学位論文

Studies on roles of the ATM-dependent DNA damage response pathway on control of development in plants

(ATM 依存的 DNA 損傷応答経路が植物の発生に果たす役割の解明)

平成 25 年 7 月　博士（理学）申請

東京大学大学院　理学系研究科

生物科学専攻

久永　哲也

Abstract

During leaf development, a decrease in cell number often associate with an increase in cell size. This phenomenon, called compensation, suggests that some system coordinates cell proliferation and cell expansion but how this is mediated at the molecular level is still unclear. The *fugu2* mutants in *Arabidopsis thaliana* exhibit typical compensation phenotypes. I reported that the *FUGU2* gene encodes FASCIATA1 (FAS1), the p150 subunit of chromatin assembly factor-1 (CAF-1). To uncover how *fas1* mutation induces compensation, I performed microarray analyses and found that many genes involved in the DNA damage response are up-regulated in *fas1*.

In the chapter I, genetic analysis showed that activation of the DNA damage response and accompanying decrease in cell number in *fas1* depend on ATAXIA TELANGIECTASIA MUTATED (ATM) but not on ATM AND RAD3 RELATED (ATR). Kinematic analysis suggested that the delay in the cell cycle leads to a decrease in cell number in *fas1* and that loss of ATM partially restores this phenotype. Consistently, both cell size phenotypes and high ploidy phenotypes of *fas1* are also suppressed by *atm*, supporting that ATM-dependent DNA damage response contributes to these phenotypes. Altogether, these data suggests that ATM-dependent DNA damage response acts as an upstream trigger in *fas1* to delay the cell cycle and promote an entry into the endocycle, resulting in compensated cell expansion.

In the chapter II, to characterize ATM-dependent DNA damage response in plants, I isolated a novel downstream factor of ATM named DNA DAMAGE INDUCIBLE1 (DDI1). My genetic analyses revealed that the *ddi1* mutation suppresses a decrease in cell number without suppressing compensated cell expansion in *fas1*

leaves. Observation of the root meristem in *fas1 ddi1* suggested that the *ddi1* mutation suppresses cell number phenotype in *fas1* through suppressing cell death. Expression analyses indicated that the expression of *DDI1* is induced in *fas1* and under genotoxic stress in an ATM-dependent manner. Furthermore, comet assay suggested that the *ddi1* mutants have defects in repairing the DNA double strand breaks via homologous recombination. These data suggests that DDI1 functions in the ATM-dependent DNA repair pathway and is involved in the control of cell death.

Altogether, my findings revealed an important connection between the DNA damage response and plant organ-size control. The DNA damage response pathway causes both the cell cycle arrest and cell death, leading to a decrease in cell number in organs. These results help us understand how plants modify their organ size when they are faced to genotoxic stress.

Table of contents

ATR-dependent DNA damage response pathway is required for the endocycle progression

DNA damage response pathway contributes to the meristem defects in *fas1* roots

Materials and methods ••••••

List of gene name

AIL6	AINTEGUMENTA LIKE6
AN3	ANGUSTIFOLIA3
ANT	AINTEGUMENTA
AP2	APETALA2
ARF2	AUXIN RESPONSIVE FACTOR2
ARGOS	AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE
ARL	ARGOS-LIKE
ATHB16	ARABIDOPSIS THALIANA HOMEOBOX16
ATM	ATAXIA TELANGIECTASIA MUTATED
ATR	ATM AND RAD3 RELATED
BB	BIG BROTHER
BIN2	BRASSINOSTEROID INSENSITIVE2
BIN4	BRASSINOSTEROID INSENSITIVE4
BOP	BLADE ON PETIOLE
BRCA	BRAST CANCER SUSCEPTIBILITY]
BRI1	BRASSINOSTEROID INSENSITIVE1
CCS52A	CELL CYCLE SWITCH 52A
CDKA;1	CYCLIN DEPENDENT KINASE A;1
CDKB1;1	CYCLIN DEPENDENT KINASE B1;1
CYCA3;1	CYCLIN A3;1
CYCB1;1	CYCLIN B1;1
CYCD3;1	CYCLIN D3;1
DDI1	DNA DAMAGE INDUCIBLE1
DEL1	DP-E2F-LIKE1
EBP1	Erb3 epidermal growth factor receptor binding protein
ELO	ELONGATA
ETG1	E2F TARGET GENE1
FAS1	FASCIATA1
GIF	GRF-INTERACTING FACTOR
GRF	GROWTH-REGUKLATING FACTOR
HPY2	HIGH PLOIDY2
JAG	JAGGED
KLU	KLUH
KN	KNOLLE
KNOX	KNOTTED-LIKE HOMEOBOX
KRP2	KIP-RELATED PROTEIN2
LEUNIG	LUG
NS	NARROW SHEATH
NUB	NUBBIN
OLI	OLIGOCELLULA
PARP	POLY (ADP-RIBOSE) POLYMERASE
RHL2	ROOT HAIRLESS2
RON2	ROTUNDA2
ROT	ROTUNDIFOLIA
RPN12	REGULATORY PARTICLE NON-TRPLE-A ATPASE 12
RPT2A	REGULATORY PARTICLE TRIPLE-A ATPASES 2A
SCR	SCARECROW

SOG1	SUPPRESSOR OF GAMMA RESPONSE 1
SPT	SPATULA
SWP	STRUWWELPETER
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, and PCF
TEB	TEBICHI
TOR	TARGET OF RAPAMYCIN
TSK	TONSOKU
WUS	WUSCHEL
XS	EXTRA SMALL SISTERS

List of abbreviations

APC/C	anaphase-promoting complex/cyclosome
BR	brassinosteroids
CAF-1	chromatin assembly factor-1
DSB	double strand break
GUS	beta-glucuronidase
HDZip I	homeodomain leucin zipper class I
HR	homologous recombination
NHEJ	non-homologous end joining
UV-B	ultra-violet light B

Chapter I

General introduction

Variation in size of organisms we see in nature is in principle dependent on their organ size variation. Each organism has species-specific size, suggesting the existence of genetic control of organ growth to form their final size and shape. The question of how organ growth is genetically controlled has been fascinating for a lot of scientists. Plant leaves are an ideal organ to study genetic control of organ growth because they have flat shape and grow into constant size under given growth conditions. Furthermore, proper control of leaf growth is important for efficient reception of sunlight and photosynthesis. Because production of our food, feed and fuel depends on plants, understanding genetic control of organ growth has us important implications for the manipulation of crop yields.

Leaf development

Leaf primordia initiate at the flank of the shoot apical meristem as a rod-shaped protrusion. Sector analyses suggest that an early leaf primordium consists of 100-150 cells in tobacco (Poethig and Sussex, 1985) and about 100 cells in cotton (Dolan and Poethig, 1998) whereas the number of founder cells in Arabidopsis is much smaller (Irish and Sussex, 1992; Schnittger et al., 1996). Analyses on a maize mutant, *narrow sheath1* (*ns1*) and *ns2* suggest that the number of founder cells is an important determinant for final organ size in monocot plants. Furthermore, Arabidopsis *struwwelpeter* (*swp*) mutants have a decreased cell number in their leaf and this decrease appears at very early stage of leaf primordia, suggesting that founder cell

number is reduced in the leaf primordia of *swp* mutants (Autran et al., 2002). After leaf initiation, a leaf primordium grows by cell proliferation. While cells divide actively throughout the whole leaf primordium during early stage, as the leaf development proceeds, cell division starts to be restricted towards the junction between the leaf blade and leaf petiole (Donnelly *et al*., 1999; Nath *et al*., 2003; White, 2006; Ichihashi *et al*., 2010, 2011; Kazama *et al*., 2010). This makes a proximal-distal gradient of cell proliferation activity, called cell cycle arrest front in the leaf blade. Recent studies suggest that arrest front does not progress gradually as previously thought, but it remains at an almost fixed position during certain period and then move quickly towards the base of the leaf blade (Kazama et al., 2010; Andriankaja et al., 2012). After the termination of cell proliferation, cells in tip region start to undergo differentiation and expansion to increase their volume, indicating that different cellular process (cell proliferation and cell expansion) occur at the same time in different regions within one organ. Finally, all cells in leaves stop to proliferate and start to expand and then they reach their final size.

Leaf size control by cell proliferation

After specification of founder cells during early stage, leaf primordia grow mainly by cell proliferation. Several genetic and molecular studies have revealed regulators of cell proliferation during the leaf growth. The APETALA2 (AP2) type transcription factors AINTEGUMENTA (ANT) and AINTEGUMENTA LIKE6 (AIL6) are expressed in young leaf primordia and promote cell proliferation (Mizukami and Fischer, 2000; Krizek, 2009; Nole-Wilson et al., 2005). While *ant* and *ail6* mutants have smaller leaves with reduced cell number, overexpression of *ANT* and *AIL6* prolongs the

period of cell proliferation, increasing cell number in leaves. ANT acts as a transcriptional activator whose overexpression maintains high level expression of cell-cycle-related genes such as *CYCLIN D3;1* (*CYCD3;1*) (Mizukami and Fischer, 2000). Strong overexpression of *CYCD3;1* is sufficient to increase cell number in leaves but final leaf size is smaller because cells fail to differentiate and expand normally (Dewitte et al., 2003). In contrast, moderate increase in the expression of *CYCD3;1* increases cell number in leaves without affecting cell size (Horiguchi et al., 2008). These data suggest that finely balanced expression of *CYCD3;1* is essential to properly shift from proliferation to differentiation during leaf development. A plant hormone, auxin, is a key upstream regulator of *ANT*. The auxin-inducible gene *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*) encodes a plant-specific protein (Hu et al., 2003). Knockdown for *ARGOS* reduces cell number associated with decreased expression of *ANT* and *CYCD3;1* in leaves, whereas overexpression of *ARGOS* leads to increase in cell number and the expression of *ANT* and *CYCD3;1*. Further, the *ant* mutation suppresses large leaf phenotype of ARGOS overexpressor. These data suggest that ARGOS, whose expression is induced by auxin, increases leaf cell number through promoting the *ANT* expression, leading to up-regulation of *CYCD3;1*. Another auxin responsive gene, *AUXIN RESPONSIVE FACTOR2* (*ARF2*) represses the *ANT* and *CYCD3;1* expression, suggesting that *ARF2* antagonaizes *ARGOS* in the control of *ANT* expression (Schruff et al., 2006). ARF2 may be important for coordination between auxin signaling and brassinosteroids (BRs) signaling (Vert et al., 2008). The BR-regulated kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) phosphorylates ARF2 upon induction by BR, leading to reduction of its DNA binding activity. Thus negative regulation of ANT expression by

ARF2 is repressed by BR-mediated phosphorylation. Analyses on the cytochrome p450 gene *KLUH* (*KLU*) suggest the existence of a novel plant hormone that controls organ growth by cell proliferation (Anastasiou et al., 2007). The *klu* mutants have reduction in leaf size with fewer cells whereas *KLU* overexpression increases leaf size via increasing cell number. *KLU* is expressed throughout young leaf primordia then it becomes limited to the leaf edge along leaf development even cells in the basal part of the leaf are still dividing. These data suggest that KLU promotes cell proliferation in a non-cell-autonomous manner. Transcriptome analyses revealed that all known plant hormone pathways are not affected in the *klu* mutant, suggesting that KLU enzyme may catalyze a reaction to produce unknown plant hormone.

The *GROWTH-REGULATING FACTOR* (*GRF*) gene family is another plant-specific group of transcription factors involved in leaf size control (van der Knaap et al., 2000). The Arabidopsis genome contains nine members of *GRFs* and all of them show elevated expression level in developing leaves (Kim et al., 2003). The *grf1 grf2 grf3* triple mutant exhibits smaller leaf phenotype due to a decrease in cell number, whereas overexpression of *GRF1* and *GRF2* increase leaf cell number, forming large leaves (Kim and Kende, 2004; Kim et al., 2003). These data suggest that GRFs redundantly promote cell proliferation during leaf development. The micro RNA miR396 targets seven of the nine GRFs. The expression of miR396 is increased along leaf maturation and overexpression of miR396 reduces the expression of several *GRFs* and *CYCLIN B1;1*, leading to a decrease in cell number (Rodriguez et al., 2010). These data suggest that GRFs and miR396 antagonistically control cell proliferation during leaf development. GRFs physically interact to putative transcriptional co-activator, GRF-INTERACTING FACTORs (GIFs) (Horiguchi et al., 2005; Kim and Kende, 2004).

Arabidopsis has three members of the GIF family. Loss-of-function mutants for *ANGUSTIFOLIA3 (AN3)/GIF1* have narrower leaves with decreased cell number, whereas the *gif1 gif2 gif3* triple mutant exhibits more severe decrease in leaf cell number, suggesting that GIFs also act redundantly during leaf development (Horiguchi et al., 2005; Kim and Kende, 2004; Lee et al., 2009).

Two related zinc finger type transcription factors, JAGGED (JAG) and NUBBIN (NUB), regulate organ growth by promoting cell proliferation (Dinneny et al., 2004, 2006; Ohno et al., 2004). The *jag* mutant exhibits early termination of cell proliferation phase during organ growth, leading to the loss of distal part of the floral organ. While *nub* single mutants exhibits only mild phenotypes, *jag nub* double mutants have strongly reduced leaf and floral organ size. In contrast, overexpression of JAG causes ectopic outgrowth of lamina from leaf petiole and ectopic formation of bracts. These data suggest that JAG mediates pattern-specific cell proliferation, rather than regulating general activity of cell proliferation during organ growth. *BLADE ON PETIOLE1* (*BOP1*) and *BOP2* encode proteins with BTB/POZ domain and ankyrin repeats and regulate leaf growth. The *bop1 bop2* double mutant has ectopic lamina tissue on the leaf petiole and ectopic bracts with increased expression of class I *KNOTTED-like homeobox* (*KNOX*) genes and *LATERAL ORGAN BOUNDARIES* genes (Ha et al., 2003, 2004, 2007; Norberg et al., 2005). Also, *bop1 bop2* double mutants show increased expression of *JAG* and *NUB*, suggesting that *BOP* genes restrict tissue growth in a region specific manner by repressing *JAG* and *NUB* (Norberg et al., 2005). On the other hand, overexpression of *BOP* genes strongly reduces organ size and this phenotype is similar to double mutants of the class I *KNOX* gene *BREVIPEDICELLUS* and BELL-like homeobox gene *BELLRINGER* (Ha et al., 2007). Altogether, *BOP* genes

seem to regulate organ growth through controlling the expression of *JAG*, *NUB* and *KNOX* genes.

The bHLH type transcription factor SPATULA (SPT) regulates organ size through controlling cell proliferation. The *spt* mutants have larger dividing zone in young leaf primordia, leading to an increase in final cell number in leaves, whereas overexpression of *SPT* decreases both cell number and cell size (Ichihashi et al., 2009). These data suggest that SPT negatively regulates final organ size by restricting the size of proliferating zone during leaf development. Another known factor which affects size of proliferating zone in leaf primordia is *ROTUNDIFORIA4* (*ROT4*) which encodes a peptide without a signal sequence for secretion. Overexpression of *ROT4* reduces the size of proliferating zone in leaf primordia specifically along the proximodistal axis, leading to shorter leaf phenotype (Narita et al., 2004; Ikeuchi et al., 2010). Local expression of *ROT4* using chimera system revealed that ROT4 perturbs positional information along proximodistal axis (Ikeuchi et al., 2010).

Control of cellular protein level appears to be important for regulation of cell proliferation. In animals, the Erb-3 epidermal growth factor receptor-binding protein EBP1 is suggested to provide a potential link between ribosome biogenesis and proliferation control (Squatrito et al., 2004). Arabidopsis also has *EBP1* and decreased levels of *EBP1* expression reduce cell number and cell size, leading to decreased leaf size. On the other hand, increased levels of *EBP1* activity make leaves larger mainly by increasing cell number, suggesting that *EBP1* primarily promotes organ growth by proliferation, possibly via stimulating ribosome biogenesis (Horvath et al., 2006). The class I TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) protein, TCP20 binds to the GCCCR element in the promoters of genes encoding ribosomal proteins

and *CYCB1;1* gene to activate their expression (Li et al., 2005). This suggests that TCP20 could coordinate ribosome biogenesis and cell division. Another mechanism that modulates cellular protein level is the protein degradation and several studies suggested the role of protein degradation in organ size control. One of the best studied strategies that underlie protein degradation is mediated by protein ubiquitination. Three types of enzymes named E1, E2 and E3 sequentially function to ubiquitinate a target protein, then the ubiquitinated protein is degraded by 26S proteasome (Vierstra, 2009). Generally, the target specificity is determined by the E3 ligase. The E3 ligase BIG BROTHER (BB) controls organ size by restricting cell proliferation (Disch et al., 2006). The *bb* mutant has larger organ size with increased cell number while over expression of BB cause severe reduction in cell number. Furthermore, the protein levels of BB increases along leaf development. These data suggest that BB acts as an intrinsic brake for growth to prevent overgrowth. Loss of function in the putative ubiquitin receptor gene *DA1* also results into larger organ size with an increased cell number (Li et al., 2008). The *da1* mutation enhances the larger organ size phenotypes in *bb*, suggesting that these factors act synergistically to restrict cell proliferation during organ growth, possibly via degradation of positive growth regulators.

Leaf size control by cell expansion

After proliferative growth, cells in organs start to grow by post-mitotic cell expansion. Cell expansion requires the synthesis of new cell wall material, water uptake into the vacuole and controlled loosening of the cell wall to permit an increase in the cell volume. Cell-wall-associated proteins called expansin are the key factors for cell wall loosening. Expansin increases cell wall extensibility by loosening the cell wall but

precise function of expansin is still unclear (Cosgrove, 2005). Overexpression of expansin promotes organ growth whereas down regulation of expansin expression reduces organ size (Cho and Cosgrove, 2000; Choi et al., 2003; Zenoni et al., 2004). Endogenous expression of expansin is increased during organ growth and decreased along cessation of organ growth. These data suggest that expansin promotes organ growth primarily via controlling cell expansion. Studies in yeast have shown that cell growth is tightly controlled by ribosome biogenesis (Cook and Tyers, 2007). TARGET OF RAPAMYCIN (TOR) kinase is an important regulator of ribosome biogenesis in yeast and it is conserved in plants. The loss-of-function mutants in Arabidopsis TOR exhibits embryonic lethality but mild downregulation of TOR function by RNAi reduce leaf size by decreasing cell size (Menand et al., 2002; Deprost et al., 2007). On the other hand, plants overexpressing TOR have larger leaves consisting of larger cells. In these plants, TOR activity correlates with altered levels of translationally active polyribosomes and *EBP1* expression levels. These data suggest that TOR promotes cell expansion via controlling ribosome biogenesis. ARGOS-LIKE (ARL) is a gene whose structure is similar to ARGOS (Hu et al., 2006). Downregulation of ARL reduce leaf size whereas overexpression of ARL increase leaf size. Changes in the leaf size in plants with modified expression of *ARL* are mainly caused by altered cell size rather than cell number, suggesting that ARL is a positive regulator of cell expansion. The expression of *ARL* is induced by exogenous application of BR and decreased in BR insensitive mutant *bri1*. Furthermore, overexpression of ARL in *bri1* partially suppress growth defects in *bri1*, suggesting that ARL functions downstream of BRI1 to mediate BR-related cell expansion during organ growth.

Several factors are known as a brake for cell expansion during leaf growth.

Reduced expression of *ARABIDOPSIS THALIANA HOMEOBOX 16* (*ATHB16*) which encodes a homeodomain leucine zipper class I (HDZip I) protein increase cell size in leaf whereas overexpression of *ATHB16* reduce cell size (Wang et al., 2003). Similarly, loss of function in *ROTUNDA2* (*RON2*)/*LEUNIG* (*LUG*) which encodes a transcriptional repressor increases leaf size by increasing cell size (Cnops et al., 2004). Therefore, ATHB16 and RON2 restrict cell expansion during later leaf development to prevent over growth.

In both plants and animal cells, nuclear ploidy level is increased by the process called endocycle. Endocycle is a modified cycle of mitotic cell cycle in which DNA replication occurs without mitosis, leading to polyploidy. Polyploid cells are found among various types of cells such as hypocotyle cells and trichome of plants. In Arabidopsis, not only trichomes but also most of differentiated cells have polyploid nuclei. A previous study showed that ploidy level correlates to cell size in pavement cells in leaf and sepal epidermis (Melaragno et al., 1993; Roeder et al., 2010), suggesting that the ploidy level is important for cell size regulation in several organs. Mutants which have lower ploidy level such as *brassinosteroid insensitive 4* (*bin4*), *hypocotyl 6* (*hyp6*), *root hairless 1* (*rhl1*) and *rhl2* often have smaller cells whereas mutants which have higher ploidy level such as *regulatory particle triple-a atpases 2a* (*rpt2a*) and *regulatory particle non-triple-a atpase 12* (*rpn12*) often have larger cells (Breuer et al., 2007; Kurepa et al., 2009; Sonoda et al., 2009; Sugimoto-Shirasu et al., 2002, 2005). Further, the tetraploid Arabidopsis plants exhibit larger organ size caused by larger cell size (Breuer et al., 2007). These data support the idea that nuclear ploidy level is important for cell size control. Although ploidy level often correlates to cell size, ploidy level is not an absolute determinant for cell size. The *rpt2a* mutants have larger

cells in leaf epidermis and petal epidermis than WT. Flow cytometry analyses revealed that ploidy level distribution is higher than WT in leaves but similar to WT in petals, suggesting that effects of ploidy level on cell size is different among different cell types (Kurepa et al., 2009). Fujikura et al. analyzed ploidy level distribution of *extra small sisters* (*xs*) mutants which have smaller cells in their leaves and found that *xs5* exhibits higher ploidy levels than WT (Fujikura et al., 2007). Furthermore, RNAi knockdown for replication licensing factor gene CDT1A exhibits similar phenotypes to *xs5*, indicating that ploidy level does not always correlate to cell size (Raynaud et al., 2005). It should be also noted that polyploidy caused by endocycle is found in limited species of annual herbal plants (Barow and Meister, 2003). Therefore, plants have several pathways to promote cell expansion; one is coupled with ploidy level and another is uncoupled with ploidy level. Recent studies have identified several regulators of endocycle. The A type cyclin, CYCLIN A2;3 represses endocycle onset (Imai et al., 2006; Boudolf et al., 2009) through interaction with CYCLIN DEPENDENT KINASE B1;1 (CDKB1;1). The transcription factor, DP-E2F-like1 (DEL1) directly represses the expression of anaphase-promoting complex/ cyclosome (APC/C) activator gene CCS52A2, inhibiting premature entry into endocycle (Lammens et al., 2008; Vlieghe et al., 2005). Plant hormone auxin and SUMO E3 ligase, HIGH PLOIDY2 (HPY2) modulates transition from mitotic cell cycle to endocycle through the regulation of cell cycle regulators (Ishida et al., 2009; Ishida et al., 2010). Although there are various factors that regulates endocycle onset, involvement of these factors in compensation is still unclear.

