

## 学位論文（要約）

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

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## 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

List of gene name .....	7
List of abbreviations .....	8
<b>Chapter I: General introduction .....</b>	<b>9</b>
Leaf development	
Leaf size control by cell proliferation	
Leaf size control by cell expansion	
Compensation gives new insight into organ size control	
Compensation is heterogeneous event	
<b>Chapter II: The ATM-dependent DNA damage response acts as an upstream trigger for compensation in the <i>fas1</i> mutation during Arabidopsis leaf development.....</b>	<b>22</b>
<b>Introduction .....</b>	<b>23</b>
<b>Results .....</b>	<b>28</b>
<i>FUGU2</i> encodes FAS1, the large subunit of CAF-1	
The <i>fas1</i> mutation up-regulates the expression of DNA damage response genes in an ATM-dependent manner	
The <i>atm</i> mutation partially suppresses the compensation phenotype in <i>fas1</i>	
The <i>atm</i> mutation partially restores the cell cycle delay in <i>fas1</i>	
The <i>atm</i> mutation partially represses the high ploidy phenotype in <i>fas1</i>	
The <i>atm</i> and <i>atr</i> mutations partially restore the meristem defects in <i>fas1</i> roots	
<b>Discussion .....</b>	<b>36</b>
ATM-dependent up-regulation of DNA damage response genes in <i>fas1</i> leaves	
ATM-dependent DNA damage response leads to compensation in <i>fas1</i>	

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** ..... 41

**Figures and tables** ..... 43

**Chapter III: The DExH type helicase DNA DAMAGE INDUCIBLE1 functions in**

**DNA repair pathway** ..... 53

**Introduction** ..... 54

**Results** ..... 57

Isolation of *DDI1* gene

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

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

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

The *ddi1* mutant has defects in DNA repair

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

**Discussion** ..... 61

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

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

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

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

**Materials and methods** ..... 65

**Figures and tables** ..... 68

**Chapter IV: General discussion** ..... 76

Which factors are involved in compensation downstream of ATM

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

Relationship among *fas1* and other compensation mutants

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

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

Conclusion remarks

**Acknowledgements** ..... 82

**References** ..... 83

## 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 HOMEODOMAIN16
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-REGULATING FACTOR
HPY2	HIGH PLOIDY2
JAG	JAGGED
KLU	KLUH
KN	KNOLLE
KNOX	KNOTTED-LIKE HOMEODOMAIN
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-TRIPLE-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* antagonizes *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 *dal* 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 HOMEODOMAIN LEUCINE ZIPPER CLASS I (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 Fisser, 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 *CYCBI;1* is strongly induced by DNA damage (Culligan et al., 2006), thus the activated DNA damage response may also account for the accumulation of *CYCBI;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 *fasI* 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 *fasI* (Fig. 9).

### **ATM-dependent up-regulation of DNA damage response genes in *fasI* 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 *fasI* (Fig. 4, Fig. 3A, B). A previous study reported that the expression of genes involved in the DNA damage response is up-regulated in *fasI* (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 *fasI atm* and *fasI atr* demonstrated that only absence of ATM suppresses the up-regulation of several DNA repair genes in *fasI* leaves, suggesting that up-regulated expression of DNA repair genes in *fasI* 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 *fasI* 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 *fasI*, the observed phenomenon can be interpreted as follows. The level of decrease in cell proliferation activity in *fasI* 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 *fasI* leaves and that the cell number of *fasI 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 *fasI*. Given that *fasI* 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 *fasI*, *fasI 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 fork 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) phytigel at 22°C 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  $\mu$ l 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.

