Genetic Analyses of Mechanisms of Compensation in Leaf Organogenesis

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Chapter 1

General Introduction

1.1 Leaf size determination

Regulation of organ size in multicellular organisms is pivotal for expressing its function appropriately. Mature leaf size corresponds to the final number of cells multiplied by the average size of cells. In some cases, the processes of cell proliferation and cell expansion do not progress independently, suggesting some interactions in leaf development. For example, γ-irradiated wheat grains exhibit no cell division after germination, but develop leaves by significantly increasing the volume of cells (Haber, 1962). Likewise, the overexpression of cyclin-dependent kinase (CDK) inhibitors or dominant-negative versions of CDKs in *Oryza sativa*, *Nicotiana tabacum*, and *Arabidopsis thaliana* causes the development of leaves that contain significantly fewer but larger cells than those of the corresponding wild-type plants (Hemerly et al., 1995; Wang et al., 2000; De Veylder et al., 2001; Boudolf et al., 2004; Barroco et al., 2006), suggesting that defect(s) in cell cycling enhances cell expansion. Furthermore, a number of Arabidopsis mutants showing such phenotypes have been reported, including *angustifolia3* (*an3*; Horiguchi et al., 2005), *aintegumenta* (*ant*; Mizukami & Fischer, 2000), *struwwelpeter* (*swp*; Autran et al., 2002), *G protein alpha subunit 1* (*gpa1;* Ullah et al., 2001), *pointed first leaf 2* (*pfl2;* Ito et al., 2000), and other mutants (Clay and Nelson, 2005; Yu et al., 2005; Exner et al., 2006; Horváth et al., 2006). These studies have suggested that the magnitude of cell expansion is affected by cell proliferation activity during leaf development in general. Tsukaya (2002, 2003, 2005, and 2006) has proposed to call this phenomenon “compensation,” and considered it important in understanding

how cell proliferation and cell expansion are coordinated during leaf development. A similar idea has been proposed by Beemster et al. (2003). However, our knowledge about compensation is superficial, and the molecular mechanisms underlying compensation are unclear. The aim of this study was to elucidate the genetic framework mediating the compensation. To this end, I focused on *an3*, also known as *grf-interacting factor1* (*gif1*; Kim and Kende, 2004), as a model case of compensation, since *an3* is one of the best characterized compensation-exhibiting mutants (Horiguchi et al., 2005). *an3* contains 70% fewer palisade cells that are 50% larger than those of wild-type plants (Horiguchi et al., 2005). *AN3* encodes a homolog of the human transcription coactivator, synovial sarcoma translocation protein (SYT) and is expressed in the basal part of the leaf primordium (Horiguchi et al., 2005), where cell proliferation takes place (Donnelly et al., 1999). During leaf development, cell proliferation occurs in the basal part of leaf primordia and cell expansion occurs in the upper part of the leaves (Donnelly et al., 1999). In leaf primordia, these two processes can be roughly distinguished by the boundary called the "arrest front" (Nath et al., 2003). Thus, when compensation takes place, the decrease in cell number and the increase in cell size should occur in the spatially distinct portions of the developing leaf primordium. Owing to this feature, compensation can be separable into two different processes: the induction process, which involves the reduction of cell proliferation, and the response process, during which cell expansion is enhanced. The originality of my Ph.D. thesis is that I investigated the induction and response of compensation separately based on the developmental genetics.

To address how cell enlargement is *induced* and how cell expansion *responds* to the inductive condition during compensation, mutants having a specific defect in each process were considered to be useful. Generally, mutants having a defect in cell proliferation/cell expansion result in a dwarf phenotype. Unfortunately, in many cases of dwarf mutants, histological analysis was insufficient for the precise comparison of cell number and cell size in the mutant and wild-type leaves; thus, what extents the defects in cell proliferation and cell expansion in these mutant contribute to changes in leaf size is unclear. Even in well-characterized dwarf mutants, the cellular basis that causes dwarfism is incorrectly described. For example, *constitutive triple response1* (*ctr1*) and *auxin resistant1* (*axr1*) have been considered to be a cell-expansion and a cell-proliferation defective mutant, respectively (Lincoln et al., 1990; Kieber et al., 1993; Solano et al., 1998). However, when I re-examined these mutants histologically, both mutants have defects in the cell number and cell size (Table 1). These discrepancies might be attributable to the experimental systems used. In many cases, the number and size of cells were measured using epidermal pavement cells in order to find a defect(s) that accounts for the change in leaf size in a particular mutant. However, the pavement cells significantly vary in size in Arabidopsis. Such variability might lead to incorrect interpretation of the mutant phenotype. Thus, it is necessary to newly identify the mutants which have a specific defect in cell number or cell size using a more reliable method. To this end, in this study, I focused on palisade cells in the subepidermal layer to determine the number and size of leaf cells since

palisade cells are relatively uniform in size and shape compared to that of epidermal cells.

