# Studies on DNA damage-induced stem cell death in *Arabidopsis thaliana*

（シロイヌナズナの DNA 損傷に応答した幹細胞特異的な細胞死の誘導機構の研究）

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## Abbreviations

Act-MYB : activator-MYB

AFB : AUXIN-RELATED F-BOX

ANAC : Arabidopsis NAC

APC/C : anaphase-promoting complex/cyclosome

ARE : auxin responsive element

ARF : AUXIN RESPONSIVE FACTOR

ARR1 : ARABIDOPSIS RESPONSE REGULATOR 1

ATM : ATAXIA TELANGIECTASIA MUTATED

ATR : ATAXIA TELANGIECTASIA MUTATED AND RAD3-RELATED

ATRIP : ATR Interacting Protein

AUX1 : AUXIN RESISTANT1

BLM : bleomycin

BRCA1 : BREAST CANCER SUSCEPTIBILITY1

BY-2 : bright yellow-2

CBF1 : COLD BINDING FACTOR 1

CCS52 : CELL CYCLE SWITCH52

CDK : cyclin-dependent kinase

CSC : columella stem cells

CSCD : columella stem cell daughters

DDR : DNA damage response

Dex : dexamethasone

DNA : deoxyribonucleic acid

DREB : DROUGHT RESPONSIVE ELEMENT BINDING

DSB : double-strand break

ERF115 : ETHYLENE RESPONSIVE FACTOR 115

ETI : effector-triggered immunity

FAS1 : FASCIATA 1

G1 phase : Gap 1 phase

G2 phase : Gap 2 phase

GFP : green fluorescent protein

GH3 : GRETCHEN HAGEN 3

GR : glucocorticoid receptor

GR2 : GLUTATHIONE REDUCTASE 2

HR : homologous recombination

HU : hydroxyurea

IAA : indole-3-acetic acid

LAX1 : LIKE-AUX1

M phase : mitotic phase

MMS : methyl methane sulphonate

MRN : MRE11-RAD50-NBS1

MS : Murashige and Skoog

NAC : NAM/ATAF/CUC

PARP : POLY(ADP-RIBOSE) POLYMERASE

PAT : polar auxin transport

PCD : programmed cell death

PEO-IAA : α-(phenylethyl-2-oxo)-IAA

PI : propidium iodide

PIN : PIN-FORMED

PLT : PLETHORA

PSK : phytosulfokine

PSK5 : PHYTOSULFOKINE 5

QC : quiescent center

RAD51 : RAD51 recombinase

RAM : root apical meristem

Rep-MYB : repressor-MYB

ROS : reactive oxygen species

RPA : replication protein A

S phase : synthesis phase

SCF-E3 : Skp, Cullin, F-box containing E3 ubiquitin ligase

SCN : stem cell niche

SCR : SCARECROW

SD : standard deviation

SE : standard error of the mean

SHR : SHORTROOT

SHY2 : SHORT HYPOCOTYL 2

SIMR : stress-induced morphogenic responses

SMR : SIAMESE-RELATED

SOG1 : SUPPRESSOR OF GAMMA RESPONSE 1

SQ : serine-glutamine

SSB : single-strand break

TAC : transit amplifying cells

TIR1 : TRANSPORT INHIBITOR RESPONSE 1

TPL : TOPLESS

UV : ultraviolet

*uvh1* : *ultraviolet hypersensitive 1*

WEE1 : WEE1 kinase

WOX5 : WUSCHEL RELATED HOMEOBOX 5

# ABSTRACT

| | Plant Growth Regulation（Masaaki Umeda） | | |
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To avoid accumulation of DNA lesions, plants have evolved robust systems for DNA damage signaling and DNA repair. Plants induce multiple responses to DNA damage, such as DNA repair, cell cycle arrest, early onset of endoreplication and stem cell death. Previous studies showed that DNA double-strand break (DSB)-induced stem cell death was observed not only in the root tip but also in shoot apices. They also showed that stem cell death is caused in a programmed manner through the ATM/ATR-SOG1 signaling pathway. However, the downstream signaling controlling the stem cell death is still unknown. In this study, I aimed to understand possible mechanisms involved in the stem cell death caused by DNA damage.

Since auxin highly accumulates in the root tip where stem cell death occurs, I investigated the effect of DNA damage on auxin signaling using the auxin signaling output marker *DR5v2:3xVENUS.* The result showed that *DR5v2:3xVENUS* expression in stem cells was reduced by DNA damage. To further understand the role of auxin signaling in the induction of stem cell death, I treated wild-type plants with the DSB inducer bleomycin and IAA and found that the induction of stem cell death was delayed than in the plants treated with bleomycin alone. In contrary, when plants were treated with bleomycin and the auxin antagonist PEO-IAA, induction of stem cell death occurred earlier than the plants treated with bleomycin alone. These results suggest that induction of stem cell death by DNA damage is associated with a reduction of auxin signaling. Moreover, when I examined the expression of DNA damage-inducible genes, I found that their induction by bleomycin treatment was suppressed by simultaneous application with IAA. In contrary, the induction was enhanced in the plants treated with bleomycin and PEO-IAA, suggesting that auxin signaling level is reversely correlated with the strength of DNA damage response. To clarify whether auxin affects the level of DNA damage or the DNA damage signaling, I checked the amount of DNA damage by the comet assay, and found that plants treated with bleomycin and IAA together had lower amount of DNA damage than plants treated with bleomycin alone. Additionally, plants treated with bleomycin and PEO-IAA together had higher amount of DNA damage than plants treated with bleomycin alone. These results suggest that auxin

protects the genome from DNA damage, and that DNA damage suppresses the auxin signaling, thereby causing a higher level of DNA damage.

Further, I found that the two Aux/IAA family genes, *IAA5* and *IAA29,* were induced by DSBs around the stem cell region in an ATM/ATR- and SOG1-dependent manner. To understand the role of IAA5 and IAA29 in the induction of stem cell death, I observed the *iaa5 iaa29* double mutant under DNA damage conditions and found no significant difference in the induction of stem cell death between *iaa5 iaa29* and wild-type. I then generated transgenic plants harboring *proWOL:XVE>>mIAA5(P59L):GFP*, which expresses a dominant mutant form of IAA5 in the stele including vascular stem cells in the root meristem. However, the result showed that there was no significant difference in the stem cell death between wild-type and transgenic lines. These results suggest that induction of *IAA5* and *IAA29* is not sufficient to reduce the auxin signaling, and/or that other factors involved in auxin-independent pathways are required to cause stem cell death under DNA damage conditions.

Recently Umeda's lab has reported that the ATM/ATR-SOG1 pathway is required for the induction of the other NAC-type transcription factors *ANAC044* and *ANAC085*, and that DNA damaged-induced G2 arrest is suppressed in the *anac044-1 anac085-1* double mutant. To understand whether ANAC044 and ANAC085 are also required for stem cell death induced by DNA damage, *anac044-1 anac085-1* was observed under DNA damage conditions. I found that stem cell death was significantly delayed in *anac044-1 anac085-1.* To further study the role of ANAC044 and ANAC085 in induction of stem cell death, I observed plants overexpressing *ANAC044* or *ANAC085* under genotoxic stress conditions. My result showed that the overexpression lines had more stem cell death than wild-type, suggesting ANAC044 and ANAC085 is required for cell death induction. Since exogenous application of IAA could suppress DNA damage-induced stem cell death, I next tested whether exogenous IAA could reduce the DNA damage sensitivity of transgenic lines overexpressing *ANAC044* and *ANAC085*. These data showed that IAA could not completely suppress stem cell death, and that the amount of stem cell death was still significantly higher in plants overexpressing *ANAC044* than that in wild-type. This suggests that ANAC044 and ANAC085 play a crucial role in DNA damage-mediated induction of stem cell death, and that this regulation is independent from the auxin-mediated pathway controlling the genome stability.

# General Introduction

## DNA damage

Maintaining genomic integrity is important not only for plants but also for all the organisms to transmit genomic information correctly to the offspring. As with other eukaryotes, plants have highly efficient genome replication and gamete formation processes, allowing accurate genomic information to be transmitted to the offspring. However, during plant growth and development, nucleotide mismatches, base insertions and deletions, and chemical modification of bases sometimes occur. Accumulation of these mutations across generations could lead to genome instability and threaten the survival of the species (Aguilera and Garcia-Muse, 2013).

DNA damage in plants is caused by both endogenous and exogenous factors. The major endogenous factors causing DNA damage are dysfunctions during DNA replication such as low replication-initiation density, untimely initiation and faulty fork progression (Aguilera and Garcia-Muse, 2013). In plants, apart from the failure of replication, the reactive oxygen species (ROS) generated during photosynthesis and other endogenous metabolic processes could damage the DNA (Hu et al., 2016). Plants being sessile organisms are constantly exposed to multiple biotic and abiotic stresses that affect growth and development. Exposure of plants to ultraviolet (UV) rays or high intensity light induces DNA damage in the form of cyclobutane pyrimidines (Rastogi et al., 2010). Recent studies have shown that stresses such as pathogen infection (Song and Bent, 2014), heavy metal stress (Achary and Panda, 2010), drought (Shim et al., 2018) and chilling (Hong et al., 2017) induce DNA damage, and DNA damage response (DDR) partially contribute to growth and developmental defects induced by these stresses.

**Sensing of DNA damage by ATM and ATR**

The initial step in DNA damage response is the recognition of damaged DNA. All eukaryotes possess conserved kinases known as ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM- AND RAD3-RELATED (ATR) (Marechal and Zou, 2013). ATM is activated in response to double-strand breaks (DSBs), whereas ATR is activated mainly in response to single-strand breaks (SSBs) and replication stress (Marechal and Zou, 2013). In mammalian cells, MRE11-RAD50-NBS1 (MRN) complex brings ATM to the damaged DNA, which activates ATM by auto-phosphorylation (Yoshiyama et al., 2013b). ATM then activates several downstream factors by phosphorylating them (Marechal and Zou, 2013) (Figure 1). In case of SSBs, replication protein A (RPA) is loaded onto the damaged SSB sites, which recruits ATRIP (ATR-Interacting Protein) and then ATR is recruited to the damaged site (Hu et al., 2016). Further interaction with 9-1-1 (RAD9, RAD1, HUS1) and RAD17 RFC activates ATR (Nisa et al., 2019) (Figure 1).

Comparing with mammalian cells, detailed activation mechanisms of ATM and ATR have not been studied well in plants. It has been shown that mutations in *ATM* and *ATR* is lethal in mammalian cells (Culligan and Britt, 2008), while *atm* and *atr* knockout *Arabidopsis* plants grow normally under normal growth conditions (Culligan and Britt, 2008). However, *atm atr* double mutants are sterile because of nuclear fragmentation during meiosis (Culligan and Britt, 2008). As expected from their preferential activation by different DNA damage types, it has been shown that *Arabidopsis atm* mutant is hypersensitive to DSBs inducers like ionizing irradiation and methyl methane sulphonate (MMS) (Culligan et al., 2004), while *atr* is hypersensitive to SSBs and replication stress inducers like hydroxyurea (HU) and aphidicolin

(Culligan et al., 2004). Though different DNA damage types activate ATM and ATR, they share most of the downstream targets. Upon DSBs induction by γ-irradiation, several hundred genes were activated immediately within 1.5 hours, and almost all of them show an ATM dependency (Culligan et al., 2004). At the same time, ATR plays a role in sustaining the expression of many genes that were induced in an ATM-dependent manner, especially the cell cycle regulators at the later time points (Culligan et al., 2004). A recent study using phosphoproteomic approach, by comparing the protein samples from γ-irradiated wild-type and *atm atr* double mutant plants, has identified that phosphorylation status of nearly 461 proteins are modified after γ-irradiation, and 134 of them are dependent on ATM/ATR (Roitinger et al., 2015). Though this study has identified many novel targets of ATM and ATR, it also indicated that phosphorylation status of many proteins is modified in response to irradiation independent of ATM/ATR, suggesting possible involvement other kinases.

One of the common phosphorylation targets of both ATM and ATR is histone H2AX protein. Upon DNA damage, ATM and ATR phosphorylate H2AX and produce gamma-H2AX (γ-H2AX), which acts as a signal post for many chromatin modifications required at the site of DNA damage (Paull et al., 2000). In Arabidopsis, both ATM and ATR are required for the normal phosphorylation of H2AX. It has been observed in *atm* plants that some residual γ-H2AX foci were still present, but in *atm atr* double mutant plants, γ-H2AX foci were completely lost upon treatment with irradiation, which shows that both ATM and ATR are required for normal phosphorylation of H2AX (Friesner et al., 2005). Moreover, mutations in the MRN complex or the RPA protein in *Arabidopsis* showed completely abolished or reduced phosphorylation of H2AX, suggesting that activation of both ATM and ATR is required for normal phosphorylation of H2AX by DNA damage (Friesner et al., 2005).

**SOG1 is the central regulator of DDR signaling downstream of ATM/ATR**

In mammalian cells, ATM and ATR activate p53, which is a central regulator that controls DNA repair, cell cycle arrest and apoptosis (Yoshiyama et al., 2013b). In contrary, those key factors involved in DNA damage response is missing in plants. Instead, a plant specific NAC transcription factor SUPPRESSOR OF GAMMA RESPONSE1 (SOG1), which is the target of ATM and ATR, has been shown to function as the central regulator of DNA damage response in plants (Yoshiyama et al., 2013b). The *sog1* mutant was initially isolated from the mutant population screened for the suppressors of the *uvh1-2* mutant, which is hypersensitive to both UV and γ-irradiation. Upon gamma irradiation, *uvh1-2* mutant exhibits a delay in producing true leaves caused by cell cycle arrest, while *uvh1-2 sog1-1* double mutant produces true leaves normally (Preuss and Britt, 2003), indicating that SOG1 is required for cell cycle arrest in response to DNA damage. Using map-based cloning approach, it was found that the mutation responsible for the *sog1-1* phenotype was due to a missense mutation in AT1G25580, which changed a highly conserved amino acid in the NAC domain (Yoshiyama et al., 2009). Yoshiyama et al. (2009) further revealed that immediate transcriptional up-regulation by γ-irradiation is dependent on ATM-SOG1 pathway. They showed that SOG1 controls the expression of several cell cycle regulators (*SMR5*, *SMR7*, *CDKB2;1*, and *KNOLLE*), and DNA repair genes (*BRCA1*, *RAD51*, and PARP1), suggesting that SOG1 is involved in cell cycle arrest and DNA repair upon DNA damage (Figure 1). In addition, they showed that SOG1 could function downstream of ATR too. They showed that the cell cycle arrest observed in *uvh1-2*, which leads to the delay in the production of true leaves in *uvh1-2*, is dependent on ATR-SOG1 pathway and independent of ATM-SOG1 pathway.

Upon DSB induction, ATM activates SOG1 by phosphorylating the five serine-glutamine (SQ) motifs present in the C-terminal region of SOG1 (Yoshiyama et al., 2013a). It has been shown that, the number of SQ motifs phosphorylated after the DNA damage determines the strength of DDR. The expression of SOG1 target genes changed incrementally depending on the number of phosphorylated SQ sites, suggesting all five SQ motifs are needed to be phosphorylated for the full activation of SOG1 (Yoshiyama et al., 2017). Later it was shown that, DNA damage-mediated phosphorylation of SOG1 is required for the binding of SOG1 to the promoters of its target genes (Ogita et al., 2018).

Though genetic interaction between ATR and SOG1 has been established already (Yoshiyama et al., 2009；Adachi et al., 2011), evidence for direct activation of SOG1 by ATR is missing. Sjogren et al. (2015) showed that Al-stress activates several cell cycle regulators and DNA damage repair genes through ATR-SOG1 pathway, leading to terminal differentiation of root tip, independent of ATM. Furthermore, they showed that ATR directly binds to and phosphorylates SOG1 *in vitro* (Sjogren et al., 2015).

Recently two studies by Ogita et al. (2018) and Bourbousse et al. (2018) have identified 146 and 310 genes as the direct targets of SOG1, respectively, and the target genes from both the studies were overlapping. The difference in the number of direct targets of SOG1 in both the studies might be due to the experimental design. In case of Ogita et al. (2018), SOG1 targets were identified after treatment with DSB inducer for a short time, whereas Bourbousse et al. (2018), identified the SOG1 targets over a 24hour time period with multiple intermittent sampling. But both the studies concur that SOG1 is a transcriptional activator controlling almost all the transcriptional regulation occurring after DNA damage. These target genes are involved in DNA repair, reduction of CDK activity, and immune response. They have also

identified that significant number of genes were downregulated by DNA damage in a SOG1 dependent manner, but SOG1 does not regulate them directly, suggesting some of the transcription factors regulated by SOG1 might be involved in the repression of genes by DNA damage. Ogita et al. (2018) have also identified a novel function for SOG1 in pathogen response. They showed that SOG1 positively contributed to the immune response against fungal infection. Additionally, they found that SOG1 specifically regulates homologous recombination (HR)-mediated DNA repair.

**DSBs induce stem cell death through ATM/ATR-SOG1 pathway**

It has been well established in mammalian cells, that DNA damage could induce cell death, especially the stem cell death. Depending upon the DNA damage, ATM/ATR activates p53 by phosphorylation to control the transcriptional induction of genes involved in apoptosis. It has been proposed that the DNA repair through error prone non-homologous end joining (NHEJ) pathway could trigger apoptosis in p53-dependent manner. In addition, it has been proposed that cell death could be a protective mechanism to avoid carcinogenesis (Roos and Kaina, 2006).

Plants induce multiple responses to DNA damage, such as DNA repair, cell cycle arrest, early onset of endoreplication and stem cell death (Yoshiyama et al., 2013b). Each response is specifically induced depending on cell types in specific tissues. For example, in *Arabidopsis* roots, DSBs induce G2 arrest in the meristematic zone, an early onset of endoreduplication in the transition zone, and cell death in stem cells (Hefner et al., 2006; Adachi et al., 2011; Curtis and Hays, 2007; Fulcher and Sablowski, 2009).