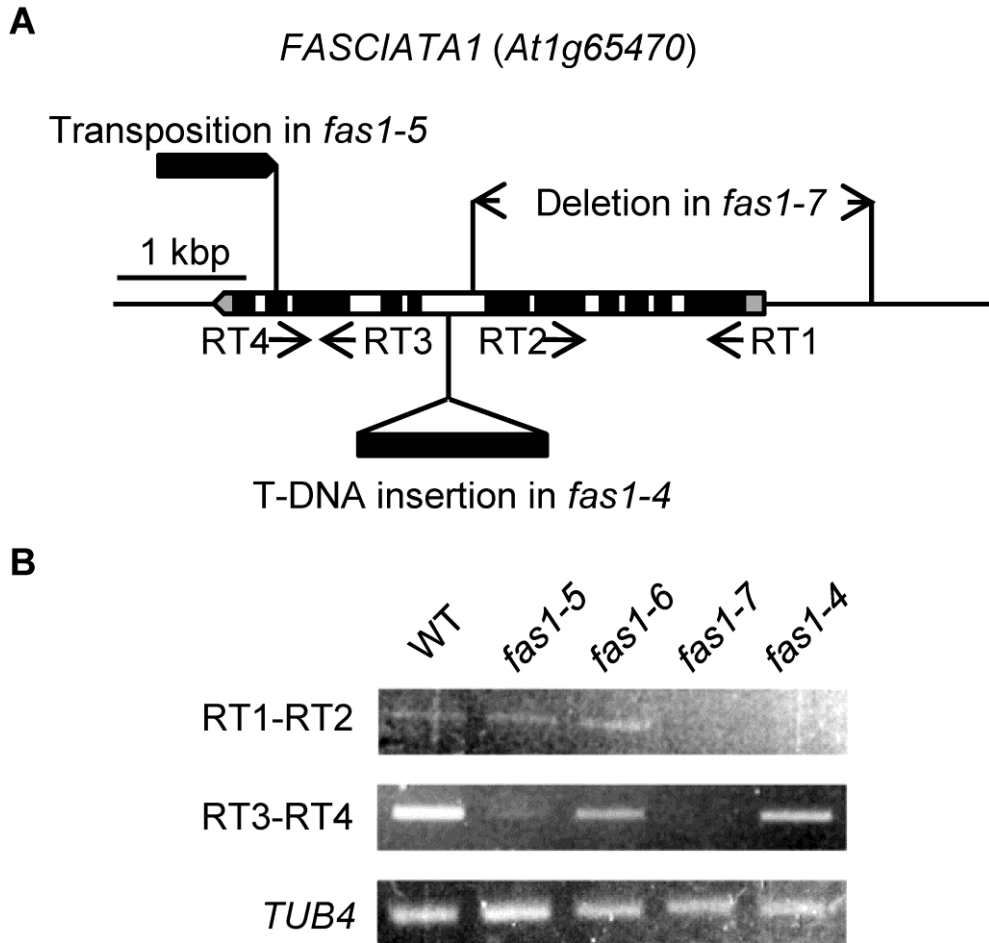
### **Microscopic analysis**

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<sub>2</sub>O) as previously described (Tsuge *et al.*, 1996). Whole leaves were observed using a stereoscopic microscope (MZ16a; Leica Microsystems, Tokyo, Japan) and individual 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  $\mu$ g/ mL propidium iodide and visualised using Leica TCS-SP5 confocal laser microscope.