Compensation gives new insights into organ size control

As I described above, organ size is mainly determined by the cell number and

the cell size. The cell number in the organ is controlled by cell proliferation whereas the cell size is controlled by post-mitotic cell expansion. Not only such cellular level controls, there is a whole organ level control in organ size control. Past studies on the wing of fruits fly (*Drosophila melanogaster*) indicates that the wing size is controlled through a mechanism called total mass checkpoint. Wings that have decreased cell proliferation activity have increase in cell size whereas wings that have increased cell proliferation activity have decreased cell size. Therefore, wing size is always constant even their cell proliferation activity is modulated (Potter and Xu, 2001). Compared to wing of flies, the increase in cell proliferation activity does not affect post-mitotic expansion in plants. Both ANT overexpression and AN3 overexpression increases their cell number in leaves but do not affect their leaf cell size (Mizukami and Fissher, 2000; Horiguchi et al., 2005). On the contrary, loss of function of *ant* and *an3* decreases leaf cell number and increases leaf cell size. This phenomenon, called compensation, suggests the existence of an interaction between cell proliferation and post-mitotic cell expansion during leaf development (Tsukaya, 2003; Beemster et al., 2003). Compensation is observed in various mutants and transgenic plants of Arabidopsis (reviewed in Horiguchi and Tsukaya 2011). Not only genetic modification but also environmental stresses which cause DNA damage such as gamma irradiation and UV-B irradiation induce compensation in wheat and Arabidopsis (Haber, 1962; Wargent et al., 2009). Such coordination mechanisms seem to be important for plastic development of plants but molecular mechanisms that coordinate cell proliferation and post-mitotic cell expansion are largely unknown.

Compensation is heterogeneous events

Several studies provide insights into the mechanisms of compensation. Ferjani et al. isolated five compensation exhibiting mutants, named *fugu*, and performed kinematic analysis on their leaf growth (Ferjani et al., 2007). These analyses revealed that dividing cells in most of the compensation exhibiting mutants have similar size to WT, indicating that compensated cell expansion occurs post-mitotically and not as a result of uncoupling of cell proliferation and cell expansion. They also revealed that there are at least three modes of compensation in the meaning of their cellular kinetics. Fujikura et al. performed genetic analyses using *oligocellula* (*oli*) mutants which have decreased cell number and normal cell size in their leaves (Fujikura et al., 2009). The single *oli* mutants have mildly decreased cell number without compensation, but the double mutants between different *oli* loci exhibit severe reduction in their cell number and compensation is induced (Fujikura et al., 2009). Based on these results, Fujikura et al. proposed a threshold theory in which compensation is induced by the extent of reduction in cell proliferation in a threshold-dependent manner (Fujikura et al., 2009). Several mutants of the *extra small sisters* (*xs*) mutants which have smaller cells in their leaves suppressed *an3*-dependent compensation (Fujikura et al., 2007). These data suggest that compensated cell expansion is driven by hyper-activation of cell expansion pathway in normal growth (Fujikura et al., 2007). Kawade et al. constructed leaves that consist of two types of cells; one is compensation-exhibiting genotype and another is non-compensation-exhibiting genotype (Kawade et al., 2010). These analysis of the chimera revealed that *an3*-dependent compensation is induced in a non-cell autonomous manner whereas *KRP2 (KIP RELATED PROTEIN2)*-dependent compensation is induced in a cell autonomous manner (Kawade et al., 2010). These studies indicate that the compensation is not a single phenomenon but contains several different pathways.

Therefore, to understand mechanisms of compensation, it is necessary to analyze compensation-exhibiting mutants other than *an3* because most of studies on compensation have done using *an3* mutant. Here I analyze a compensation-exhibiting mutant *fugu2* (Ferjani et al., 2007) which has several different characteristics from *an3*, such as different kinetics of cell number increase and different leaf shape. Based on my results, I will discuss one of the mechanisms to induce compensation during leaf development.

Chapter II

The ATM-dependent DNA damage response acts as an upstream trigger for compensation in the *fas1* mutation during Arabidopsis leaf development

INTRODUCTION

Organ size is determined by cell number and cell size, both of which can be modulated by various physiological and environmental changes. To reach proper organ size under various growth conditions, cell proliferation and post-mitotic cell expansion must be highly coordinated during organogenesis. Despite the importance of these processes in the control of organ size, the underlying mechanisms are not well understood. Plant leaves usually reach constant size under given growth condition, making them an excellent model system to study organ-size control. All cells in a young leaf primordium actively proliferate at the beginning but as leaf development proceeds, cell proliferation starts to be restricted towards the junction between the leaf blade and leaf petiole (Donnelly *et al*., 1999; Nath *et al*., 2003; White, 2006; Ichihashi *et al*., 2010, 2011; Kazama *et al*., 2010), generating a proximal–distal gradient of cell proliferation activity in the leaf blade. Subsequently, cells that terminate proliferation in the distal region start post-mitotic expansion whereas cells in the proximal region still continue to proliferate (Beemster *et al*., 2005). The leaf eventually reaches its appropriate final size when all cells stop proliferation and expansion.

During leaf development, a defect in cell proliferation often triggers enhanced cell expansion. When wheat seedlings are irradiated with gamma rays, they develop leaves that have fewer but larger cells than non-irradiated seedlings (Haber, 1962). This phenomenon, called compensation, has been reported in various mutants and transgenic plants of *Arabidopsis thaliana* (Arabidopsis, hereafter) (for reviews, see Tsukaya, 2002; Beemster et al., 2003; Horiguchi and Tsukaya, 2011). For example, a loss-of-function mutation in a transcriptional coactivator ANGUSTIFOLIA3 (AN3) or overexpression of a cyclin-dependent kinase inhibitor KIP RELATED PROTEIN2

(KRP2) both cause a compensation phenotype (De Veylder *et al*., 2001; Horiguchi *et al*., 2005; Ferjani *et al*., 2007). Kinematic analysis of various compensation mutants revealed that the size of dividing cells in most of these mutants is similar to that of wild-type (WT) plants, strongly suggesting that compensated cell expansion is not a result of uncoupling between cell proliferation and cell expansion (Ferjani *et al*., 2007). It is thought, instead, that some regulatory system operates post-mitotically to coordinate cell proliferation and cell expansion. Such a system should be an advantage for plants to support their highly plastic development but molecular mechanisms underlying this control are largely unknown.

Several recent reports have begun to provide insights into the mechanism of compensation. A study on Arabidopsis *oligocellula* (*oli*) mutants suggested that compensated cell expansion is induced by the extent of reduction in cell proliferation in a threshold-dependent manner (Fujikura *et al*., 2009). Moreover, several different mode of compensation, in terms of duration and rate of cell expansion, is observed in various *fugu* mutants, implying that compensation might be mediated through multiple mechanisms (Ferjani *et al*., 2007). Chimera analysis also revealed that excess cell expansion induced by the *an3* mutation is non-cell-autonomous whereas the expansion induced by *KRP2* overexpression is cell-autonomous (Kawade *et al*., 2010). Genetic analysis using *extra small sisters* (*xs*) and the compensation-exhibiting *an3* mutants showed that compensated cell expansion is governed by hyperactivation of cell expansion pathways required for normal cell expansion (Fujikura *et al*., 2007). Among many multicellular organisms, a positive correlation exists between cell size and nuclear ploidy level (Nagl, 1976; Melaragno *et al*., 1993). Nuclear ploidy is increased by a process called endocycle or endoreduplication cycle in which nuclear DNA is

replicated without mitosis. Several Arabidopsis mutants have defects in the endocycle progression and in many cases, these defects are accompanied by altered cell size, suggesting that ploidy regulation is important for the control of cell size (Sugimoto-Shirasu *et al*., 2005; Breuer *et al*., 2007; Kurepa *et al*., 2009; Sonoda *et al*., 2009). Some, but not all, mutants exhibiting compensation also show higher ploidy phenotypes, suggesting that an increase in ploidy may play some roles in compensated cell expansion (Ferjani *et al*., 2007).

Chromatin assembly factor-1 (CAF-1) is a histone chaperon consisting of three subunits (p150, p60 and p48 in humans) and it functions in the nucleosome assembly, recruiting histones H3-H4 onto a newly synthesised DNA chain (Smith and Stillman, 1989, 1991; Shibahara and Stillman, 1999; Tagami *et al*., 2004). CAF-1 is well conserved among eukaryotes but the consequence of CAF-1 disruption is not identical among different organisms. For example, cultured human cells with defective CAF-1 do not proceed into the cell cycle and instead undergo apoptosis (Hoek and Stillman, 2003; Ye *et al*., 2003; Nabatiyan and Krude, 2004). The CAF-1 mutation in *Drosophila* similarly leads to complete arrest of the cell cycle and subsequent lethality (Song *et al*., 2007). Therefore, the CAF-1 activity appears to be essential for the survival of animals and insects. In contrast, the CAF-1 mutation only delays cell cycle progression in yeasts and these defects are accompanied by heterochromatin silencing (Kaufman *et al*., 1997; Enomoto and Berman, 1998). In Arabidopsis *FASCIATA1* (*FAS1*) and *FAS2* encode the large and middle subunit of CAF-1, respectively. The *fas1* and *fas2* mutants were originally isolated as mutants exhibiting stem fasciation (Leyser and Furner, 1992) but they also exhibit other developmental defects including abnormal phyllotaxy, abnormal structure of shoot and root apical meristem, and

serrated leaves (Kaya *et al*., 2001). It is hypothesised that these phenotypes are caused by ectopic expression of key meristem regulators such as *WUSCHEL* (*WUS*) and *SCARECROW* (*SCR*) due to the compromised chromatin assembly (Kaya *et al*., 2001). The *fas1* and *fas2* mutants also exhibit various abnormalities within the nucleus, some of which might result from open chromatin conformation in these mutants. These include increased DNA double strand breaks, increased frequencies of T-DNA insertion and homologous recombination, and loss of telomere and 45s rDNA repeat sequences (Endo *et al*., 2006; Kirik *et al*., 2006; Ono *et al*., 2006; Schönrock *et al*., 2006; Mozgová *et al*., 2010). At the cellular level, *fas1* and *fas2* leaves appear to display typical compensation phenotypes since they have fewer but larger cells (Exner *et al*., 2006; Ramirez-Parra and Gutierrez, 2007). Given that DNA damaging treatment partially phenocopies these phenotypes, it is speculated that the DNA damage response activates the cell cycle checkpoint, promoting the exit from the mitotic cycle into the endocycle (Ramirez-Parra and Gutierrez, 2007). In support of this idea, Adachi et al (2011) has recently shown, using Arabidopsis roots and culture cells, that cells arrested by DNA damage switch into the endocycle and differentiate.

Various endogenous and exogenous stresses cause damage to genomic DNA and eukaryotic organisms have mechanisms to respond to these damages. Two related kinases, ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3 RELATED (ATR), are essential for the DNA damage response in mammals. ATM is activated by double strand breaks whereas ATR is activated by single strand breaks or stalled replication forks (Harper and Elledge, 2007). These kinases are known to activate several downstream regulators to elicit cellular responses such as cell cycle arrest and DNA repair. Both ATM and ATR are widely conserved among eukaryotes

(Harper and Elledge, 2007; Garcia *et al*., 2003; Culligan *et al*., 2004). For example, gene expression analysis of gamma-irradiated Arabidopsis plants revealed that hundreds of DNA damage response genes, including *POLY* (*ADP-RIBOSE*) *POLYMERASE1* (*At-PARP1*) (Doucet-Chabeaud *et al*., 2001), *At-PARP2* (Babiychuk *et al*., 1998), *At-RAD51* (Doutriaux *et al*., 1998) and *BREAST CANCER SUSCEPTIBILITY1* (*At-BRCA1*) (Lafarge and Montane, 2003), are expressed in an ATM-dependent manner (Culligan *et al*., 2006).

A previous study reported that the *fugu2* mutants display typical compensation phenotypes (Ferjani *et al*., 2007). In this study I show that *FUGU2* encodes FAS1 and the two *fugu2* alleles have mutations in the *FAS1* locus. Microarray analyses showed that genes up-regulated in the leaf primordia of *fas1* largely overlap with a group of genes that respond to the genotoxic stress. Subsequent genetic analyses with *fas1 atm* and *fas1 atr* double mutants further revealed that ATM-mediated DNA damage response triggers the cell cycle delay, an entry into the endocycle and compensated cell expansion in *fas1*. These results suggest that ATM-dependent DNA damage response is one of the upstream triggers for compensated cell expansion in Arabidopsis.

RESULTS

FUGU2 encodes FAS1, the large subunit of CAF-1

The three alleles of *fugu2*, *fugu2-1*, *fugu2-2* and *fugu2-3*, were originally isolated from a screen of mutants with altered leaf size (Horiguchi *et al*., 2006). To gain molecular insights into compensation, I performed map-based cloning of the *FUGU2* gene using the *fugu2-1* allele. Sequencing of genomic DNA revealed that *fugu2-1* has a transposition in the tenth exon of *FAS1*, the large subunit of CAF-1 (Kaya *et al*., 2001) and that *fugu2-3* has a 3.5 kbp deletion from the promoter region to the sixth intron (Fig. 1A). Although I failed to identify the precise molecular lesion in *fugu2-2*, these data suggest that *FUGU2* encodes FAS1. I also examined the mRNA levels of *FAS1* in *fugu2* mutants using semi-quantitative RT-PCR and found that the level of *FAS1* transcripts is partially reduced in *fugu2-1* and *fugu2-2* or undetectable in *fugu2-3* (Fig. 1B). These results confirm that the *FUGU2* locus corresponds to *FAS1*, thus I renamed *fugu2-1*, *fugu2-2* and *fugu2-3* as *fas1-5*, *fas1-6* and *fas1-7*, respectively.

To further substantiate that the *fas1* mutation causes compensation, I reexamined the leaf phenotype of *fas1-4* (SAIL_662_D10) previously reported by Exner *et al*. (2006). The *fas1-4* mutant has a T-DNA insertion in the sixth intron of *FAS1* and accordingly our RT-PCR analysis failed to detect the first half of the *FAS1* transcript (Fig. 1B). As shown in Fig. 2A, B, *fas1-4* has narrower and more serrated leaves than WT. An examination of subepidermal palisade cells in the first leaf also confirmed that *fas1-4* has fewer cells (40%) than WT but the size of individual cells is on average 150% larger compared to WT (Fig. 2C, D). As a consequence, *fas1-4* leaves are smaller than WT but only by 70% (Fig. 2D), clearly indicating that *fas1-4* exhibits a typical compensation phenotype. Although the level of *FAS1* transcripts

varies between *fas1-5*, *fas1-6* and *fas1-7*, the degree of compensation is comparable in these alleles (data not shown). Thus, I used the *fas1-4* and *fas1-5* alleles for further analyses.

The *fas1* mutation up-regulates the expression of DNA damage response genes in an ATM-dependent manner

During the development of first leaves, cells in the leaf primordium stop dividing at 10 days after sowing (Ferjani *et al*., 2007). Therefore, the molecular response that causes compensated cell expansion is expected to be up-regulated around this developmental stage. To explore the basis that induces compensation, Tsukaya lab performed microarray analysis using total RNA extracted from the first pair of WT and *fas1-5* leaves at 10 days after sowing. These data from two independent experiments suggested that 67 genes are up-regulated and 118 genes are down-regulated in *fas1-5* leaf primordia by more than 3-fold. I then examined these expression changes using semi-quantitative RT-PCR and found that 46 genes are up-regulated in the leaf primordia of *fas1-4* and *fas1-5* (Fig. 3) while 39 genes are down-regulated (data not shown).

To obtain an overview of these expression profiles, I compared these results to publicly available microarray datasets using the Genevestigator V3 tool (https://www.genevestigator.com/gv/index.jsp). I found that genes up-regulated in *fas1* largely overlap with those that respond to known DNA damage treatments such as UV-B irradiation or bleomycin and mitomycin C treatment (Fig. 3A). I also compared my data to microarray data obtained from gamma-irradiated plants (Culligan *et al*., 2006) and found that many of genes up-regulated in *fas1* overlap with those that

respond to gamma radiation (Fig. 3B). To validate this trend, I tested whether the expression of several known DNA repair genes, including *PARP1*, *PARP2*, *RAD51* and *BRCA1*, is upregulated in *fas1-4* leaf primordia. As expected, my real-time RT-PCR analysis detected significant up-regulation of these DNA repair genes in *fas1-4* (Fig. 4), further supporting that the DNA damage response is activated in developing leaves of *fas1*.

Two closely related kinases, ATM and ATR, are the central players of the DNA damage response in Arabidopsis (Garcia *et al*., 2003; Culligan *et al*., 2004). To test whether they participate in the DNA damage response in *fas1* mutants, I generated the *fas1-4 atm-2* and *fas1-4 atr-2* double mutants. As shown in Fig. 3, the *atm-2* mutation almost completely abolishes the up-regulation of *PARP1*, *PARP2*, *RAD51* and *BRCA1* genes in *fas1-4* while the *atr-2* mutation has very little effects (Fig. 4). These results suggest that the *fas1* mutation activates the ATM-dependent DNA damage response, leading to the up-regulation of DNA repair genes.

The *atm* mutation partially suppresses the compensation phenotype in *fas1*

To explore the link between ATM-dependent DNA damage response and compensation of *fas1* leaves, I examined whether the *atm* mutation interferes with the compensation phenotype in *fas1*. As predicted, *atm-2* single mutants do not have any obvious defects in leaf morphology (Fig. 5A, B) and their leaf cell number and size are approximately the same as those in WT (Fig. 5C, D). When this mutation is introduced into *fas1-4*, it partially restores the growth defects of *fas1-4* leaves (Fig. 5A, B, D). Quantitative analysis of cell number and cell size in the *fas1-4 atm-2* double mutants revealed that the *atm-2* mutation partially rescues the decreased cell number phenotype

and that this recovery is associated with suppression of the compensated cell expansion phenotype in *fas1-4* leaves (Fig. 5C, D).

My gene expression data suggest that the ATR-dependent pathway does not play major roles in the activation of DNA damage response genes in *fas1* (Fig. 4). Consistently, the *atr-2* mutation does not restore the leaf growth defects in *fas1-4* and instead it retards the growth further (Fig. 5A, B, D). At the cellular level, I found that *atr-2* does not rescue the cell number phenotype in *fas1-4* but independently suppresses the compensated cell expansion (Fig. 5C, D). These results suggest that the ATM-mediated DNA damage response participates in triggering compensation in *fas1* while ATR-mediated pathway contributes to compensated cell expansion through some other mechanisms.

The *atm* mutation partially restores the cell cycle delay in *fas1*

The mature *fas1-4* leaves have less than 40% of palisade cells compared to WT (Fig. 2D, 5D). To test whether this is caused by delays in the cell cycle progression or premature termination of cell production during leaf development, I performed the kinematic analysis on the first leaf of WT and *fas1-4* harvested at 5 to 12 days after sowing. As shown in Fig. 6A, WT leaves show steady increase in cell number from day 5 and they stop producing new cells by day 12. Cell number in *fas1-4* leaves is strongly reduced at day 5, indicating that the cell cycle progression is already perturbed during early primordium development (Fig. 6A). Although *fas1-4* cells continue to produce new cells up to day 12, the rate of cell production between day 5 and day 8 is slightly reduced in *fas1-4* leaves (Fig. 6A). I calculated the slope of the graph in Fig 6A to estimate the rate of cell production and found that the slope

drops from 1.5 in WT to 1.3 in *fas1*, implying that the *fas1* mutation has prolonged effects on the cell cycle progression. These results suggest that the decreased cell number phenotype of *fas1-4* primarily results from delayed cell cycle progression rather than premature termination of cell production.

To further investigate how cell production is perturbed in *fas1-4*, I performed flow cytometry analysis using the first pair of leaves from 8-day-old plants. Both WT and *fas1-4* leaf cells contain only 2C and 4C nuclei at this stage but the proportion of 4C to 2C nuclei is much higher in *fas1-4* compared to WT (Fig. 6B). Interestingly, similar phenotypes are also described for mutants arrested at the G2/M phase of the mitotic cell cycle, for example *e2f target gene 1* (Takahashi et al, 2008), implying that the duration of G2/M phase is also prolonged in *fas1-4*. I therefore analysed the expression of S phase genes *CYCLINA3;1* (*CYCA3;1*) and *histone H4*, G2/M phase genes *CYCB1;1* and *CYCB1;2* and M phase gene *KNOLLE* (*KN*) (Breuer *et al*., 2007; Takahashi *et al*., 2008). My real-time RT-PCR analysis revealed that the expression of *CYCA3;1* and *histone H4* is comparable between WT and *fas1-4* whereas the expression of *CYCB1;1*, *CYCB1;2* and *KN* is elevated in *fas1-4* (Fig. 6C). These results support the view that the *fas1* mutation delays the cell cycle progression at the G2/M phase.

To explore whether ATM-dependent DNA damage response leads to the cell cycle delay in *fas1*, I examined whether the *atm* mutation interferes with the cell cycle phenotypes in *fas1-4*. The duration and rate of cell production are similar between WT and *atm-2* (Fig. 6A). Compared to *fas1-4*, *fas1-4 atm-2* leaves contain more cells at day 5 and the rate of cell production, as estimated by the slope of the graph in Fig 6A, is restored to 1.4 in *fas1-4 atm-2* leaves, suggesting that ATM-dependent DNA damage

response contributes to the perturbation of cell production in *fas1-4*. Consistently, my flow cytometry analysis and RT-PCR analysis of cell cycle genes suggested that the *atm* mutation also partially recovers the cell cycle delay at the G2/M phase (Fig. 6B, C). I should note that the expression of *CYCB1;1* is strongly induced by DNA damage (Culligan et al., 2006), thus the activated DNA damage response may also account for the accumulation of *CYCB1;1* transcripts in *fas1*.