For the reasons mentioned above, I participated in a large-scale screening of leaf-size mutants. As a result, I found that mutants isolated in this screening could be classified into several groups according to their cell number and size (Fig. 1; reported by Horiguchi et al., 2006a). Among these groups, I successfully identified specific classes of mutants defective either in cell proliferation or cell expansion in leaves (Fig. 1). Some mutants have a specific decrease in cell number and have relatively normal cell size in leaves compared to the wild type (shown in Fig. 1, highlighted in blue). These mutants should serve as a powerful tool to analyze in the induction process of compensation. On the other hand, I found another mutant class characterized by a specific defect in cell size and a relatively normal number of cells in the leaves (Fig. 1, highlighted in red). This category of mutant was considered suitable for an analysis of the response process of compensation. Taken together, these mutants are ideal genetic tools to analyze the *induction* and *response* processes of compensation. Using these resources, I analyzed possible mechanisms of compensation.

My Ph.D. thesis consists of five chapters including this chapter (General Introduction, Chapter 1), Materials & Methods (Chapter 2), Results from analyses of the “induction” process (Chapter 3) and “response” process (Chapter 4), and General Discussion (Chapter 5). In the following section, I briefly summarize the contents of Chapters 3 and 4.

Table 1 Characterization of leaf organization in hormonal mutants

		Mesophyll cells		Pavement cells		
Genotype	Leaf area (mm^2)	Area (μm^2)	Number	Area (μm^2)	Number	Stomatal index
Wild-type	48.0 ± 3.6 (100)	4186 ± 444 (100)	11510 ± 1194 (100)	3603 ± 208 (100)	22762 (100)	17.7 ± 0.7
aba2-1	20.6 ± 2.7* (43)	3093 ± 300* (74)	6524 ± 919* (57)	2285 ± 170* (63)	16603 (73)	19.4 ± 0.4*
aba3-1	33.4 ± 3.8* (70)	3388 ± 393* (81)	9450 ± 1149* (82)	3096 ± 309* (86)	19737 (87)	19.1 ± 0.4*
axr1-3	28.8 ± 2.4*(60)	3116 ± 454* (75)	9636 ± 884* (84)	2666 ± 376* (74)	18368 (81)	17.7 ± 0.6
axr1-12	22.5 ± 2.7* (47)	2540 ± 369* (61)	9343 ± 1229* (81)	2033 ± 402* (56)	17965 (79)	16.9 ± 0.4*
arf2-1	69.7 ± 5.0* (145)	4595 ± 666 (110)	14672 ± 1715* (128)	4541 ± 247* (126)	28026 (123)	18.9 ± 0.4*
arf7-1	40.8 ± 1.8* (85)	3390 ± 389* (81)	12522 ± 1145 (109)	3444 ± 292 (96)	19996 (88)	17.5 ± 0.6
arf7-1 arf19-1	21.4 ± 3.4* (45)	1824 ± 100* (44)	12054 ± 2298 (105)	1752 ± 162.3* (49)	19955 (88)	17.5 ± 0.6
ctr1-12	5.5 ± 0.9* (12)	2085 ± 238* (50)	2975 ± 286* (26)	1631 ± 173* (45)	6037 (27)	18.9 ± 1.1*
ein3-1	57.5 ± 5.5* (120)	4432 ± 421 (106)	12993 ± 1471* (113)	3847 ± 230 (107)	25051 (110)	17.3 ± 0.5
eto1-1	25.0 ± 4.0* (52)	2856 ± 465* (68)	8494 ± 1410* (74)	2686 ± 323* (75)	12760 (56)	17.4 ± 0.9
etr1-1	65.1 ± 6.7* (136)	5067 ± 612* (121)	12828 ± 1304* (112)	4885 ± 722* (134)	21597 (94.9)	16.5 ± 0.7*
jar1-1	36.6 ± 5.6* (76)	3545 ± 310* (85)	10111 ± 1088* (88)	2865 ± 312* (80)	22422 (99)	18.3 ± 0.7

Characterization of leaf organization in hormonal mutants.
Leaves from 25-day-old plants were used. Data are means ± SD (n = 8 for each line); relative values compared to the wild type are shown in parentheses. For each leaf, the average cell area was determined by measuring 20 palisade cells or all epidermal cells within a 0.4-μm^2 area. The total number of palisade cells was determined by dividing the leaf area by the palisade cell density for each leaf. The total number of epidermal cells was estimated by dividing the mean leaf area by the mean epidermal cell size (pavement plus guard cells). The stomatal index (SI) was determined by the following formula: SI = [S/(E + S)] 100 where S is the number of stomata per unit area and E is the number of epidermal cells per unit area (Mishra, 1997). Asterisks indicate significant differences from the wild type (Student's *t*-test, $P < 0.05$). (Reproduced from Horiguchi, Fujikura, Ferjani, Ishikawa, Tsukaya, 2006b)