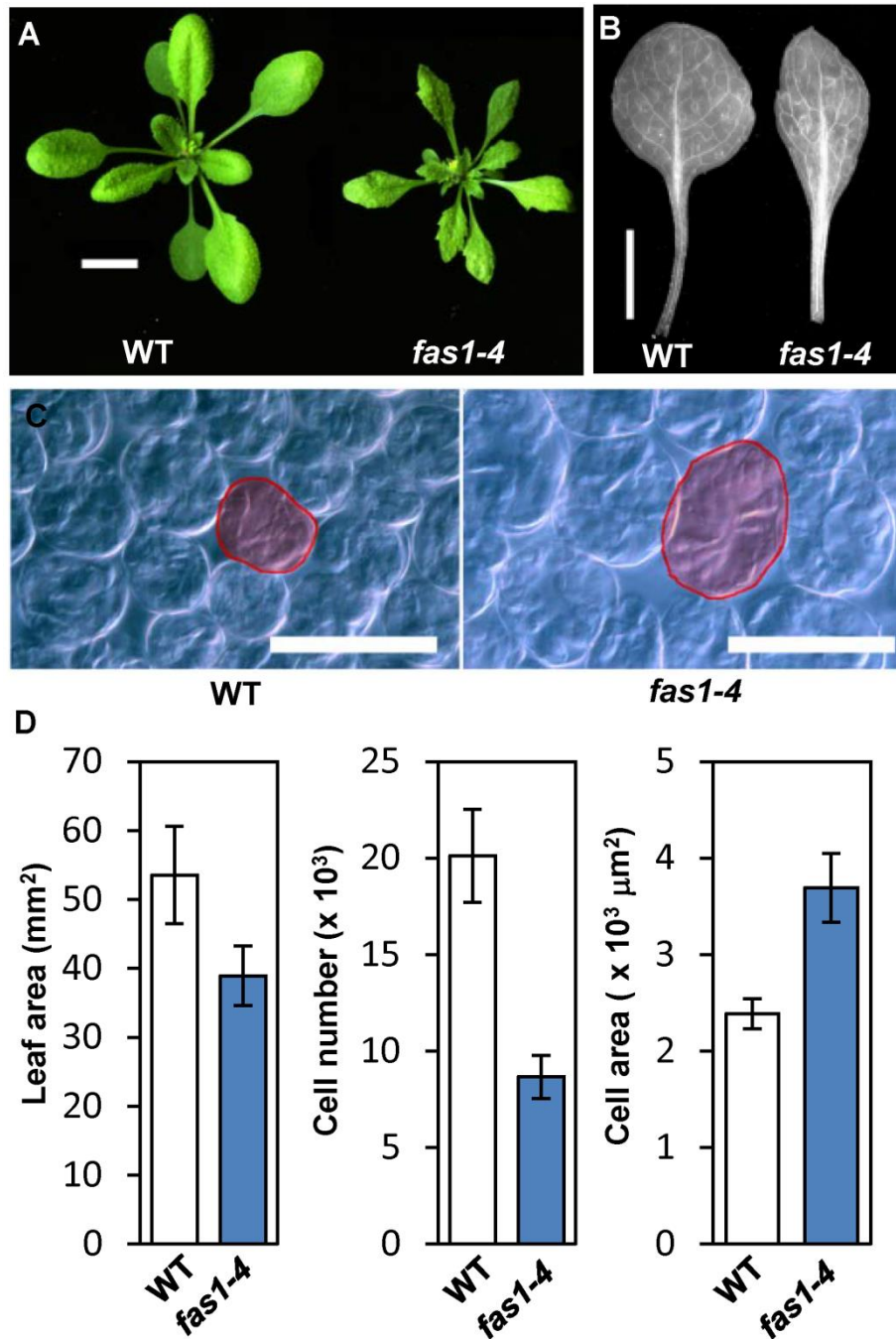
### **Ploidy measurements**

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  $EI = (0 \times \%2C) + (1 \times \%4C) + (2 \times \%8C) + (3 \times \%16C) + (4 \times \%32C)$  (Sterken *et al.*, 2012) and averaged over at least three technical replicates.

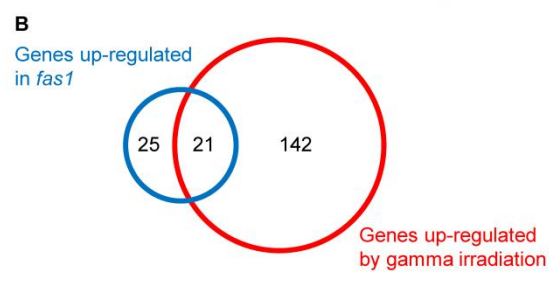
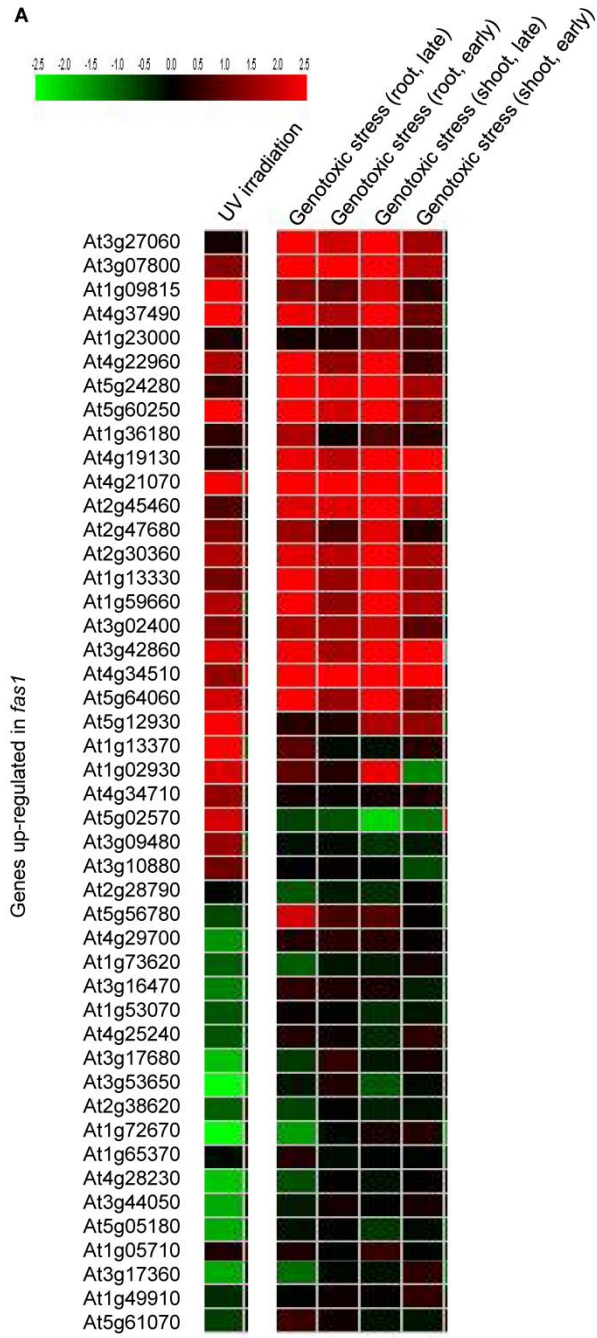
**Figures**