Curtis and Hays (2007) had shown for the first time in plants that DNA damage could

induce stem cell death. They have shown that replication stress induced by UVB-radiation exposure could induce cell death specifically in vascular stem cells and transit-amplifying cells in Arabidopsis root. Later, Fulcher and Sablowski (2009) had shown that DSBs induced by γ-irradiation and chemicals such as DSBs-inducer zeocin could induce cell death at stem cell niche in both at root and shoot apices. They found that stem cell death in response to DSBs was mediated by ATM/ATR. They observed that dying vascular initials did not show apoptotic features like cell shrinkage, chromatin condensation and nuclear fragmentation, while vascular initials had denser cytoplasm with lot of vesicles resembling autophagic vesicles (Fulcher and Sablowski., 2009). Later, Furukawa et al. (2010) showed that both UVB- and γ-irradiation-induced cell death was dependent on SOG1, and cell death could be induced by either ATM-SOG1 or ATR-SOG1 pathways. They showed that both in *atm* and *atr* single mutants, cell death was delayed but not abolished. The delay in *atr* mutant was comparatively less than *atm* mutant. In addition, cell death was not observed in *sog1* plants (Furukawa et al., 2010), suggesting activation of one of the pathways, either ATM-SOG1 or ATR-SOG1, is enough to induce stem cell death and SOG1 is indispensable.

**Physiological relevance of stem cell death induced by DNA damage**

The reason why plants induce cell death in response to DNA damage, specifically at stem cells and their daughter cells is still unknown. A possible hypothesis is that when DNA damage is less and not so severe, cell cycle is arrested and the damaged DNA is repaired, while when DNA damage is severe, plants induce terminal differentiation or cell death to arrest the passing of the damaged DNA to a daughter cell (Hu et al., 2016). Since stem cells are the source for the mitotically active cells which later becomes differentiated cells, the removal of dead

stem cells and replenishment of stem cells is very important for the continued growth of plants (Heyman et al., 2013) (Figure 2). It has been showed that ETHYLENE RESPONSIVE FACTOR 115 (ERF115), a proteolytic target of CELL CYCLE SWITCH52A2 (CCS52A2), induces the quiescent center (QC) cell division, in a brassinosteroid-dependent manner, through transcriptional activation of *PHYTOSULFOKINE 5* (*PSK5*). Upon DNA damage, ERF115 is stabilized, which induces QC cell division through PSK signaling pathway, to allow the plants to replenish the stem cell niche (Heyman et al., 2013). Therefore, stem cell death upon DNA damage could be important for maintaining genome integrity in stem cells, enabling plants to survive under DNA stress.

To produce stem cells with undamaged proper genome, it is necessary to maintain genome integrity in QC cells. It has been shown that low mitotic activity of QC cells not only helps the QC to maintain quiescence but also to maintain its genome integrity (Cruz-Ramirez et al., 2013). It has been shown that RETINOBLASTOMA-RELATED (RBR) maintains quiescence of the QC by repressing the SCARECROW (SCR) activity, which is required for asymmetric cell division. Reduction in the RBR level could induce asymmetric division of the QC and produce new stem cells, suggesting the loss of quiescence by QC cells. Though reduced quiescence of the QC did not affect the plant root architecture or stem cell maintenance, it affected the sensitivity of QC to DNA damage-inducing agents; upon treatment with zeocin, cell death was induced in QC cells of the mutants in which RBR activity was reduced, while the QC in wild-type plants was highly tolerant to DNA damage, and cell death was not observed (Cruz-Ramirez et al., 2013).

A recent study by Hong et al. (2017) has shown that stem cell death induced by DNA damage could be a protective mechanism, which allows the plant to survive under chilling and

drought stress and recover robustly once the stress is relieved. They showed that chilling stress induces DNA damage in root stem cells, and DNA damage suppresses several PINs leading to the reduction of auxin maxima in the QC, which triggers a round of cell division in columella stem cells (CSC) leading to the production of new columella stem cell daughters (CSCD). The newly generated CSCDs undergo cell death before entering the cell differentiation. Auxin transport is blocked due to the cell death at CSCDs, leading to re-establishment of auxin maxima at the QC again, which helps to protect the stem cells. To further verify the protective mechanism of this cell death at CSCDs, they exposed the plants to chilling stress first and then they exposed the plants to drought stress. They found that plants that have cell death at CSCDs were able to tolerate the drought stress better than the plants without cell death at CSCDs, suggesting the cell death induced at CSCDs is a protective mechanism.

**Aim of my study**

Previous studies have demonstrated that DNA damage-induced stem cell death in roots requires the active ATM/ATR-SOG1 pathway (Fulcher and Sablowski, 2009; Furukawa et al., 2010). However, the signaling pathway downstream of SOG1 that controls the induction of stem cell death is still unknown. In this study, I aim to understand the possible mechanisms involved in the stem cell death caused by DNA damage. In the first chapter of my thesis, I study the effect of DNA damage on auxin signaling, and show that auxin signaling is involved in maintaining the genome integrity. In the second chapter, I study the possible role of two transcription factors, ANAC044 and ANAC085, in the induction of stem cell death in response to DNA damage.

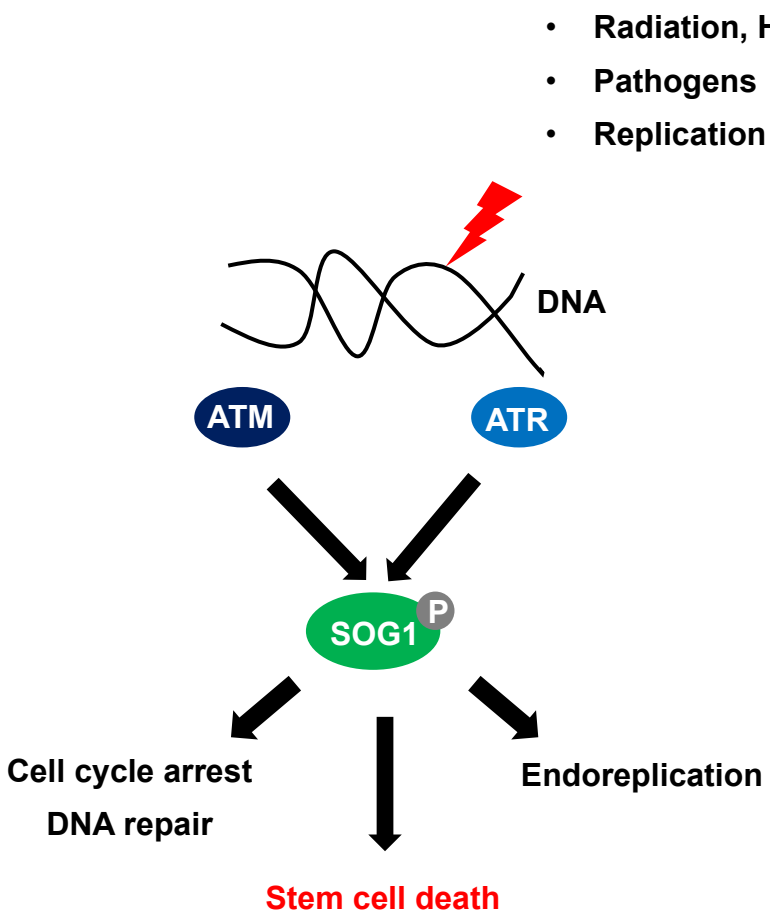



**Figure 1. DNA damage response in *Arabidopsis***

Multiple factors (*e.g.* radiation, heavy metals, pathogen, replication errors, and ROS) could induce DNA damage in plants, which is sensed by kinases, ATM and ATR. Both ATM and ATR could activate SOG1, a plant-specific NAC transcription factor, by phosphorylation. Once activated, SOG1 induces multiple responses to DNA damage, such as cell cycle arrest, DNA repair, endoreplication and stem cell death.

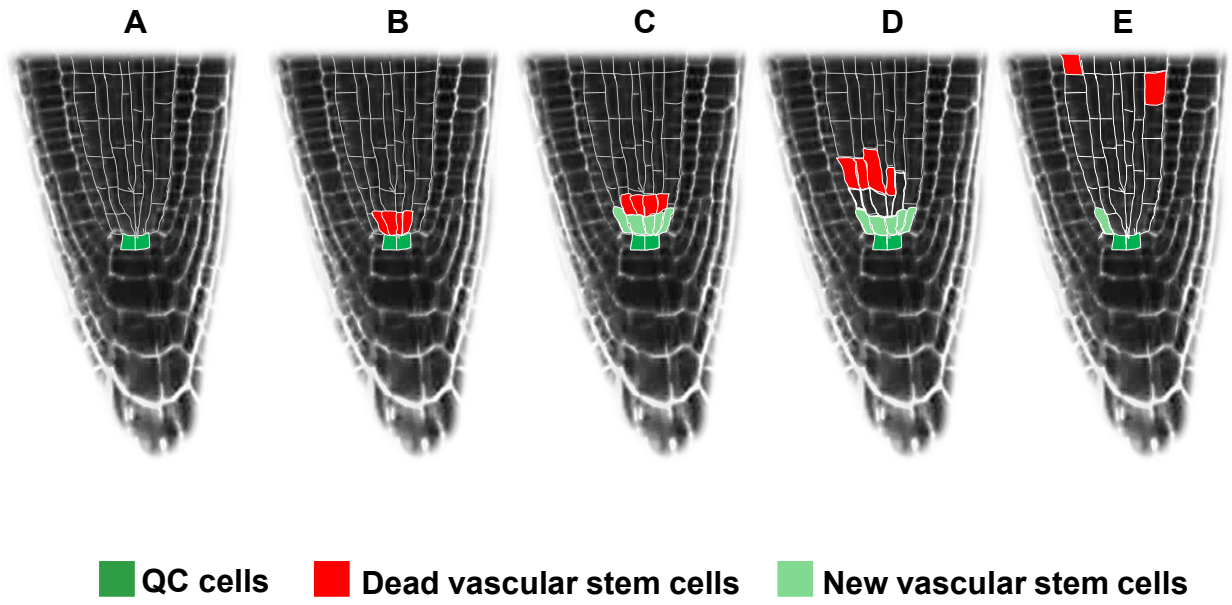



**Figure 2. Stem cell death could be a protective mechanism**

(A) QC cells are tolerant to DNA damage due to reduced cell cycle activity and higher expression of DDR genes even under normal conditions, which allows QC to maintain the integrity of its genome (Cruz-Ramirez et al., 2013). (B) Stem cells are susceptible to DNA damage, and cell death is induced in response to DNA damage. (C) Cell death in stem cells induces cell division in QC cells and produces new stem cells with undamaged genome. (D) Newly produced stem cells divide and produce new transit amplifying cells (TACs). (E) Actively dividing TACs allow the plants to replace dead cells with newly produced cells, thereby enabling plants to recover from DNA damage and maintain genome integrity. Therefore, DNA damage induced stem cell death could be a protective mechanism that helps to maintain genome integrity of plants (Heyman et al., 2013).

# Chapter I

# Auxin signaling protects the genome from DNA damage

# Introduction

*Arabidopsis* root has three distinct developmental zones; namely meristematic zone, transition zone, and elongation/differentiation zone (Figure 1). In the meristematic zone, cells actively divide multiple time, increasing the number of cells available for growth. In the transition zone, cells lose their ability to divide and increase in size. In the elongation/differentiation zone, cells elongate more rapidly and acquire their specialized functions (Petricka et al., 2012). Within the meristematic zone, there is a group of mitotically less active cells, known as quiescent center (QC) (Figure 1). These QC cells form a stem cell niche together with the surrounding shootward and rootward stem cells. Shootward stem cells produces vascular, endodermal, cortical, epidermal and lateral root cap cells, while rootward stem cells produces columella root cap (van den Berg et al., 1997). The QC is essential for maintaining stem cells in an undifferentiated state. It has been shown that stem cells adjacent to a dead QC cell becomes differentiated (van den Berg et al., 1997), suggesting that QC produces a signal and communicates with the surrounding stem cells to maintain their undifferentiated state. It has been shown that two pathways controlled by transcription factors, PLETHORA (PLT) and SHORTROOT (SHR)/SCARECROW (SCR) specify QC identity (Aida et al., 2004; Sabatini et al., 2003). It has been shown that WOX5, which is specifically expressed in QC, is required for maintaining QC identity and it moves from QC into columella stem cells (CSC) to maintain the undifferentiated state of CSC (Sarkar et al., 2007; Pi et al., 2015).

Plant hormone auxin is known to play key roles in almost all the aspects of plant growth and development. Auxin is mainly synthesized in the shoots and transported to root tips through

long distance transport via phloem cells (Overvoorde et al., 2010). Once auxin is delivered to the root tips, auxin is transported between cells by the mechanism known as polar auxin transport (PAT). The polar auxin transport is achieved through coordinated activities of several plasma membrane associated proteins involved in influx and efflux. Auxin efflux is carried out by PIN-FORMED (PIN) proteins and ABC transporters, while influx is carried out by AUXIN RESISTANT1 (AUX1) and LIKE-AUX1 (LAX1) (Petrasek and Friml, 2009). The PAT establishes an auxin gradient in the root tip, and is crucial for forming the auxin maxima in the CSC. This auxin gradient is important for proper patterning of roots (Aida et al., 2004). PLETHORA (PLT) and BABY BOOM (BBM) transcription factors are expressed in gradient fashion in the root tips, with high protein level in the stem cell niche that coincide with auxin maximum, which is crucial for the QC cell specification and stem cell maintenance (Aida et al., 2004; Galinha et al., 2007; Petersson et al., 2009). Indole-3-acetic acid (IAA), the common naturally occurring auxin in plants, is known to be mainly synthesized from L-tryptophan (Trp), and TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) flavin-containing monooxygenase family proteins play a crucial role in IAA biosynthesis in plants (Zhao et al., 2001; Tao et al., 2008; Stepanova et al., 2008). IAA is perceived in nucleus by auxin receptors, TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) proteins, which are included in SKP1-CULLIN-F-box ($SCF^{TIR1/AFB}$) E3 ligase complex (Tan et al., 2007). Auxin signalling is transcriptionally regulated by the AUXIN RESPONSE FACTOR (ARF) transcription factors (Guilfoyle & Hagen, 2001; Tiwari et al., 2003). However, under low auxin level, AUXIN/INDOLE ACETIC ACID (Aux/IAA) proteins associate with ARFs, thereby repressing their transcriptional activity (Abel et al., 1994; Dreher et al., 2006). In contrary, when auxin is abundant,

$SCF^{TIR1/AFB}$ polyubiquitinates Aux/IAA proteins, leading to their protein degradation by 26S proteasome (Gray et al., 2001; Maraschin et al., 2009). Degradation of Aux/IAA proteins releases ARF proteins, allowing the transcriptional regulation of auxin-responsive genes (Weijers et al., 2005).

In response to environmental stresses, plants modify their development through altering plant hormone signaling pathways. Auxin signaling being involved in almost all aspects of plant growth and development, is an important target for plants to modify their developmental program in response to stresses (Kazan, 2013). Several biotic and abiotic stresses have been shown to modify auxin signaling by altering auxin synthesis, perception and signaling. *MicroRNA393* has been shown to regulate transcripts coding for auxin receptors TIR1, AFB1, AFB2 and AFB3. It has been shown that *miR393* was induced in response to bacterial pathogens, salt stress and nitrate levels and reduces the mRNA level of TIR1/AFBs, leading to the suppression of auxin signaling (Vidal et al., 2010; Navarro et al., 2013; Iglesias et al., 2014). Chilling stress has been shown to affect the PAT by affecting trafficking and membrane localization of PIN2 and PIN3 (Shibasaki et al., 2009). Almost all the stresses are known to change the redox status of the cells, and changes in the redox status affects membrane localization of PINs (Yu et al., 2013) and stability of PLTs (Yamada et al., 2020). It has been shown that mutation in plastid-localized GLUTATHIONE REDUCTASE 2 (GR2) leads to root growth inhibition due to impaired polar auxin transport and reduced expression of several PLTs (Yu et al., 2013). A recent study showed that drought and cold stress-responsive transcription factors CBF1 and DREB2B directly bind to the promoters of *AUX/IAA* genes, *IAA5* and *IAA19*, and induce their expression in response to abiotic stresses, leading to the suppression of auxin signaling (Shani et al., 2017).

The effect of DNA damage on auxin signaling is relatively unknown, although a couple of studies have reported contradicting results. A previous report showed that DNA damage increases auxin signaling in the stele (Ricaud et al., 2007). However, a recent study found no detectable change in the auxin maximum in the QC (Johnson et al., 2017). Additionally, Hong et al. (2017) showed that chilling stress lead to reduction of auxin signaling in the QC and the stem cell niche, through ATM and ATR-mediated response, suggesting that DNA damage response is involved in the chilling stress-induced reduction of auxin signaling. Given the contradicting results of these studies, effect of DNA damage on auxin signaling is still elusive.

It has been previously demonstrated that, DNA damage-induced stem cell death requires active ATM/ATR-SOG1 pathway (Fulcher and Sablowski, 2009; Furukawa et al., 2010). However, the signaling pathways downstream of SOG1 is still unknown. Since auxin signaling plays a key role in stem cell maintenance, in this chapter, I studied the effect of DNA damage on auxin signaling and found that DNA damage decreases auxin signaling in *Arabidopsis* roots through ATM/ATR-SOG1 pathway. Moreover, I found that reduction in the auxin signaling causes accumulation of DNA damage, suggesting that auxin signaling protects the genome from DNA damage. Furthermore, I found that exogenous auxin application suppresses stem cell death upon DNA damage, suggesting that reduction of auxin response in stem cells is crucial for the induction of stem cell death. Additionally, I found that, upon DSB treatment, AUX/IAA family genes *IAA5* and *IAA29* were induced in stem cell populations where cell death occurs. Although I could not establish a clear role of IAA5 and IAA29 in the induction of stem cell death, it is still possible that IAA5 and IAA29 could contribute to the suppression of auxin signaling in the stem cells along with some unknown mechanisms to maintain genome integrity in stem cells.