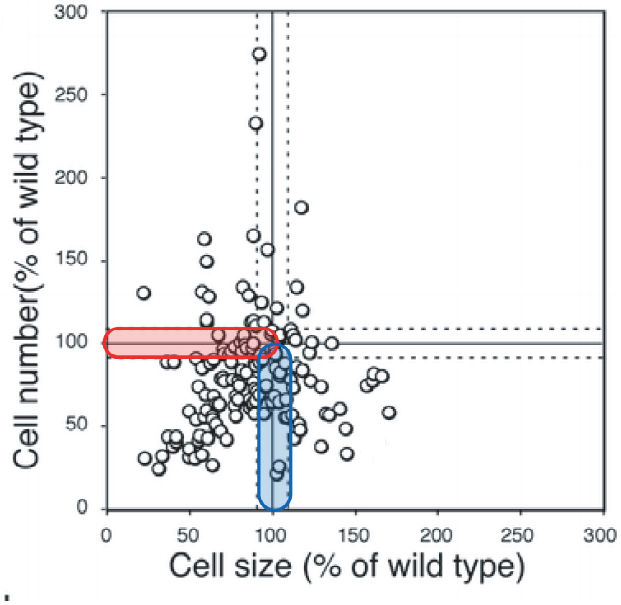

Figure 1
Characterization of mutants with altered leaf size: Changes in cell number and size in individual mutant lines.
Each mutant line and wild-type plants (Col-0) were grown for 25 days at 22ºC under 16 h light/8 h dark conditions. Total palisade cell number in the subepidermal layer and the area of palisade cells observed from a paradermal viewpoint were determined using at least six first leaves. Values are expressed relative to the corresponding wild-type values. Dotted vertical and horizontal lines indicate ±10% levels. (Reproduced from Horiguchi et al., 2006a)

1.2 Brief summary of this study

In Chapter 3, I discuss the important hallmarks of the "induction" process. As mentioned above, compensation is closely linked to cell proliferation levels, suggesting that the induction of aberrant cell enlargement during compensation is triggered by a decrease in cell number. However, impaired cell proliferation does not always induce compensation (e.g., Narita et al., 2004). To clarify the relationship between the induction of compensation and the state of cell proliferation, in Chapter 3, I isolated several mutants with a decreased leaf cell number that do not show compensation and named these mutants "*oligocellula* (*oli*)". I then conducted genetic analyses by using *oli* mutants and *an3* and discovered a unique rule governing the relationship between cell number and cell size. Based on several findings from these analyses, I discuss the possible mechanisms of the induction of compensation.

In Chapter 4, I discuss the mechanisms of the *response* process of compensation. I attempted to genetically dissect the response process of compensation and therefore isolated several mutants that have a specific defect in cell expansion and have a normal number of leaf cells compared to the wild type. I named these mutants "*extra-small sisters* (*xs*)". If aberrant cell expansion observed in *an3* is the result of hyper-activation of a subset of cell expansion systems that functions in normal leaf development, these *xs* mutations should suppress the aberrant cell enlargement observed in *an3*. Genetic analyses using *xs* mutants and *an3* revealed that some *xs* mutations completely suppress abnormal cell enlargement observed in *an3*. Based on the results

obtained in this chapter, I discuss the genetic framework(s) of the response process of compensation.

In Chapter 5, based on the data obtained through this study, I overview the processes of compensation from the viewpoint of coordination between cell proliferation and cell expansion in organogenesis, and also discuss differences in size regulation strategies in different organisms by considering leaves in Arabidopsis and wings in Drosophila, as examples. Finally, based on the data obtained in this study, I discuss future issues required to reveal the mechanism(s) for multicellularity.

Chapter 2

Materials and Methods

2.1 Plant Materials

The Columbia-0 (Col-0) accession was used as the wild-type genetic background of *Arabidopsis thaliana* (L.) Heynh. in this study. A null allele of *an3-3* (Horiguchi et al., 2005) was used as a representative mutant showing compensation. The *oligocellula* (*oli*) and *extra-small sisters* (*xs*) mutants were obtained from a collection of mutants that were identified from the M2 progeny of a fast-neutron-mutagenized population as small-leaf mutants in the Col-0 background (Horiguchi et al., 2006a, b) and were backcrossed three times with Col-0. To characterize these *oli* and *xs* mutants, I confirmed their phenotypes at least three times. Various combinations of double mutants were produced from *an3*, *oli*, and *xs*. Several putative double mutants were identified in the F2 generation based on their phenotypes. When double mutant phenotypes were obscure, individuals showing the phenotype of either single mutant were selected from the F2 generation, and in the subsequent generation, individuals showing a new phenotype, which segregated in the expected ratio, were selected as putative double-mutant strains. The putative double mutants were crossed again with one of their parental single mutants and the resultant F1 plants were tested to determine whether they showed the phenotype of the parental single mutant.

