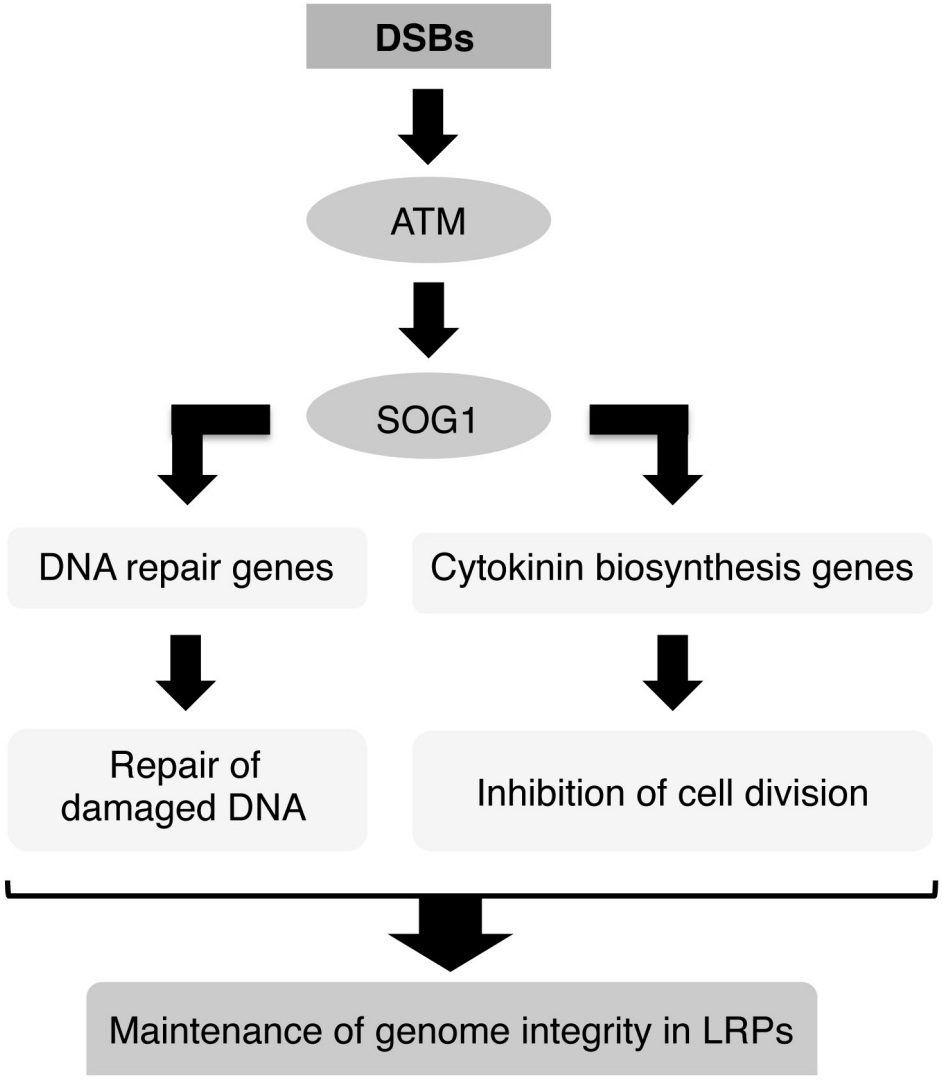

(A)
WT
(B)
ipt3-2;5-1;7-1
Relative expression level
control
2.5 μM zeocin
5.0 μM zeocin
RAD51
BRCA1
PARP2
RAD17

Figure S5


DSBs
ATM
SOG1
DNA repair genes
Cytokinin biosynthesis genes
Repair of damaged DNA
Inhibition of cell division
Maintenance of genome integrity in LRPs