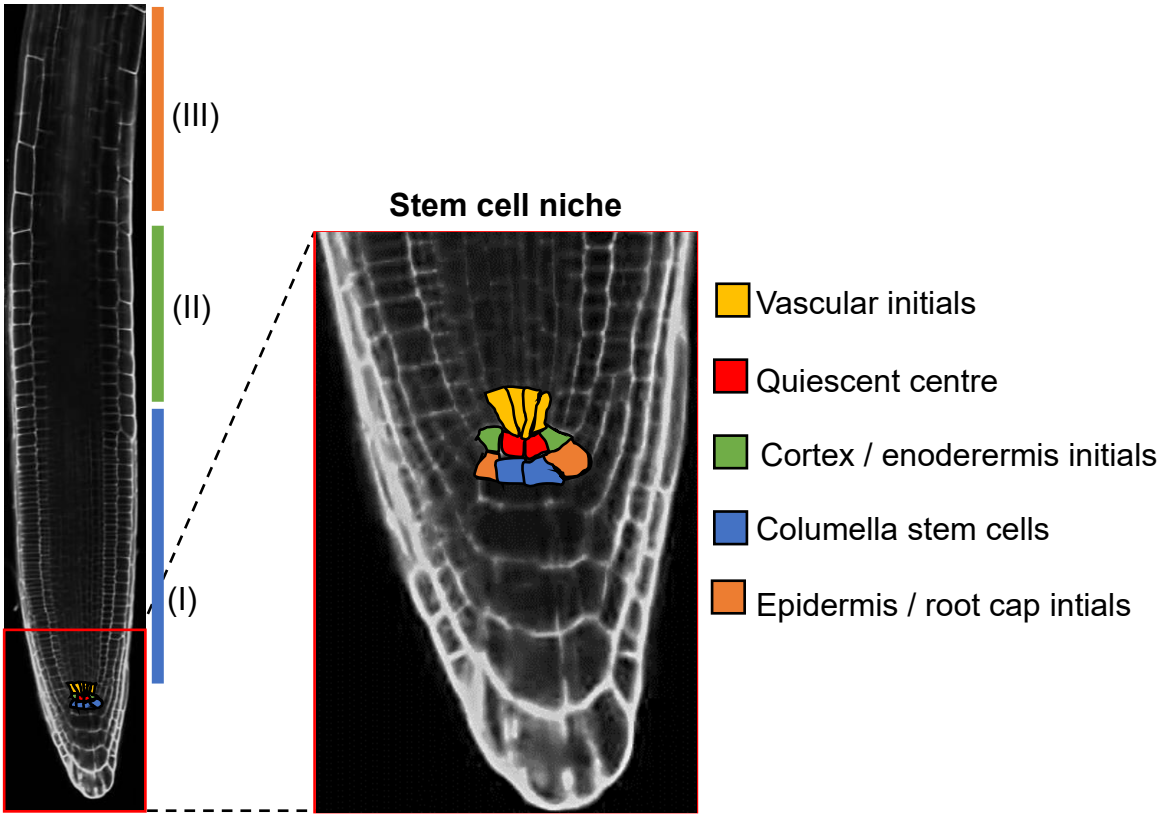



**Figure 1. *Arabidopsis* root structure**

*Arabidopsis* root has three distinct developmental zones; namely (I) meristematic zone, (II) transition zone, and (III) elongation/differentiation zone. Stem cell niche is located within the meristematic zone. In the middle of stem cell niche, there is a group of mitotically less active cells, known as quiescent center (QC), which is surrounded by stem cells.

# Results

## DNA damage induces cell death in stem cells

It has previously been shown that DNA damage induces cell death in root and shoot stem cells in *Arabidopsis* (Curtis and Hays, 2007; Fulcher and Sablowski, 2009; Furukawa et al., 2010). In *Arabidopsis* roots, vascular initials and their early progenies as well as columella stem cells are preferentially killed by DNA damage (Fulcher and Sablowski, 2009). To understand the mechanisms involved in the induction of stem cell death, 5-day-old *Arabidopsis* Col-0 wild-type plants were treated with bleomycin, a DSB inducer. It has been previously shown that bleomycin directly binds to the minor groove of the B-form DNA and changes its structure, which leads to the cleavage of DNA (Hecht, 1999). The induction of cell death in root tips was monitored after bleomycin treatment. Seedlings were stained with propidium iodide (PI) which stained the dead cells and cell walls, and root tips were photographed with confocal microscopy. It was found that cell death started to occur between 12 to 18 hours of treatment with 0.75 μg/ml bleomycin treatment (Figure 1A). Initially, cell death was observed in vascular stem cells and their progenies known as transit amplifying cells (TAC). At 24 hours of treatment with 0.75 μg/ml bleomycin, cell death has further expanded into root apical meristems, and cell death was also induced in columella stem cells (Figure 1A).

## DNA damage-induced stem cell death is mediated by ATM/ATR-SOG1 pathway

ATM and ATR kinases primarily sense DNA damage (Garcia et al., 2003; Culligan et al., 2004). These two kinases phosphorylate and activate the plant-specific transcription factor SOG1 (Yoshiyama et al., 2013a; Sjogren et al., 2015). Previously, it has been shown that cell

death in root tips upon DNA damage requires the activation of DNA damage signals by ATM, ATR and SOG1 (Fulcher and Sablowski, 2009; Furukawa et al., 2010). To confirm whether ATM/ATR-SOG1 pathway is involved in the induction of stem cell death by DSBs, the induction of cell death was monitored after bleomycin treatment in *atm-2*, *atr-2* and *sog1-101* mutants. Five-day-old seedlings of wild-type, *atm-2*, *atr-2* and *sog1-101* were transferred to MS plates with 0.75 μg/ml bleomycin. It was found that stem cell death was induced in wild-type plants after 24 hours of bleomycin treatment. In contrary, stem cell death was not observed in the *atm-2*, *atr-2* or *sog1-101* until 24 hours (Figure 1B). These results suggest that DNA damage-induced stem cell death is a programmed response and is mediated by ATM, ATR and SOG1.

**DNA damage suppresses auxin signaling**

To understand the pathways controlling the induction of stem cell death downstream of ATM/ATR-SOG1, I looked for the genes that could be involved in the stem cell death among the genes regulated by DNA damage, using the public available microarray data. I found that several *PIN* genes were downregulated by DNA damage. PINs are required for polar auxin transport (PAT), a key mechanism involved in establishing the auxin maxima in root tips. Since auxin signaling plays key role in maintaining stem cells in the root tip, I studied the effect of DNA damage on auxin signaling. Firstly, I verified the microarray data using quantitative RT-PCR (qRT-PCR). It was found that while comparing with untreated seedlings, expression level of *PIN1*, *PIN3* and *PIN4* were reduced nearly 40 % in the seedlings treated with bleomycin for 6 hours, while *PIN2* and *PIN7* were not altered (Figure 2A). To verify the qRT-PCR data, I checked the expression of PINs under DNA damage condition using *pPIN:PIN:GFP* reporter

lines. It was found that expression levels of PIN1:GFP and PIN4:GFP was reduced significantly, after treatment with bleomycin, while the expression levels of PIN2:GFP and PIN7:GFP were not altered significantly (Figure 2B). Since, PIN1, PIN3 and PIN4 are involved in downward flow of auxin, and establishment of auxin maxima around the stem cell niche, these results suggest that DNA damage suppresses downward auxin transport into the root tips, causing reduction of auxin in the stem cell niche of the roots.

To examine whether DNA damage affects auxin signaling in root stem cells, 5-day-old seedlings expressing *DR5v2:n3xVenus*, which is a marker for auxin signaling output, were transferred to MS plate with or without 0.75 μg/ml bleomycin. Auxin signaling level was monitored after 0, 6 and 12 hours treatment with bleomycin. It was found that auxin signaling started to decrease in the vasculature after 6 hours, and there was a significant reduction in the auxin signaling level after 12 hours of treatment (Figure 3). To exclude the possibility that induction of cell death might have led to the reduction of *Venus* fluorescence in the vascular stem cells, seedlings used to study the *DR5v2:n3xVenus* level were counter-stained with PI and checked for induction of stem cell death. It was found that stem cell death has not been initiated in root stem cells after 12 hours of bleomycin treatment, while the reduction of *Venus* fluorescence was observed in the vascular stem cells. This result suggests that DNA damage-induced reduction of the auxin signaling in root stem cells occurs before the induction of stem cell death.

**Auxin signaling regulates DNA damage response by regulating the amount of DNA damage**

Since DNA damage suppresses auxin signaling in root tips, I modified the auxin

signaling level by exogenous application of IAA and PEO-IAA, and studied the DNA damage response to understand the effect of auxin signaling on DNA damage response. PEO-IAA is an auxin signaling antagonist, which binds to auxin receptors, thereby competing with endogenous IAA and suppressing the auxin signaling. To examine the effect of auxin signaling level on DNA damage response, I checked the expression of genes involved in DNA repair, *RAD51* and *PARP2*, and the gene involved in cell cycle arrest, *SMR5*, by qRT-PCR using the RNA samples isolated from root tips of seedlings treated with bleomycin, bleomycin plus IAA, and bleomycin plus PEO-IAA. I found that the treatment with bleomycin and IAA together suppressed the induction of *RAD51*, *PARP2*, and *SMR5* compared to seedlings treated with bleomycin alone (Figure 4A). In addition, I found that treatment with bleomycin and PEO-IAA together enhanced the induction of those genes comparing to seedlings treated with bleomycin alone (Figure 4A). To further verify the qRT-PCR data, the promoter-reporter lines, *pRAD51:GFP*, *pPARP2:GFP* and *pSMR5:GFP* were monitored after treatments with bleomycin, IAA, and bleomycin with IAA together. Consistent with qRT-PCR analysis, treatment with bleomycin and IAA together suppressed the induction of *RAD51*, *PARP2*, and *SMR5* comparing to seedlings treated with bleomycin alone (Figure 4B). These results indicate that auxin signaling level affects the induction of DNA damage-responsive genes.

Previous studies have shown that the strength of DNA damage response is directly proportional to the amount of DNA damage (Yoshiyama et al., 2017). Since auxin signaling level could regulate the DNA damage response, I next checked whether auxin signaling level also regulates the amount of DNA damage. To reveal this, I quantified the amount of DNA damage in root tips of the seedlings treated with bleomycin, bleomycin plus IAA, and bleomycin plus PEO-IAA, using alkaline comet assay (Figure 5A). For comet assay, root tips

of 0.5 cm were collected from the seedlings after 6 hours of the above-mentioned treatments. Root tips were chopped with a razor blade to isolate nuclei in a phosphate buffer, followed by filtration to remove the unwanted debris. Nuclei were mixed with molten agarose and coated on a slide. After drying the agarose, nuclei were lysed and electrophoresed in a alkali solution, and nuclei were stained with SYBR gold. I found that treatment with bleomycin and IAA together significantly reduced the amount DNA damage comparing to seedlings treated with bleomycin alone. In contrary, I found that treatment with bleomycin and PEO-IAA together increased the amount of DNA damage comparing to seedlings treated with bleomycin alone (Figure 5B). These results indicate that higher auxin signaling level protects the genome from DNA damage.

**Auxin regulates onset of stem cell death**

Since DNA damage suppresses auxin signaling which regulates the genome integrity, it is possible that genome integrity of stem cells might be highly compromised by DNA damage, leading to further accumulation of DNA damage and promoting stem cell death. To verify this possibility, I examined the effect of auxin signaling on DNA damage-induced stem cell death. Five-day-old wild-type seedlings were treated with 0.75 μg/ml bleomycin, 0.75 μg/ml bleomycin plus 5 nM IAA, or 0.75 μg/ml bleomycin plus 5 μM PEO-IAA. I studied the induction of cell death by confocal microscopy. In bleomycin treated plants, cell death started to occur around 12 h after treatment and progressed from stem cell niche to TAC as treatment time increased (Figure 6A). Interestingly, in the plants treated with bleomycin and IAA together, onset of cell death was delayed. Cell death started around 18 hours of treatment and the progression into the TAC is much slower than the plants treated with bleomycin alone (Figure

6A). In contrary, in the plants treated with bleomycin and PEO-IAA together, cell death occurred earlier than the plants treated with just bleomycin (Figure 6A). The area of cell death was measured for 24 hours-treated root tips using Image J software. It was found that amount of cell death was significantly higher in roots treated with both bleomycin and PEO-IAA than those with bleomycin alone (Figure 6B), while cell death area was significantly lower in the roots treated with bleomycin and IAA together (Figure 6B). These data suggest that auxin signaling level regulates the onset of DNA damage-induced stem cell death.

### ***AUX/IAAs*, *IAA5* and *IAA29* are induced through the ATM/ATR-SOG1 pathway in response to DNA damage**

Since DNA damage suppresses auxin signaling, I searched for the genes related to auxin signaling among the genes regulated by DNA damage using microarray datasets available in public database. I found that *AUX/IAA* genes, *IAA5* and *IAA29*, were induced in wild-type plants, while the expression of those genes was unaltered in the *sog1-1* plants. Other *AUX/IAA* genes except *IAA5* and *IAA29* were not induced by DNA damage (Figure 7A). To confirm the induction of *IAA5* and *IAA29*, plants expressing *pIAA5:GFP* and *pIAA29:GFP* were generated in wild-type, *atm-2*, *atr-2* and *sog1-1* backgrounds. I found that upon treatment with bleomycin, *IAA5* and *IAA29* were induced at the stem cell niche in wild-type background. However, *IAA5* and *IAA29* were not induced in *atm-2*, *atr-2* and *sog1-1* backgrounds (Figure 7B), indicating the ATM/ATR-SOG1-mediated pathway is required for the induction of *IAA5* and *IAA29* in response to DNA damage.

IAA5 and IAA29 belong to AUX/IAA family of proteins. AUX/IAA proteins bind to ARFs (Auxin response factor) and suppress the expression of auxin inducible genes, thus

exerting negative effect on the auxin signaling. Since the induction of *IAA5* and *IAA29* by DNA damage was specifically observed in the stem cell niche (Figure 7B), auxin signaling may be decreased locally in the stem cell niche after treatment with bleomycin. To study whether reduction of auxin signaling by IAA5 and IAA29 is involved in induction of stem cell death by DNA damage, I studied cell death phenotype of *iaa5 iaa29* double knockout mutant after treatment with bleomycin. I found that there was no significant difference in the induction of stem cell death between *iaa5 iaa29* and wild-type (Figure 8B).

To further study the role of IAA5 and IAA29 in stem cell death induced by DNA damage, I made transgenic plants expressing *proWOL:XVE>>mIAA5(P59L):GFP* (*mIAA5*), which expresses the dominant mutant of *IAA5* in the stele including vascular stem cells after the induction with estradiol (Figure 9A). Both wild-type and three independent *mIAA5* transgenic lines were treated with both bleomycin to induce DNA damage and estradiol to induce *mIAA5*. I found that there was no difference in the stem cell death between wild-type and mIAA5 transgenic lines (Figure 9B). These results suggest that induction of *IAA5* and *IAA29* is not sufficient to reduce the auxin signaling, and/or that other factors involved in auxin-independent pathways are required to cause stem cell death under DNA damage conditions.