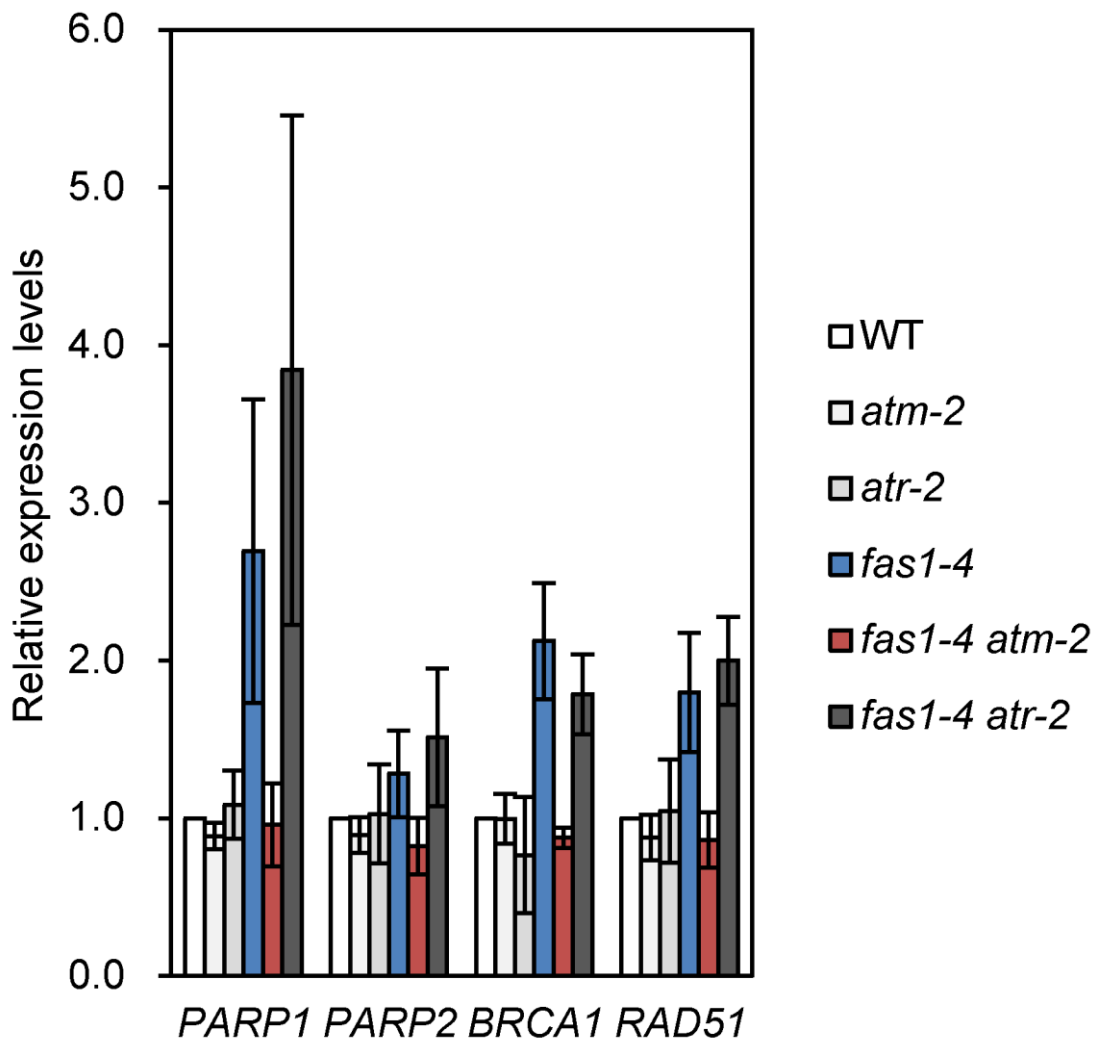
**Figure 1. Cloning of the *FUGU2* gene.** (A) Mutation points of each *fas1* allele are indicated. The exon, intron, untranslated region and intergenic region are indicated by a black box, white box, grey box and solid line, respectively. Arrows indicate the primers used for RT-PCR. (B) Semi-quantitative 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.