2.2 Growth conditions

The seeds were sown on rockwool (Nitto Boseki Co., Ltd. Tokyo. Japan) moistened with 0.1% (w/v) of HYPONeX™ solution (HYPONeX JAPAN Co., Ltd. Osaka. Japan) and grown for 3 weeks under a 16-h-light/8-h-dark fluorescent illumination cycle (ca. 48 mmol m^{-2} s^{-1}) at 22ºC as described previously (Horiguchi et al., 2005). The first set of leaves was used for microscopic observation. To determine root and hypocotyl lengths, surface-sterilized seeds were sown on plates containing Murashige and Skoog medium supplemented with 1% (w/v) sucrose and Gamborg's B5 vitamins (Gamborg et al., 1968; Murashige and Skoog, 1962) solidified with 0.4% (w/v) Gellan gum. The seeds were then incubated at 4ºC in darkness for 3 days. After this cold treatment, the seedlings were grown on vertically placed plates to measure root growth for 8 days under continuous fluorescent illumination (*ca.* 26 mmol m^{-2} s^{-1}) at 22ºC. For hypocotyl measurement, the surface-sterilized seeds were incubated on plates for 2 hours after the cold treatment with fluorescent illumination (*ca.* 26 mmol m^{-2} s^{-1}) and then transferred in darkness and kept for 8 days before the measurements.

2.3 Measurements of leaves and cells

Whole leaves and leaf cells were observed under a stereoscopic microscope (MZ16a) and a Nomarski differential interference contrast microscope

(DMRX/E; both from Leica Microsystems, Tokyo, Japan), respectively. Leaves, roots, and hypocotyls were fixed in a formalin-acetic acid-alcohol (FAA) solution at 4ºC for 12 hours, cleared with a chloral hydrate solution containing 200 g chloral hydrate, 20 g glycerol, and 50 ml H_2O (Tsuge et al., 1996), and observed under the microscopes. The samples were then photographed under the microscopes, and the cell size and leaf size were determined using Image J software (http://rsb.info.nih.gov/ij/, National Institutes of Health, Bethesda, MD, USA). For each leaf, more than 20 palisade cells in the subepidermal layer in the center of the leaf blade between the midvein and the leaf margin were analyzed. To measure the epidermal cell size, I created a replicate image of the adaxial epidermis using the dried-gel method (Horiguchi et al., 2006b) and determined the pavement cell size using Image J.

2.4 Measurements of ploidy levels

To measure the nuclear DNA content, the first set of leaves from 21-day-old seedlings grown on rockwool were used. Leaves were minced with a razor blade in 500 µl of extraction buffer containing 2 mM magnesium chloride, 10 mM Tris(hydroxymethyl)aminomethane, 1% (v/w) ß-mercaptoethanol, 40 mg ml^{-1} polyvinylpyrrolidone, 0.1 mg ml^{-1} ribonuclease A, and 0.1% (v/w) Triton X-100 at pH 8.0 (Galbraith et al., 1983). The suspension was filtered through a 20-µm nylon filter and incubated at 37ºC for 10 min. Then 50 µl of propidium iodide solution [1 mg ml^{-1} propidium iodide (Sigma, MO, USA) and 12.5 mg ml^{-1}

of 1,4-diazabicyclo (2.2.2) octane (Sigma, MO, USA) in phosphate-buffered saline (pH 7.4)] were added into the suspension containing nuclei, and the mixture was incubated at 4ºC for 1 h. At least 5000 nuclei per sample were analyzed using flow cytometry EPICS XL (Beckman Coulter Inc., CA, USA).

2.5 Genetic mapping

The *XS* loci were genetically mapped using various genetic markers (simple sequence length polymorphism, cleaved amplified polymorphisms, and small insertion/deletions) according to the sequence information available at The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/index.jsp). Mapping lines were produced by crossing between *xs* mutants and L*er*.

Chapter 3

Induction Process of Compensation

3.1 Summary

Although compensation is generally induced following the reduction of cell number in leaves, a reduction in leaf cell number does not always induce compensation, as illustrated by the presence of mutants showing a specific reduction in cell number. To address this discrepancy, I isolated six lines of mutants named *oligocellula* (*oli*), which had a slight but apparent decrease in cell number but did not show compensation. I then produced double mutants among the *oli* mutants. As a result, the leaf cell number in *oli* double mutants was remarkably decreased compared to each parental single *oli* mutant, and the leaf cell size was obviously increased. This result indicated the possibility that the induction of compensation depends on a decrease in cell number in a threshold-dependent manner. To address whether further decreases in cell number affect cell size when compensation is induced, I produced double mutants between *angustifollia3* (*an3*), a typical compensation-exhibiting mutant, and the *oli* mutants. As a result, most of the double mutants between *oli* and *an3* had much fewer but larger leaf cells than *an3*. Exceptionally, the *oli1 an3* double mutant had a similar number and size of leaf cells compared to *an3*. These results indicated that a further decrease in cell number can induce additional cell enlargement when cell number falls below a threshold level. It also revealed that the *OLI1* and *AN3* genes function in cell proliferation in the same pathway.