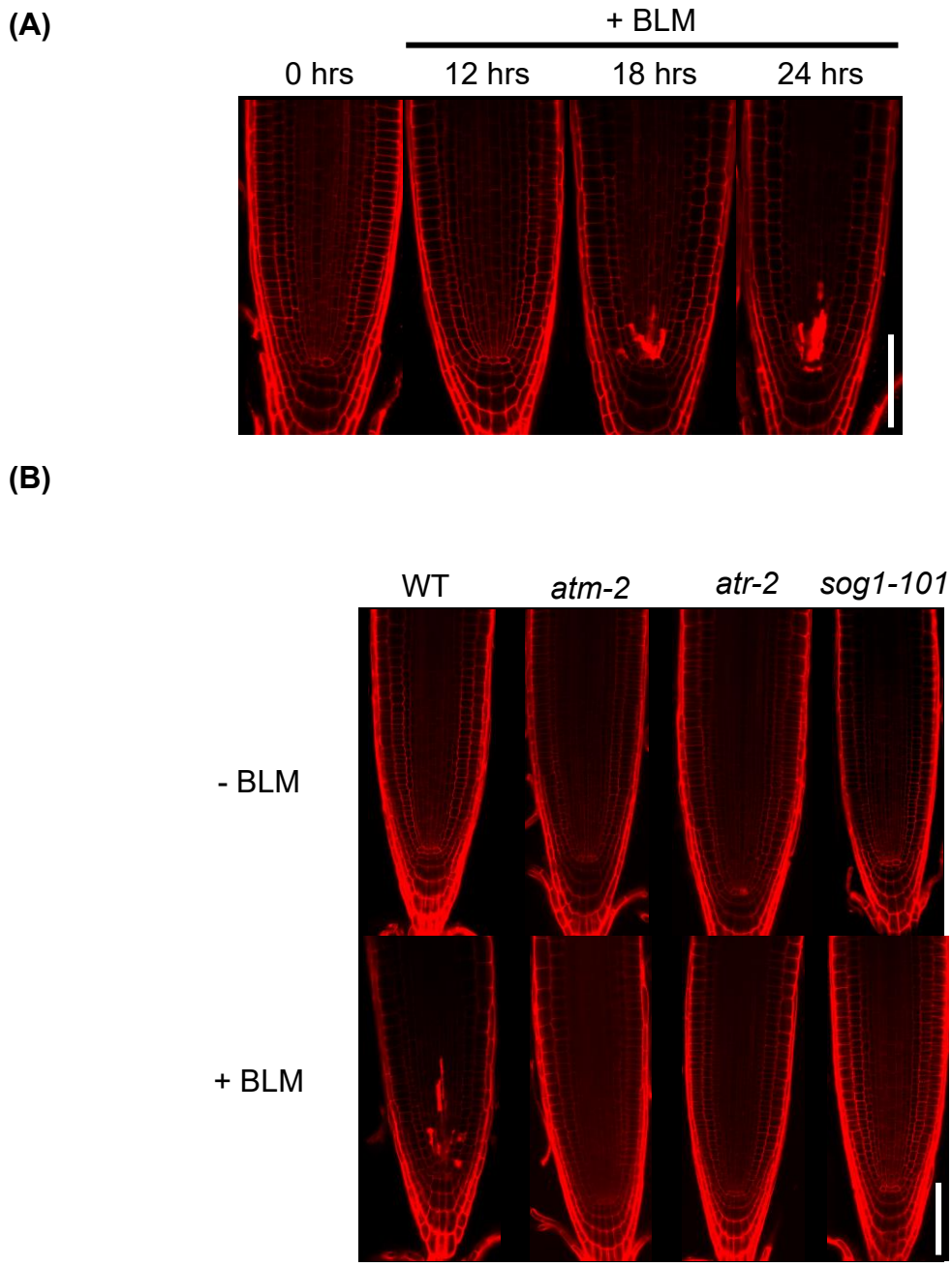



**Figure 1. DNA damage-induced stem cell death is mediated by ATM/ATR-SOG1 pathway.** (A) Five-day-old wild-type (WT) seedlings were treated with 0.75 μg/ml bleomycin (+BLM) and induction of cell death was observed after 0, 12, 18 and 24 hours treatment. Seedlings were stained with propidium iodide (PI) and visualized by confocal microcopy. Scale bar = 100 μm. (B) Five-day-old WT, *atm-2*, *atr-2* and *sog1-101* seedlings were treated with (+ BLM) or without (- BLM) 0.75 μg/ml bleomycin and cell death was observed after 24 hours. Scale bar = 100 μm.

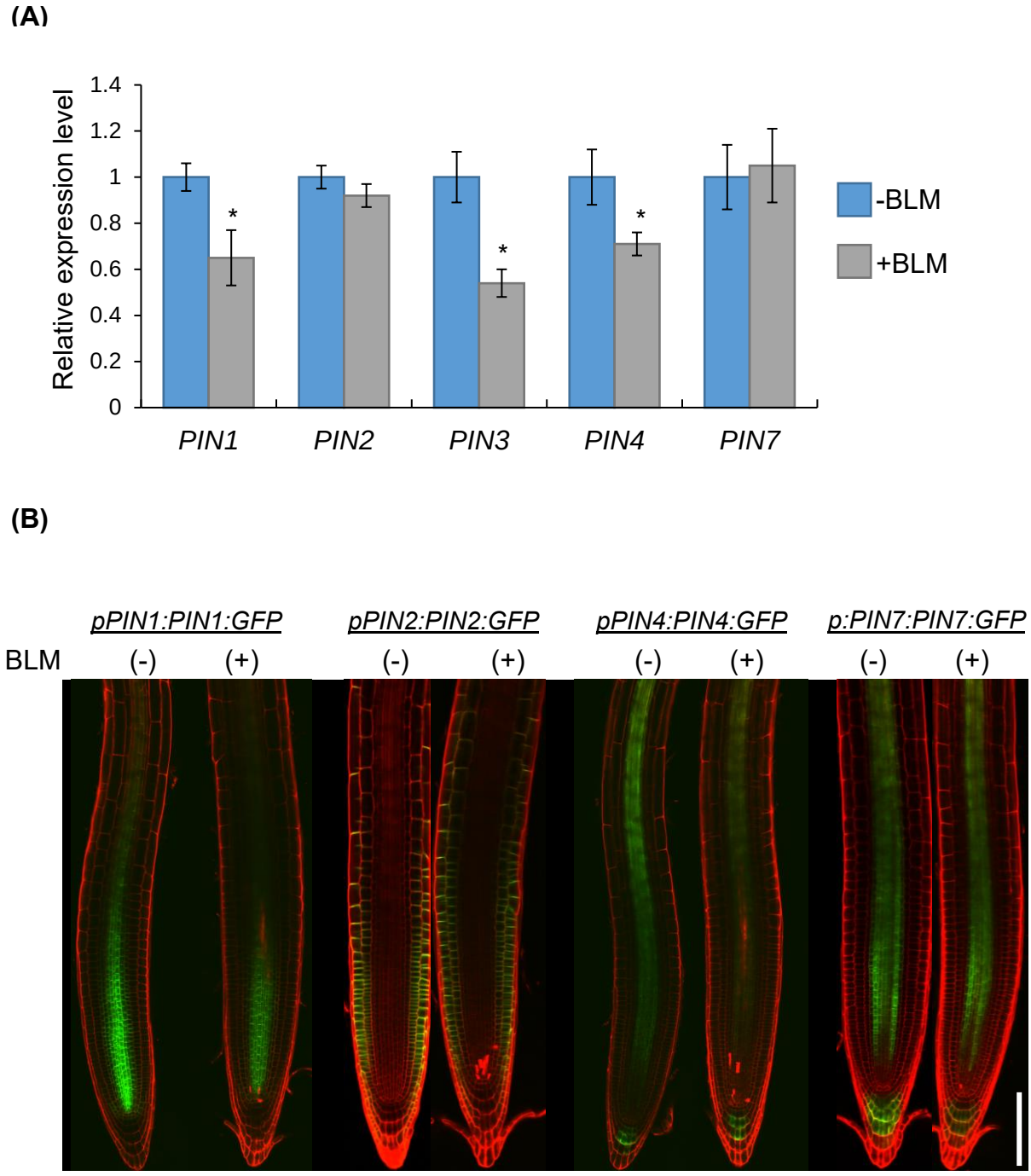



**Figure 2. DNA damage suppresses the polar auxin transport.**

(A) Five-day-old wild-type (WT) plants were treated with (+ bleomycin) or without (- bleomycin) 0.75 μg/ml bleomycin for 6 hours, and total RNA was isolated from root tips. mRNA levels were normalized with *ACTIN2*, and control value was set to be 1. Error bar indicates SE, statistical significance was checked using student's *t*-test (*, $P < 0.05$). (B) Five-day-old seedlings expressing *pPIN1:PIN1-GFP*, *pPIN2:PIN2-GFP*, *pPIN4:PIN4-GFP* and *pPIN7:PIN7-GFP* were treated with (+) or without (-) 0.75 μg/ml bleomycin (BLM) for 12 hours. Scale bar = 100 μm.

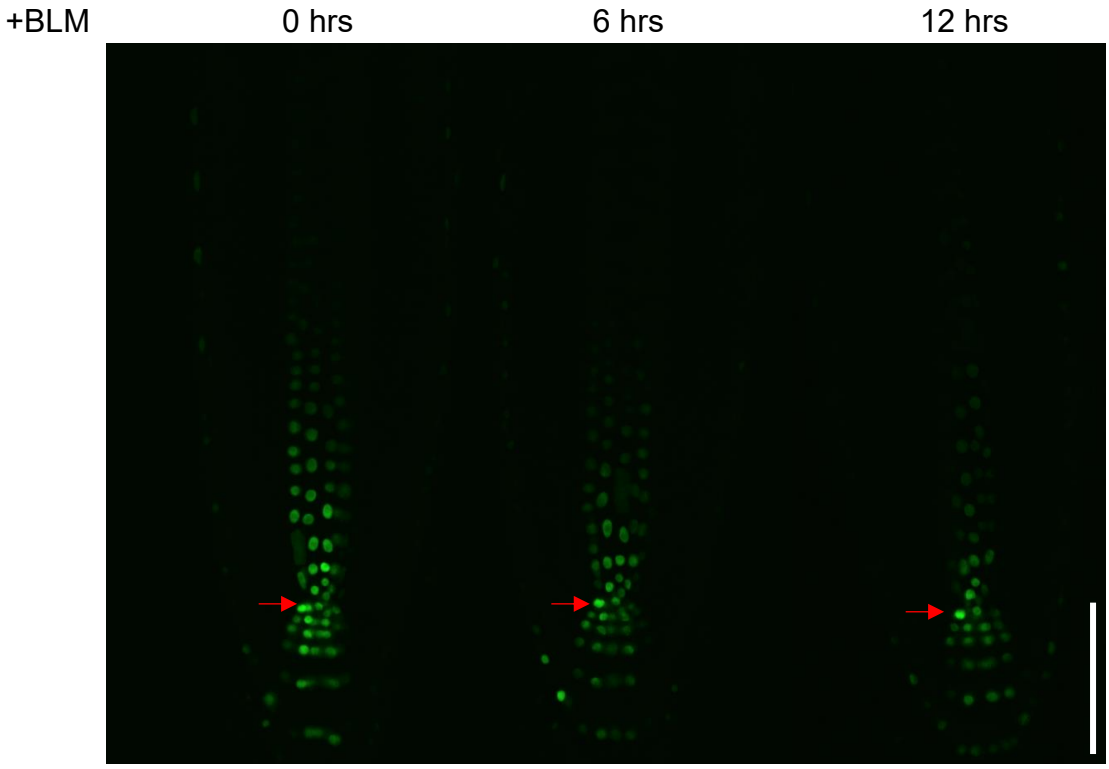



**Figure 3. DNA damage suppresses auxin signaling.**

Five-day-old seedlings expressing *DR5v2:n3xVenus* was treated with 0.75 μg/ml bleomycin (BLM), and flourescence was observed after 0, 6 and 12 hours using confocal microscope. Arrows indicate QC cells. Scale bar = 100 μm.

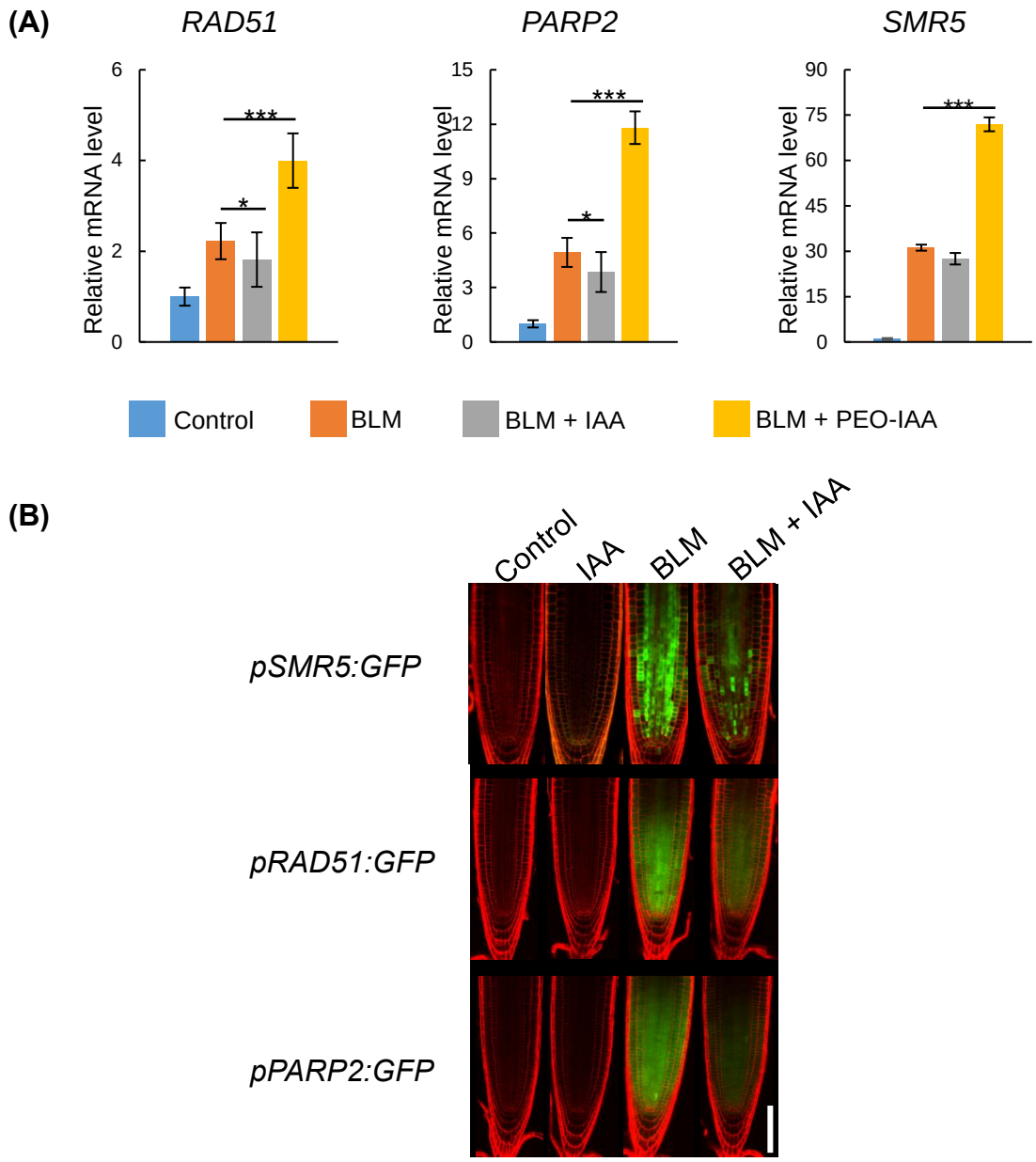



**Figure 4. Auxin signaling suppresses DNA damage response.**

(A) Five-day-old wild-type seedlings were treated with 0.75 μg/ml bleomycin (BLM), 5 nM IAA, 5 μM PEO-IAA, 0.75 μg/ml bleomycin plus 5 nM IAA, 0.75 μg/ml bleomycin plus 5 μM PEO-IAA for 6 hours, and total RNA was isolated from root tips. mRNA levels were normalized with *ACTIN2*, and the control value was set to be 1. Error bar indicates SE, statistical significance was checked using student's *t*-test (*, $P < 0.05$, ***, $P < 0.001$). (B) Five-day-old seedlings expressing *pSMR5:GFP*, *pRAD51:GFP* and *pPARP2:GFP* were treated with 5 nM IAA , 0.75 μg/ml bleomycin, and 0.75μg/ml bleomycin plus 5 nM IAA for 12 hours, and GFP fluorescence was observed using confocal microscope. Scale bar = 100 μm.

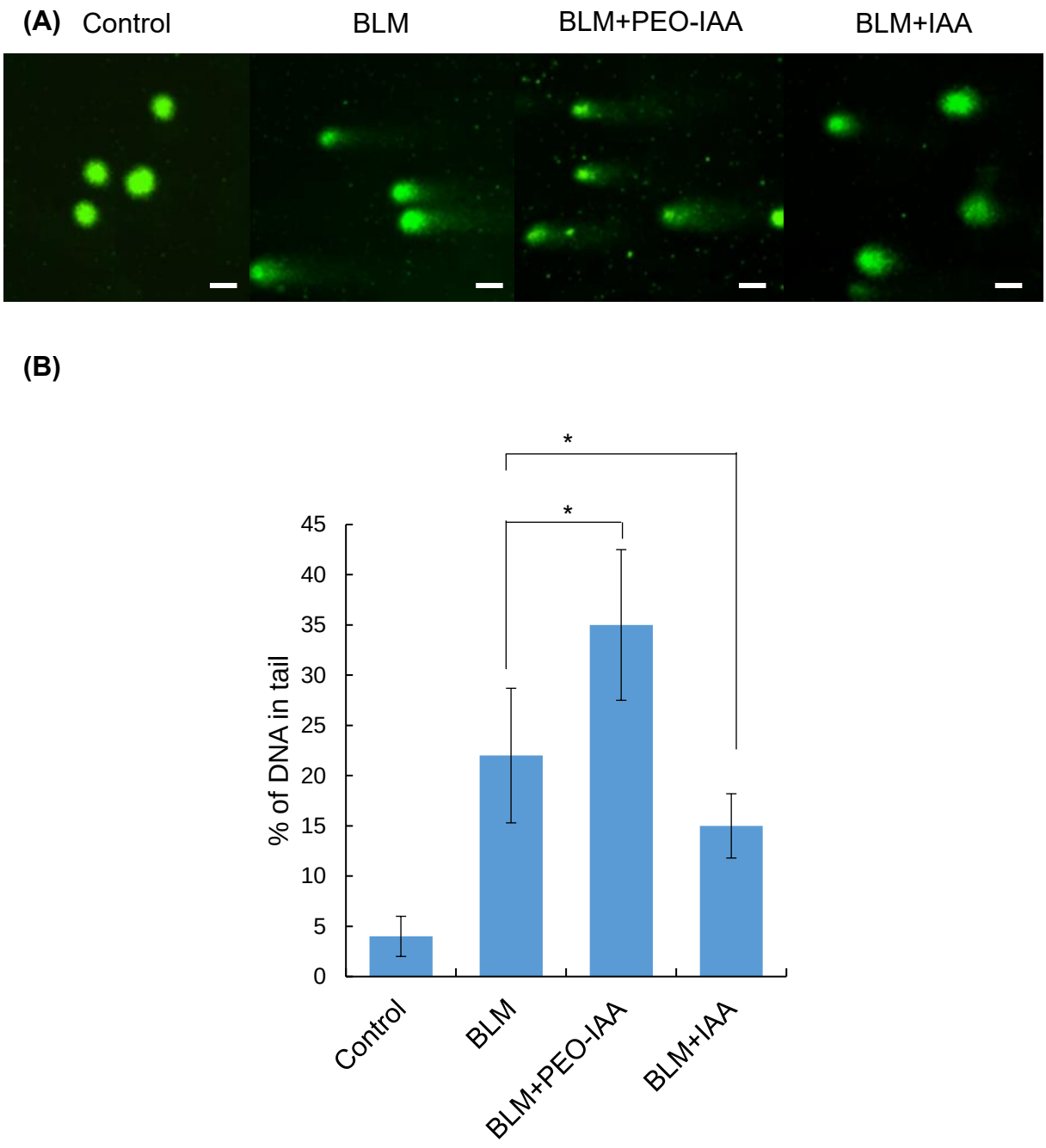



**Figure 5. Auxin signaling suppresses the accumulation of DNA damage.**

(A) Five-day-old wild-type seedlings were treated with 0.75 μg/ml bleomycin (BLM), 0.75 μg/ml bleomycin plus 5 nM IAA, 0.75μg/ml bleomycin plus 5 μM PEO-IAA for 6 hours, and nuclei was isolated from root tips. Amount of DNA damage was analyzed by alkali comet assay. Representative nuclei were shown in (A). Scale bar = 10μm. (B) 25 nuclei per sample were measured. Error bar indicates SD. Statistical significance was analyzed using student's *t*-test (*, $P < 0.05$).