The *atr-2* mutation does not restore the cell number defects in fully mature leaves from 21-day-old *fas1-4* seedlings (Fig. 5D). Similarly, the impact of the *atr-2* mutation to the cell production phenotype in *fas1-4* is minor in 5 to 12-day-old seedlings although I occasionally see a limited degree of recovery in *fas1-4 atr-2* (Fig. 6A). my flow cytometry analysis suggested that the *atr-2* mutation may partially recover the G2/M progression defects in *fas1-4* (Fig. 6B) but these results are not consistent with my RT-PCR data showing that *atr-2* does not modify the expression of S phase and G2/M phase and M phase genes in *fas1-4* (Fig. 6C). Together, these results suggest that ATM-dependent DNA damage response pathway primarily contributes to the cell cycle delay in *fas1*.

The *atm* mutation partially represses the high ploidy phenotype in *fas1*

In Arabidopsis leaves, cell size often correlates with the nuclear ploidy level (Melaragno *et al*., 1993). Consistently, enhanced cell expansion in *fas1* is associated with its higher ploidy phenotypes (Exner *et al*., 2006; Ferjani *et al*., 2007; Ramirez-Parra and Gutierrez, 2007). Given that the DNA damaging chemical, zeocin, phenocopies these phenotypes, it is thought that the DNA damage promotes the endocycle and accompanied cell expansion. To examine whether the high ploidy

phenotypes of *fas1* are induced through the ATM-dependent DNA damage response, I performed flow cytometry analysis using fully mature leaves and calculated the endoreduplication index (Sterken *et al*., 2012). The nuclear ploidy level of cells in first leaves of 21-day-old WT plants ranges from 2C to 32C, indicating that many cells in WT leaves have undergone several rounds of endocycles (Fig. 7A). Compared to this, cells in first leaves of 21-day-old *fas1-4* plants display higher ploidy phenotypes with their endoreduplication index significantly higher than WT (Fig. 7A, B). The ploidy distribution in the *atm-2* single mutant is indistinguishable from WT but when this mutation is introduced into the *fas1-4* background, it rescues high ploidy phenotype in *fas1-4* (Fig. 7A, B). These results strongly suggest that ATM-dependent DNA damage response participates in the induction of high ploidy phenotypes in *fas1-4*.

Unexpectedly, my flow cytometry also revealed that the *atr-2* mutation alone gives reproducible defects in the endocycle progression (Fig. 7A, B), suggesting that ATR-mediated DNA damage response pathway is required for the DNA repair during the endocycle. The *fas1-4 atr-2* double mutants display ploidy distribution intermediate between *fas1-4* and *atr-2*, and consequently their endoreduplication index is similar to that of WT (Fig. 7A, B).

The *atm* and *atr* mutations partially restore the meristem defects in *fas1* roots

The *fas1* mutants display severe defects in the structure of shoot and root meristem (Leyser and Furner, 1992; Kaya et al., 2001). Given that these defects are associated with ectopic expression of meristem regulators such as *WUS* and *SCR*, misexpression of these genes is thought to cause the *fas1* phenotypes (Kaya et al., 2001). Since the ATM-mediated DNA damage response pathway triggers the cell cycle

defects in *fas1* leaves, I asked whether the same pathway also contributes to the meristem defects in *fas1*. As described in Kaya et al (2001), the typical arrangement of initial cells and columella cells found in WT is lost in *fas1-4* mutants (Fig. 8A, B). In contrast, the *fas1-4 atm-2* roots have similar arrangement of initial and columella cells to WT (Fig. 8A, B), indicating that the *atm* mutation suppresses these aspects of the *fas1-4* phenotypes. In addition, I found that the size of root meristem in *fas1-4* is shorter than WT and that the *atm-2* mutation suppresses this phenotype (Fig. 8A, C). Interestingly, I also noticed that the *atr-2* mutation restores both of these meristem defects in *fas1-4* roots (Fig. 8). These data suggest that both ATM- and ATR-dependent DNA damage response pathway have a contribution to the defects in the root meristem of *fas1*.

DISCUSSION

In this study I demonstrate that the *fas1* mutants display compensation phenotypes through activation of the ATM-dependent DNA damage response pathway. My data suggest that the ATM-dependent DNA damage response leads to the delay in the mitotic cell cycle and promotion of the endocycle, finally resulting in the induction of compensated cell expansion in *fas1* (Fig. 9).

ATM-dependent up-regulation of DNA damage response genes in *fas1* leaves

My microarray analysis and subsequent RT-PCR analysis revealed that the expression of DNA damage response genes is up-regulated in developing leaves of *fas1* (Fig. 4, Fig. 3A, B). A previous study reported that the expression of genes involved in the DNA damage response is up-regulated in *fas1* (Schönrock *et al*., 2006) but it was not clear in which organs DNA damage response take place because total RNA extracted from whole seedlings was used for the analysis. In this study, I used total RNA extracted from leaf primordia. My data largely agree with data in Schönrock *et al*. (2006) and suggest that leaf primordium is at least one of the organs in which DNA damage response is activated by the loss of *FAS1* function.

DNA damage response largely consists of DNA repair and cell cycle arrest, and gamma irradiation induces up-regulation of hundreds of genes involved in these processes (Culligan *et al.*, 2006). With gamma irradiation, ATM is responsible for the up-regulation and ATR has almost no roles in the process (Culligan *et al*., 2006). My expression analysis using *fas1 atm* and *fas1 atr* demonstrated that only absence of ATM suppresses the up-regulation of several DNA repair genes in *fas1* leaves, suggesting that up-regulated expression of DNA repair genes in *fas1* leaves depends

only on ATM. Therefore, the transcriptional response of several DNA repair genes appears to be regulated similarly in gamma irradiation and loss of *FAS1* function. A previous study reported that *fas1* has an increased amount of endogenous double strand breaks (Endo *et al*., 2006). Thus, it is likely that the accumulated double strand breaks by the *fas1* mutation activates the ATM-mediated DNA damage response.

ATM-dependent DNA damage response leads to compensation in *fas1*

My genetic analysis revealed that the *atm* mutation partially suppresses the decrease in cell number in *fas1* (Fig. 5D). Furthermore, kinematic analysis suggested that the cell number phenotype of *fas1* is a result of the cell cycle delay and that the *atm* mutation also partly restores this phenotype (Fig. 6A). My flow cytometry analysis and gene expression analysis also showed that young *fas1* leaves contain more cells at the G2/M phase compared to WT and that this phenotype is also partly dependent on ATM (Fig. 6B, C). Together, these data suggest that ATM-dependent DNA damage response triggers the G2/M arrest in *fas1*, leading to a decrease in cell number. My flow cytometry analysis on fully mature leaves indicated that the *fas1* mutants display higher ploidy phenotypes in an ATM-dependent manner (Fig. 7), suggesting that activation of the ATM-mediated DNA damage response pathway also leads to the promotion of endocycles. Since the *atm* mutation suppresses both ploidy and compensated cell expansion phenotypes in *fas1*, my data support the potential role of the endocycle in compensated cell expansion. Based on our current knowledge on the function of ATM, it is unlikely that ATM directly regulates the endocycle. Instead, I speculate that ATM is required for the sequential process, i.e. DNA damage response, leading to endoreduplication. Interestingly, a recent study by Adachi *et al*. (2011)

showed that Arabidopsis culture cells or root cells arrested by DNA damage transit into the endocycle and undergo cell expansion prematurely.

It is intriguing that in contrast to the partial recovery of cell number, compensated cell expansion in *fas1* is completely suppressed by the *atm* mutation (Fig. 5C, D). Fujikura *et al.* (2009) proposed a threshold theory in which compensation is triggered only when the down-regulated cell proliferation activity is below a certain threshold. If this theory is applied to *fas1*, the observed phenomenon can be interpreted as follows. The level of decrease in cell proliferation activity in *fas1* caused by the ATM-dependent cell cycle arrest is enough to trigger compensated cell expansion. However, when ATM is disrupted, cell proliferation activity is partially restored and this recovery is sufficient to prevent exceeding the threshold, thereby leading to a complete suppression of compensation.

I should also note that the *atm* mutation does not fully restore the growth defects of *fas1* leaves and that the cell number of *fas1 atm* leaves is still less than that of WT leaves (Fig. 5). These data suggest that additional mechanisms also contribute to the cell cycle arrest and subsequent compensation in *fas1*. Given that *fas1* mutants display various pleiotropic phenotypes (Kirik *et al*., 2006; Ono *et al*., 2006; Schönrock *et al*., 2006; Mozgová *et al*., 2010), some of these defects may have downstream consequences in the cell cycle progression.

ATR-dependent DNA damage response pathway is required for the endocycle progression

Compared to *fas1*, *fas1 atr* seem to have slightly increased number of cells in developing young leaves (Fig. 6A) but they have similar number of cells in fully

mature leaves (Fig. 5D), suggesting that ATR has only a minor effect on the cell number phenotype in *fas1*. Further, induced expression of G2/M phase genes in *fas1* is not suppressed by *atr*, supporting that ATR is not primarily involved in the cell cycle delay at the G2/M phase. Why my flow cytometry data are not consistent with these views and appear to show that *atr* partly cancels the accumulation of 4C nuclei in young *fas1* leaf cells is not clear (Fig. 6B). One possibility that might explain this discrepancy is that some proportion of 4C nuclei I detect by flow cytometry might have actually entered into the endocycle, especially in *fas1*, and that with the role of ATR in the endocycle, as discussed below, the *atr* mutation may block this progression into the endocycle, resulting in the apparent reduction of 4C nuclei in *fas1 atr*. Since the transition into the endocycle is controlled both transcriptionally and post-translationally (Komaki et al., 2012), *fas1* cells might be able to enter the endocycle while keeping the transcript level of G2/M genes relatively high (Fig. 6C).

In contrast to the minor effect on the cell number phenotype, compensated cell expansion in *fas1* is completely suppressed by *atr*, suggesting that ATR has more direct impacts on compensated cell expansion in *fas1*. My flow cytometry analysis on fully mature leaves revealed that *atr* mutants have a decreased ploidy compared to WT (Fig. 7). It is known that molecular function of ATR is to sense DNA replication folk stress, therefore ATR seems to be required to deal with replication stresses associated with the successive progression of endocycles.

DNA damage response pathway contributes to the meristem defects in *fas1* roots

The *fas1* mutant was isolated as a mutant that has stem fasciation phenotypes (Leyser and Furner, 1992) and a previous study suggested that correct chromatin

assembly by the CAF-1 complex is important to regulate the expression of genes required for meristem maintenance (Kaya et al., 2001). In contrast, my observation in this study revealed that the meristem defects in *fas1* roots are partially suppressed by *atm* and *atr*, suggesting that the DNA damage response pathway contributes to the meristem defects in *fas1* (Fig. 8). Although I did not investigate the DNA damage response in roots further, the *fas1* mutation may induce the damage response similar to shoots, leading to the G2/M arrest and/or premature onset of the endocycle in the root meristem. Adachi *et al*. (2011) reported that both ATM- and ATR-dependent pathway participates in the DNA damage response and the induction of endocycles in Arabidopsis roots. My data are consistent with this and further support that both ATM and ATR pathways contribute to the DNA damage response in the root meristem. It will be interesting to test whether the DNA damage response also participates in fasciation or other shoot meristem defects in *fas1*. I did not address these questions because the *fas1-4* allele I used in this study does not display strong shoot phenotypes under my growth condition.

In conclusion my study showed that one mechanism to induce compensation is mediated through the ATM-dependent DNA damage response pathway. Whether this type of compensatory mechanism also operates under normal growth conditions will be an interesting question for future studies.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild type accession used in this study was Columbia. As previously described, allelic *fugu2* mutants were in the Columbia background (Horiguchi *et al*., 2006; Ferjani *et al*., 2007). The *fas1-4* mutants (SAIL_662_D10) were obtained from the Arabidopsis Biological Resource Center. The *atm-2* and *atr-2* mutants in the Columbia background were kind gifts from Kevin Culligan and Anne Britt (University of California, Davis, CA, USA). For histological analyses, plants were grown either on rock wool or on plates containing Murashige and Skoog salts, pH 5.8, 1% (w/v) sucrose and 0.5% (w/v) phytagel at 22ºC under a 16-h light/8-h dark photoperiod.

Microarray analysis

For the microarray analysis, plants were harvested at 10 days after sowing and total RNA was extracted from the first pair of leaf primordia using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). Microarray analysis was performed for two independent biological materials by using ATH1 expression array (Affymetrix Japan, Tokyo, Japan). Array data were processed and analysed with Microarray Suites 5.0 software (Affymetrix Japan).

Quantitative real-time RT-PCR analysis

Plants were harvested at 10 days after sowing and total RNA was extracted from leaf primordia, using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse-transcribed using the Prime Script RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). Each cDNA sample was diluted 1:9 in

water and 1 μl of this dilution was used as a PCR template. Quantitative real-time RT-PCR was performed using the THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan) on an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The gene-specific primer sets used in this study are listed in Supplemental Table 1. Expression levels were normalised with respect to those of *ACTIN2* and averaged over at least three technical and three biological replicates.

Microscopic analysis

To measure leaf area, cell number and cell size, leaves from healthily grown plants were collected and fixed with formalin/acetic acid/alcohol (FAA) and cleared with chloral solution (200 g chloral hydrate, 20 g glycerol and 50 ml dH_2O) as previously described (Tsuge *et al*., 1996). Whole leaves were observed using a stereoscopic microscope (MZ16a; Leica Microsystems, Tokyo, Japan) and indivudual leaf cells were visualized using a microscope equipped with Nomarski differential interference contrast (DMRX E; Leica Microsystems). To observe the root meristem structure, roots were stained with 10 μg/ mL propidium iodide and visualised using Leica TCS-SP5 confocal laser microscope.

Ploidy measurements

Ploidy levels were quantified by flow cytometry (PA-I, Partec) as described previously (Sugimoto-Shirasu *et al*., 2005). At least 7000 nuclei isolated from the first pair of leaves were used for each ploidy measurement. Endoreduplication index (EI) was calculated as EI = (0×%2C) + (1×%4C) + (2×%8C) + (3×%16C) + (4×%32C) (Sterken *et al*., 2012) and averaged over at least three technical replicates.

Figures

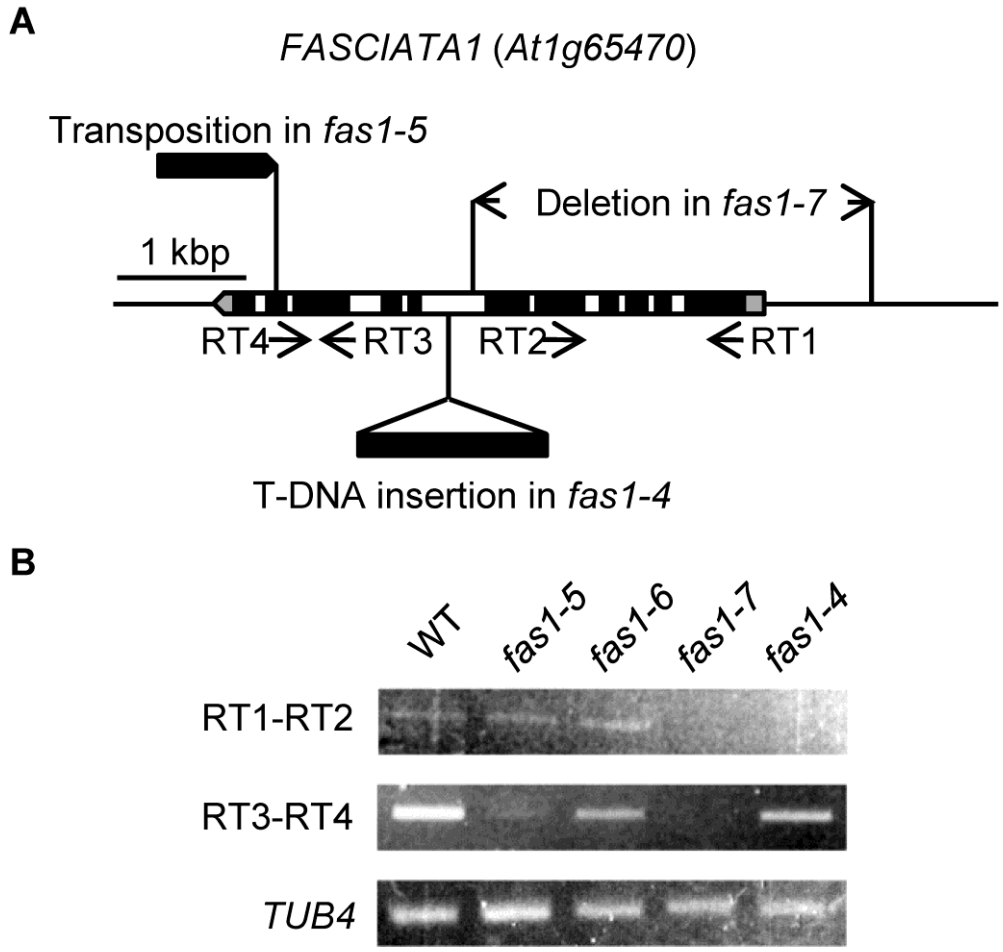

Figure 1. Cloning of the *FUGU2* gene. (A) Mutation points of each *fas1* allele are indicated. The exon, intron, untranslated region and intergenic region are indicated by a black box, white box, grey box and solid line, respectively. Arrows indicate the primers used for RT-PCR. (B) Semi-quantitative RT-PCR analysis of *FAS1* expression in wild-type (WT) and each *fas1* allelic mutant. cDNA fragments amplified with the RT1-RT2 primer set are indicated in the upper row, and those of the RT3-RT4 primer set are given in the middle row. The lower row shows *TUB4* expression as an internal control.

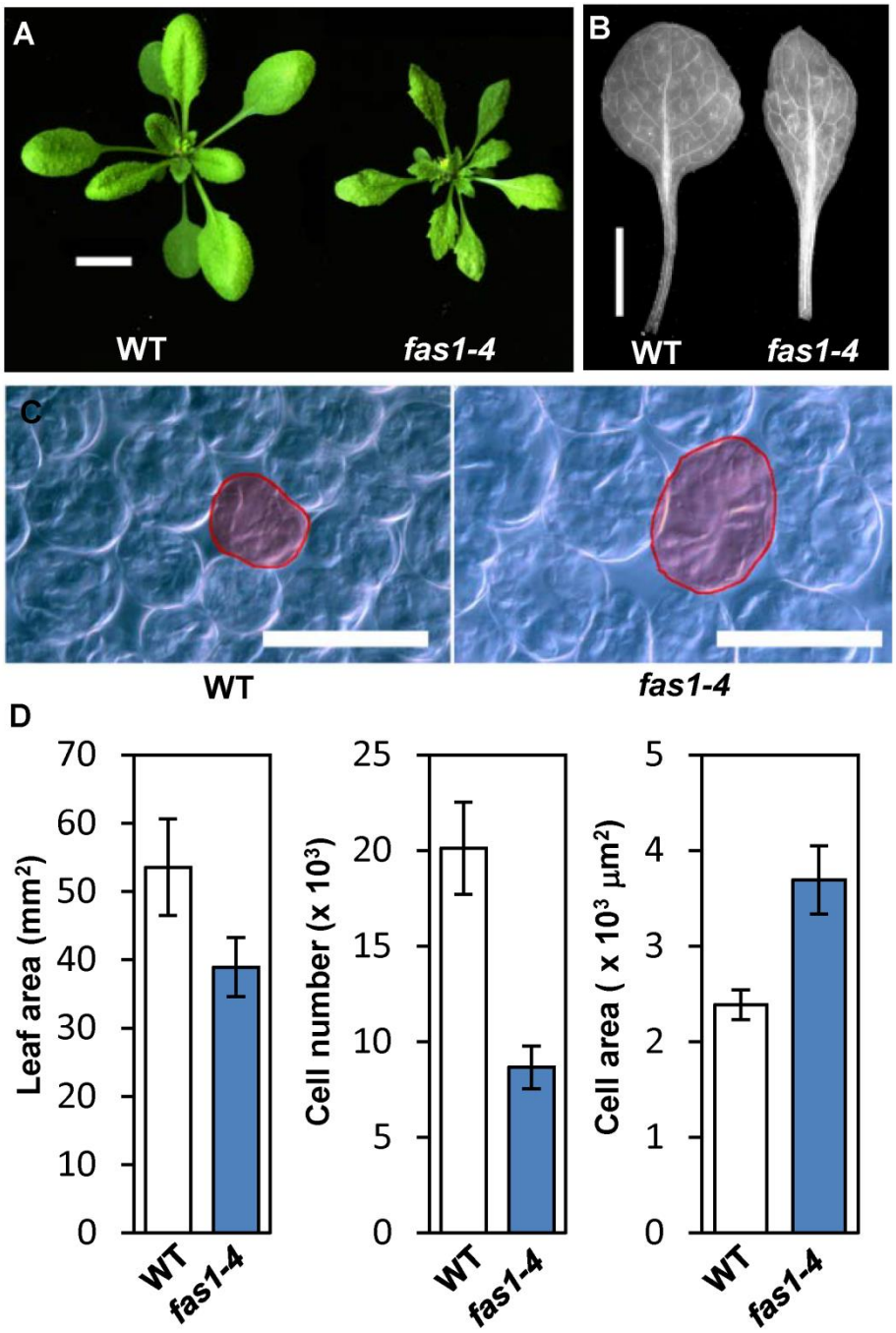

Figure 2. Leaf phenotype of *fugu2/fas1* mutants. (A) Whole rosette of WT (left) and *fas1-4* plants (right) at 25 days after sowing. Bar = 10 mm. (B) First leaves of WT (left) and *fas1-4* plants (right) at 25 days after sowing. Bar = 10 mm. (C) Palisade cells in the first leaf of WT (left) and *fas1-4* (right). Typical cells are marked in red. Bar = 100 µm. (D) Leaf area, subepidermal palisade cell number per leaf and projected cell area (left to right, respectively). First leaves of plants grown on rock wool at 25 days after sowing were used for the analysis. n = 8, mean ± SD.