3.2 Introduction

In this chapter, I analyzed mechanism(s) of the “induction” process of compensation. Considering the phenotypic time series of compensation, the primary cue appears to be the defect in cell proliferation, and the abnormal cell enlargement is a secondary phenotype. This idea is also supported by the fact that primary defect of compensation-exhibiting mutants is cell proliferation, since the responsible genes of these mutants are expressed mainly in meristematic tissue including leaf primordia and/or play a role in cell proliferation (e.g. Horiguchi et al., 2005; Autran et al., 2002; De Veylder et al., 2001). Thus, it is suggested that the induction of compensation is closely linked to cell proliferation levels. However, not all few-cell mutants exhibit compensation as represented by *rot4-1D* (Narita et al., 2004). Many other mutants have a decreased number of leaf cells with smaller sizes than the wild type (e.g., the *curly leaf* mutant reported by Kim et al. 1998). Thus, I thought that the induction of compensation requires not only a decrease in cell number but also some special conditions. Prior to this study, however, nothing was known about the conditions required for the induction of compensation. To clarify this issue, I thought that mutants with specific defects in cell proliferation at various levels are necessary to identify the condition(s) required to induce compensation. However, after detailed observations of several known mutants, I found that no such mutants existed except for the above-mentioned *rot4-1D*. Therefore, I isolated and used several mutants specifically impaired in cell proliferation at several levels with normal cell size in leaves, and named them

oligocellula (*oli*). I then applied *oli* mutants as genetic tools to control cell number for elucidation of the cue(s) that is required for induction of compensation.

3.3 Results

3.3.1 Identification of *oligocellula* mutants

To clarify the mechanisms of the induction process of compensation, it is necessary to identify the mutants that have a specific defect in cell proliferation. However, as mentioned above, such mutants were found to be lacking. While there are several mutants that have a defect both in cell proliferation and cell expansion, these mutants are inconvenient for genetic analyses of the induction process of compensation since compensation consists of these two events, i.e., impaired cell proliferation and increased cell expansion. Fortunately, in the laboratory in which I conducted this Ph.D. study, vast collections of mutants with altered leaf size or shape were available, and the characterization of these mutants in terms of the leaf cell number and cell size had been carried out previously (Horiguchi et al., 2006a,b; see Chapter I). Through the establishment of mutant collections, I first isolated several lines of mutants that were impaired in cell proliferation and had relatively normal cell sizes in leaves from 147 mutant lines, as described in Chapter I. To confirm their phenotypes, these mutant leaves were subjected to histological analysis several times; finally, I selected six mutant lines that were regarded as cell-proliferation-specific

mutants. I named these mutants *oligocellula* (*oli*) and used these *oli* mutants to analyze the induction process of compensation (Fig. 1).

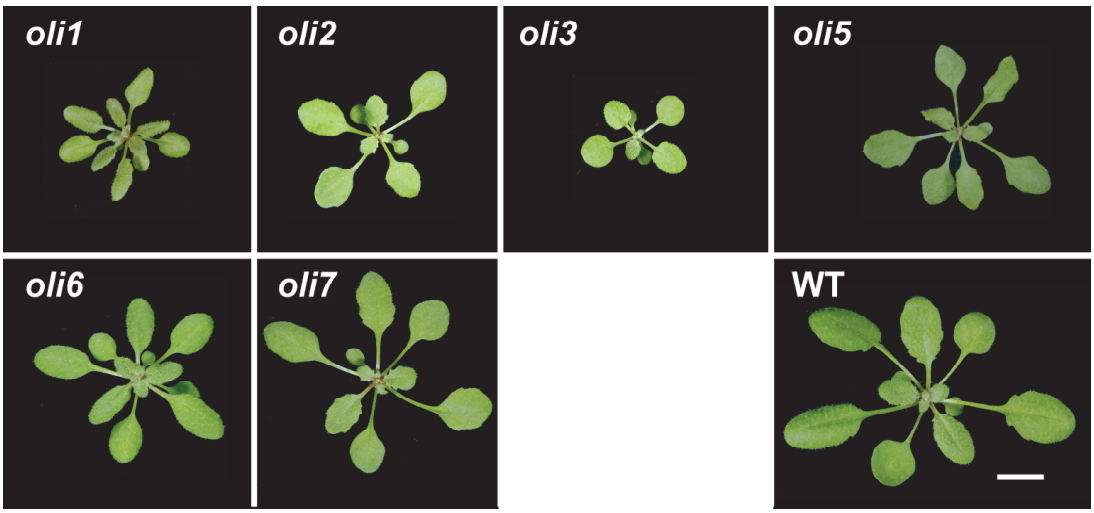

Figure 1
Rosette phenotypes of *oli* mutants.
Plants grown for 3 weeks with 16-h-light/8-h-dark fluorescent illumination cycle at 22ºC are shown. Bar = 10 mm.