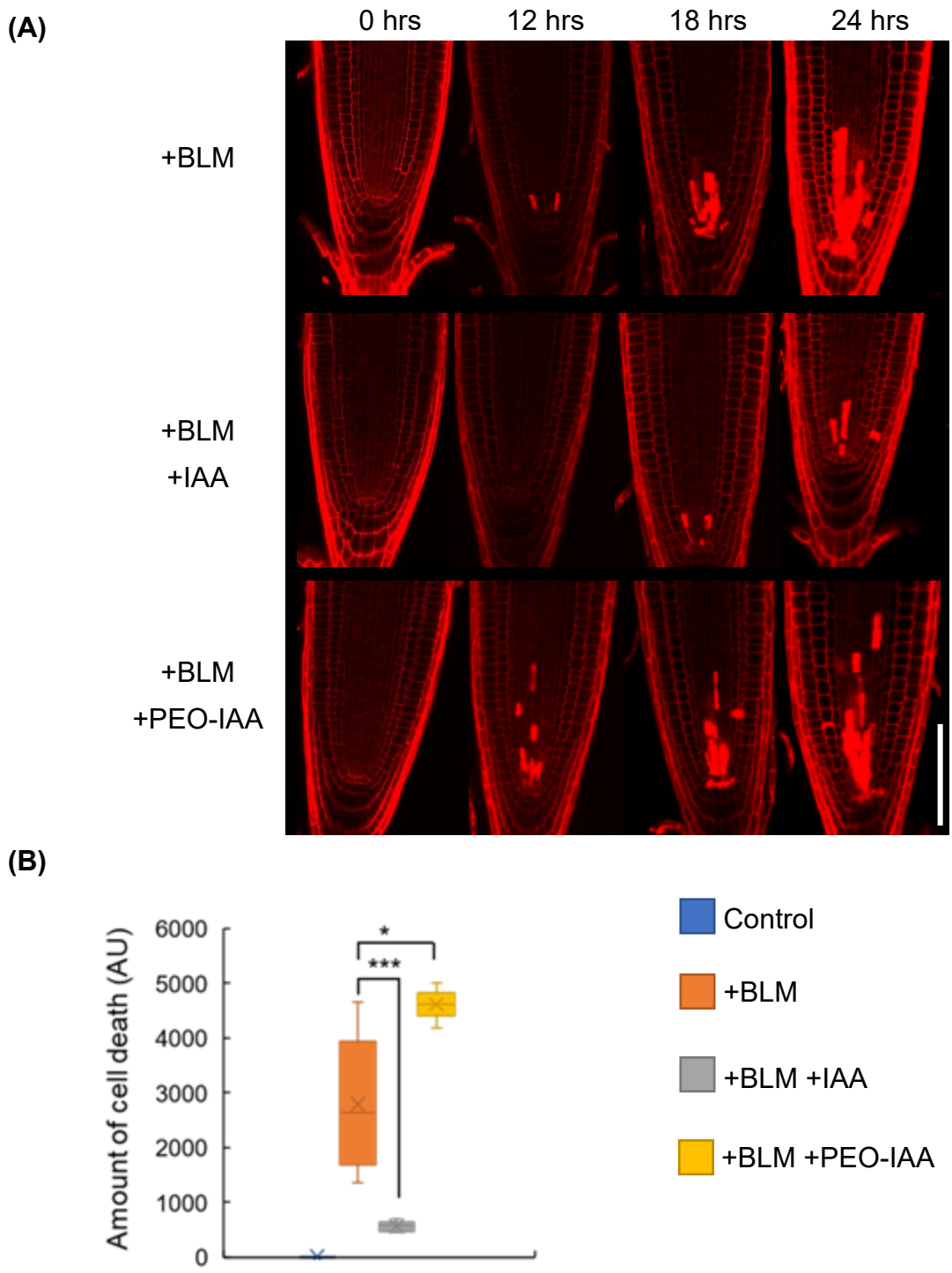



**Figure 6. Auxin regulates onset of stem cell death under DNA damage.**

(A) Five-day-old wild-type (WT) seedlings were treated with 0.75 µg/ml bleomycin (+BLM), 0.75 µg/ml bleomycin plus 5 nM IAA (+BLM +IAA), and 0.75 µg/ml bleomycin plus 5 µM PEO-IAA (+BLM +PEO-IAA) for 12, 18 and 24 hours. Root tips were stained with PI, and observed by confocal microscopy. Scale bar = 100 µm. (B) Cell death was measured from samples treated for 24 hours. Error bar indicates SE, statistical significance was checked using student's *t*-test (*, $P < 0.05$, ***, $P < 0.001$) ($n \geq 11$).

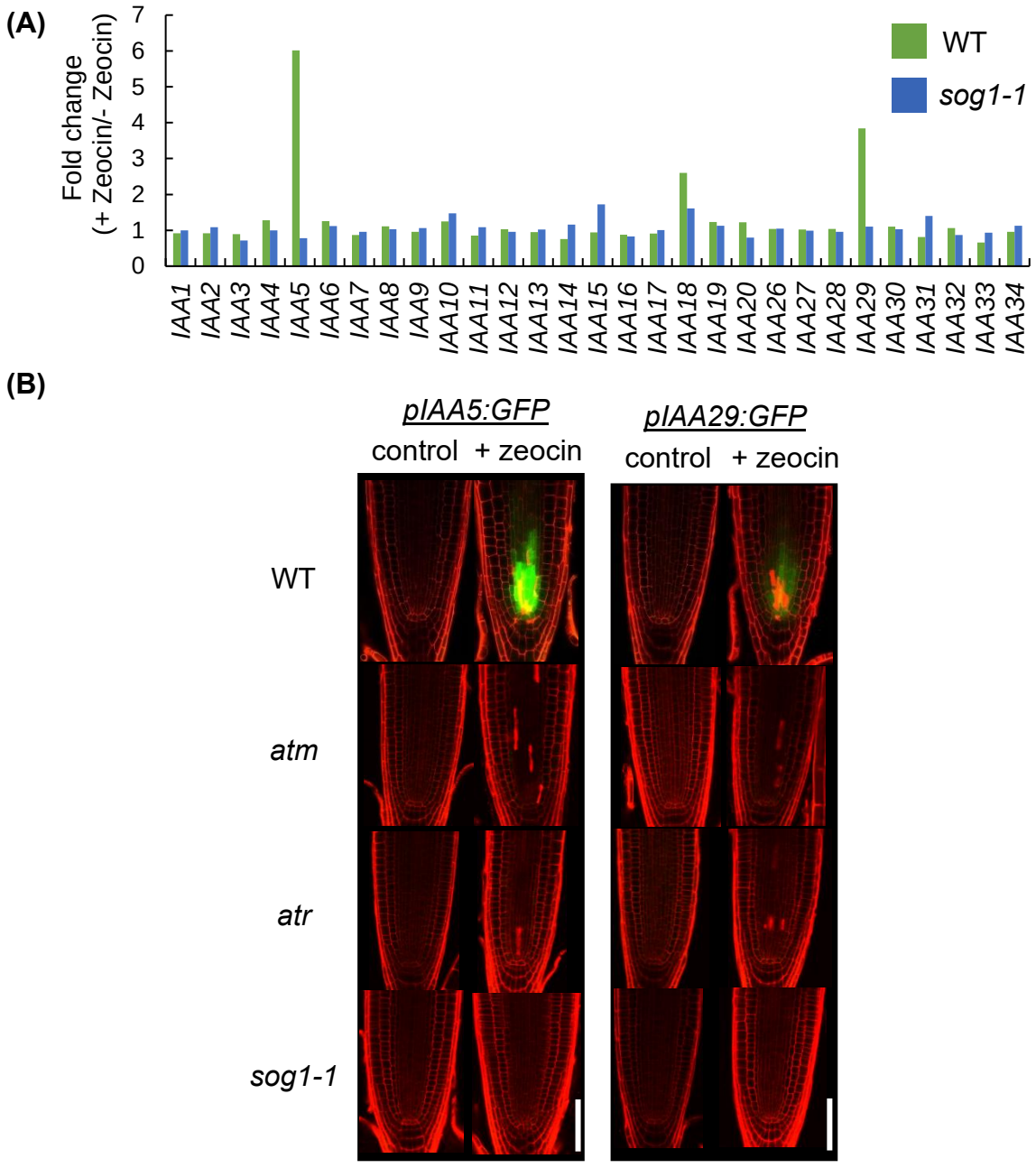



**Figure 7. AUX/IAAs, *IAA5* and *IAA29* are induced through ATM/ATR-SOG1 pathway in response to DNA damage**

(A) The expression level of all known *AUX/IAA* genes were extracted for zeocin treated and untreated samples from microarray datasets (Ogita et al., 2018). Changes in the expression was calculated as fold change between zeocin treated and untreated samples. Response of all *AUX/IAA* genes to DNA damage in wild-type (WT) and *sog1-1* was shown. (B) Five-day-old seedlings expressing *pIAA5:GFP* and *pIAA29:GFP* in wild-type, *atm-2*, *atr-2* and *sog1-1* background were treated with (+ zeocin) or without (- zeocin) 10 µM zeocin for 18 hours. The GFP fluorescence in root tips counterstained with PI was observed. Scale bar = 100 µm.

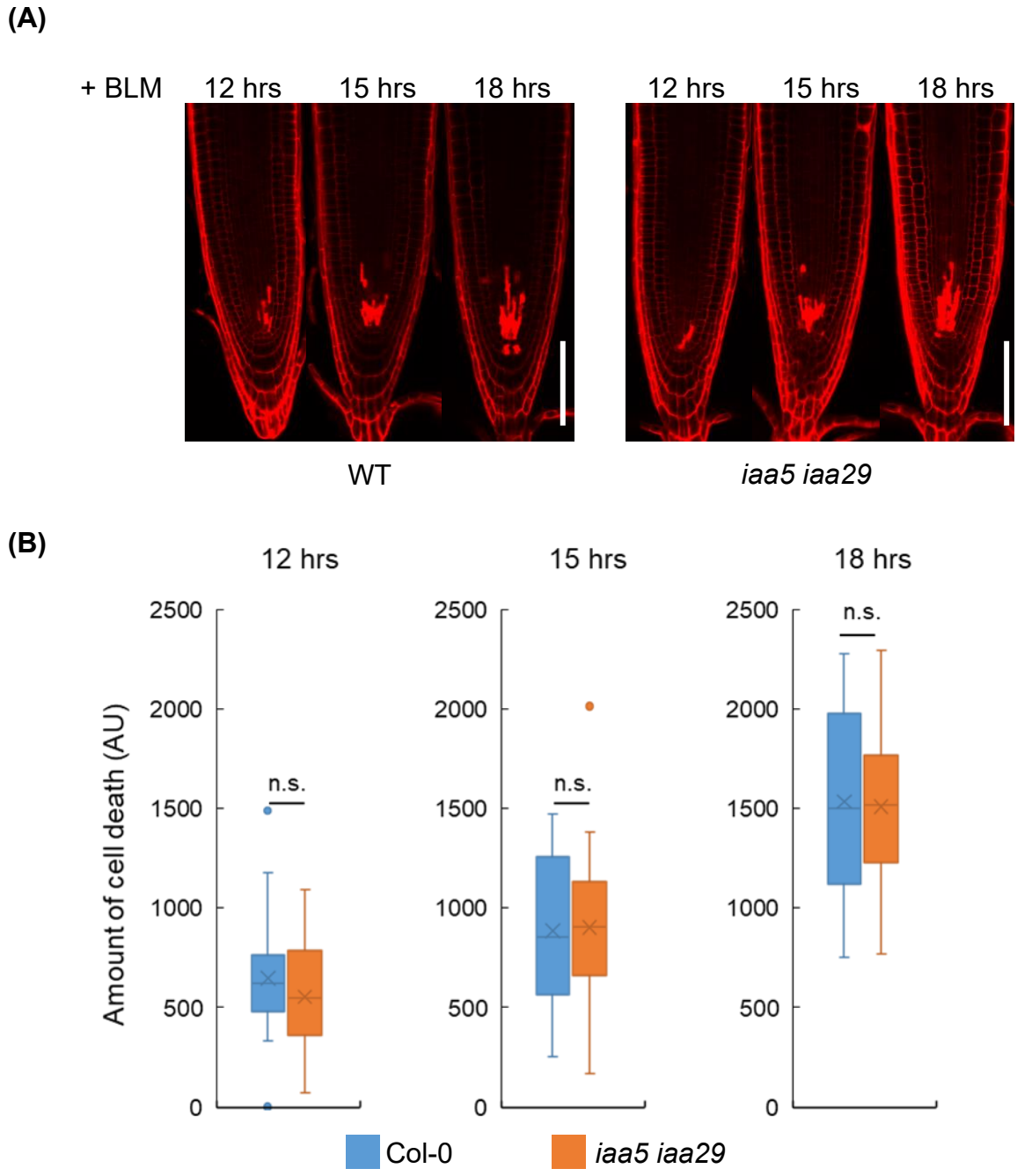



**Figure 8. IAA5 and IAA29 are not sufficient for the induction of stem cell death**

(A) Five-day-old wild-type (WT) and *iaa5 iaa29* were treated with 0.75 µg/ml bleomycin (BLM) and cell death was observed after 12, 15 and 18 hours. Root tips were stained with PI and cell death was observed using confocal microscope. Scale bar = 100 µm. (B) Amount of cell death area was measured using Image J software. Statistical significances were measured using student's *t*-test ($n \geq 22$). 'n.s.' indicates no significance.

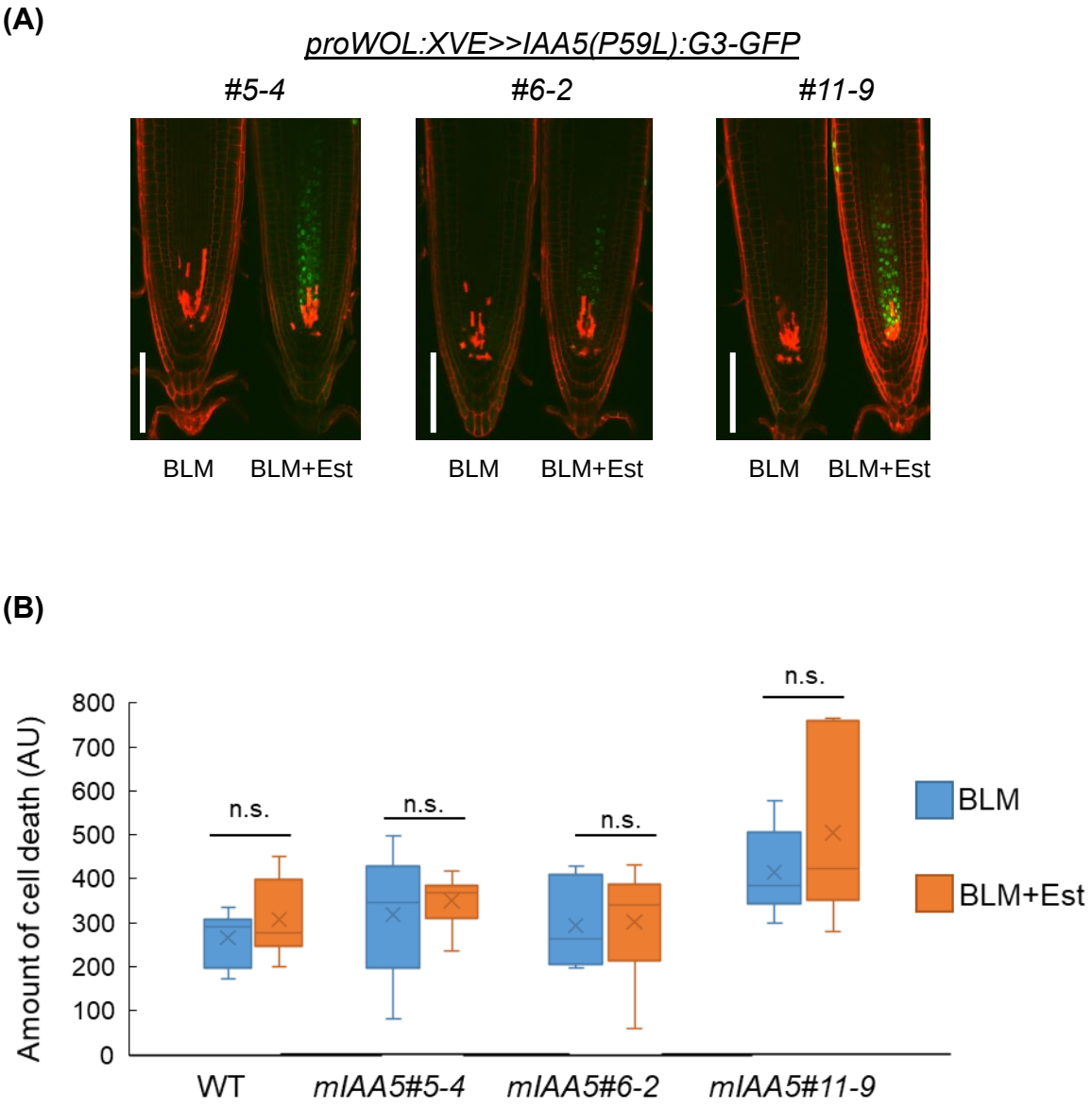



**Figure 9. Induction of *IAA5* and *IAA29* in vascular stem cells is not sufficient to induce stem cell death**

(A) The transgenic plants expressing *pWOL:XVE>>IAA5(P59L):G3-GFP* (lines #5-4, #6-2, #11-9) were treated with 0.75 μg/ml bleomycin (BLM) and/or 10 μM estradiol (Est) for 12 hours. Scale bar = 100 μm. (B) Five-day-old plants expressing *pWOL:XVE>>IAA5(P59L):G3-GFP* (lines #5-4, #6-2, #11-9) were treated with 0.75 μg/ml bleomycin (BLM) and 0.75μg/ml bleomycin plus 10 μM estradiol (BLM+Est), respectively. Cell death was observed after 12 hours treatment. Statistical significance was calculated by student's *t*-test ($n \geq 11$). 'n.s.' indicates no significance.