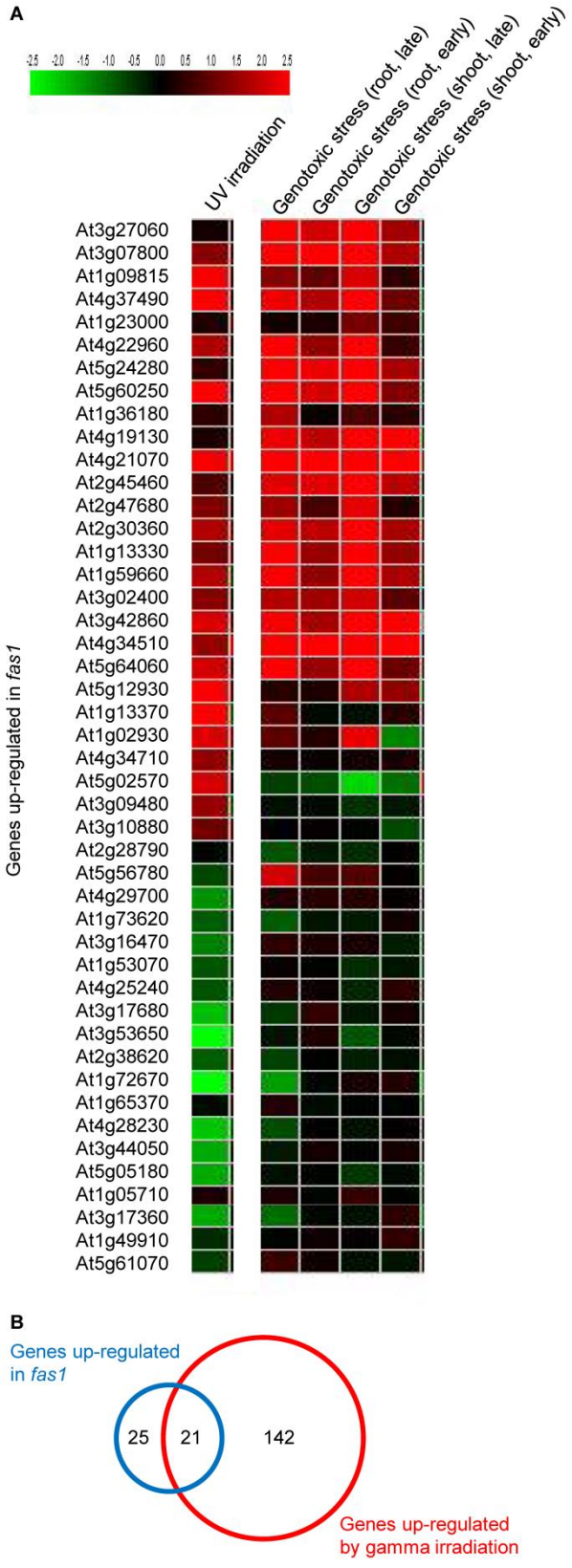
A
-2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5
UV irradiation
Genotoxic stress (root, late)
Genotoxic stress (root, early)
Genotoxic stress (shoot, late)
Genotoxic stress (shoot, early)
Genes up-regulated in fas1
At3g27060
At3g07800
At1g09815
At4g37490
At1g23000
At4g22960
At5g24280
At5g60250
At1g36180
At4g19130
At4g21070
At2g45460
At2g47680
At2g30360
At1g13330
At1g59660
At3g02400
At3g42860
At4g34510
At5g64060
At5g12930
At1g13370
At1g02930
At4g34710
At5g02570
At3g09480
At3g10880
At2g28790
At5g56780
At4g29700
At1g73620
At3g16470
At1g53070
At4g25240
At3g17680
At3g53650
At2g38620
At1g72670
At1g65370
At4g28230
At3g44050
At5g05180
At1g05710
At3g17360
At1g49910
At5g61070
B
Genes up-regulated in fas1
25
21
142
Genes up-regulated by gamma irradiation

Figure 3. Many genes up-regulated in *fas1* are also up-regulated in WT under various genotoxic treatments. Expression of 46 genes up-regulated in *fas1* was compared against publically available microarray datasets using Genevestigator V3. (A) Each column represents gene expression under different genotoxic conditions. For the first column, WT plants were irradiated with UV for 15 min and RNA was extracted from whole seedlings (Ulm *et al.*, 2004). For the next four columns, WT plants were treated with 1.5 μg/ml bleomycin and 22 μg/ml mitomycin for 3 hrs, and total RNA was extracted from roots or shoots at 3 hrs (early) or 12 hrs (late) after the treatment (Kiliam *et al.*, 2007). (B) A Venn diagram indicating the overlap between genes up-regulated in *fas1* and those up-regulated by gamma irradiation. WT plants were irradiated with 100 Gy gamma rays for 1.5 hrs and RNA was extracted from whole seedlings (Culligan *et al*., 2006).

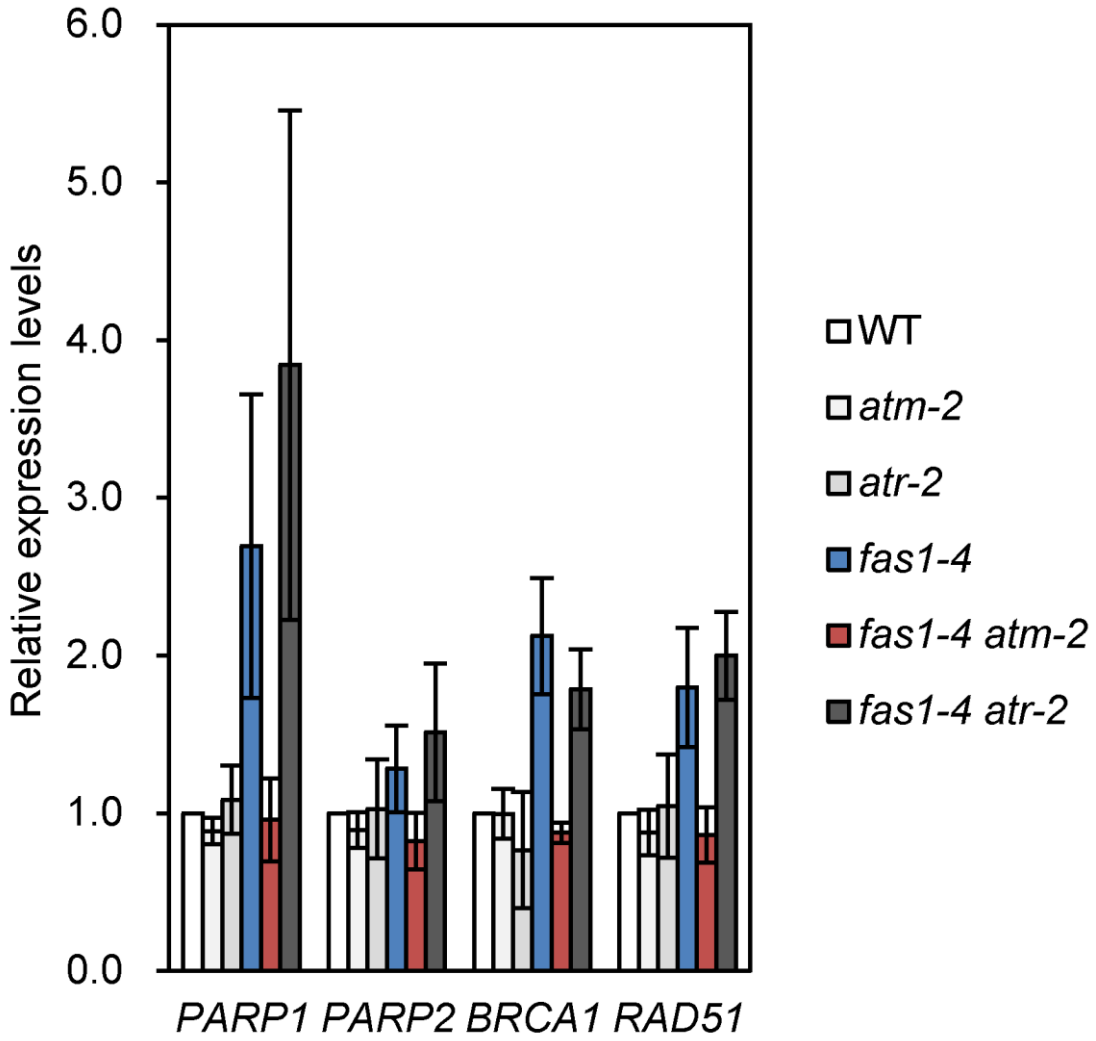

Figure 4. Expression of DNA damage response genes in *fas1-4 atm-2* and *fas1-4 atr-2*. RT-PCR analysis of DNA repair genes (*RAD51*, *BRCA1*, *PARP1* and *PARP2*) in WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. Total RNA prepared from the first leaf pair at 10 days after sowing was reverse-transcribed and amplified by RT-PCR. All values were normalised against the expression level of the *ACTIN2* gene and expressed relative to WT level. We used three biological replicates and error bars indicate SD.

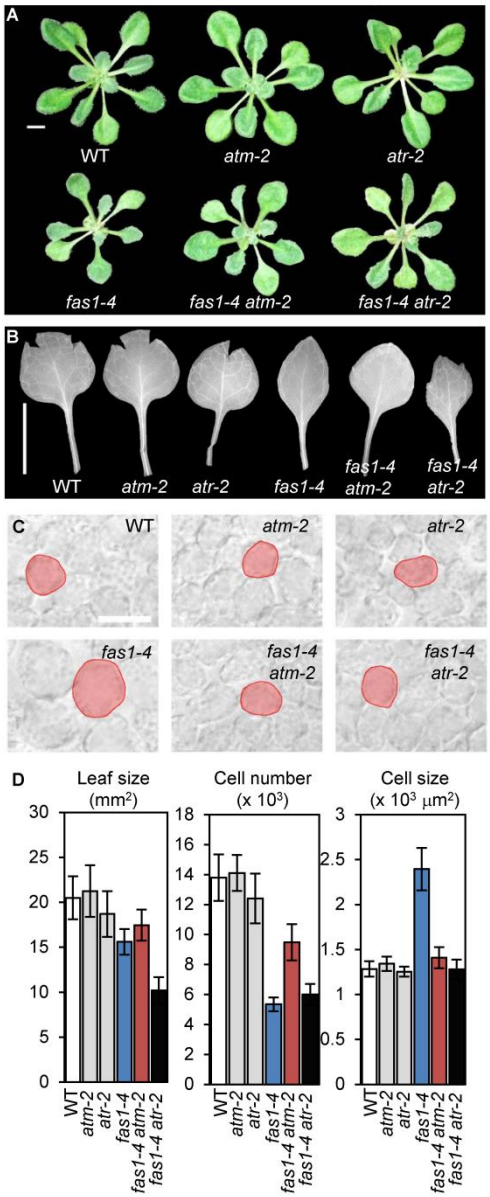

Figure 5. Leaf size, cell number and cell size of *fas1-4 atm-2* and *fas1-4 atr-2*. (A) Whole rosette of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* at 21 days after sowing. Bar = 10 mm. (B) First leaf of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* at 21 days after sowing. Bar = 10 mm. (C) Palisade cells in first leaf of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. Typical cells are marked in red. Bar = 100 μm. (D) Bar graphs indicating leaf area, cell number per leaf and projected cell area of the subepidermal palisade layer (left to right, respectively). First leaves of plants grown on phytagel plates were harvested at 21 days after sowing and used for the analysis. n = 8, mean ± SD

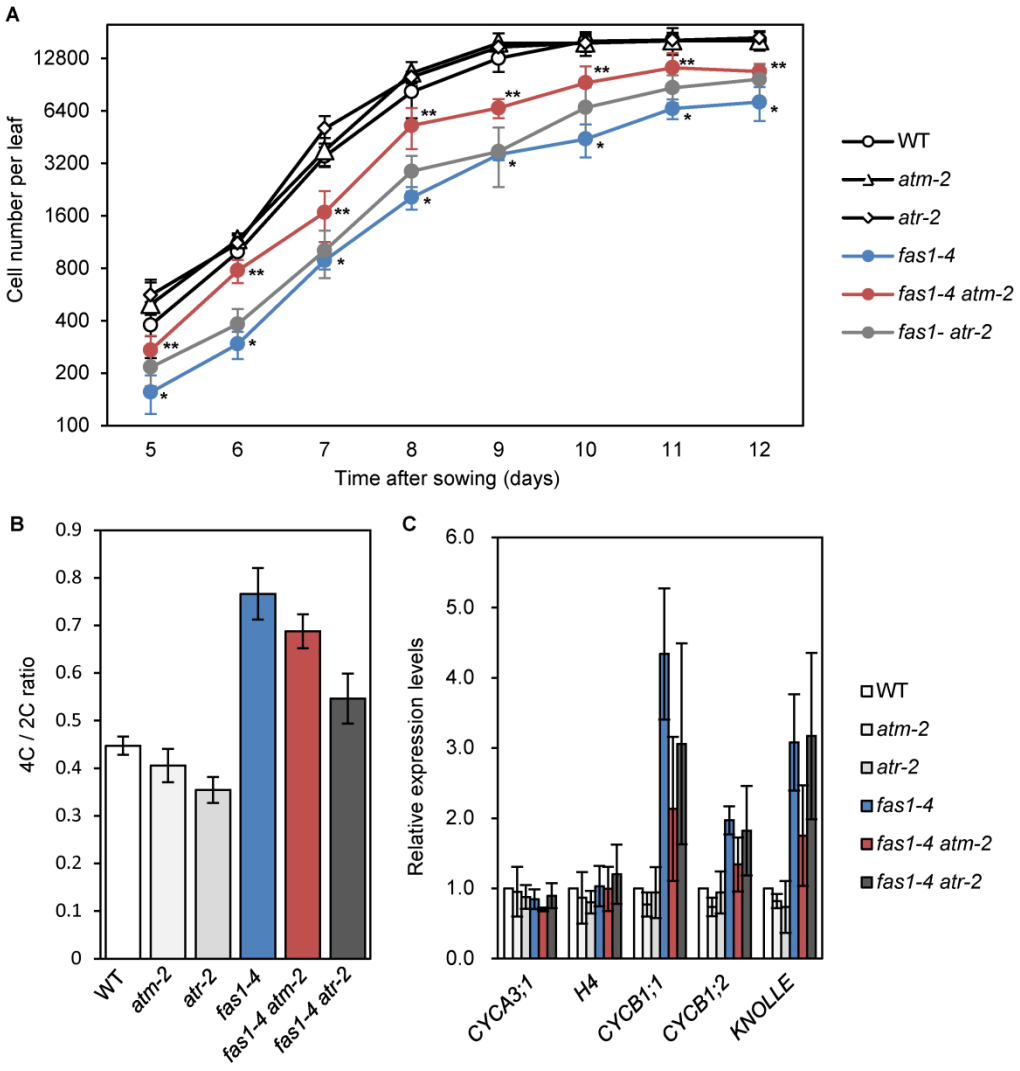

Figure 6. ATM contributes to the cell cycle delay in *fas1-4*. (A) Kinematic analysis of leaf cell number of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. The average cell number per leaf was calculated as described in De Veylder *et al*. (2001). *n* = 8, mean ± SD. *: P < 0.01 between *fas1-4* and *fas1-4 atm-2* (Student's *t*-test). (B) Flow cytometry analysis of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. First leaves of plants at 8 days after sowing were used. n = 3, mean ± SD. (C) RT-PCR analysis of S phase (*CYCA3;1* and *histone H4*) and G2/M phase (*CYCB1;1*, *CYCB1;2* and *KNOLLE*) genes in WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. Total RNA prepared from the first leaf pair at 10 days after sowing was reverse-transcribed and amplified by RT-PCR. All values were normalised against the expression level of the *ACTIN2* gene and expressed relative to WT level. We used three biological replicates and error bars indicate SD.

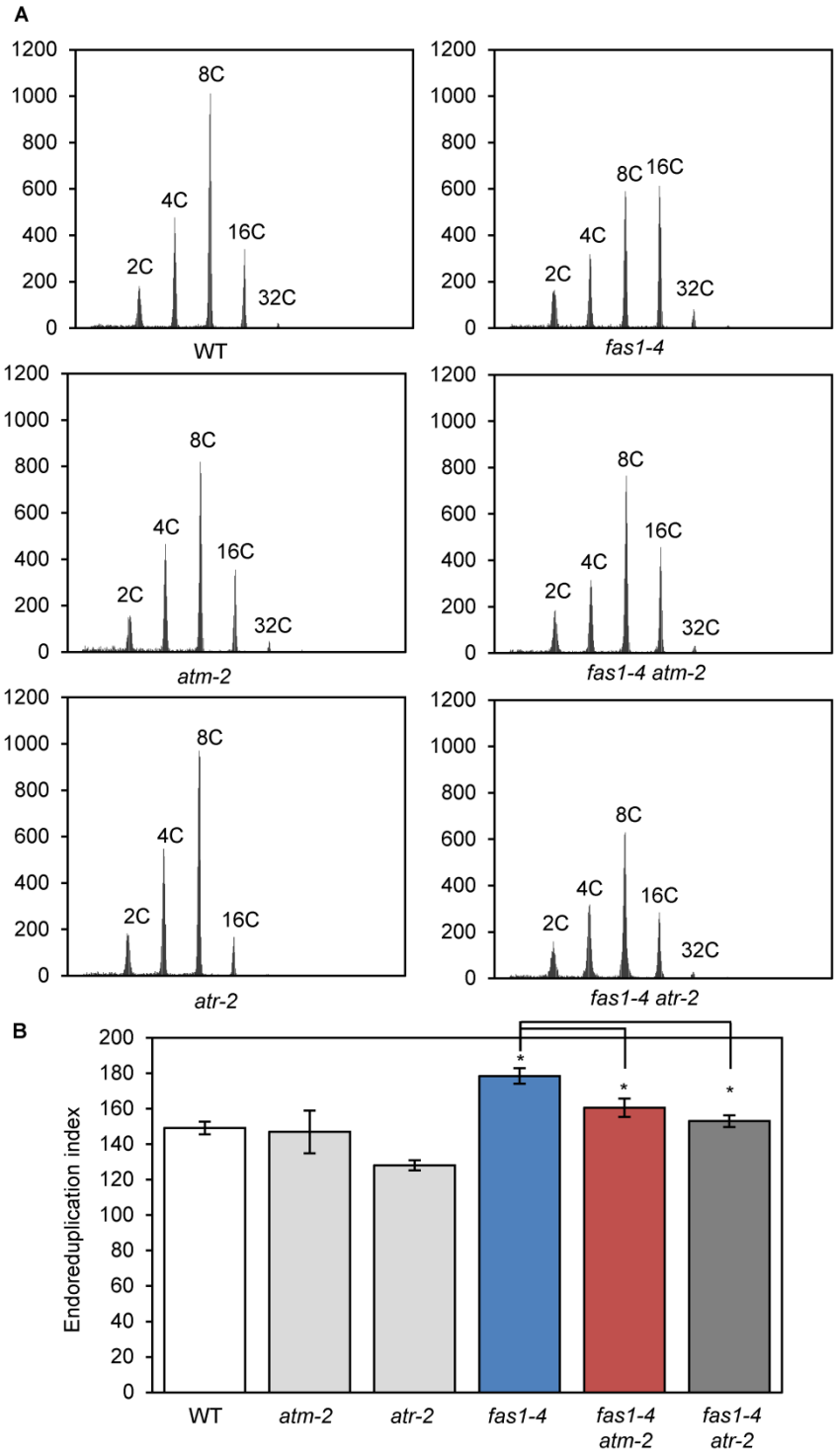

Figure 7. Ploidy level distribution of *fas1-4 atm-2* and *fas1-4 atr-2*. (A) Ploidy level distribution of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* nuclei from first leaf of plants at 21 days after sowing. The most representative data are shown. (B) The endoreduplication index (EI) of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. n = 3, mean ± SD. * : $P < 0.0.1$ (Student's *t*-test)

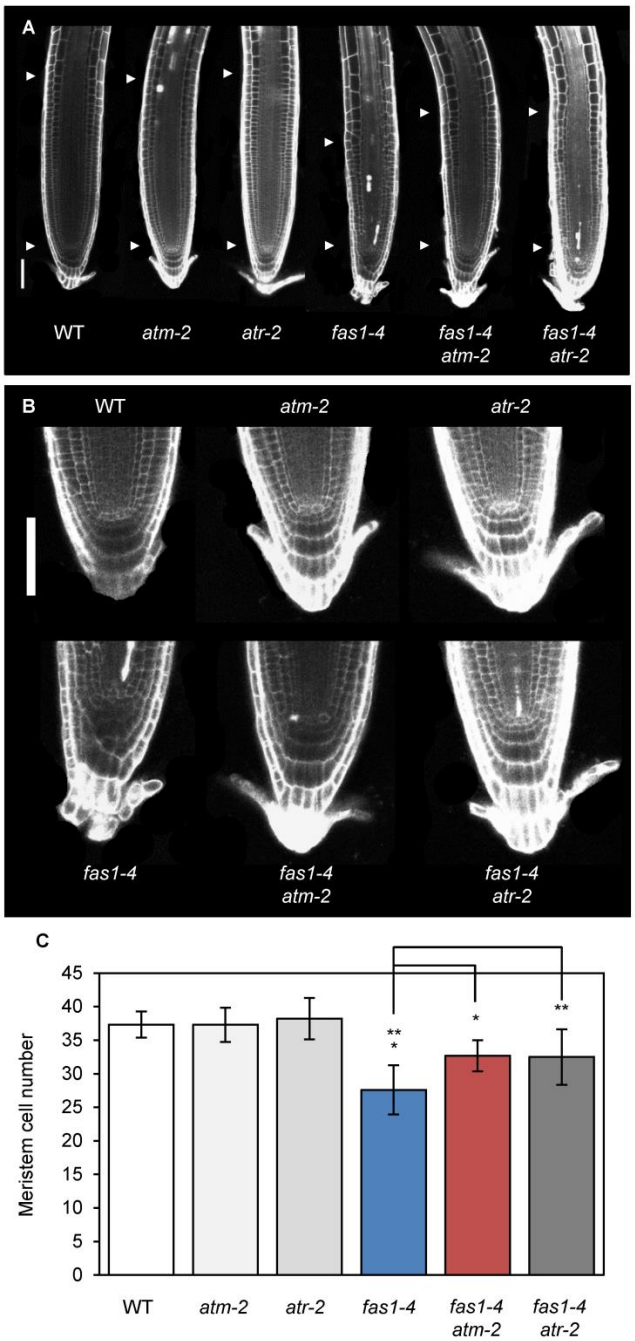

Figure 8. Root meristem phenotypes of *fas1-4 atm-2* and *fas1-4 atr-2*. (A) Root meristem of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* at 9 days after sowing. Bar = 100 μm. Arrowheads mark the position of meristems (B) Initial cells and columella cells of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* at 9 days after sowing. Bar = 100 μm. (C) The number of cortex cells in the root meristem was counted in 9-day-old WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* roots. n = 10, mean ± SD. * : $P < 0.01$, ** : $P < 0.05$ (Student's *t*-test)

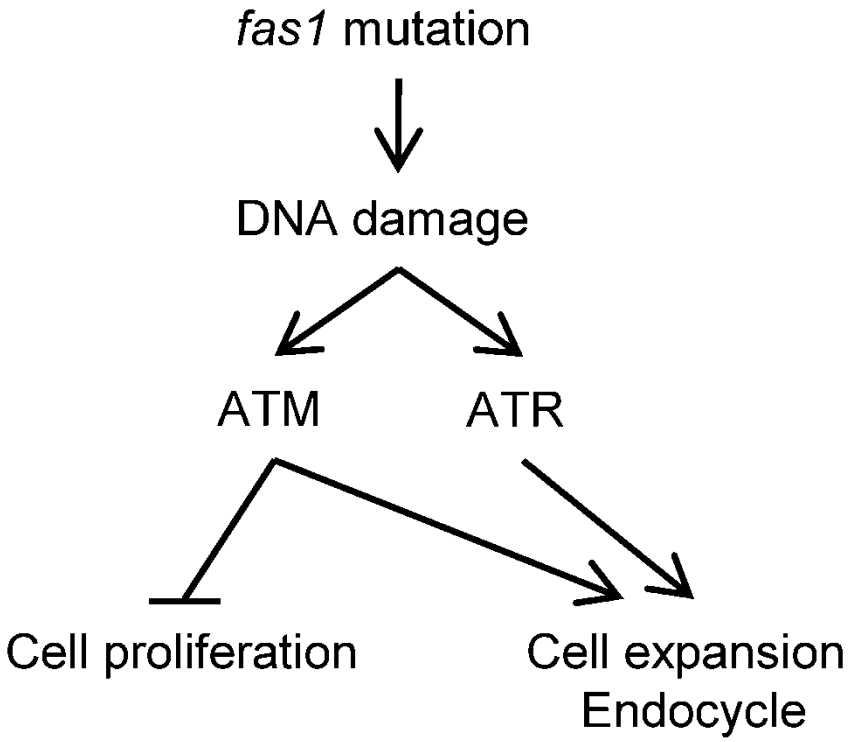

Figure 9. A scheme diagram that explains how compensation is induced in *fas1* leaves.