3.3.2 Characterization of *oligocellula* mutants

Based on the segregation patterns of backcrossed F2 populations and allelism tests among *oli* mutants, all *oli* mutations were found to be non-allelic, single recessive mutations (data not shown). Figure 1 shows the rosette phenotype of the *oli* mutant plants. *oli1* and *oli6* have small-rosette phenotypes with proportional size changes. *oli2*, *oli5*, and *oli7* share a similar feature in leaf shape, including pointed and light green leaves. *oli3* has normal-shaped leaves except for football-shaped cotyledons. Histologically, the number of palisade cells observed from a paradermal view ranged from 56.6 ± 6.3% (*oli3*) to 78.9 ± 1.6% (*oli2*) of the wild-type values (Fig. 2C). In contrast, the size of palisade cells was not significantly different from that of the wild type (Fig. 2B). Leaf size was not always significantly smaller than that of the wild type (Fig. 2A). In *oli2*, *oli5*, and *oli7*, the density of palisade cells in leaves ranged from 51.7% (*oli7*) to 70.2% (*oli2*) of the wild-type value. These results indicate that the small-rosette phenotype observed in *oli* mutants was due to a reduction in cell number in leaves and that the *OLI* genes play a specific or preferential role in cell proliferation processes rather than cell expansion, at least in palisade tissues.

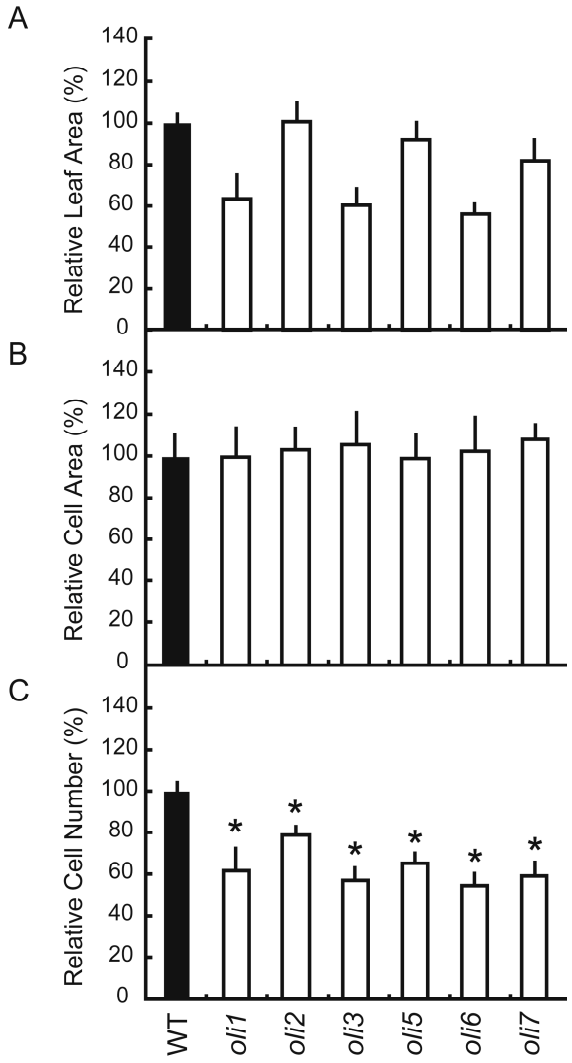

Figure 2

Leaf sizes and the sizes and numbers of palisade tissue cells of *oli* mutants.

First leaves from 3-week-old plants were observed by microscopic analysis. (A) Leaf sizes of the *oli* mutants and the wild type (n = 8). (B) Sizes of palisade cells observed from a paradermal view (n ≥ 240 using eight leaves). (C) Total number of palisade cells in the subepidermal layer of mature first leaves (n ≥ 8). The results are expressed relative to wild-type values ± s.d. Asterisks indicate significant differences from the wild type (Student's *t*-test, $P < 0.05$).

I also examined the root cell length in *oli* mutants to determine whether *oli* mutations cause defects in other organs (Fig. 3). The root length of *oli2*, *oli5*, and *oli6* was shorter than that of wild-type plants by 78.6 ± 0.6%, 64.7 ± 1.7%, and 86.0 ± 1.6%, respectively. In contrast, *oli1*, *oli3*, and *oli7* had relatively normal root lengths compared to the wild type. These results suggest that the *OLI2, OLI5*, and *OLI6* genes play a role in cell proliferation both in leaf and root development and that *OLI1*, *OLI3*, and *Oli7* have a function in cell proliferation, mainly in the leaf primordia.