# Discussion

In response to DNA damage, plants induce multiple responses such as DNA repair, cell cycle arrest and cell death in a tissue- and cell type-specific manner. In *Arabidopsis* roots, DNA damage induces G2 arrest in the meristematic zone, an early onset of endoreduplication in the transition zone, and cell death in stem cells, respectively (Hefner et al., 2006; Adachi et al., 2011; Fulcher and Sablowski, 2009). Previous studies have established that induction of stem cell death by DNA damage is mediated by the ATM/ATR-SOG1 pathway (Furukawa et al., 2010). However, it was not well understood how stem cell death upon DNA damage is controlled. In my study, I found that reduction of auxin signaling is crucial for the induction of stem cell death.

Because of its eminent role in plant growth and development, auxin signaling is a crucial vehicle through which plants impart environmental cues and signals into their developmental program (Kazan, 2013). Multiple biotic and abiotic stresses have been shown to modulate auxin signaling to enhance stress tolerance (Navarro et al., 2013; Iglesias et al., 2014; Vidal et al., 2010; Shibasaki et al., 2009; Shani et al., 2017). Additionally, stresses other than DNA damage have also been shown to induce cell death in various tissues. For example, programmed cell death (PCD) induced during pathogen infection known as hypersensitive response (Lam et al., 2001), drought-induced PCD in leaves to promote senescence (Gechev and Hille, 2005) and salinity-induced PCD in the root tips have been shown previously (Yazdani and Mahdieh, 2012). Almost all these cell deaths are initiated to remove the damaged tissue, which might help in the survival of the organism (Petrov et al., 2015). Auxin has been shown to suppress the cell death induced by elicitors like harpin N, and thaxtomin A and $H_2O_2$

(Chang et al., 2015; Awwad et al., 2019; Kerchev et al., 2015). This suggests that the mechanisms of auxin-mediated cell death suppression may be a common system to help plants to survive under stressful conditions.

I found that DNA damage suppresses PAT through suppression of *PIN1*, *PIN3* and *PIN4*. I also found that auxin signaling was reduced in the vasculature and stem cells. But a direct link between ATM/ATR-SOG1-mediated DNA damage response pathway and suppression of auxin signaling is still lacking. Previously, it has been shown that DNA damage changes the ROS balance in root tips and leads to the accumulation $H_2O_2$ in the root tips in a ATM/ATR-SOG1 dependent manner (Chen and Umeda, 2015). It has been reported that changes in the ROS balance could suppress the PAT by affecting the trafficking and membrane localization of PINs (Yu et al., 2013). In addition, apoplastic ROS has been shown to suppress the auxin signaling through direct oxidation of IAA (Blomster et al., 2011). Thus, it may be possible that DNA damage-induced accumulation of $H_2O_2$ might play a role in the suppression of auxin signaling by DNA damage.

In this study, I found that higher auxin signaling helps the cells to protect their genome while reduction in auxin signaling affects their genome integrity. A recent study showed that tobacco BY2 cells grown in the absence of IAA accumulated higher amount DNA damage even in the absence of any DNA damage-inducing agent, while addition of IAA suppressed the amount of DNA damage, suggesting that auxin signaling mediated through canonical TIR1/AFB pathway helps protecting genome integrity (Hasegawa et al., 2018). They found that reduction in auxin signaling led the chromatin to loosen, which increased the chromatin accessibility for DNA-damaging agent to induce higher amount of DNA damage. Exposure of plants to higher levels of boron induces DNA damage. A recent study showed that exposure to

higher level of boron led to hyperacetylation of chromatin (Sakamoto et al., 2018). Acetylation of chromatin is associated with open chromatin structure. Since open chromatin structure seems to increase accessibility for DNA damaging agent, hyperacetylation due to exposure to higher amount of boron might cause accumulation of DNA damage (Sakamoto et al., 2018). In addition, it has been shown that hyperacetylation is positively correlated with increased amount of DSBs (Sakamoto et al., 2018). Auxin had been shown to regulate chromatin acetylation and deacetylation levels to regulate several gene expression (Weiste and Droge-Laser, 2014). Therefore, it is possible that auxin signaling could regulate the genome integrity by regulating chromatin acetylation levels.

If auxin works solely through above mentioned modulation of chromatin structure (open/close) to regulate genome integrity, then it means auxin does not control the stem cell death induced by DNA damage directly, it regulates the cell death indirectly by regulating the strength of entire DNA damage response. At the same it is still possible, that auxin might regulate the stem cell death directly through some unknown mechanism in parallel to its action on genome stability. For example, Harpin N, an elicitor isolated from *Erwinia amylovora* has been shown to induce effector-triggered immunity (ETI)-mediated programmed cell death. And, auxin was found to be suppressing the harpin induced cell death, but it did not suppress the other immune responses like induction of defense genes (Chang et al., 2015). Further analysis showed that auxin blocked the harpin induced bundling and detaching of actin filaments from plasma membrane. This actin filament bundling and detaching was perceived as membrane damage and programmed cell death was induced (Chang et al., 2015). It is possible that similar mechanism might also be involved in stem cell death.

As shown in the hypothetical model (Figure 10), I expected that induction of *IAA5* and

*IAA29* in and around stem cell niche might lead to reduction of local auxin signaling level and cause excessive amount of DNA damage accumulation in stem cell niche, that might lead the cells to decide that it's an unrepairable level of DNA damage, and induce cell death. I could not see any difference in the amount of stem cell death between wild-type and *iaa5 iaa29* knockout mutant, or with plants overexpressing the mutated stable version of *IAA5*. These results suggest that, in addition to IAA5 and IAA29, other factors are required to reduce the auxin signaling sufficiently to induce stem cell death. In addition, it is possible that IAA5 and IAA29 do not have a role in DNA damage-induced stem cell death, or that auxin signaling level alone cannot determine the induction of stem cell death and some other factors might be involved. In the second part of my thesis, I describe role of two such proteins, ANAC044 and ANAC085. I conclude the first part my thesis, by emphasizing that DNA damage suppresses the auxin signaling and suppression of auxin signaling induces genome instability and leads to accumulation of DNA damage.

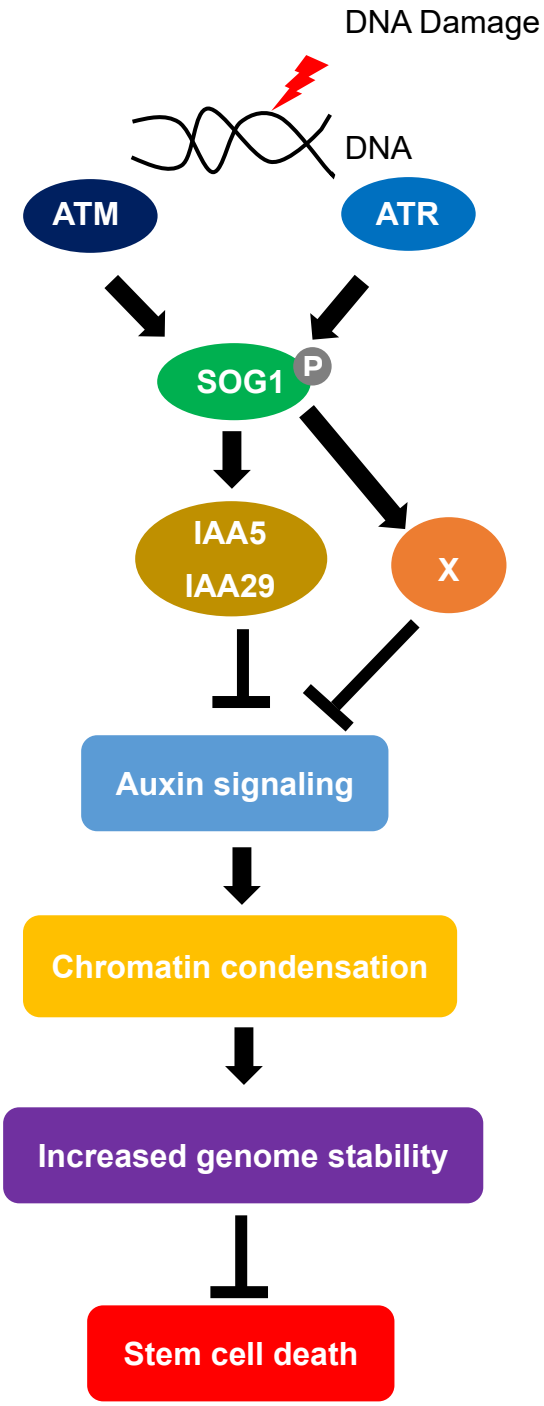



**Figure 10. Hypothetical model for auxin signaling-mediated stem cell death induced by DNA damage**

ATM/ATR-SOG1 pathway suppresses auxin signaling under DNA damage. Suppression of auxin signaling in stem cells is accomplished through induction of *IAA5* and *IAA29* in and around stem cell niche, and possibly through some still unknown pathway. Decrease in auxin signaling may leads to open chromatin structure which increases genome instability and accumulation of higher amount of DNA damage in stem cells, causing to the induction of stem cell death.

# Materials and Methods

## Plant growth conditions

*Arabidopsis thaliana* (Columbia-0) plants were grown vertically under continuous light conditions at 22 °C on half strength Murashige and Skoog (MS) medium containing plates supplemented with 0.5 × MS salts, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES), 1 % sucrose, and 1.2 % phytoagar (pH 5.8).

For cell death observation and measurement, five-day-old seedlings were transferred onto half strength MS medium containing 0.75 μg/mL bleomycin, a DSB inducer. To modify auxin signaling, the above-mentioned medium was supplemented with either 5 nM IAA or 5 μM PEO-IAA. Both IAA and PEO-IAA were dissolved in 100% ethanol.For induction of genes, which were, expressed using an inducible system, above-mentioned media was supplemented with either 10 μM estradiol or 10 μM dexamethasone, depending upon the transgenic plants and expression system used in those plants.

## Plant materials and constructs

*atm-2* (Garcia et al., 2003), *atr-2* (Culligan et al., 2004), *sog1-1* (Yoshiyama et al., 2009) *sog1-101* (Ogita et al., 2018), *DR5v2:n3xVenus* (Liao et al., 2015), *pRAD51:GFP*, *pSMR5:GFP*, and *pPARP2:GFP* (Ogita et al., 2018) have been described previously. For *iaa5* (CS9578), and *iaa29* (SALK_152235C), mutant lines were obtained from TAIR and homozygous mutants were isolated using PCR based genotyping. Primers are listed in the Table I.

## Quantitative RT-PCR

Five-day old seedlings were transferred to half MS media with required supplements. Root tips was collected and frozen immediately with liquid nitrogen. Total RNA was isolated using Trizol and 1 μg of total RNA was used to synthesize complementary DNA (cDNA). cDNA was prepared from total RNA with ReverTra Ace (Toyobo) according to the manufacturer's instructions. For qRTPCR, Thunderbird SYBR qPCR Mix (Toyobo) was used with 10 μM primers and 25 times diluted cDNA sample was used as template.

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* was used to normalize the expression values. Primer sequences are listed in Table I.

**Generation of constructs**

The promoter region of *IAA5* and *IAA29* was amplified and cloned into pDONR221 entry vector by BP recombination. Primers used for amplification are listed in the Table I. To generate *pIAA5:GFP* and *pIAA29:GFP*, entry clones were mixed with pGWB4 (Nakagawa et al., 2007) destination vector and LR reaction was performed.

To create *pWOL:XVE>>IAA5(P59L):G3-GFP*, initially coding region of *IAA5* was amplified from cDNA samples and cloned into pDONR221 using BP reaction. Primers are listed in the Table I. Then entry clones were mixed with R4pGWB650 (Nakagawa et al., 2008) and p1R4-pWOL:XVE (Siligato et al., 2016) and LR reaction was performed.

**Generation of transgenic plants**

All constructs were transferred into *Agrobacterium tumifaciens* GV3101 strain using

electroporation. Four-week-old *Arabidopsis* plants at flowering stage were infected with agrobacterium, and T0 seeds were collected from the infected plants after seed maturation (Harrison et al., 2006). T1 plants were selected on half strength MS medium containing 200 mg/L cefotaxime and appropriate antibiotic. Generally, kanamycin (50 µg/ml), hygromycin (25 µg/ml) and basta (20 µM) were used for antibiotic selection.

**Comet assay**

Five-day-old *Arabidopsis* seedlings were treated with 0.75 µg/ml bleomycin for 6 hours. Root tips of 0.5 cm was collected from around 50 seedlings and chopped with razor blade for 30 seconds in cold phosphate buffer saline (PBS). Comet assay was performed using Comet Assay Kit (Trevigen) with some modifications (Reis et al., 2017). 10 µl of nuclear solution was mixed with 100 µl of molten low melting agarose that was maintained at 37ºC. It was spread onto comet slide immediately. Slides were incubated at 4 ºC in dark for 30 min. Slides were then subjected to electrophoresis (300 mM NaOH, 1 mM EDTA at pH 13) at 1 V/cm for 15 min. Slides were soaked in 1% Triton-X100 in PBS. Then, slides were washed with 96 %ethanol for 5 min and slides were dried for 1 h at 37 ºC. After drying, slides were stained with SYBR gold for 30 min and slides were dried for 37 ºC for 30 min. Fluorescence was observed using confocal microscope. Image J was used to analyze the images.

**Table 1. List of primers**

| Genes | Primer sequences | Usage |
|---|---|---|
| *PIN1* | 5’-GGAACATTGAAATGCCAGCTC-3’ | qRT-PCR |
| | 5’-GGAACATTGAAATGCCAGCTC-3’ | |
| *PIN2* | 5’-ATCGTTCCTTTTGTTTTCGCC-3’ | |
| | 5’-GGTCGTATCGCCTTTTATTTGC-3’ | |
| *PIN3* | 5’-ATAGTTCAGGCCGCATTACC-3’ | |
| | 5’-ACCGAAAGCTTATAACCCGAG-3’ | |
| *PIN4* | 5’-ATCCCACGATTCTAAGCACTG-3’ | |
| | 5’-GCTTTGCTTATTTCCTCGTTACC-3’ | |
| *PIN7* | 5’-TGGCGGTGAGATTCTTTACTG-3’ | |
| | 5’-TGTACTCAAGATTGCGGGATG-3’ | |
| *RAD51* | 5’-GATCACGGGAGCTCGATAAA-3’ | |
| | 5’-GCGGAACTCACCATATAACTCTG-3’ | |
| *PARP2* | 5’-AGCCTGAAGGCCCGGGTAACA-3’ | |
| | GCTGTCTCAGTTTTGGCTGCCG-3’ | |
| *SMR5* | 5’-TTGCCGGATACCAGCATAC-3’ | |
| | 5’-GCGGCTGAAAATATCCCTTC-3’ | |
| *IAA5* | 5’-CGGCTGAGAAAGAAACCCTAC-3’ | Genotyping |
| | 5’-AAGGCTCACTCACATTCACATG-3’ | |
| *IAA29* | 5’-GTAGCCAGTCACCCTCTTTCC-3’ | |
| | 5’-CGAACACAACCTTTTCCAAAG-3’ | |
| *IAA5* | 5’-ggggacaactttgtatagaaaagttgAAGCATATGCAACAACTCTGCACAC-3’ | For cloning promoter |
| | 5’-ggggactgctttttttgtacaaacttgCTTTGATGTTTTTGATTGAAAGTATTG-3’ | |
| *IAA29* | 5’-ggggacaactttgtatagaaaagttgCATATGTCAATGTCAAGCATACAGC-3’ | |
| | 5’-ggggactgctttttttgtacaaacttgTTCTAAGGCAGCTTCGTCTTTGATG-3’ | |
| *IAA5* | 5’-aaaaagcaggctttATGGCGAATGAGAGTAATAATCTTG-3’ | For cloning coding region |
| | 5’-agaaagctgggtaTCCTCTGTTACATGATCTCTTCATAA-3’ | |

# Chapter II

## Role for ANAC044 and ANAC085 in DNA damage-induced stem cell death

# Introduction

*Arabidopsis* induces several responses to DNA damage such as DNA repair, cell cycle arrest and cell death in a tissue- and cell type-specific manner. In *Arabidopsis* roots, DSBs induce G2 arrest in the meristematic zone, an early onset of endoreduplication in the transition zone, and cell death in stem cells (Hefner et al., 2006; Adachi et al., 2011; Fulcher and Sablowski, 2009). Since stem cells are the source for the mitotically active cells, which later becomes differentiated cells, the induction of cell death in stem cells is considered as a protective survival mechanism.