Chapter III

The DExH type helicase DNA DAMAGE INDUCIBLE1 functions in DNA repair pathway

INTRODUCTION

DNA damage response is important for correct transduction of genomic information

Genomic information of organisms is constantly threatened by various damages. Not only exogenous sources such as ionizing radiation and excess amount of ultraviolet light, but also intrinsic factors such as reactive oxygen species from normal cellular metabolism damages genomic DNA. These factors cause various types of lesions including mismatch base pairs, chemical alteration of DNA bases, replication folk stress, single strand breaks and double strand breaks (DSBs). Among these lesions, DSBs are most dangerous because unrepaired DSBs can be lethal for cells. Eukaryotic organisms have evolved DNA damage response to maintain the genomic integrity, and DSBs are mainly repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ rejoins the loose ends of DSB thus can be accompanied by deletion of genomic information (reviewed in Ciccia and Elledge, 2010). In contrast, HR uses intact homologous sequences of sister chromatid as a template to repair DSBs therefore less error-prone than NHEJ (reviewed in Ciccia and Elledge, 2010). Molecular mechanisms of NHEJ and HR are extensively studied in yeasts and mammals (reviewed in Ciccia and Elledge, 2010). In plants, such mechanisms are studied by identifying homologs of genes involved in HR in mammals and yeasts such as *FANCONI ANEMIA COMPLEMENTATION GROUP M* (*FANCM*), *MEIOTIC RECOMBINATION 11* (*MRE11*), *RADIATION SENSITIVE 51* (*RAD51*), *STRUCTURAL MAINTENANCE OF CHROMOSOMES 6B* (*SMC6B*) and *X-RAY REPAIR CROSS COMPLEMENTING 3* (*XRCC3*) (Roth et al., 2012). Function of these genes is confirmed by using transgenic plants which express functional *β-glucuronidase* (*GUS*) gene after HR events (Roth et

al., 2012). A recent study suggested that small RNA around DSB is transcribed and required for DSB repair (Francia et al., 2012; Michalik et al., 2012; Wei et al., 2012).

The link between the DNA damage response and development

As shown in chapter I, *fas1* mutants exhibit several developmental defects via ATM-dependent DNA damage response pathway. Previous studies reported that *tonsoku/mgoun3/brushy1* (*tsk/mgo3/bru1*), *tebichi* (*teb*), *e2f target gene1* (*etg1*) and *meristem disorganization 1* (*mdo1*) mutants exhibit pleiotropic developmental phenotypes and activated DNA damage response that resembles *fas1* (Suzuki et al., 2004; Takeda et al., 2004; Guyomarc'h et al., 2004; Inagaki et al., 2006, 2009; Takahashi et al., 2008, 2010; Hashimura and Ueguchi 2011). Several studies using a drug, zeocin, which causes DSBs, showed that DSBs induce early entry into the endocycle in the Arabidopsis root meristem and cause cell death in vascular stem cells of roots and the inner tissue of shoot apical meristem (Adachi et al., 2011; Fulcher and Sablowski 2009). Both of these phenotypes are not observed in *atm atr* mutants. These data suggest that hyperactivation of DNA damage response affects plant development, but it is still unclear which factors cause cell cycle arrest and which factors cause cell death. Several reports identified downstream factors of ATM. For example, recent research identified that a NAC (for NAM, ATAF1/2 and CUC2) type transcription factor SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) is a functional orthologue of mammalian p53, which activates hundreds of genes involved in DNA repair after DNA damage treatment (Yoshiyama et al., 2009). WEE1 kinase, conserved among eukaryotes, is one of the inhibitors of CDKA;1 and phosphorylates CDKA;1 after DNA damage to inhibit its activity, leading to cell cycle arrest during DNA repair (De Schutter et al.,

2007). It is still unclear which factors activate the cell death pathway in plants.

DEAD/DEAH type helicases are involved in various RNA related processes

DEAD/DEAH type helicases are a group of helicase which belong to super family 2 and they unwind various types of RNAs in an ATP-dependent manner. For example, mammalian DExH-box protein DHX29 is required for translation initiation of several mRNAs which has highly structured 5' UTR (Pisareva et al., 2008; Parsyan et al., 2009). Spliceosome, which catalyzes splicing of introns from pre-mRNA, consist of several DEAD/DEAH type RNA helicases (reviewed in Cordin et al., 2012). Tudor-domain containing 9 (Tdrd9) from mice interacts with piwi protein and is involved in producing piRNA that represses transposable elements in germ line (Shoji et al., 2009). These data suggest that DEAD/DEAH box type helicases function in various pathways involved in unwinding RNA-RNA or RNA-protein interactions.

In this chapter, I describe the isolation of a mutant of *DDI1* (*DNA DAMAGE INDUCIBLE1*) which encodes a DExH type helicase. Loss-of-function mutation of *ddi1* partially suppresses several DNA damage-related phenotypes in *fas1*, suggesting that *DDI1* functions downstream of the DNA damage response. Expression analysis revealed that the expression of *DDI1* is up-regulated by DNA damage treatment in both root and shoot meristem. The *ddi1* mutants accumulate more DNA double strand breaks than WT and exhibit lower efficiency of homologous recombination under both normal and DNA damage stress conditions. These data collectively suggest that *DDI1* is involved in the DNA repair.

RESULTS

Isolation of *DDI1* gene

In chapter I, I revealed that ATM-dependent DNA damage response acts as an up-stream trigger for compensation in *fas1* but it is still unclear which factors have an important role downstream of ATM. To further characterize the ATM-dependent DNA damage response, I investigated functional roles of genes whose expression is up-regulated in leaf primordia of *fas1*. I isolated loss-of-function mutants of these genes and generated double mutants between *fas1-4* to analyze their leaf phenotypes. Among them, I focused on one gene which encodes a protein with a DExH box type helicase domain and a CCCH type zinc finger domain (Fig. 10A, B). This gene was named *C3H31* based on the genome-wide classification of CCCH type zinc finger in Arabidopsis (Wang et al., 2008). Here, I renamed this gene *DDI1* for *DNA DAMAGE INDUCIBLE1* because several transcriptome studies suggest that the expression of this gene is induced by DNA damage (Culligan et al., 2006; Yoshiyama et al., 2009). I obtained two allelic mutants of *ddi1* mutants from public resource. The *ddi1-1* (SALK_127155) allele has a T-DNA insertion in the fifth intron of *DDI1* locus whereas the *ddi1-2* (SAIL_877_H01) allele has a T-DNA insertion in the first intron (Fig. 10A). Semi-quantitative RT-PCR analysis revealed that full-length mRNA of *DDI1* is not detectable in both alleles (Fig. 10C).

The *ddi1* mutation partially suppresses the decreased cell number phenotype in *fas1* leaves

My quantitative reverse transcription (RT)-PCR analysis revealed that the expression of *DDI1* is up-regulated 2-fold in *fas1-4* background in an ATM-dependent manner

(Figure 11). To reveal the function of DDI1 in *fas1* mutants, I analyzed the leaf phenotypes of *ddi1* and *fas1 ddi1* mutants. Single *ddi1-1* mutants show no obvious defects in leaf morphology under the normal growth condition (Fig. 12A, B). Similarly, cell number and cell size in *ddi1-1* leaves are approximately same as those in WT (Fig. 12C, D). When this mutation is introduced into *fas1-4*, it partially restores the growth defects of *fas1-4* leaves (Fig. 12A, B, D). Since this phenotypic rescue is similar to that I described for *fas1-4 atm-2*, I further examined whether the *ddi1-1* mutation also restores cellular phenotype of *fas1-4* leaves. Quantitative analysis of cell number and cell size in the *fas1-4 ddi1-1* double mutants revealed that the *ddi1-1* mutation partially restores the decreased cell number phenotype in *fas1-4* (Fig. 12D) whereas cell size of *fas1-4 ddi1-1* is similar to that of *fas1-4* (Fig. 12C, D).These data suggest that DDI1 is one of the factors acting downstream of ATM and is involved in some mechanism that causes reduction of cell number in *fas1-4* leaves.

The *ddi1* mutation partially suppresses cell death phenotype in the root meristem of *fas1*

I examined whether the *ddi1* mutation restores the short root phenotype in *fas1* because the *atm* mutation partially suppresses this phenotype and *fas1-4 ddi1-1* mutants partially mimics *fas1-4 atm-2* mutants in their leaf phenotype. As expected, the *ddi1-1* mutation partially suppresses the short root phenotype of *fas1-4* (Fig. 13A, C). To further characterize this phenotype, I observed the root meristem of these mutants using confocal microscopy. As described in chapter I, *fas1-4* has smaller meristem than WT, abnormal structure of initial and columella cells and dead cells in stele cells. Counting cortex cell number in the root meristem revealed that the *ddi1-1* mutation does not

affect the meristem size of *fas1* (Fig. 13D). In contrast, the *ddi1-1* mutation partially restores abnormal arrangement of initial and columella cells and cell death phenotype (Fig. 13B, E), suggesting that the *ddi1* mutation contributes to the short root phenotype in *fas1* through abnormal arrangement of meristem cells and cell death in stele cells. These data suggest that DDI1 functions downstream of the ATM-dependent DNA damage response and contribute to developmental defects in *fas1* mutants.

The expression of *DDI1* is induced in *fas1* and by genotoxic stress

Co-expression database suggest that the *DDI1* co-expresses with the genes involved in DNA repair (Table 1). Several study reported that the expression of *DDI1* is up-regulated after DNA damage in ATM-dependent manner (Culligan et al., 2006, Yoshiyama et al., 2009). These data suggest that the *DDI1* functions downstream of ATM-dependent DNA damage response. To further investigate whether DDI1 functions downstream of the ATM-dependent DNA damage response, I examined the expression of the *DDI1* gene. Quantitative reverse transcription (RT)-PCR analysis revealed that the expression of *DDI1* in WT plants is up-regulated 6-fold 24 hours after the zeocin treatment (Fig. 14A). In contrast, the expression of *DDI1* is not up-regulated in *atm-2* by zeocin treatment, indicating that the expression of *DDI1* is induced by genotoxic stress in an ATM-dependent manner. Next, I constructed transgenic plants carrying the *DDI1pro: GUS* to examine the spatial expression pattern of *DDI1* in vivo. Consistent with my quantitative RT-PCR data, I detected only weak expression of *DDI1* from plants grown under normal condition (Fig. 14B, D). However, strong signal is detected from zeocin-treated plants (Fig. 14C, E). Interestingly, the GUS signal of zeocin treated plants is limited to actively dividing cells such as those in the root and shoot apical

meristem or leaf primordia (Fig. 14C, E).

The *ddi1* mutant has defects in DNA repair

To further explore the function of DDI1, I examined co-expression database (ATTED-II, http://atted.jp/) and found that *DDI1* co-expresses with genes involved in the DNA repair via HR pathway, suggesting that DDI1 may function in the HR pathway. Therefore I carried out a comet assay to compare the level of DNA double strand breaks in nucleus of WT and *ddi1-1*. In the case of WT, the amount of DSBs is increased when they are treated with zeocin (Fig. 15A, B). In contrast, *ddi1* has almost same amount of DSBs as zeocin-treated WT even under normal growth condition (Fig. 15B). Furthermore, zeocin treatment increases the amount of DSBs in *ddi1-1* mutants (Fig. 15B). These data suggest that *DDI1* is required for the efficient repair of DSBs.

The *ddi1* mutation reduces dead cells in the root meristem under genotoxic stress

Expression analysis and comet assay suggest that DDI1 functions in the DNA repair pathway, then I examined the developmental phenotype of *ddi1-1* under genotoxic stress. I treated WT and *ddi1* plants with zeocin and observed their root meristem using SYTOX Green staining which specifically stains dead cells. In mock treatment, both WT and *ddi1-1* mutants do not have any dead cells in their root meristems (Fig. 16A, C). On the other hand, zeocin treatment causes cell death in stele cells of WT, suggesting zeocin treatment mimics the *fas1* phenotype (Fig. 16B, E). Consistent with the fact that *ddi1-1* mutation suppresses the cell death phenotype in *fas1-4* root, *ddi1-1* mutants have less dead cells than WT after zeocin treatment (Fig. 16D, E). These data suggest that *ddi1* mutants are insensitive to the genotoxic stress.

DISCUSSION

The *ddi1* mutation partially suppresses the *fas1* phenotype

Genetic analysis revealed that the *ddi1-1* mutation partially suppresses the leaf phenotype in *fas1-4* (Fig. 12). As described in chapter I, the *atm-2* mutation partially suppresses the leaf phenotype in *fas1-4*. Previous transcriptome data and my expression analyses revealed that the expression of *DDI1* is up-regulated after DNA damage in an ATM-dependent manner (Culligan et al., 2006; Fig. 14). Therefore, DDI1 seems to function downstream of the ATM-dependent DNA damage response. Histological analysis revealed that restoration of the leaf size phenotype by the *ddi1* mutation is mainly caused by restoration in cell number (Fig. 12D). On the other hand, the *ddi1* mutation does not affect cell size phenotypes in *fas1* mutants (Fig. 12C, D). Compared to this, *atm* mutation restores leaf phenotype in *fas1* by recovering both cell number and cell size phenotype (chapter II). One possible explanation for these differences is that DDI1 is one of the downstream factors of ATM and compensation is still active by other downstream factors in *fas1 ddi1*.

DDI1 contributes to the cell death phenotype in *fas1* root

As described in chapter I, *fas1* exhibits shorter root phenotypes caused by several defects in their root meristem such as abnormal arrangement of initial and columella cells, cell death in vascular cells and early transition from mitotic cycle to endocycle. My observation in chapter I revealed that the *atm* mutation suppresses all of these phenotypes in the root meristem, leading to restoration of root length phenotype. In contrast, the *ddi1* mutation partially suppresses abnormal cell arrangement phenotype and cell death phenotype but not early transition from mitotic cell cycle to endocycle

(Fig. 13). These data suggest that ATM-dependent DNA damage response pathway contributes to root meristem phenotype in *fas1* and DDI1 is one of the downstream factors which contribute to cell death phenotype and meristem structure phenotype. Generally, the outputs of the DNA damage response are mainly separated to two parts, cell cycle arrest and DNA repair. The fact that the *ddi1* mutation does not suppress earlier transition from mitotic cycle to endocycle in *fas1* suggests that DDI1 is not required for the cell cycle arrest downstream of the DNA damage response. Co-expression database suggest that *DDI1* co-expresses with genes involved in the DNA repair, implying that DDI1 functions in DNA repair pathway (Table 1). One possibility that explains the *fas1 ddi1* phenotype is as below. In the root meristem of *fas1*, ATM senses the DNA damage and activates DNA repair. If the DNA damages are correctly repaired, cells will survive, but if they are not repaired, cell death will occur. In *fas1 ddi1* double mutants, DNA repair may not work efficiently because they lack functional DDI1, leading to cell survival with accumulation of DNA damage.

Cell death may contributes to a decrease in cell number in *fas1* leaves

Based on the observation of root meristem in *fas1*, I speculate that cell death also occurs in the shoot apical meristem and early leaf primordia in *fas1*. Previously, Fulcher and Sablowski reported that DNA damage treatment causes cell death in the shoot meristem as well as in the root meristem (Fulcher and Sablowski 2009), supporting this idea. The *ddi1* mutation suppresses cell death phenotype in the *fas1* root meristem without suppressing the cell cycle transition phenotype. Therefore, cell number restoration in leaf of *fas1 ddi1* might be caused by suppression of cell death in the shoot meristem or young leaf primordia rather than suppression of the cell cycle

delay. Interestingly, the *ddi1* mutation has only minor effects on cell size phenotype in *fas1* while the *atm* mutation suppresses cell size phenotype in *fas1*. These differences might be explained as below. A decrease in cell number in *fas1* leaves may be caused by both effects in cell cycle delay and cell death, and compensated cell expansion might be triggered only by sensing cell cycle delay not by simply sensing total amount of cells. In the case of *fas1 atm*, both cell cycle delay and cell death are restored, leading to restoration of cell size phenotype. On the other hand, *ddi1* only affects cell death phenotype and cell cycle is still delayed, keeping the compensation phenotype.

DDI1 may be involved in DNA repair pathway, especially in homologous recombination

My analysis revealed that the expression of *DDI1* is induced at least 24 hour after DNA damage treatment in the root meristem, shoot meristem and young leaf primordia (Fig. 14). Comet assay revealed that *ddi1* mutants accumulate more DNA double strand breaks compared to WT under normal growth condition (Fig. 15). These data collectively suggests that DDI1 functions in the DNA repair pathway. I used zeocin which causes DNA double strand breaks. In general, DSBs are repaired by NHEJ or HR. DDI1 co-expresses with genes involved in HR, suggesting that DDI1 is also involved in the HR pathway. The *ddi1* mutant has less dead cells compared to WT after DNA damage treatment (Fig. 16). These data seem to be inconsistent with the data that *ddi1* have more DSBs. This may be caused by difference of tissue I used in these experiments. I observed root meristem to detect cell death and used whole seedlings to extract nucleus for comet assay. In *ddi1* mutants, cell death rarely occurs and cells survive with a lot of DSBs, making tissues containing a lot of DNA damage. Therefore

we detect more DSBs from *ddi1* seedlings than those of WT.

The DDI1 protein has DExH box type helicase domain and CCCH type zinc finger domain (Fig. 10). Blast search suggested that this type of protein is conserved only among vascular plants. Therefore, DDI1 might be a plant-specific factor for DNA repair. Other proteins which have DExH domain are reported to be involved in various RNA related processes such as mRNA splicing, initiation of translation and generation of small RNA in mice germ line (Shoji et al., 2009; Pisareva et al., 2008; Parsyan et al., 2009; Cordin et al., 2012). Furthermore, a recent report suggests that region around DSBs are transcribed and processed into 21 nt small RNA, and these small RNAs are important for the correct repair for DSBs (Francia et al., 2012; Michalik et al., 2012; Wei et al., 2012). Thus, it is also plausible that DDI1 might be involved in the generation of small RNA.

MATERIALS and METHODS

Plant materials and growth conditions

The Arabidopsis (*Arabidopsis thaliana*) wild-type accession used in this study was Columbia. As described in chapter I, allelic *fas1* mutants were in the Columbia background. The *fas1-4* mutants (SAIL_662_D10), the *ddi1-1* mutants (SALK_127155) and the *ddi1-2* mutants (SAIL_877_H01) were obtained from the Arabidopsis Biological Resource Center. The *atm-2* mutants in the Columbia background were kind gifts from Kevin Culligan and Anne Britt (University of California, Davis, CA, USA). For histological analyses, plants were grown either on rock wool or on MS plates (Murashige and Skoog salts, pH 5.8, 1% (w/v) sucrose and 0.5% (w/v) phytagel) at 22ºC under a 16-h light/8-h dark photoperiod. For DNA damage treatment, plants were grown on MS plates at 22ºC under continuous light condition for 5 days, then transferred to MS plates with or without 10 μg/mL zeocin (Life Technologies) and incubated 24 hours.