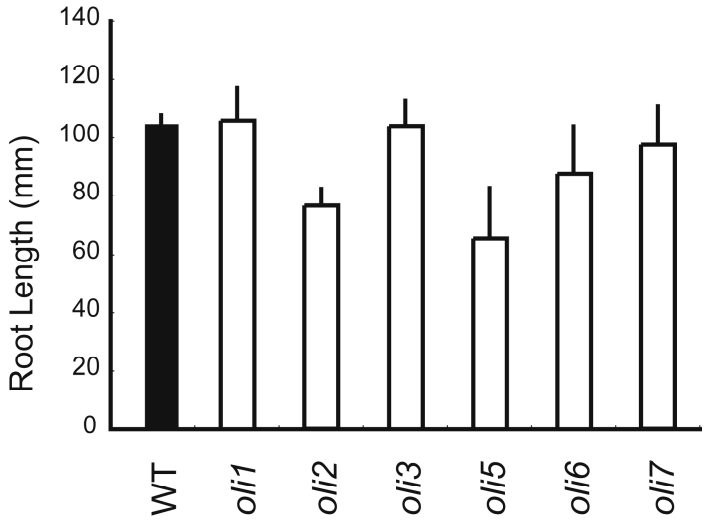

Figure 3
Root length of *oli* mutants.
Wild-type plants and *oli* mutant seedlings were collected 8 days after sowing, and the lengths of the primary roots were measured (n = 10). The results are mean ± s.d. expressed relative to wild-type values.

3.3.3 Threshold model: the induction of compensation requires a reduction in cell number below a threshold

As mentioned above, both *oli* and *an3* have a decreased cell number in the leaves; however, *an3* exhibits compensation while *oli* mutants do not. *an3* and other compensation-exhibiting mutants show a significant decrease in cell number (*an3* has about 30% and the *KRP2* over-expressor has about 10% of cells; Horiguchi et al., 2005; De Veylder et al., 2001) whereas *oli* mutants only have a slight decrease in cell number, ranging from 56.6 ± 6.3% (*oli3*) to 78.9 ± 1.6% (*oli2*). Thus, it is plausible that the level of decrease in cell number may be a critical cue to induce compensation. In other words, I postulated that the induction of compensation may require a substantial reduction in cell number below a certain threshold level. If this is the case, the further decrease in cell number by producing double mutants among *oli* mutants below such a threshold level could exhibit compensation (Fig. 4). Thus, I produced double mutants among several different *oli* mutants to decrease the cell number to various levels to examine the above idea (Fig. 5).

As a result, the number of palisade cells in all *oli* double mutants produced in this study was clearly decreased compared to that of each parental single mutant (Fig. 5), suggesting that these *OLI* genes may have a function in cell proliferation in different pathways during leaf organogenesis. In addition, as expected, the sizes of palisade cells in *oli* double mutants were increased compared to those of each parent, supporting the idea of threshold-dependent triggering of compensation (Fig. 5). For example, the number of palisade cells

in *oli1* and *oli3* single mutants and *oli1 oli3* double mutants were 57.2%, 56.7%, and 36.0%, respectively, of wild-type values, and the leaf cell size in the *oli1 oli3* double mutants was 144.9% of wild-type values whereas both *oli1* and *oli3* single mutants had normal cell sizes (Fig. 5C and 5D). Results of combinations between *oli1* and *oli2, oli1* and *oli6,* and *oli1* and *oli7* were similar, supporting the threshold hypothesis. Hence, I concluded that the induction of compensation requires a decrease in cell number below certain threshold levels.

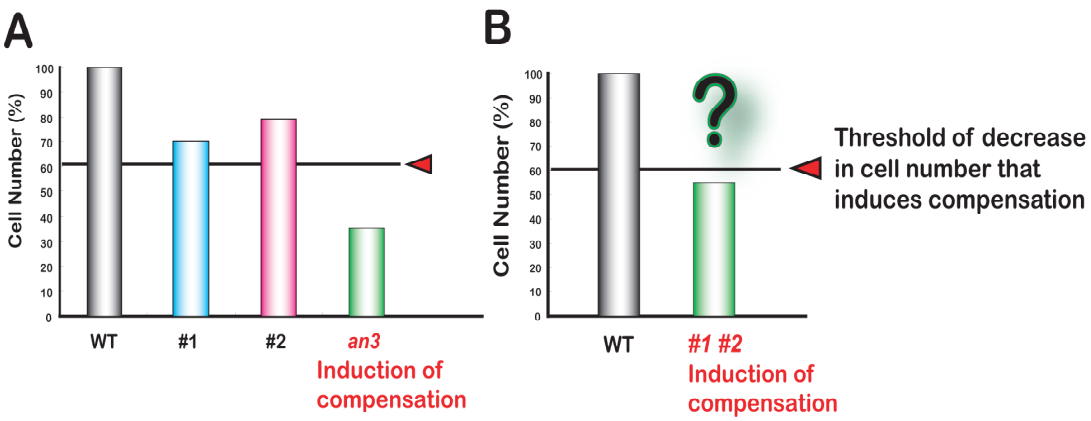

Figure 4
Working hypothesis: Threshold Model.
Compensation may be induced only when cell number is reduced below a threshold level.
(A) Decrease in cell numbers in mutants #1 and #2 may be insufficient for induction of compensation.
(B) Decrease in cell number in double mutants between #1 and #2 may be sufficient to reach the threshold and to exhibit compensation.