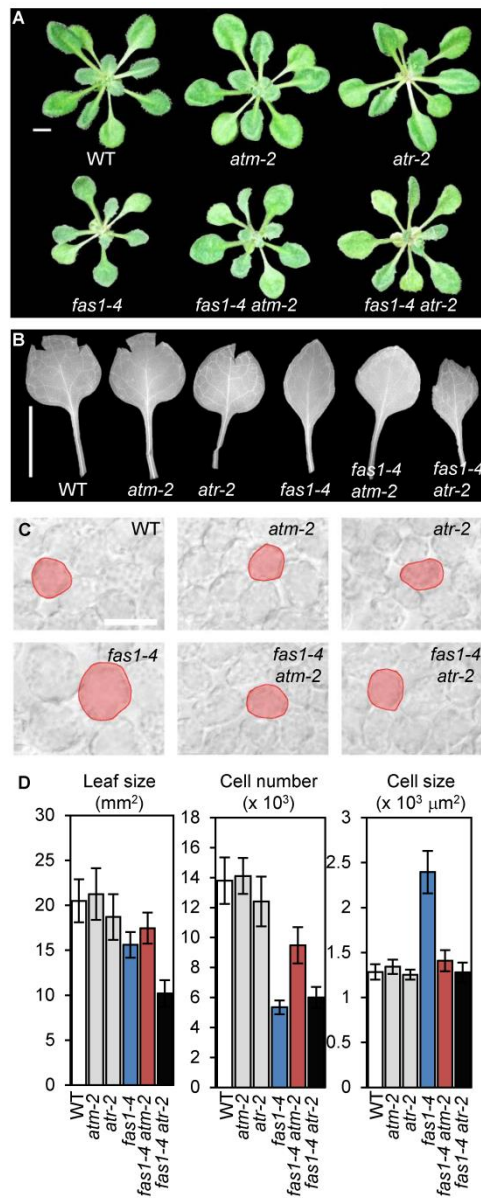
**Figure 2. Leaf phenotype of *fugu2/fas1* mutants.** (A) Whole rosette of WT (left) and *fas1-4* plants (right) at 25 days after sowing. Bar = 10 mm. (B) First leaves of WT (left) and *fas1-4* plants (right) at 25 days after sowing. Bar = 10 mm. (C) Palisade cells in the first leaf of WT (left) and *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  $\pm$  SD.