Cloning of *DDI1pro: GUS*

The putative *DDI1* promoter was amplified by PCR (PrimeSTAR HS DNA Polymerase, TaKaRa) from Columbia DNA using the primers DDI1p-Fw (5’-CACCGAGAAGTTGTGTCAGAACTC-3’) and DDI1p-Rv (5’-TGTTGTCGTACGCGGAGCAC-3’) and subcloned to pENTR (pENTR™/D-TOPO® Cloning Kit, Life Technologies). The subcloned promoter was transferred to pGWB533 (Nakagawa et al., 2007) by LR reaction (LR Clonase, Life Technologies).

Histochemical GUS-staining

Histochemical GUS-staining was performed based on the protocol described by Malamy and Benfey (1997). Seedlings were fixed in 90% acetone on ice for 15 minutes, stained in the GUS buffer with 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-d-glucuronide for 3 hours, mounted in chloral hydrate solution (200 g chloral hydrate, 20 g glycerol and 50 ml dH2O), and observed using an Olympus BX51 fluorescence microscope.

Quantitative real-time RT-PCR analysis

Plants were harvested at 10 days after sowing and total RNA was extracted from leaf primordia, using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse-transcribed using the Prime Script RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). Each cDNA sample was diluted 1:9 in water and 1 μl of this dilution was used as a PCR template. Quantitative real-time RT-PCR was performed using the THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan) on an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The gene-specific primer sets used in this study are listed in Supplemental Table 1. Expression levels were normalised with respect to those of ACTIN2 and averaged over at least three technical and three biological replicates.

Microscopic analysis

To measure leaf area, cell number and cell size, leaves were fixed with formalin/acetic acid/alcohol (FAA) and cleared with chloral solution (200 g chloral hydrate, 20 g glycerol and 50 ml dH2O) as previously described (Tsuge et al., 1996). Whole leaves were observed using a stereoscopic microscope (MZ16a; Leica Microsystems, Tokyo, Japan) and indivudual leaf cells were visualized using a

microscope equipped with Nomarski differential interference contrast (DMRX E; Leica Microsystems). To observe root meristem structure, roots were stained with 10 μg/ mL propidium iodide (PI) and visualized using Leica TCS-SP5 confocal laser microscope.

Comet assay

Plant seedlings are chopped using razor blade in 1 X PBS, 20 mM EDTA solution. Then, nuclei are isolated using mesh. Neutral comet assay to detect DNA double strand breaks are performed using Comet Assay Reagent Kit for Single Cell Gel Electrophoresis Assay (TREVIGEN).

FIGURES

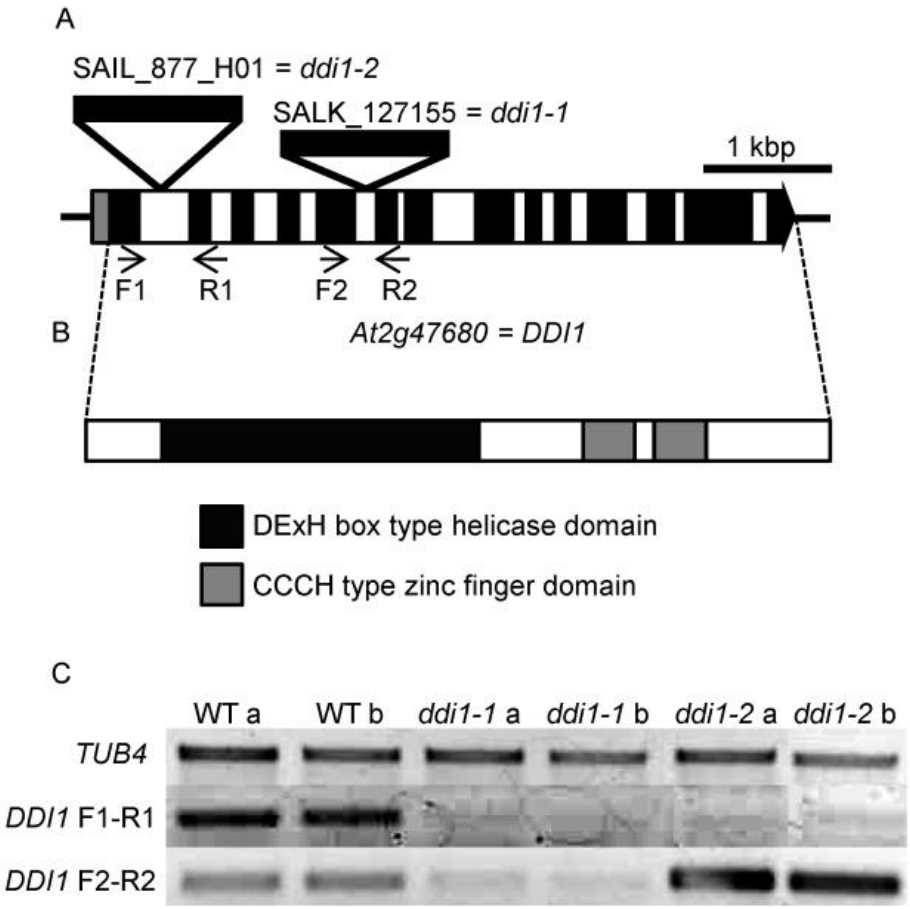

Figure 10. Gene structure of *DDI1*. (A) Mutation points of each *ddi1* allele are indicated. The exon, intron, untranslated region and intergenic region are indicated by a black box, white box, grey box and solid line, respectively. Arrows indicate the primers used for RT-PCR in (C). (B) Protein structure of DDI1 is indicated. The DExH box type helicase domain and CCCH type zinc finger domain are indicated by a black box and grey box, respectively. (C) Semi-quantitative RT-PCR analysis of *DDI1* expression in wild-type (WT) and each *ddi1* allelic mutant. cDNA fragments amplified with the F1-R1 primer set are indicated in the middle row, and those of the F2-R2 primer set are given in the lower row. The upper row shows *TUB4* expression as an internal control. I used two biological replicates for each genotypes.

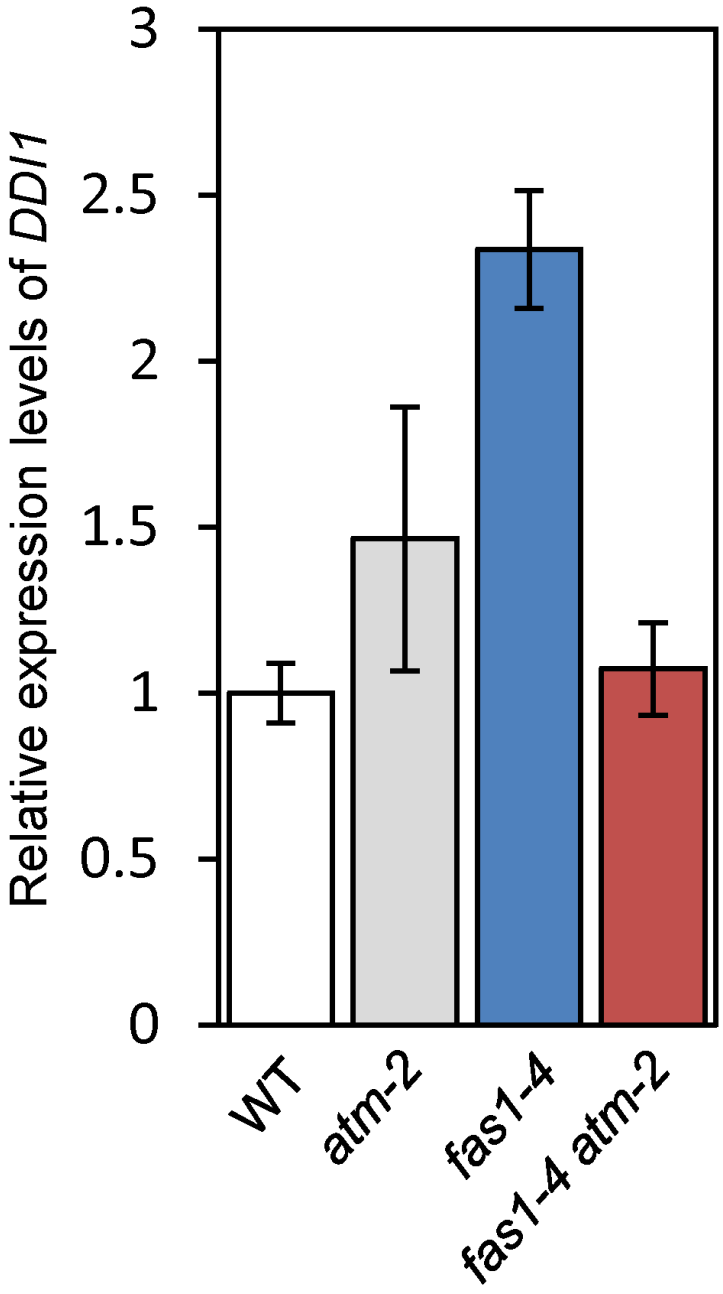

Figure 11. The expression of *DDI1* in *fas1* and *fas1 atm*. RT-PCR analysis of *DDI1* gene in WT, *atm-2*, *fas1-4* and *fas1-4 atm-2*. Total RNA prepared from the whole seedlings at 6 days after sowing was reverse-transcribed and amplified by RT-PCR. All values were normalised against the expression level of the *ACTIN2* gene and expressed relative to WT level. We used three biological replicates and error bars indicate SE.

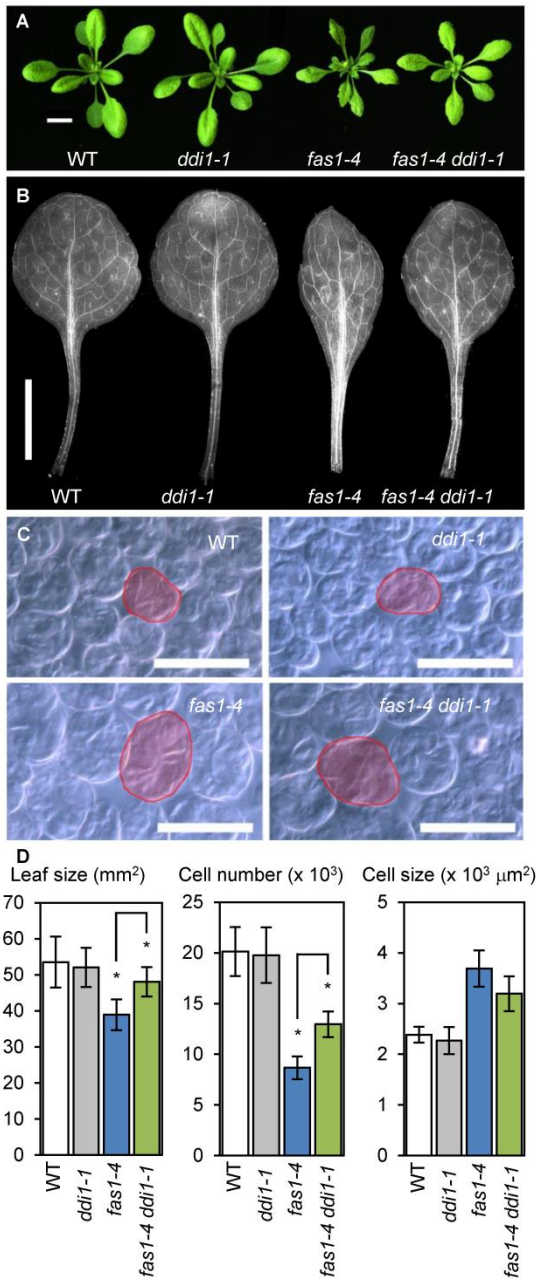

Figure 12. Leaf phenotype of *fas1 ddi1* mutants. (A) Whole rosette of WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* at 21 days after sowing. Bar = 10 mm. (B) First leaf of WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* at 21 days after sowing. Bar = 5 mm. (C) Palisade cells in first leaf of WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* at 21 days after sowing. Typical cells are marked in red. Bar = 100 μm. (D) Bar graphs indicating leaf area, cell number per leaf and projected cell area of the subepidermal palisade layer (left to right respectively). First leaves of plants grown on rock wool were harvested at 21 days after sowing and used for the analysis. n = 8, mean±SD. *: $P < 0.01$ (Student's *t*-test).

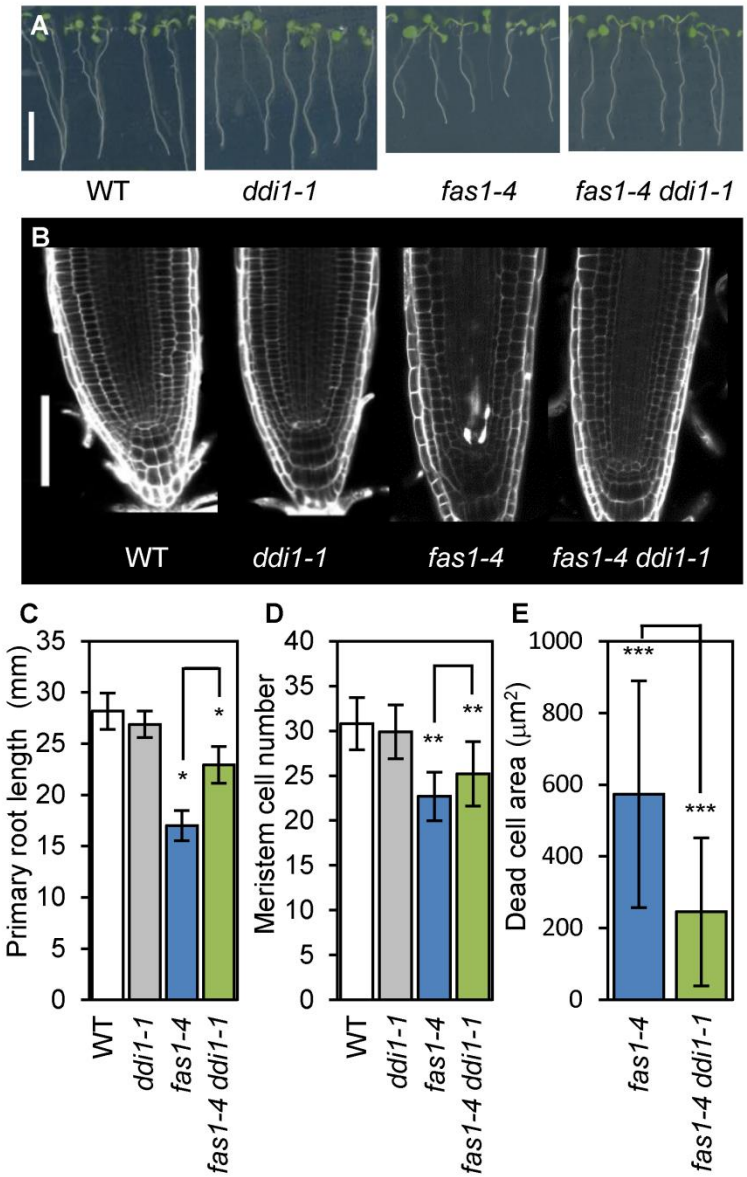

Figure 13. Root phenotype of *fas1 ddi1* mutants. (A) Whole seedling of WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* at 7 days after sowing. Bar = 10 mm. (B) Root meristem of WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* at 7 days after sowing. Bar = 100 μm. (C) The primary root length was measured in 7-day-old WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* seedlings. n = 8, mean ±SD.*: $P < 0.01$ (Student's *t*-test). (D) The number of cortex cells in the root meristem was counted in 7-day-old WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* roots. n = 8, mean ± SD. **: no significance (Student's *t*-test). (E) The area of dead cells in stele cells was counted in 7-day-old WT, *ddi1-1*, *fas1-4* and *fas1-4 ddi1-1* roots. n = 8, mean ± SD. ***: $P < 0.05$ (Student's *t*-test)

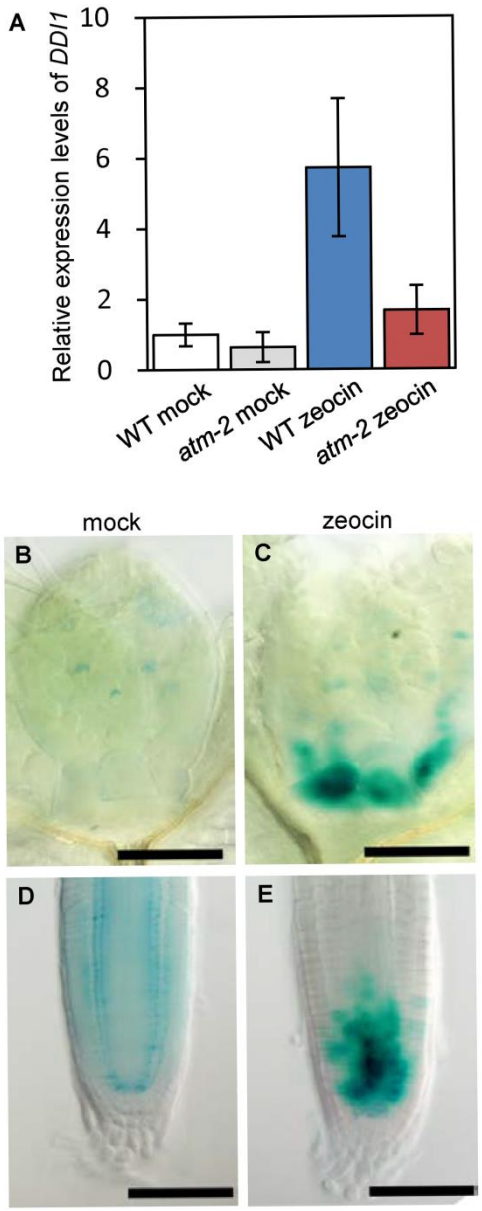

Figure 14. Expression analyses of *DDI1*. (A) RT-PCR analysis of *DDI1* gene in WT and *atm-2* under mock or zeocin treatment. Total RNA prepared from the whole seedlings at 6 days after sowing was reverse-transcribed and amplified by RT-PCR. All values were normalised against the expression level of the *ACTIN2* gene and expressed relative to mock treated WT level. We used three biological replicates and error bars indicate SE. (B) and (C) Expression patterns of *DDI1pro::GUS* in leaf primordia of mock treated plants (B) and zeocin treated plants (C). (D) and (E) Expression patterns of *DDI1pro::GUS* in root meristem of mock treated plants (D) and zeocin treated plants (E). Bars in (B), (C), (D) and (E) = 100 μm.

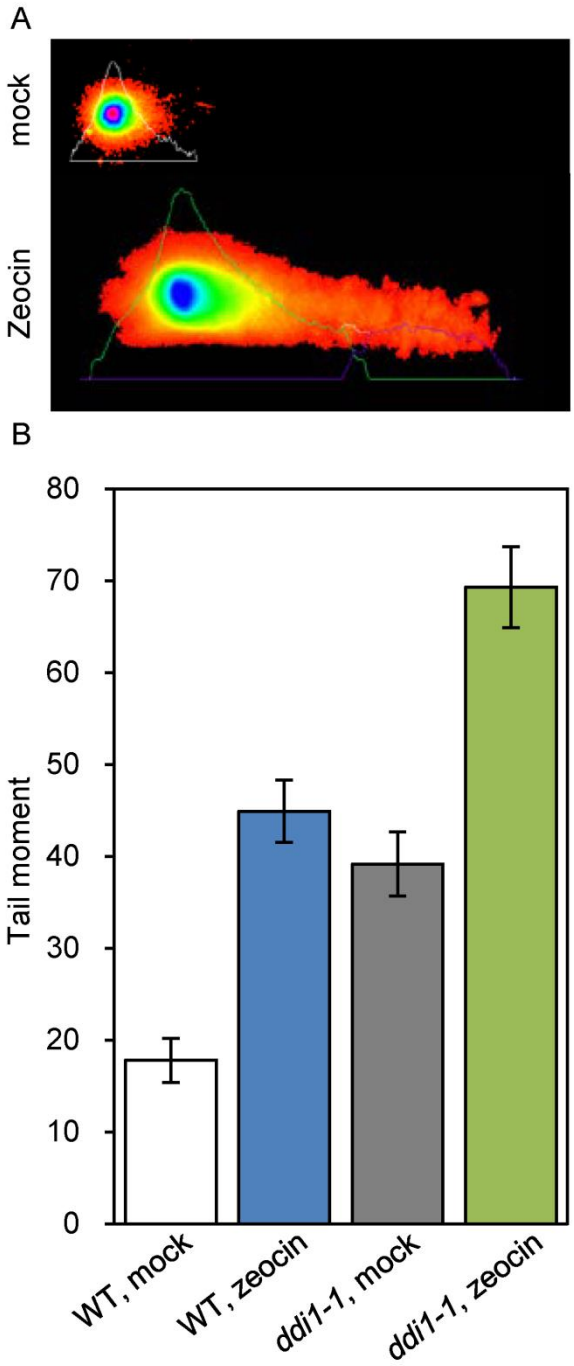

Figure 15. The *ddi1* mutants has more DSBs than WT. (A) Fluorescent images of WT nuclei under normal growth condition and genotoxic stress condition. Images are modified using Comet Score software. (B) The amount of DSBs in WT and *ddi1-1* under normal growth condition and genotoxic stress condition are quantified by calculating tail moment. n > 100, mean + SE.