In mammalian and yeast cells, it has been proposed that DNA damage-induced cell cycle delay, known as checkpoint mechanism, allows the cells to repair the lesions. Once DNA damage is repaired, the cell cycle resumes progression. In contrary, in case of cells with irreparable amounts of DNA damage, cell death is induced to remove the cells with mutated DNA (Pucci et al., 2000). The molecular mechanism through which cells decide that DNA damage is irreparable and the underlying mechanism that decides to induce cell death are still largely unknown in plants (Surova and Zhivotovsky, 2013). In plants, few studies have shown that there is some relationship between cell cycle arrest and cell death. It was shown that exposure of synchronized BY-2 cells to cryptogein, a proteinaceous fungal elicitor induces cell cycle arrest at G1 and G2 phases and subsequent cell death (Kadota et al., 2004). In addition, cells treated with elicitor are arrested at G2 phase, and then few hours later cell death was induced. Plant hormones cytokinin and ethylene have been shown to induce cell cycle arrest at G1 and G2/M phase, respectively, and subsequent cell death in BY-2 cells (Suda et al., 2009; Herbert et al., 2001). These results suggest that induction of cell death might be regulated by

cell cycle progression, and cell cycle arrest might be a prerequisite for cell death induction.

In animal cells, DSBs induces both G1 and G2 arrest, through ATM/ATR-p53 pathway. However, in plants ATM/ATR-SOG1 pathway induces only G2 arrest in response to DSBs (Umeda et al., 2019). ATM/ATR-SOG1 pathway induces G2 arrest through two parallel actions. One is suppression of CDK activity and the other is repression of G2/M genes through repression of ACT-MYB and activation of Rep-MYP. It has been shown that ATR-SOG1 pathway is required for DNA damage induced degradation of CDKB2;1, which is required for G2/M transition (Adachi et al., 2011). ATM-SOG1 pathway directly induces several CDK inhibitors like *SMR4*, *SMR5*, *SMR7*, *WEE1* and *KRP6* (Ogita et al., 2018 and Bourbousse et al., 2018). MYB3R transcription factors are regulators of G2/M gene expression. MYB3R4 is an activator, MYB3R3, 5 are repressor of G2/M genes. While MYB3R1 acts as an activator or repressor, depending upon its interacting partners (Kobayashi et al., 2015). DNA damage has been shown to suppress the expression of Act-MYB in a SOG1 dependent manner (Chen et al., 2017). It has been shown that in normal condition Rep-MYB is phosphorylated by CDK and degraded by ubiquitin proteasome pathway. Under DNA damage conditions, due to reduced CDK activity, Rep-MYB is not phosphorylated, which makes Rep-MYB stable and leading to repression of G2/M genes. A recent study by Bourbousse et al. (2018) also showed that Rep-MYB functions in a SOG1-dependent manner to suppress G2/M genes under DNA damage condition, suggesting that Rep-MYB is crucial for G2 arrest in response to DNA damage.

Recently, it has been shown that two of the closest relatives of SOG1, *ANAC044* and *ANAC085* were induced by DNA damage in a SOG1-dependent manner. ANAC044 and ANAC085 were shown to be required for G2/M arrest induced by DNA damage (Takahashi et al., 2019). It was reported that in *anac044 anac085* double knockout mutant, suppression of

G2/M-specific genes at a later time point (around 24 hours after induction of DNA damage) did not occur, and this expression pattern of G2/M-specific genes resembled that in the *myb3r3* and *myb3r5* mutants. In addition, *anac044 anac085 myb3r3* triple mutant was tolerant to DNA damage, similar to *myb3r3* single mutant, suggesting that ANAC044, ANAC085 and MYB3R3 might be working in the same pathway. Moreover, in the *anac044 anac085* mutant, DNA damage-induced accumulation of Rep-MYB did not occur, suggesting that ANAC044 and ANAC085 control the G2/M arrest through the regulation of the protein stability of Rep-MYB. In the second part of this thesis, I tried to understand whether ANAC044 and ANAC085-mediated G2 arrest has any role in the induction of stem cell death by DNA damage.

# Results

## ANAC044 and ANAC085 are required for DNA damage-induced stem cell death

To understand whether ANAC044 and ANAC085 have any role in DNA damage-induced stem cell death, the expression of *ANAC044* and *ANAC085* was studied under DNA damage condition. Five-day-old wild-type and *sog1-101* plants were treated with 0.75 µg/ml bleomycin for 6 hours, and total RNA was extracted from root tips. I found that *ANAC044* and *ANAC085* expression was induced in response to bleomycin in the wild-type plants but not in *sog1-101* plants, suggesting that ATM/ATR-SOG1-mediated pathway induces *ANAC044* and *ANAC085* in response to DNA damage (Figure 1A).

Previously, it has been shown that ANAC044 and ANAC085 are required for DNA damage-induced G2 arrest (Takahashi et al., 2019). To understand whether ANAC044 and ANAC085 are also required for stem cell death, five-day-old wild-type and *anac044-1 anac085-1* seedlings were treated with 0.75 µg/ml bleomycin, and cell death induction was observed after 24 hours, 48 hours and 72 hours of treatment. I found that wild-type plants showed severe cell death in vascular and columella stem cells after 24 hours of treatment. However, in *anac044-1 anac085-1* mutant, stem cell death was not induced at all even after 72 hours of treatment, suggesting ANAC044 and ANAC085 are required for DNA damage-induced stem cell death (Figure 1B). Moreover, treatment with 0.75 µg/ml bleomycin for 72 hours lead to complete loss of meristem in wild-type, whereas *anac044-1 anac085-1* was able to maintain its meristem structure (Figure 1B).

## Overexpression of *ANAC044* enhances stem cell death

Since *anac044-1 anac085-1* did not induce any cell death in response to DNA damage, I further studied the role of ANAC044 and ANAC085 in stem cell death. I generated the overexpression plants of *ANAC044* fused with glucocoluticoid receptor (GR) under the control of *Cauliflower mosaic virus 35S* promoter (*35S:ANAC044-GR*). Five-day-old wild-type seedlings and transgenic plants expressing *35S:ANAC044-GR* were treated with 10 μM dexamethasone and induction of stem cell death was studied after 15 hours of treatment. I found that there was no induction of stem cell death in both wild-type and *35S:ANAC044-GR* (Figure 2A), suggesting that *ANAC044* overexpression by itself cannot induce stem cell death. Next, *35S:ANAC044-GR* seedlings were treated with 0.75 μg/ml bleomycin, and induction of cell death was studied after 15 hours of treatment. It was found that there was no significant difference in amounts of cell death between wild-type and *35S:ANAC044-GR* plants (Figure 2B). In contrary, when *35S:ANAC044-GR* plants were treated with bleomycin and dexamethasone together, cell death area was significantly higher in *35S:ANAC044-GR* than in wild-type seedlings (Figure 2C). These results suggested that ANAC044 enhances DNA damage-induced stem cell death, and ANAC044 requires the activation of DNA damage signaling to induce stem cell death.

**Overexpression of *ANAC044* could partially overcome the auxin-mediated suppression of cell death**

In the first part of this thesis, I have found that DNA damage suppresses auxin signaling and application of exogenous auxin suppressed the DNA damage response through reducing the amount of DNA damage. Since *35S:ANAC044-GR* plants showed higher amounts of cell death than wild-type, I studied whether auxin could suppress the higher amounts of cell

death found in *35S:ANAC044-GR*. Five-days-old wild-type and *35S:ANAC044-GR* plants were treated with 0.75 μg/ml bleomycin plus 10 μM dexamethasone, or 0.75 μg/ml bleomycin, 10 μM dexamethasone plus 10 nM IAA. I found that cell death was suppressed in both wild-type and *35S:ANAC044-GR* seedlings treated with bleomycin, dexamethasone plus IAA than seedlings treated with bleomycin plus dexamethasone (Figure 3A). In addition, I found that among the plants treated with 0.75 μg/ml bleomycin, 10 μM dexamethasone plus 10 nM IAA, *35S:ANAC044-GR* showed significantly higher cell death than wild-type (Figure 3B), suggesting that induction of *ANAC044* could induce significant amount of stem cell death, even with the treatment with IAA.

**ANAC044 and ANAC085 induce stem cell death in parallel with auxin-controlled maintenance of genome integrity**

My results thus far suggest that both auxin-mediated control of genome integrity and ANAC044 and ANAC085-mediated pathway contribute to induction of stem cell death. Since suppression of auxin signaling enhanced the DNA damage response and cell death, I examined whether suppression of auxin signaling in *anac044-1 anac085-1* could induce cell death. Wild-type and *anac044-1 anac085-1* seedlings were treated with 0.75 μg/ml bleomycin plus 5 μM PEO-IAA, and cell death was observed after 30 hours of the treatment. I found that PEO-IAA enhanced the amounts of cell death in wild-type comparing to bleomycin alone treated sample (Figure 4A). However, in *anac044-1 anac085-1,* there was no significant induction of stem cell death after 30 hours of treatment with both bleomycin and bleomycin plus PEO-IAA treated samples (Figure 4B). Although small amounts of cell death were found in both bleomycin and bleomycin plus PEO-IAA treated samples of *anac044-1 anac085-1* double mutant, this cell

death does not occur in stem cells, rather it was found mostly in proximal meristem (Figure 4). These results suggest that suppression of auxin signaling cannot induce stem cell death in *anac044-1 anac085-1*, and that induction of stem cell death requires ANAC044 and ANAC085. In addition, it was suggested that ANAC044 and ANAC085 contribute to induction of stem cell death in parallel with auxin-controlled maintenance of genome integrity.

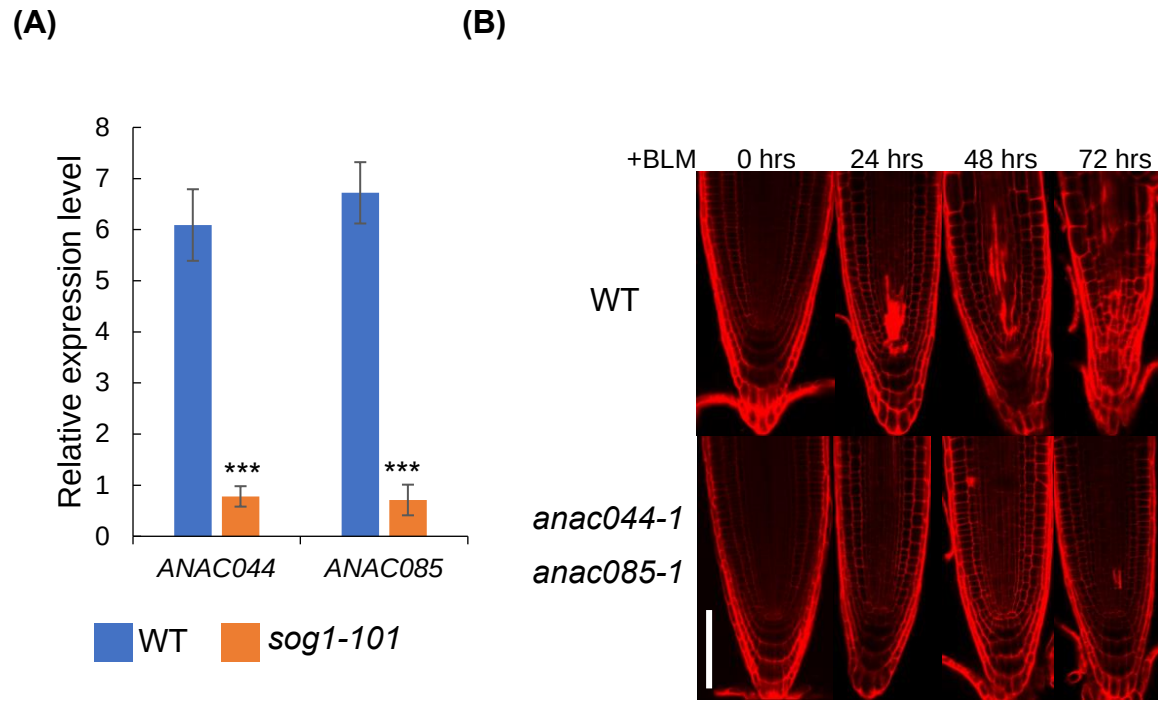



**Figure 1. ANAC044 and ANAC085 are required for induction of DNA damage-induced stem cell death.**

(A) Five-day-old wild-type and *sog1-101* plants were treated with 0.75 μg/ml bleomycin for 6 hours. Total RNA was isolated from root tips. The expression level was normalized with *ACTIN2* level. Statistical significance was calculated using student's *t*-test (***, $P < 0.001$). (B) Five-day-old wild-type (WT) and *anac044-1 anac085-1* seedlings were treated with 0.75 μg/ml bleomycin (BLM) for 0, 24, 48 and 72 hours. Scale bar = 100 μm

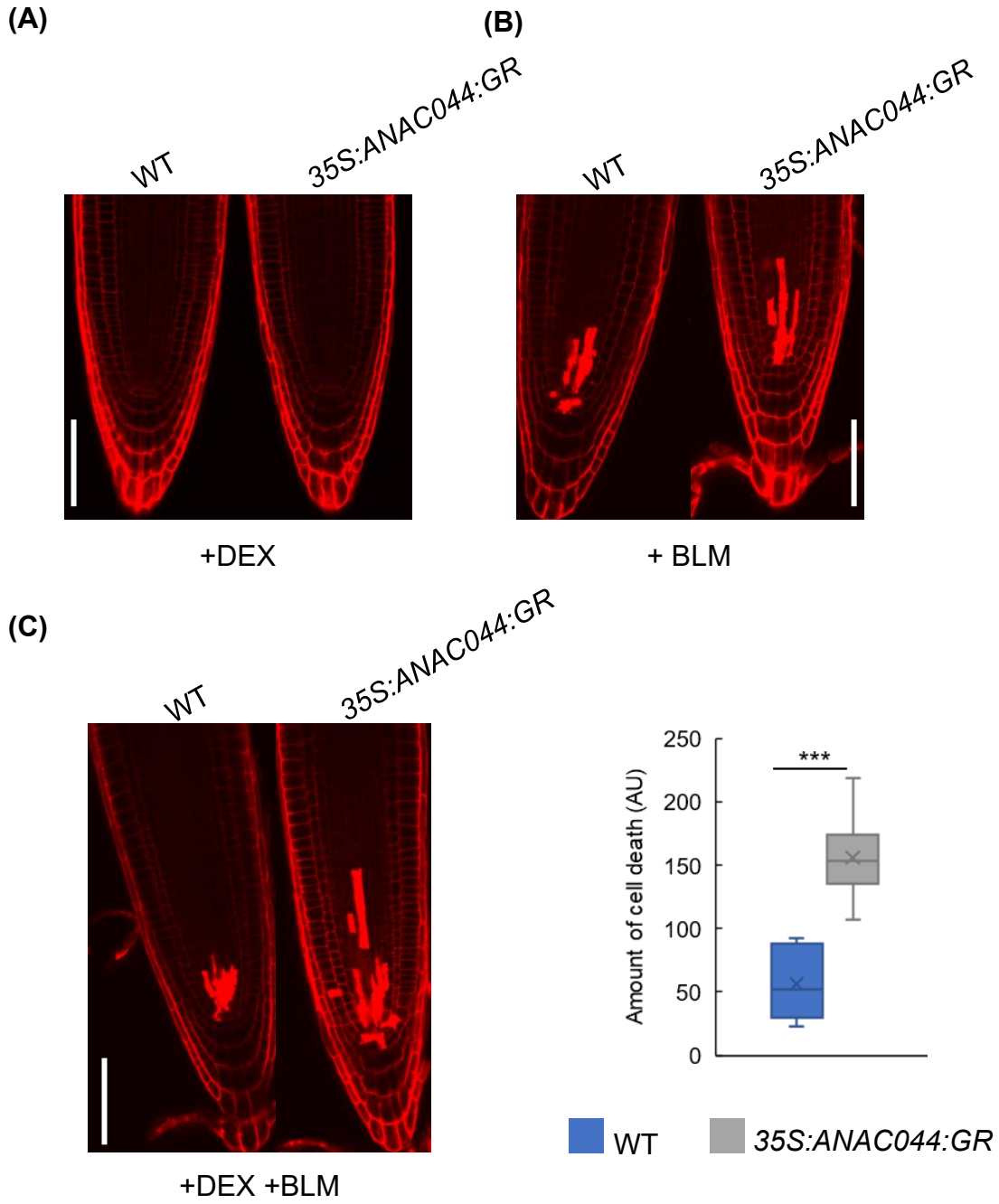



**Figure 2. Overexpression of *ANAC044* enhances stem cell death**

(A) Five-day-old wild-type (WT) and transgenic plants expressing *35S:ANAC044-GR* were treated with 10 µM dexamethasone (DEX) and cell death was observed after 15 h. (B) Five-day-old wild-type (WT) and *35S:ANAC044-GR* were treated with 0.75 µg/ml bleomycin (BLM) and cell death was observed after 15 hours. (C) Five-day-old wild-type and *35S:ANAC044-GR* were treated with 0.75 µg/ml bleomycin plus 10 µM dexamethasone (+DEX +BLM) and cell death was observed after 15 hours. Cell death was measured using Image J and statistical significance was calculated by student's *t*-test (n > 15; ***, $P < 0.001$). AU = arbitrary unit. Scale bar = 100 µm.