**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).

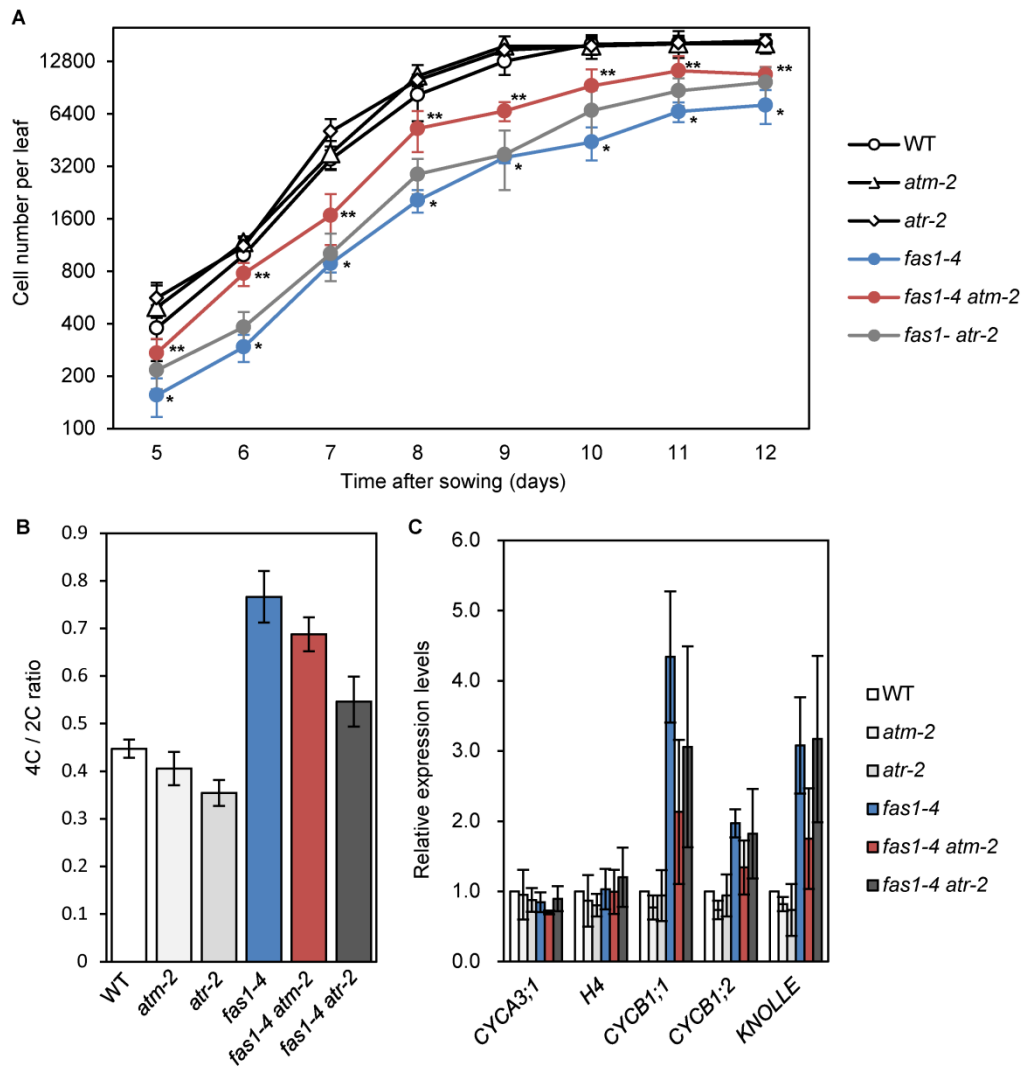


**Figure 4. Expression of DNA damage response genes in *fas1-4 atm-2* and *fas1-4 atr-2*.** RT-PCR analysis of DNA repair genes (*RAD51*, *BRCA1*, *PARP1* and *PARP2*) in WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. Total RNA prepared from the first leaf pair at 10 days after sowing 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.