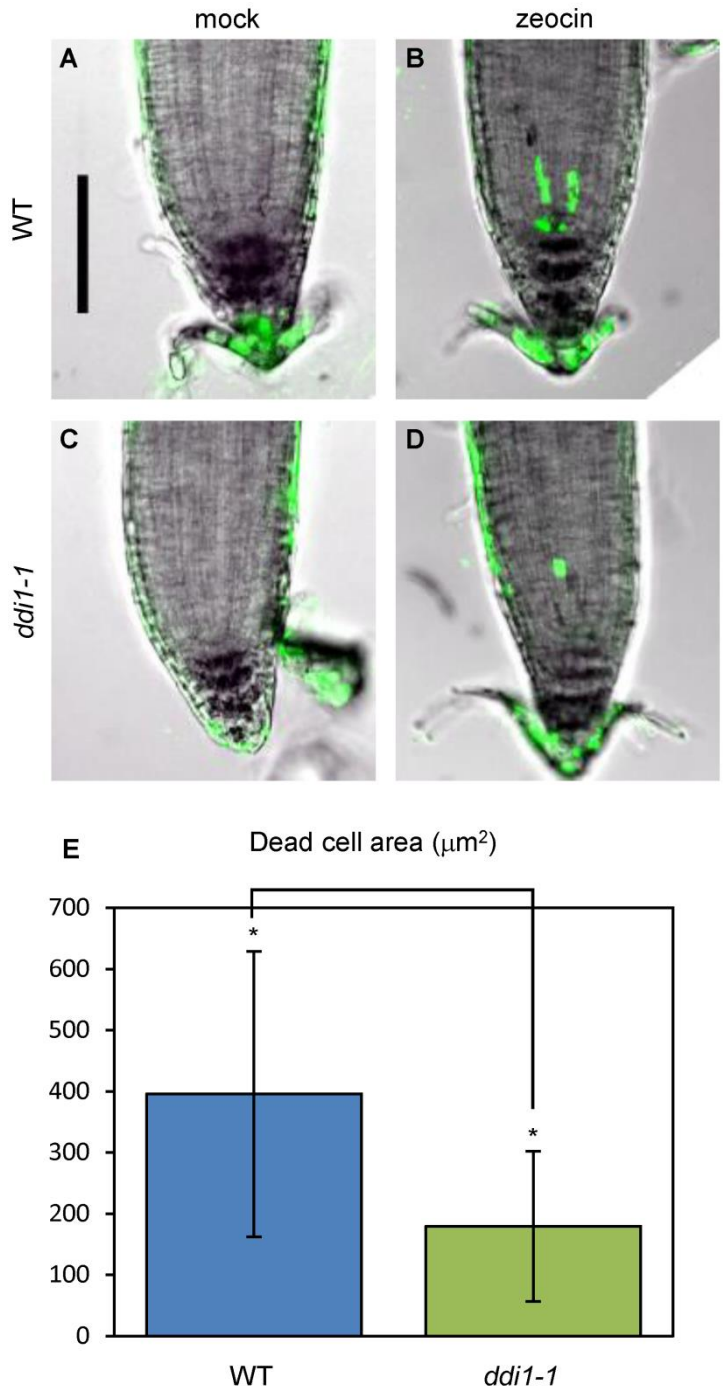

Figure 16. *ddi1* mutants are resistant to genotoxic stress. (A) and (B) Root meristem of WT plants at 7 days after sowing treated with mock (A) and zeocin (B). (C) and (D) Root meristem of *ddi1-1* plants at 7 days after sowing treated with mock (C) and zeocin (D). Root meristems in (A), (B), (C) and (D) were stained with SYTOX Green. Bar = 100 μm. (E) Dead cell area was measured as area which has green fluorescence in stele cells. n = 10, mean ± SD. *: $P < 0.05$ (Student's *t*-test).

Table 1. List of top 20 genes co-expressed with *DDI1*

	AGI code	Gene name	Involved in DNA repair
1	At1g08260	TIL1	○
2	At4g17260		
3	At1g13330	AHP2	○
4	At4g21070	BRCA1	○
5	At4g29170	MND1	○
6	At1g09815	POLD4	
7	At3g07800		
8	At5g45400	RPA70C	○
9	At5g64060	NAC103	
10	At5g20850	RAD51	○
11	At2g31320	PARP1	○
12	At4g02390	PARP2	○
13	At5g48720	XRI1	○
14	At4g19130		
15	At5g66130	RAD17	○
16	At4g28950	ROP9	
17	At2g45460		
18	At2g46610	RS31a	
19	At3g27060	TSO2	○
20	At4g37490	CYCB1;1	

Chapter IV

General discussion

Which factors are involved in compensation downstream of ATM

In the chapter I, I revealed that one of the up-stream triggers for compensation is hyper activation of ATM-dependent DNA damage response. During leaf development in *fas1*, cells suffer from DNA damage caused by lack of CAF-1 activity, leading to an activation of ATM-dependent DNA damage response. This response triggers cell cycle arrest and subsequently promotes cell expansion and endocycle onsets, resulting into fewer and larger cells. How does ATM-dependent DNA damage response promote these cellular processes? Massive studies have been carried out in yeasts and mammals to clarify the downstream factors of DNA damage response. In mammals, ATM is known to activate a transcription factor, p53 by its phosphorylation. The phosphorylated p53 promotes the transcription of its targets involved in DNA repair, cell cycle arrest and apoptosis (Ciccia and Elledge, 2010). Genomic information suggests that there are no homologues of p53 in Arabidopsis, however recent study suggests that a NAC type transcription factor SOG1 might function as a functional orthologue of p53 (Yoshiyama et al., 2009). SOG1 is suggested to act as a downstream factor of ATM- and ATR-dependent DNA damage response, and is required for premature transition from mitotic cell cycle to endocycle upon DNA damage in Arabidopsis root (Yoshiyama et al., 2009; Adachi et al., 2011). This suggests that SOG1 is a good candidate which is involved in triggering compensation in *fas1,* and genetic analysis between *fas1* and *sog1* should provide more information. WEE1 is another factor known to function

downstream of ATM- and ATR-dependent DNA damage response in both animals and plants. The WEE1 kinase inhibits CDKA;1 activity through its phosphorylation (De Schutter et al., 2007). It is still unclear whether WEE1 is involved in triggering compensation in *fas1*, however plants with dominant negative type CDKA;1 exhibits compensation and they have serrated leaves, the phenotype also seen in *fas1* (Dissmeyer et al., 2009). Therefore, it is possible that cell cycle arrest in *fas1* may be a result of CDKA;1 inactivation by WEE1. There are several questions that need to be addressed in the future. 1: Does the *wee1* mutation suppress compensation in *fas1*? 2: Does the ectopic expression of WEE1 in leaves cause compensation? 3: Is CDKA;1 inactivated in *fas1* leaves? SOG1 and WEE1 are good candidates for downstream factor of ATM-dependent DNA damage response pathway involved in triggering compensation in *fas1*.

Activation of ATM-dependent DNA damage response in *fas1* not only inhibits cell proliferation but also promote endocycle onset, leading to the high ploidy phenotype in *fas1*. Therefore, compensated cell expansion in *fas1* may be driven by ploidy-dependent cell growth. APC/C is known to be an important regulator for endocycle onset. Activity of APC/C is regulated by activator proteins such as CCS52A1 and CCS52A2 and repressor proteins such as UVI4 and GIG1 (Lammens et al, 2008; Larson-Rabin et al, 2009; Kasili et al, 2010; Mathieu-Rivet et al, 2010; Iwata et al., 2011; Heyman et al., 2011). Furthermore, the expression level of CCS52A1 is regulated by GTL1 whereas that of CCS52A2 is regulated by DEL1 (Breuer et al., 2012; Lammens et al.,2008). To understand the regulation of endocycle onset during compensated cell expansion in *fas1*, expression analyses of these endocycle regulators in *fas1* and genetic analyses between *fas1* and mutants of these genes should be useful.

How is compensation in *fas1* regulated at an organ level?

As described in introduction, cell proliferation and cell expansion occur at the same time in different position of one leaf during its development. Therefore, to get proper final leaf size, cell proliferation and cell expansion must be tightly coordinated. Kawade et al. revealed that there are two different pathways to achieve this coordination (Kawade et al., 2010); one is cell-autonomous pathway in the *KRP2*-dependent compensation and another is non-cell-autonomous pathway in the *an3*-dependent compensation. In cell-autonomous pathway, compensated cell expansion might be achieved as below. Individual cells in leaf primordia would memorize their cell proliferation activity, and then they activate compensated cell expansion only when cell proliferation activity becomes lower. On the other hand, in non-cell-autonomous pathway, compensation might be achieved through cell to cell communication. When cell proliferation activity gets lower, cells in the proliferative phase would produce signalling molecules that promote post-mitotic cell expansion. As described in chapter I, compensation in *fas1* is triggered by cell cycle arrest caused by DNA damage response. There seem to be much variation of amount of DNA damage among cells in *fas1*. Therefore, extent of the DNA damage response varies among each cell, leading to different extent of cell cycle arrest. If compensated cell expansion in *fas1* is a result of early transition from mitotic cycle to endocycle, compensation in *fas1* should be regulated in cell-autonomous manner. Chimera analyses as shown in Kawade et al. (2010) should help testing this possibility. Furthermore, it is interesting to explore the relationship among amount of DNA damage, ploidy level and cell size at the cellular level. The *fas1* mutants with chimera system which marks cells with DNA damage

could be useful to analyse relationship between cell size and DNA damage response in cellular level.

Relationship among *fas1* and other compensation mutants

Many mutants that have defects in cell proliferation cause compensation. Previous studies suggested that compensation is heterogeneous phenomena. Each mutant may have different cause for decreased cell proliferation activity and each mutant may have different pathway to increase their cell size. Is there any common mechanism for balancing cell proliferation and cell expansion to control organ size? To answer this question, not only analyses focused on a specific mutant to understand molecular basis for compensation in each mutant but also comparative analyses using several mutants should be done. For example, as Fujikura et al (2007) have performed on a series of *xs* mutants for their influence on *an3*-dependent compensated cell enlargement, genetic analyses between *fas1* and *xs* mutants to explore which xs mutations supress compensation in *fas1* could help to understand the control of cell expansion in compensation.

How does the DNA damage response pathway affect meristem defects in *fas1*?

Genetic analysis revealed that DNA damage response pathway contributes to meristem defects in *fas1*. The *fas1* mutant exhibits early transition phenotype, cell death phenotype and abnormal arrangement of initial cells and columella cells. The *atm* mutation suppresses all of these phenotypes in *fas1* root, whereas the *ddi1* mutation suppresses cell death phenotype and partially suppresses abnormal arrangement phenotype in *fas1* root. Downstream cellular processes of the DNA damage response

can be separated to cell cycle arrest and cell death. Early transition phenotype may be a result of cell cycle arrest. Based on the observation of the *fas1 ddi1* double mutant, abnormal arrangement of meristematic cells might be caused by additive result of cell cycle arrest and cell death. In the root meristem of *fas1*, cells suffered by DNA damage cause cell cycle arrest or cell death. Cell death in meristem would make gaps and these gaps might perturb positional cues that are important for the maintenance of proper meristem structure. On the other hand, cell cycle arrest in the meristem might also affect positional cues. A recent study revealed that cell division and cell growth are tightly coordinated in floral meristem (Schiessl et al., 2012). These authors assume that this coordination is important to maintain the proper flow of auxin. Therefore, cell cycle arrest in the *fas1* root meristem may change cell size in meristem and then perturb auxin flow, leading to abnormal meristem structure.

Interestingly, the *ddi1* mutation partially suppresses a decrease in cell number in *fas1* leaves without suppressing the cell size phenotype. Considering the observation of the root meristem phenotype, cell death may occur also in leaf primordia of *fas1*. ATM might contribute to both cell cycle arrest and cell death in leaf primordia of *fas1*, whereas DDI1 may contribute to only cell death. Altogether, I assume that a decrease in cell number caused by cell death may not contribute to the induction of compensated cell expansion. In other words, compensation might be induced through sensing cell proliferation activity rather than simply counting the final cell number.

Relationship between *fas1* and other mutants with constitutive activation of DNA damage response

Other mutants that exhibit constitutive activation of DNA damage response

such as *etg1*, *teb* and *mdo1* showed different genetic interaction with *atm* and *atr* (Takahashi et al., 2008; Inagaki et al., 2009; Hashimura and Ueguchi 2011). All of these mutants share pleiotropic developmental phenotype with *fas1*. I showed *atm* and *atr* generally suppress developmental phenotypes of *fas1*, however, developmental phenotypes of *mdo1* are enhanced by *atm* and those of *etg1* and *teb* are enhanced by *atr* (Takahashi et al., 2008; Inagaki et al., 2009; Hashimura and Ueguchi 2011). One possibility is that they have different amount of endogenous DNA damage. This possibility can be tested using exogenous DNA damage agents. If this is the case, exogenous DNA damage treatment to *fas1 atm* might mimic the *etg1 atr*, *teb atr* and *mdo1 atm* phenotypes.

Conclusion remarks

In this thesis I revealed that ATM-dependent DNA damage response pathway is one of the trigger for compensation. The ATM-dependent DNA damage response pathway causes cell cycle arrest and promotes an endocycle onset. It is unclear whether this pathway is used in other compensation exhibiting mutants but insights from this thesis will be useful for comparative analyses with other compensation exhibiting mutants to deepen our understanding for organ size control. Furthermore, I isolated a novel, plant-specific gene involved in DNA repair and associated cell death. I also found that cell death partially contributes to the meristem defects in *fas1*. These results shed new light on the relationship between DNA damage stress and plant development. More generally, this study advances our understanding of the strategy that plants takes against the environmental stress.

Acknowledgements

I wish to express my sincere appreciation to Dr. Hirokazu Tsukaya (University of Tokyo, Japan) and Dr. Keiko Sugimoto (RIKEN, Japan) for their invaluable suggestions and guidance in this work. I would also like to express my deepest appreciation to Dr. Ali Ferjani (Tokyo Gakugei University, Japan) and Dr. Gorou Horiguchi (Rikkyo University) for their generous guidance, insightful suggestions and encouragement. I would like to thank Dr. Mitsutomo Abe, Dr. Hiroyuki Hirano, Dr. Munetaka Sugiyama and Dr. Takashi Ueda (University of Tokyo, Japan) for helpful advice and for critically reviewing the manuscript. I am also grateful to Dr. Taku Demura (RIKEN, Japan), Dr. Usio Fujikura (Potsdam University, Germany), Dr. Hiroo Fukuda (University of Tokyo, Japan), Dr. Naoko Ishikawa (University of Tokyo, Japan) and Dr. Minoru Kubo (University of Freiburg, Germany) for carrying out the microarray analyses of the *fas1* mutant, to Dr. Anne Britt and Dr. Kevin Culligan (University of California, Davis) for providing *atm-2* and *atr-2* mutants, to Arabidopsis Biological Resource Center for providing *fas1-4*, *ddi1-1* and *ddi1-2* mutants. Finally, I am deeply grateful to all the present and former members of Tsukaya laboratory (University of Tokyo, Japan) and Sugimoto laboratory (RIKEN, Japan) for fruitful discussion, technical support and encouragement throughout my study.

References

Adachi S, Minamisawa K, Okushima Y, Inagaki S, Yoshiyama K, Kondou Y, Kaminuma E, Kawashima M, Toyoda T, Matsui M Kurihara D, Matsunaga S, Umeda M (2011) Programmed induction of endoreduplication by DNA double-strand breaks in Arabidopsis. Proc Natl Acad Sci U S A. **108:** 10004-10009.

Anastasiou E, Kenz S, Gerstung M, MacLean D, Timmer J, Fleck C, Lenhard M (2007) Control of plant organ size by KLUH/CYP78A5-dependent intercellular signaling. Dev Cell **13:** 843-856.

Andriankaja M, Dhondt S, De Bodt S, Vanhaeren H, Coppens F, De Milde L, Muhlenbock P, Skirycz A, Gonzalez N, Beemster GTS, Inze D (2012) Exit from proliferation during leaf development in Arabidopsis thaliana: a not-so-gradual process. Dev Cell **22:** 64-78.

Autran D, Jonak C, Belcram K, Beemster G, Kronenberger J, Grandjean O, Inzé D, Traas J (2002) Cell numbers and leaf development in Arabidopsis: a functional analysis of the STRUWWELPETER gene. EMBO J **21:** 6036-6049.

Babiychuk E, Cottrill PB, Storozhenko S, Fuangthong M, Chen Y, O'Farrell MK, Van Montagu M, Inzé D, Kushnir S (1998) Higher plants possess two structurally different poly(ADP-ribose) polymerases. Plant J **15:** 635-645.

Barow M, Meister A (2003) Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. Plant Cell Environ **26:** 571-584.

Beemster G, Fiorani F, Inzé D (2003) Cell cycle: the key to plant growth control? Trends Plant Sci **8:** 154-158.

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inze D, Vuylsteke M (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiol **138:** 734-743.

Boudolf V, Lammens T, Boruc J, Van Leene J, Van Den Daele H, Maes S, Van Isterdael G, Russinova E, Kondorosi E, Witters E, De Jaeger G, Inze D, De Veylder L (2009) CDKB1;1 forms a functional complex with CYCA2;3 to suppress endocycle onset. Plant Physiol **150:** 1482-1493.

Breuer C, Morohashi K, Kawamura A, Takahashi N, Ishida T, Umeda M, Grotewold E, Sugimoto K (2012) Transcriptional repression of the APC/C activator CCS52A1 promotes active termination of cell growth. EMBO J **31:** 4488-4501.

Breuer C, Stacey N, West C, Zhao Y, Chory J, Tsukaya H, Azumi Y, Maxwell A,

Roberts K, Sugimoto-Shirasua K (2007) BIN4, a novel component of the plant DNA topoisomerase VI complex, is required for endoreduplication in Arabidopsis. Plant Cell **19:** 3655-3668.

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proc Natl Acad Sci U S A **97:** 9783-9788.

Choi DS, Lee Y, Cho HT, Kende H (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. Plant Cell **15:** 1386-1398.

Ciccia A, Elledge SJ (2010) The DNA damage response: making it safe to play with knives. Mol Cell **40:** 179-204.

Cnops G, Jover-Gil S, Peters JL, Neyt P, De Block S, Robles P, Ponce MR, Gerats T, Van Lijsebettens M, Micol JL (2004) The rotunda2 mutants identify a role for the LEUNIG gene in vegetative leaf morphogenesis. J Exp Bot **55:** 1529-1539.

Cook M, Tyers M (2007) Size control goes global. Curr Opin Biotechnol **18:** 341-350.

Cordin O, Hahn D, Beggs JD (2012) Structure, function and regulation of spliceosomal RNA helicases. Curr Opin Cell Biol **24:** 431-438.

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol **6:** 850-861.

Culligan K, Tissier A, Britt A (2004) ATR regulates a G2-phase cell-cycle checkpoint in Arabidopsis thaliana. Plant Cell **16:** 1091-1104.

Culligan KM, Robertson CE, Foreman J, Doerner P, Britt AB (2006) ATR and ATM play both distinct and additive roles in response to ionizing radiation. Plant J **48:** 947-961.

De Schutter K, Joubes J, Cools T, Verkest A, Corellou F, Babiychuk E, Van Der Schueren E, Beeckman T, Kushnir S, Inze D, De Veylder L (2007) Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. Plant Cell **19:** 211-225.

De Veylder L, Beeckman T, Beemster G, Krols L, Terras F, Landrieu I, van der Schueren E, Maes S, Naudts M, Inzé D (2001) Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell **13:** 1653-1668.

Deprost D, Yao L, Sormani R, Moreau M, Leterreux G, Nicolaie M, Bedu M, Robaglia C, Meyer C (2007) The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. EMBO Rep **8:** 864-870.

Dewitte W, Riou-Khamlichi C, Scofield S, Healy JMS, Jacqmard A, Kilby NJ,

Murray JAH (2003) Altered cell cycle distribution, hyperplasia, and inhibited differentiation in arabidopsis caused by the D-type cyclin CYCD3. Plant Cell **15:** 79-92.

Dinneny JR, Weigel D, Yanofsky MF (2006) NUBBIN and JAGGED define stamen and carpel shape in Arabidopsis. Development **133:** 1645-1655.

Dinneny JR, Yadegari R, Fischer RL, Yanofsky MF, Weigel D (2004) The role of JAGGED in shaping lateral organs. Development **131:** 1101-1110.

Disch S, Anastasiou E, Sharma VK, Laux T, Fletcher JC, Lenhard M (2006) The E3 ubiquitin ligase BIG BROTHER controls Arabidopsis organ size in a dosage-dependent manner. Curr Biol **16:** 272-279.

Dissmeyer N, Weimer AK, Pusch S, De Schutter K, Kamei CLA, Nowack MK, Novak B, Duan GL, Zhu YG, De Veylder L, Schnittger A (2009) Control of cell proliferation, organ growth, and DNA damage response operate independently of dephosphorylation of the Arabidopsis Cdk1 Homolog CDKA;1. Plant Cell **21:** 3641-3654.

Dolan L, Poethig RS (1998) Clonal analysis of leaf development in cotton. Am J Bot **85:** 315-321.

Donnelly P, Bonetta D, Tsukaya H, Dengler R, Dengler N (1999) Cell cycling and

cell enlargement in developing leaves of Arabidopsis. Dev Biol **215:** 407-419.

Doucet-Chabeaud G, Godon C, Brutesco C, de Murcia G, Kazmaier M (2001) Ionising radiation induces the expression of PARP-1 and PARP-2 genes in Arabidopsis. Mol Genet Genomics **265:** 954-963.

Doutriaux MP, Couteau F, Bergounioux C, White C (1998) Isolation and characterisation of the RAD51 and DMC1 homologs from Arabidopsis thaliana. Mol Gen Genet **257:** 283-291.

Endo M, Ishikawa Y, Osakabe K, Nakayama S, Kaya H, Araki T, Shibahara K, Abe K, Ichikawa H, Valentine L, Hohn B, Toki S (2006) Increased frequency of homologous recombination and T-DNA integration in Arabidopsis CAF-1 mutants. EMBO J **25:** 5579-5590.

Enomoto S, Berman J (1998) Chromatin assembly factor I contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci. Genes Dev **12:** 219-232.

Exner V, Taranto P, Schönrock N, Gruissem W, Hennig L (2006) Chromatin assembly factor CAF-1 is required for cellular differentiation during plant development. Development **133:** 4163-4172.

Ferjani A, Horiguchi G, Yano S, Tsukaya H (2007) Analysis of leaf development in

fugu mutants of Arabidopsis reveals three compensation modes that modulate cell expansion in determinate organs. Plant Physiol **144:** 988-999.

Francia S, Michelini F, Saxena A, Tang D, de Hoon M, Anelli V, Mione M, Carninci P, di Fagagna FD (2012) Site-specific DICER and DROSHA RNA products control the DNA-damage response. Nature **488:** 231-235.

Fujikura U, Horiguchi G, Ponce M, Micol J, Tsukaya H (2009) Coordination of cell proliferation and cell expansion mediated by ribosome-related processes in the leaves of Arabidopsis thaliana. Plant J **59:** 499-508.

Fujikura U, Horiguchi G, Tsukaya H (2007) Dissection of enhanced cell expansion processes in leaves triggered by a defect in cell proliferation, with reference to roles of endoreduplication. Plant Cell Physiol **48:** 278-286.