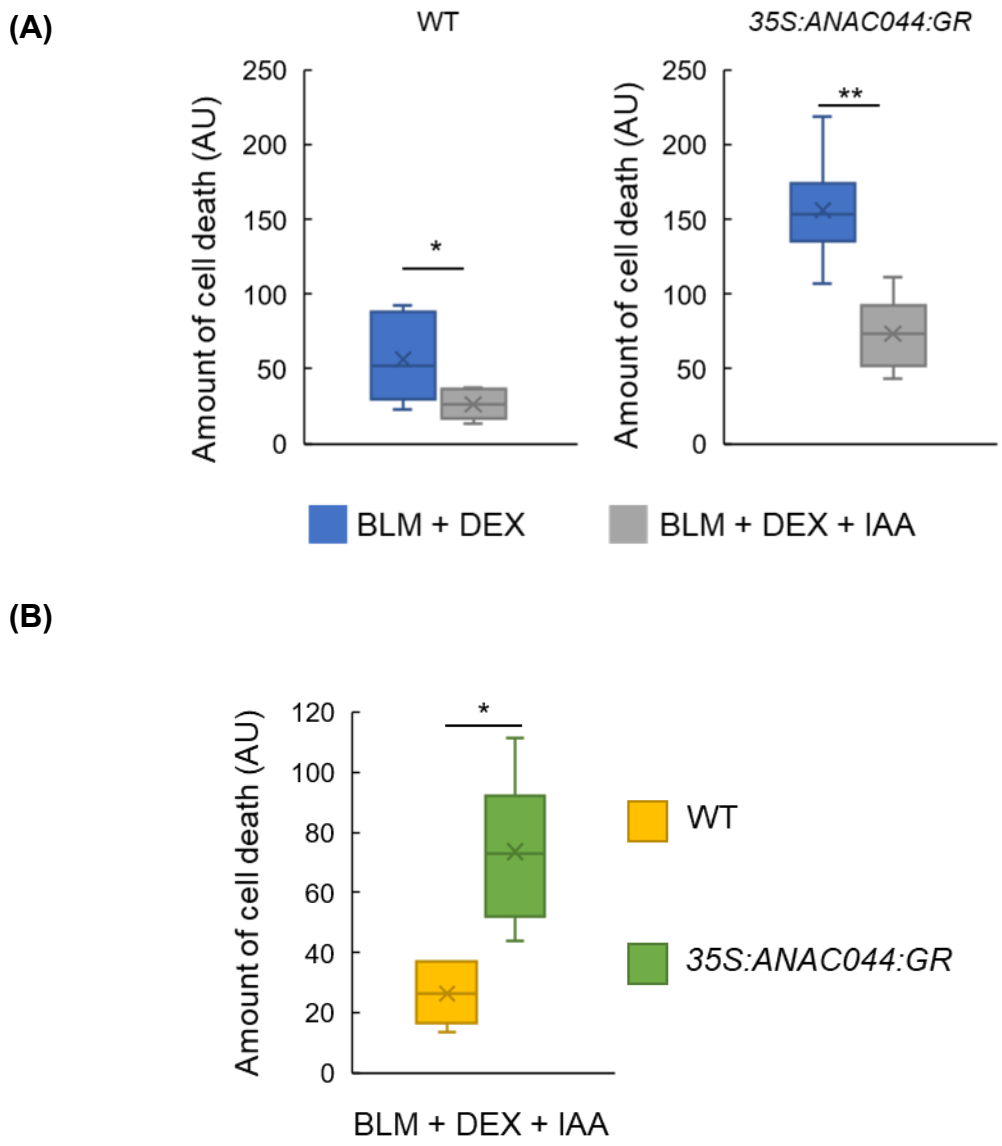



**Figure 3. Overexpression of *ANAC044* could partially overcome the auxin-mediated suppression of cell death**

(A) Five-day-old wild-type and *35S:ANAC044-GR* plants were treated with 0.75 µg/ml bleomycin plus 10 µM dexamethasone (BLM+DEX) and 0.75 µg/ml bleomycin, 10 µM dexamethasone plus 10 nM IAA (BLM+DEX+IAA) for 20 hours, and cell death was measured using Image J software. Statistical significance was measured using student's *t*-test (n > 15; *, $P < 0.05$, **, $P < 0.01$). (B) Five-day-old wild-type and *35S:ANAC044-GR* plants were treated with 0.75 µg/ml bleomycin, 10µM dexamethasone plus 10 nM IAA (BLM+DEX+IAA) for 20 hours, and cell death was measured using Image J software. Statistical significance was measured using student's *t*-test (n > 15; **, $P < 0.01$). AU = arbitrary unit.

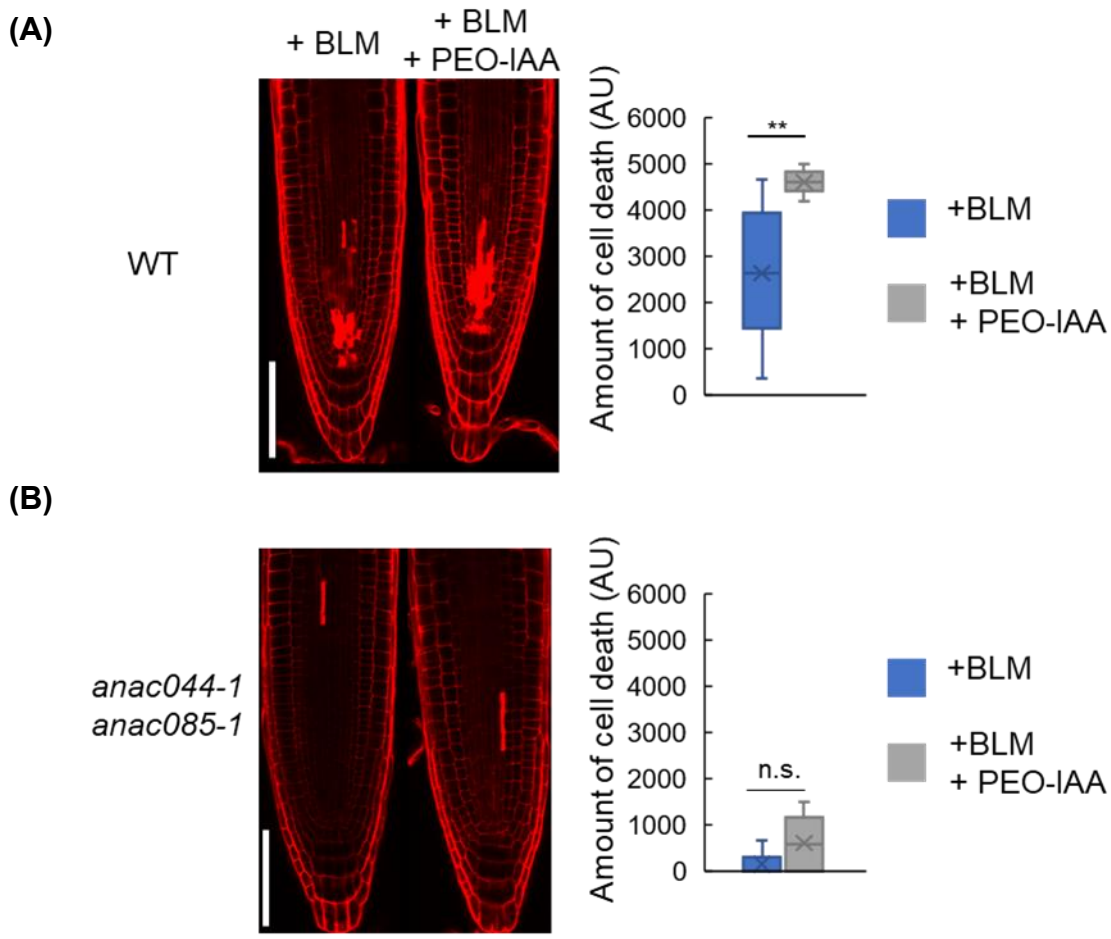



**Figure 4. Suppression of auxin signaling could not induce cell death in *anac044-1 anac085-1***

(A) Five-days-old wild-type (WT) and (B) *anac044-1 anac085-1* plants were treated with 0.75 µg/ml bleomycin (+BLM) and 0.75 µg/ml bleomycin plus 5 µM PEO-IAA (+BLM +PEO-IAA). Cell death was observed after 30 hours of treatment. Statistical significance was measured using student's *t*-test (n > 15; **, $P < 0.01$). 'n.s.' indicates no significance. AU = arbitrary unit. Scale bar = 100 µm.

# Discussion

In *Arabidopsis*, DNA damage induces stem cell death through the ATM/ATR-SOG1 pathway (Furukawa et al., 2010). It has been proposed that when DNA damage is accumulated beyond repair, plants induce stem cell death to avoid the passing of damaged genome to the daughter cells (Hu et al., 2016). The signaling pathways that functions downstream of ATM/ATR-SOG1 and controls the induction of stem cell death is still unknown. Here, I show that two NAC-type transcription factor proteins ANAC044 and ANAC085 induced by DNA damage through ATM/ATR-SOG1 pathway are required for DNA damage-induced stem cell death.

In this study, I found that induction of *ANAC044* in the absence of DNA damage did not induce any cell death. ANAC044 and ANAC085 belongs to NAC transcription factor family. NAC family proteins are known for its ability to make homo- and hetero-dimer with other NAC proteins (Olsen et al., 2005). Therefore, ANAC044 and ANAC085 might form homo- or hetero-dimers with other NAC proteins under DNA damage conditions to induce stem cell death. ANAC044 and ANAC085 are highly similar to SOG1 at sequence level (Gladman et al., 2016). Therefore, it is possible that ANAC044 and ANAC085 might form a complex with SOG1 and/or other NAC-type transcription factor proteins to regulate DNA damage-induced stem cell death.

In the mammalian cells, it has been proposed that DNA damage-induced cell cycle arrest allows the cells to repair the DNA lesions, and cell cycle resumes normally once DNA damage is repaired. In contrary, in case of unrepairable amounts of DNA, cells induce cell death (Pucci et al., 2010). However, how cells determine that particular amounts of DNA damage are unrepairable is still unknown. Previously, it has been proposed that duration of the

cell cycle arrest could act as a cue for the cells to induce cell death (Borges et al., 2008). Existence of these types of mechanisms in plants is still unknown to data. It has been previously shown that DNA damage-induced G2 arrest was not observed in *anac044-1 anac085-1* (Takahashi et al., 2019). Since stem cell death was not observed in *anac044-1 anac085-1*, it is possible that ANAC044 and ANAC085-mediated G2 arrest might be prerequisite for induction of stem cell death by DNA damage.

*ANAC044* and *ANAC085* have been shown to be induced by DNA damage conditions through ATM/ATR-SOG1 pathway. In addition, it has been shown that *ANAC044* and ANAC085 are induced independent of ATM/ATR-SOG1 pathway in response to heat stress to induce G2 arrest, while ANAC044 and ANAC085 did not show any involvement with osmotic stress, which induced G1 arrest, suggesting that ANAC044 and ANAC085 could work as a common module under different environmental stresses that induce G2 arrest (Takahashi et al., 2019). It would be interesting if future studies could show a link between cell death induced by different stresses and cell cycle arrest induced by ANAC044 and ANAC085 at G2 phase. For example, cryptogein and ethylene has been shown to induce cell cycle arrest at G2 and subsequent cell death. It would be interesting to see whether cryptogein, a fungal elicitor and ethylene, a plant growth hormone, could use ANAC044/ANAC085 to induce G2 arrest.

# Materials and Methods

## Plant growth conditions

Plant growth and DNA damage treatment used were described in the first part of Materials and Methods. Briefly, *Arabidopsis* seedlings were grown in continuous light conditions at 22 °C on half strength MS medium supplemented with 0.5 × MS salts, 0.5 g/L MES, 1 % sucrose, and 1.2 % phytoagar (pH 5.8). For cell death observation, five-day-old seedlings were transferred onto half strength MS medium containing 0.75 μg/mL bleomycin. To modify auxin signaling, either 5 nM IAA or 5 μM PEO-IAA was used. For induction of *ANAC044* in the *35S:ANAC044-GR* line, 10 μM dexamethasone was used.

## Plant materials and constructs

*anac044-1 anac085-1* and *35s:ANAC044:GR* have been described previously by Takahashi et al. (2019).

## Quantitative RT-PCR

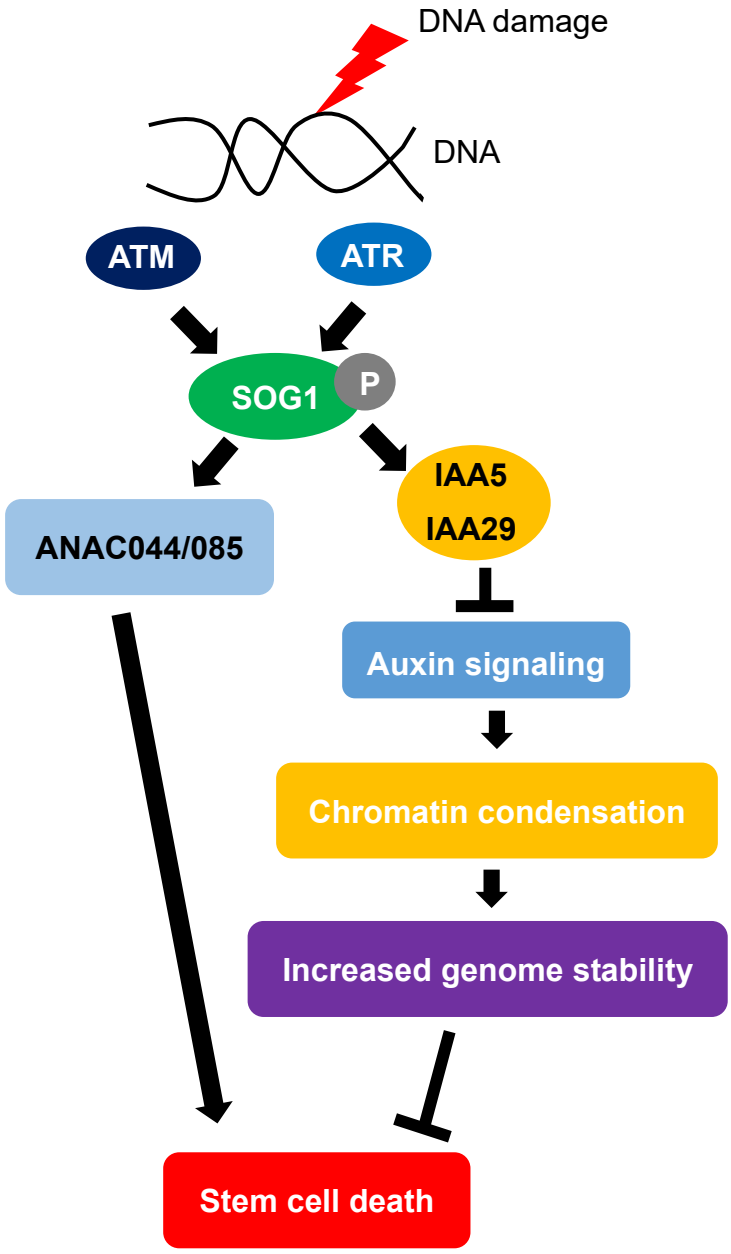
Total RNA was extracted using Trizol from root tips of five-day-old seedlings treated with or without bleomycin. cDNA was prepared from 1 μg of total RNA with ReverTra Ace (Toyobo) according to the manufacturer's instructions. For qRT-PCR, Thunderbird SYBR qPCR Mix (Toyobo) was used with 10 μM primers and 25 times diluted cDNA samples were used as template.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* was used to normalize the expression values.

**Table 1. List of primers**

| Genes | Primer sequences | Usage |
|---|---|---|
| *ANAC044* | 5’-GAGTTCATTCGTCCTGTCACTG-3’ | qRT-PCR |
| | 5’-TGTTCCATATGCCTGCACTGTC-3’ | |
| *ANAC085* | 5’-AGCACACCGAAAACTAGTAC-3’ | |
| | 5’-CTTCAATAACACTCACATTCCC-3’ | |

# Conclusion

Plants are constantly exposed to multiple endogenous and exogenous stresses that might cause DNA damage. Accumulation of DNA damage due to impaired DNA repair, could lead to genome instability and pose a serious threat to survival of species. Plants have evolved a robust repair mechanism to overcome the DNA damage. Plant cells sense DNA damage through conserved ATM/ATR sensor kinase proteins, which pass the signal to SOG1 transcription factor by phosphorylating it. SOG1 acts as a central regulator of DNA damage response. Plants induce multiple responses to DNA damage, such as DNA repair, cell cycle arrest, early onset of endoreplication, and stem cell death. Previous studies have shown that ATM/ATR-SOG1-mediated pathway is required for DSB-induced stem cell death. However, the regulatory pathways of stem cell death downstream of SOG1 was unknown. In the first part of my thesis, I showed that DSBs suppress auxin signaling. Suppression of auxin signaling leaded to accumulation of DNA damage, thereby causing stem cell death. In the second part of my thesis, I showed that *ANAC044* and *ANAC085* were induced by SOG1. Additionally, I found that ANAC044 and ANAC085 are required for induction of stem cell death, possibly working in parallel to the auxin-mediated control of genome integrity.




**Model for DNA damage-induced stem cell death**

DNA damage sensed by ATM/ATR activates SOG1, which controls stem cell death through two possible pathways. SOG1 suppresses auxin signaling, leading to open chromatin structure, which causes accumulation of DNA damage in stem cells and then enhances stem cell death. In parallel, SOG1 directly activates ANAC044 and ANAC085 in response to DNA damage, which is required for induction of stem cell death.

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