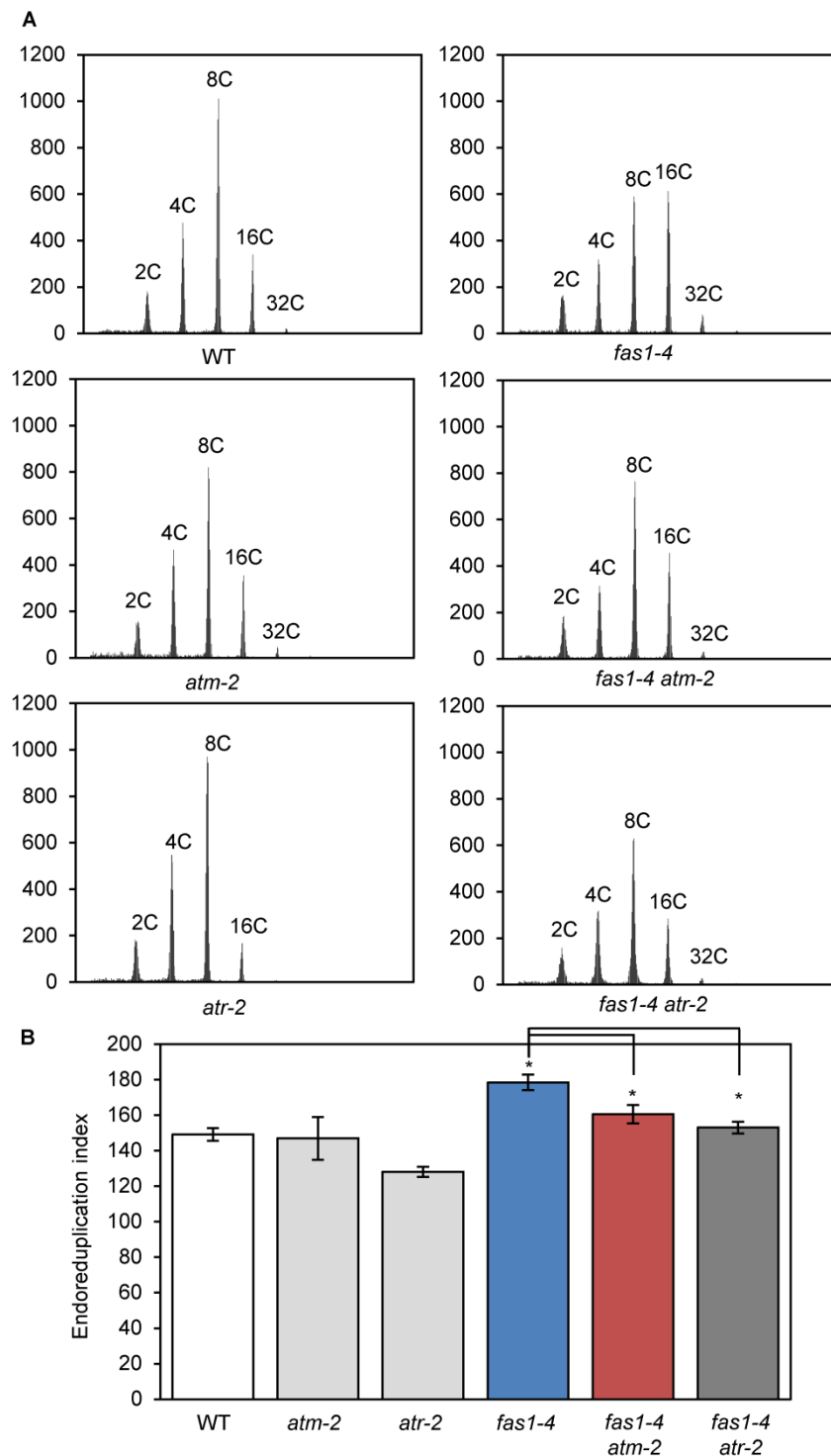


**Figure 5. Leaf size, cell number and cell size of *fas1-4 atm-2* and *fas1-4 atr-2*.** (A) Whole rosette of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* at 21 days after sowing. Bar = 10 mm. (B) First leaf of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2* at 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  $\mu\text{m}$ . (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 phytigel plates were harvested at 21 days after sowing and used for the analysis.  $n = 8$ , mean  $\pm$  SD