Fulcher N, Sablowski R (2009) Hypersensitivity to DNA damage in plant stem cell niches. Proc Natl Acad Sci U S A **106:** 20984-20988.

Garcia V, Bruchet H, Camescasse D, Granier F, Bouchez D, Tissier A (2003) AtATM is essential for meiosis and the somatic response to DNA damage in plants. Plant Cell **15:** 119-132.

Guyomarc'h S, Vernoux T, Traas J, Zhou DX, Delarue M (2004) MGOUN3, an Arabidopsis gene with TetratricoPeptide-Repeat-related motifs, regulates

meristem cellular organization. J Exp Bot **55:** 673-684.

Ha CM, Jun JH, Nam HG, Fletcher JC (2004) BLADE-ON-PETIOLE1 encodes a BTB/POZ domain protein required for leaf morphogenesis in Arabidopsis thaliana. Plant Cell Physiol **45:** 1361-1370.

Ha CM, Jun JH, Nam HG, Fletcher JC (2007) BLADE-ON-PETIOLE1 and 2 control Arabidopsis lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. Plant Cell **19:** 1809-1825.

Ha CM, Kim GT, Kim BC, Jun JH, Soh MS, Ueno Y, Machida Y, Tsukaya H, Nam HG (2003) The BLADE-ON-PETIOLE 1 gene controls leaf pattern formation through the modulation of meristematic activity in Arabidopsis. Development **130:** 161-172.

Haber A (1962) Nonessentiality of concurrent cell divisions for degree of polarization of leaf growth. 1. Studies with radiation-induced mitotic inhibition. Am J Bot **49:** 583-589.

Harper JW, Elledge SJ (2007) The DNA damage response: ten years after. Mol Cell **28:** 739-745.

Hashimura Y, Ueguchi C (2011) The Arabidopsis MERISTEM DISORGANIZATION 1 gene is required for the maintenance of stem cells

through the reduction of DNA damage. Plant J **68:** 657-669.

Heyman J, Van den Daele H, De Wit K, Boudolf V, Berckmans B, Verkest A, Kamei CLA, De Jaeger G, Koncz C, De Veylder L (2011) Arabidopsis ULTRAVIOLET-B-INSENSITIVE4 maintains cell division activity by temporal inhibition of the anaphase-promoting complex/cyclosome. Plant Cell **23:** 4394-4410.

Hoek M, Stillman B (2003) Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication in vivo. Proc Natl Acad Sci U S A **100:** 12183-12188.

Horiguchi G, Fujikura U, Ferjani A, Ishikawa N, Tsukaya H (2006) Large-scale histological analysis of leaf mutants using two simple leaf observation methods: identification of novel genetic pathways governing the size and shape of leaves. Plant J **48:** 638-644.

Horiguchi G, Gonzalez N, Beemster GTS, Inze D, Tsukaya H (2009) Impact of segmental chromosomal duplications on leaf size in the grandifolia-D mutants of Arabidopsis thaliana. Plant J **60:** 122-133.

Horiguchi G, Kim G, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of Arabidopsis thaliana. Plant J **43:** 68-78.

Horiguchi G, Tsukaya H (2011) Organ size regulation in plants: insights from compensation. Front Plant Sci 2:24.

Horvath BM, Magyar Z, Zhang Y, Hamburger AW, Bako L, Visser RGF, Bachem CWB, Bogre L (2006) EBP1 regulates organ size through cell growth and proliferation in plants. EMBO J **25:** 4909-4920.

Hu Y, Poh HM, Chua NH (2006) The Arabidopsis ARGOS-LIKE gene regulates cell expansion during organ growth. Plant J **47:** 1-9.

Hu YX, Xie O, Chua NH (2003) The Arabidopsis auxin-inducible gene ARGOS controls lateral organ size. Plant Cell **15:** 1951-1961.

Ichihashi Y, Horiguchi G, Gleissberg S, Tsukaya H (2010) The bHLH transcription factor SPATULA controls final leaf size in Arabidopsis thaliana. Plant Cell Physiol **51:** 252-261.

Ichihashi Y, Kawade K, Usami T, Horiguchi G, Takahashi T, Tsukaya H (2011) Key proliferative activity in the junction between the leaf blade and leaf petiole of Arabidopsis. Plant Physiol **157:** 1151-1162.

Ikeuchi M, Yamaguchi T, Kazama T, Ito T, Horiguchi G, Tsukaya H (2011) ROTUNDIFOLIA4 regulates cell proliferation along the body axis in

Arabidopsis shoot. Plant Cell Physiol **52:** 59-69.

Imai K, Ohashi Y, Tsuge T, Yoshizumi T, Matsui M, Oka A, Aoyama T (2006) The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in Arabidopsis endoreduplication. Plant Cell **18:** 382-396.

Inagaki S, Nakamura K, Morikami A (2009) A link among DNA replication, recombination, and gene expression revealed by genetic and genomic analysis of TEBICHI gene of Arabidopsis thaliana. PLoS Genet **5**: e1000613.

Inagaki S, Suzuki T, Ohto M, Urawa H, Horiuchi T, Nakamura K, Morikami A (2006) Arabidopsis TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. Plant Cell **18:** 879-892.

Irish VF, Sussex IM (1992) A fate map of the Arabidopsis embryonic shoot apical meristem. Development **115:** 745-753.

Ishida T, Adachi S, Yoshimura M, Shimizu K, Umeda M, Sugimoto K (2010) Auxin modulates the transition from the mitotic cycle to the endocycle in Arabidopsis. Development **137:** 63-71.

Ishida T, Fujiwara S, Miura K, Stacey N, Yoshimura M, Schneider K, Adachi S, Minamisawa K, Umeda M, Sugimoto K (2009) SUMO E3 ligase HIGH

PLOIDY2 regulates endocycle onset and meristem maintenance in Arabidopsis. Plant Cell **21:** 2284-2297.

Iwata E, Ikeda S, Matsunaga S, Kurata M, Yoshioka Y, Criqui MC, Genschik P, Ito M (2011) GIGAS CELL1, a novel negative regulator of the anaphase-promoting complex/cyclosome, is required for proper mitotic progression and cell fate determination in Arabidopsis. Plant Cell **23:** 4382-4393.

Kasili R, Walker JD, Simmons LA, Zhou J, De Veylder L, Larkin JC (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana trichomes. Genetics **185:** 257-268.

Kaufman P, Kobayashi R, Stillman B (1997) Ultraviolet radiation sensitivity and reduction of telomeric silencing Saccharomyces cerevisiae cells lacking chromatin assembly factor-I. Genes Dev **11:** 345-357.

Kawade K, Horiguchi G, Tsukaya H (2010) Non-cell-autonomously coordinated organ size regulation in leaf development. Development **137:** 4221-4227.

Kaya H, Shibahara K, Taoka K, Iwabuchi M, Stillman B, Araki T (2001) FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. Cell **104:** 131-142.

Kazama T, Ichihashi Y, Murata S, Tsukaya H (2010) The mechanism of cell cycle arrest front progression explained by a KLUH/CYP78A5-dependent mobile growth factor in developing leaves of Arabidopsis thaliana. Plant Cell Physiol **51:** 1046-1054.

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. Plant J **50:** 347-363.

Kim JH, Choi DS, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in Arabidopsis. Plant J **36:** 94-104.

Kim JH, Kende H (2004) A transcriptional coactivator, AtGIF1, is involved in regulating leaf growth and morphology in Arabidopsis. Proc Natl Acad Sci U S A **101:** 13374-13379.

Kirik A, Pecinka A, Wendeler E, Reiss B (2006) The chromatin assembly factor subunit FASCIATA1 is involved in homologous recombination in plants. Plant Cell **18:** 2431-2442.

Komaki S, Sugimoto K (2012) Control of the plant cell cycle by developmental and environmental cues. Plant Cell Physiol **53:** 953-964.

Krizek BA (2009) AINTEGUMENTA and AINTEGUMENTA-LIKE6 act redundantly to regulate Arabidopsis floral growth and patterning. Plant Physiol **150:** 1916-1929.

Kurepa J, Wang S, Li Y, Zaitlin D, Pierce A, Smalle J (2009) Loss of 26S proteasome function leads to increased cell size and decreased cell number in Arabidopsis shoot organs. Plant Physiol **150:** 178-189.

Lafarge S, Montané MH (2003) Characterization of Arabidopsis thaliana ortholog of the human breast cancer susceptibility gene 1: AtBRCA1, strongly induced by gamma rays. Nucleic Acids Res **31:** 1148-1155.

Lammens T, Boudolf V, Kheibarshekan L, Zalmas LP, Gaamouche T, Maes S, Vanstraelen M, Kondorosi E, La Thangue NB, Govaerts W, Inze D, De Veylder L (2008) Atypical E2F activity restrains APC/C-CCS52A2 function obligatory for endocycle onset. Proc Natl Acad Sci U S A **105:** 14721-14726.

Larson-Rabin Z, Li Z, Masson PH, Day CD (2009) FZR2/CCS52A1 expression is a determinant of endoreduplication and cell expansion in Arabidopsis. Plant Physiol **149:** 874-884.

Lee BH, Ko J-H, Lee S, Lee Y, Pak J-H, Kim JH (2009) The Arabidopsis GRF-INTERACTING FACTOR gene family performs an overlapping function in determining organ size as well as multiple developmental properties. Plant

Physiol **151:** 655-668.

Leyser H, Furner I (1992) Characterization of 3 shoot apical meristem mutants of Arabidopsis thaliana. Development **116:** 397-403.

Li CX, Potuschak T, Colon-Carmona A, Gutierrez RA, Doerner P (2005) Arabidopsis TCP20 links regulation of growth and cell division control pathways. Proc Natl Acad Sci U S A **102:** 12978-12983.

Li Y, Zheng L, Corke F, Smith C, Bevan MW (2008) Control of final seed and organ size by the DA1 gene family in Arabidopsis thaliana. Genes Dev **22:** 1331-1336.

Malamy JE, and Benfey PN (1997) Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development **124:** 33–44.

Mathieu-Rivet E, Gevaudant F, Sicard A, Salar S, Do PT, Mouras A, Fernie AR, Gibon Y, Rothan C, Chevalier C, Hernould M (2010) Functional analysis of the anaphase promoting complex activator CCS52A highlights the crucial role of endo-reduplication for fruit growth in tomato. Plant J **62:** 727-741.

Melaragno JE, Mehrotra B, Coleman AW (1993) Relationship between endopolyploidy and cell size in epidermal tissue of Arabidopsis. Plant Cell **5:** 1661-1668.

Menand B, Desnos T, Nussaume L, Berger F, Bouchez D, Meyer C, Robaglia C (2002) Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. Proc Natl Acad Sci U S A **99:** 6422-6427.

Michalik KM, Bottcher R, Forstemann K (2012) A small RNA response at DNA ends in Drosophila. Nucleic Acids Res **40:** 9596-9603.

Mizukami Y, Fischer R (2000) Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. Proc Natl Acad Sci U S A **97:** 942-947.

Mozgová I, Mokros P, Fajkus J (2010) Dysfunction of chromatin assembly factor 1 induces shortening of telomeres and loss of 45S rDNA in Arabidopsis thaliana. Plant Cell **22:** 2768-2780.

Nabatiyan A, Krude T (2004) Silencing of chromatin assembly factor 1 in human cells leads to cell death and loss of chromatin assembly during DNA synthesis. Mol Cell Biol **24:** 2853-2862.

Nagl W (1976) DNA endoreduplication and polyteny understood as evolutionary strategies. Nature **261:** 614-615.

Nakagawa T, Suzuki T, Murata S, Nakamura S, Hino T, Maeo K, Tabata R, Kawai T, Tanaka K, Niwa Y, Watanabe Y, Nakamura K, Kimura T, Ishiguro S

(2007) Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. Biosci Biotechnol Biochem **71:** 2095-2100.

Narita NN, Moore S, Horiguchi G, Kubo M, Demura T, Fukuda H, Goodrich J, Tsukaya H (2004) Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in Arabidopsis thaliana. Plant J **38:** 699-713.

Nath U, Crawford B, Carpenter R, Coen E (2003) Genetic control of surface curvature. Science **299:** 1404-1407.

Nole-Wilson S, Tranby TL, Krizek BA (2005) AINTEGUMENTA-like (AIL) genes are expressed in young tissues and may specify meristematic or division-competent states. Plant Mol Biol **57:** 613-628.

Norberg M, Holmlund M, Nilsson O (2005) The BLADE ONPETIOLE genes act redundantly to control the growth and development of lateral organs. Development **132:** 2203-2213.

Ohno CK, Reddy GV, Heisler MGB, Meyerowitz EM (2004) The Arabidopsis JAGGED gene encodes a zinc finger protein that promotes leaf tissue development. Development **131:** 1111-1122.

Ono T, Kaya H, Takeda S, Abe M, Ogawa Y, Kato M, Kakutani T, Mittelsten Scheid O, Araki T, Shibahara K (2006) Chromatin assembly factor 1 ensures the stable maintenance of silent chromatin states in Arabidopsis. Genes Cells **11:** 153-162.

Parsyan A, Shahbazian D, Martineau Y, Petroulakis E, Alain T, Larsson O, Mathonnet G, Tettweiler G, Hellen CU, Pestova TV, Svitkin YV, Sonenberg N (2009) The helicase protein DHX29 promotes translation initiation, cell proliferation, and tumorigenesis. Proc Natl Acad Sci U S A **106:** 22217-22222.

Pisareva VP, Pisarev AV, Komar AA, Hellen CU, Pestova TV (2008) Translation initiation on mammalian mRNAs with structured 5'UTRs requires DExH-box protein DHX29. Cell **135:** 1237-1250.

Poethig RS, Sussex IM (1985) The developmental morphology and growth dynamics of the tobacco leaf. Planta **165:** 158-169.

Potter CJ, Xu T (2001) Mechanisms of size control. Curr Opin Genet Dev **11:** 279-286.

Ramirez-Parra E, Gutierrez C (2007) E2F regulates FASCIATA1, a chromatin assembly gene whose loss switches on the endocycle and activates gene expression by changing the epigenetic status. Plant Physiol **144:** 105-120.

Raynaud C, Perennes C, Reuzeau C, Catrice O, Brown S, Bergounioux C (2005) Cell and plastid division are coordinated through the prereplication factor AtCDT1. Proc Natl Acad Sci U S A **102:** 8216-8221.

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in Arabidopsis thaliana by microRNA miR396. Development **137:** 103-112.

Roeder AHK, Chickarmane V, Cunha A, Obara B, Manjunath BS, Meyerowitz EM (2010) Variability in the control of cell division underlies sepal epidermal patterning in Arabidopsis thaliana. PLoS Biol **8:** e1000367.

Roth N, Klimesch J, Dukowic-Schulze S, Pacher M, Mannuss A, Puchta H (2012) The requirement for recombination factors differs considerably between different pathways of homologous double-strand break repair in somatic plant cells. Plant J **72:** 781-790.

Schiessl K, Kausika S, Southam P, Bush M, Sablowski R (2012) JAGGED controls growth anisotropy and coordination between cell size and cell cycle during plant organogenesis. Curr Biol **22:** 1739-1746.

Schnittger A, Grini PE, Folkers U, Hulskamp M (1996) Epidermal fate map of the Arabidopsis shoot meristem. Dev Biol **175:** 248-255.

Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ (2006) The AUXIN RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division, and the size of seeds and other organs. Development **133:** 251-261.

Schönrock N, Exner V, Probst A, Gruissem W, Hennig L (2006) Functional genomic analysis of CAF-1 mutants in Arabidopsis thaliana. J Biol Chem **281:** 9560-9568.

Shibahara K, Stillman B (1999) Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell **96:** 575-585.

Shoji M, Tanaka T, Hosokawa M, Reuter M, Stark A, Kato Y, Kondoh G, Okawa K, Chujo T, Suzuki T, Hata K, Martin SL, Noce T, Kuramochi-Miyagawa S, Nakano T, Sasaki H, Pillai RS, Nakatsuji N, Chuma S (2009) The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. Dev Cell **17:** 775-787.

Smith S, Stillman B (1989) Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA-replication in vitro. Cell **58:** 15-25.

Smith S, Stillman B (1991) Stepwise assembly of chromatin during DNA-replication in vitro. EMBO J **10:** 971-980.

Song Y, He F, Xie G, Guo X, Xu Y, Chen Y, Liang X, Stagljar I, Egli D, Ma J, Jiao R (2007) CAF-1 is essential for Drosophila development and involved in the maintenance of epigenetic memory. Dev Biol **311:** 213-222.

Sonoda Y, Sako K, Maki Y, Yamazaki N, Yamamoto H, Ikeda A, Yamaguchi J (2009) Regulation of leaf organ size by the Arabidopsis RPT2a 19S proteasome subunit. Plant J **60:** 68-78.

Squatrito M, Mancino M, Donzelli M, Areces LB, Draetta GF (2004) EBP1 is a nucleolar growth-regulating protein that is part of pre-ribosomal ribonucleoprotein complexes. Oncogene **23:** 4454-4465.

Sterken R, Kiekens R, Boruc J, Zhang F, Vercauteren A, Vercauteren I, De Smet L, Dhondt S, Inzé D, De Veylder L, RussinovaE, Vuylsteke M (2012) Combined linkage and association mapping reveals CYCD5;1 as a quantitative trait gene for endoreduplication in Arabidopsis. Proc Natl Acad Sci U S A **109:** 4678-4683.

Sugimoto-Shirasu K, Roberts G, Stacey N, McCann M, Maxwell A, Roberts K (2005) RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. Proc Natl Acad Sci U S A **102:** 18736-18741.

Sugimoto-Shirasu K, Stacey NJ, Corsar J, Roberts K, McCann MC (2002) DNA

topoisomerase VI is essential for endoreduplication in Arabidopsis. Curr Biol **12:** 1782-1786.

Suzuki T, Inagaki S, Nakajima S, Akashi T, Ohto M, Kobayashi M, Seki M, Shinozaki K, Kato T, Tabata S, Nakamura K, Morikami A (2004) A novel Arabidopsis gene TONSOKU is required for proper cell arrangement in root and shoot apical meristems. Plant J **38:** 673-684.

Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y (2004) Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell **116:** 51-61.

Takahashi N, Lammens T, Boudolf V, Maes S, Yoshizumi T, De Jaeger G, Witters E, Inze D, De Veylder L (2008) The DNA replication checkpoint aids survival of plants deficient in the novel replisome factor ETG1. EMBO J **27:** 1840-1851.

Takahashi N, Quimbaya M, Schubert V, Lammens T, Vandepoele K, Schubert I, Matsui M, Inze D, Berx G, De Veylder L (2010) The MCM-binding protein ETG1 aids sister chromatid cohesion required for postreplicative homologous recombination repair. PLoS Genet **6:** e1000817.

Takeda S, Tadele Z, Hofmann I, Probst AV, Angelis KJ, Kaya H, Araki T, Mengiste T, Scheid OM, Shibahara K, Scheel D, Paszkowski J (2004) BRU1, a novel link between responses to DNA damage and epigenetic gene silencing in

Arabidopsis. Genes Dev **18:** 782-793.

Tsuge T, Tsukaya H, Uchimiya H (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in Arabidopsis thaliana (L) Heynh. Development **122:** 1589-1600.

Tsukaya H (2002) Interpretation of mutants in leaf morphology: Genetic evidence for a compensatory system in leaf morphogenesis that provides a new link between cell and organismal theories. Int Rev Cytol **217:** 1-39.

Tsukaya H (2003) Organ shape and size: a lesson from studies of leaf morphogenesis. Curr Opin Plant Biol **6:**57-62.

Ulm R, Baumann A, Oravecz A, Máté Z, Adám E, Oakeley EJ, Schäfer E, Nagy F (2004) Genome-wide analysis of gene expression reveals function of the bZIP transcription factor HY5 in the UV-B response of Arabidopsis. Proc Natl Acad Sci U S A **101:** 1397-1402.

van der Knaap E, Kim JH, Kende H (2000) A novel gibberellin-induced gene from rice and its potential regulatory role in stem growth. Plant Physiol **122:** 695-704.

Vert G, Walcher CL, Chory J, Nemhauser JL (2008) Integration of auxin and brassinosteroid pathways by Auxin Response Factor 2. Proc Natl Acad Sci U S A **105:** 9829-9834.

Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol **10:** 385-397.

Vlieghe K, Boudolf V, Beemster GTS, Maes S, Magyar Z, Atanassova A, Engler JD, De Groodt R, Inze D, De Veylder L (2005) The DP-E2F-like gene DEL1 controls the endocycle in Arabidopsis thaliana. Curr Biol **15:** 59-63.

Wang D, Guo YH, Wu CG, Yang GD, Li YY, Zheng CC (2008) Genome-wide analysis of CCCH zinc finger family in Arabidopsis and rice. BMC Genomics **9:** 44.

Wang Y, Henriksson E, Soderman E, Henriksson KN, Sundberg E, Engstrom P (2003) The Arabidopsis homeobox gene, ATHB16, regulates leaf development and the sensitivity to photoperiod in Arabidopsis. Dev Biol **264:** 228-239.

Wargent JJ, Gegas VC, Jenkins GI, Doonan JH, Paul ND (2009) UVR8 in Arabidopsis thaliana regulates multiple aspects of cellular differentiation during leaf development in response to ultraviolet B radiation. New Phytol **183:** 315-326.

Wei W, Ba ZQ, Gao M, Wu Y, Ma YT, Amiard S, White CI, Danielsen JMR, Yang YG, Qi YJ (2012) A role for small RNAs in DNA double-strand break repair. Cell **149:** 101-112.

White DWR (2006) PEAPOD regulates lamina size and curvature in Arabidopsis. Proc Natl Acad Sci U S A **103:** 13238-13243

Ye X, Franco A, Santos H, Nelson D, Kaufman P, Adams P (2003) Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. Mol Cell **11:** 341-351.

Yoshiyama K, Conklin PA, Huefner ND, Britt AB (2009) Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage. Proc Natl Acad Sci U S A **106:** 12843-12848.

Zenoni S, Reale L, Tornielli GB, Lanfaloni L, Porceddu A, Ferrarini A, Moretti C, Zamboni A, Speghini S, Ferranti F, Pezzotti M (2004) Downregulation of the Petunia hybrida alpha-expansin gene PhEXP1 reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. Plant Cell **16:** 295-308.