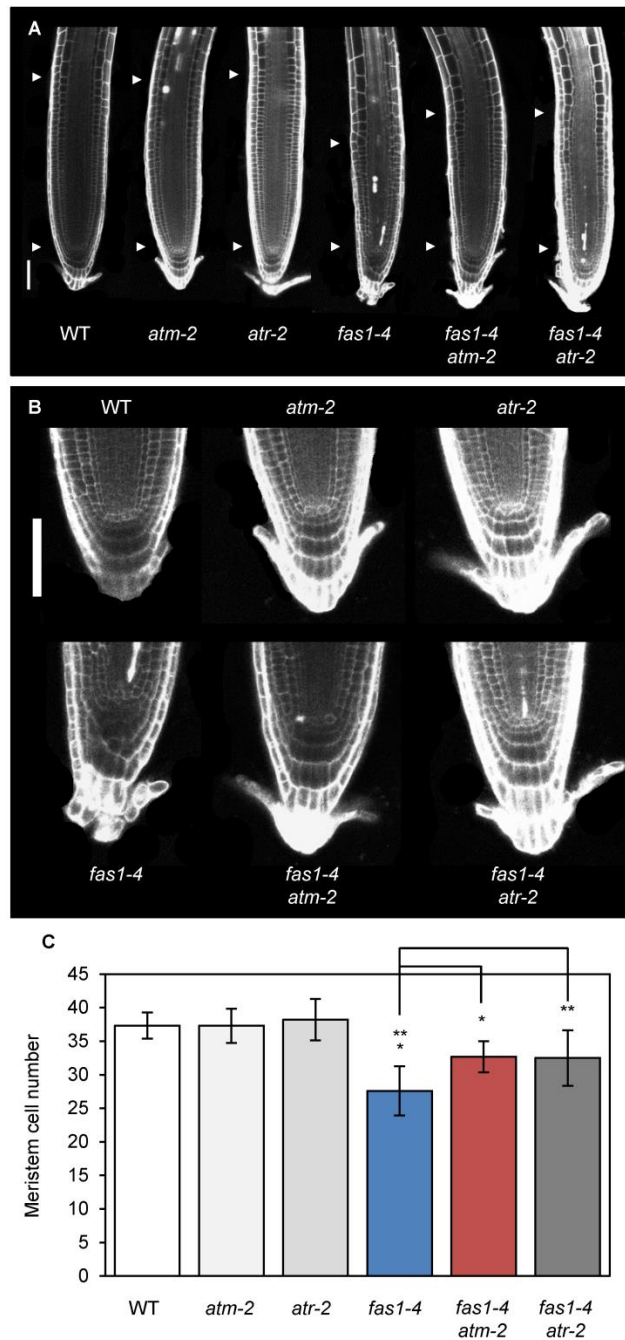




**Figure 6. ATM contributes to the cell cycle delay in *fas1-4*.** (A) Kinematic analysis of leaf cell number of WT, *atm-2*, *atr-2*, *fas1-4*, *fas1-4 atm-2* and *fas1-4 atr-2*. The average cell number per leaf was calculated as described in De Veylder *et al.* (2001).  $n = 8$ , mean  $\pm$  SD. \*:  $P < 0.01$  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  $\pm$  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  $\pm$  SD. \*:  $P < 0.01$  (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  $\mu$ m. 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  $\mu$ m. (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  $\pm$  SD. \* :  $P < 0.01$ , \*\* :  $P < 0.05$  (Student's *t*-test)

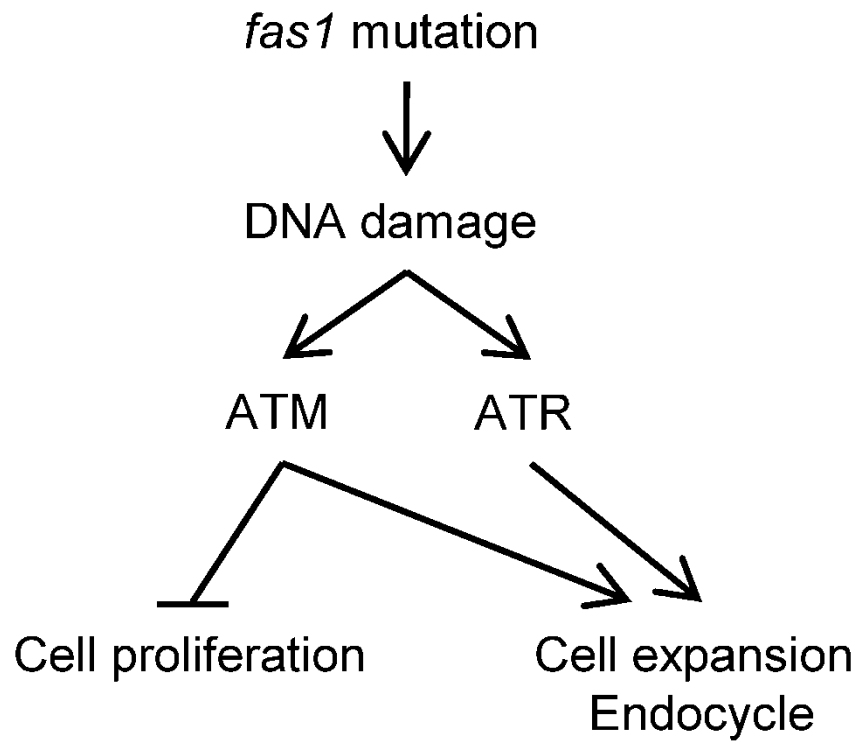


Figure 9. A scheme diagram that explains how compensation is induced in *fas1* leaves.

### 第3章

本章については、5年以内に雑誌等で刊行予定のため、  
非公開。

## 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 *fasI*, 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 *fasI*, and genetic analysis between *fasI* 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 *fasI* 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 *fasI* 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 *fasI*. Therefore, extent of the DNA damage response varies among each cell, leading to different extent of cell cycle arrest. If compensated cell expansion in *fasI* is a result of early transition from mitotic cycle to endocycle, compensation in *fasI* 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 *fasI* 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 suppress 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.

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