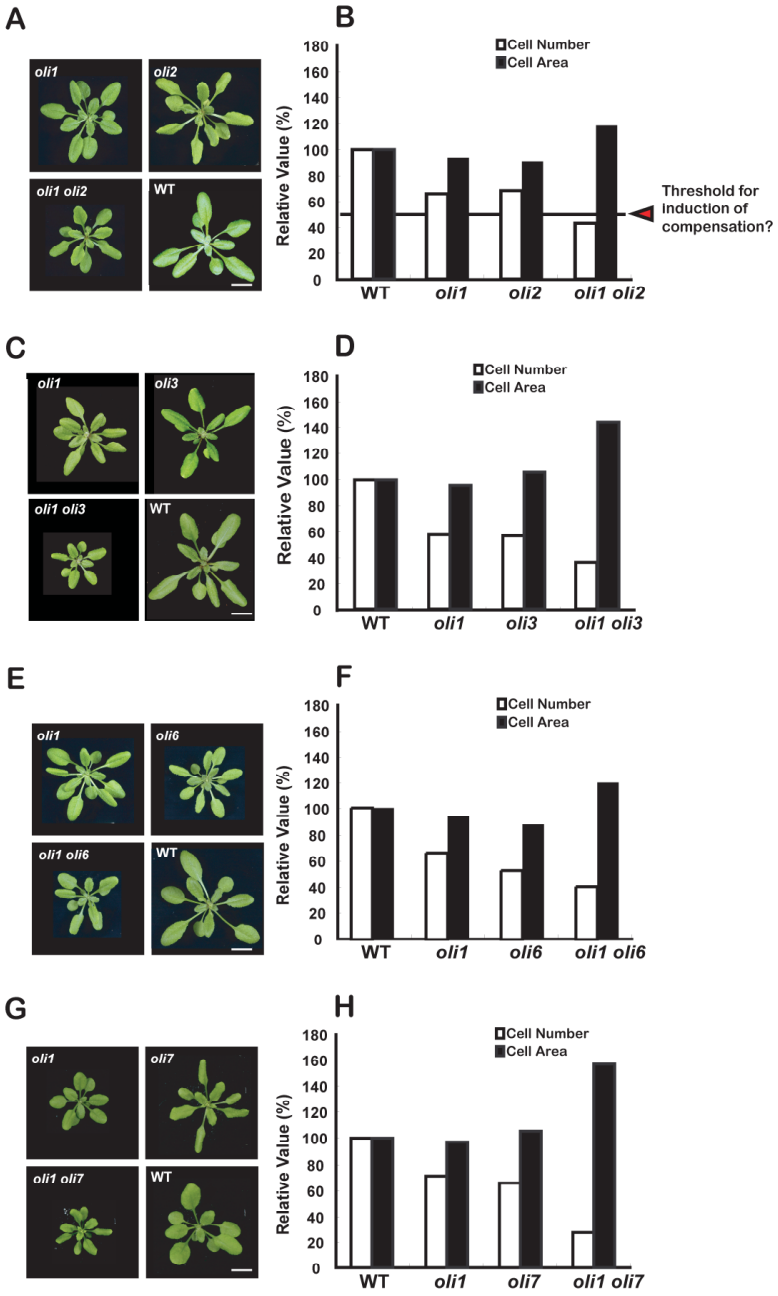

Figure 5

Characterization of double mutants among *oli* mutants.

(A, C, E, G) Rosette phenotypes of *oli* double mutants. Plants were grown for 3 weeks under a 16-h-light/8-h-dark fluorescent illumination cycle at 22ºC. Bars: 10 mm. (B, D, F, H) Number and size of leaf cells in *oli* double mutants. First leaves from 3-week-old plants were analyzed microscopically. The results are mean ± s.d. expressed relative to wild-type values (n = 8 for cell number and n ≥ 240 from 8 leaves for cell size).

3.3.4 Further decreases in cell number enhance cell enlargement when compensation is induced

As shown above, the induction of compensation depends on a decrease in cell number below a certain threshold level. How do further decreases in cell number affect cell size after compensation has been induced? One possibility is that the cell size is constant against further decreases in cell number upon the induction of compensation. The other possibility is that the size of leaf cells increases in a threshold-dependent staircase pattern, or in proportion to further decreases in cell number after compensation is induced. To clarify which is the case, I introduced each *oli* mutation into the null-allele *an3* background that showed typical compensation (Figs. 6, 7). Double mutants between *an3* and *oli* mutants produced a small rosette with some morphological changes. Double mutants between *an3* and *oli2*, *oli5* and *oli7* shared common features, i.e., narrow and sharply pointed leaves (Fig. 6). On the other hand, the other *oli an3* double mutants exhibited additive phenotypes of parental mutants with respect to the shape or size of the rosette leaves (Fig. 6). Although the gross morphology was varied, all *oli an3* double mutants showed a further decrease in cell number and had greatly enlarged palisade cells compared to *an3* single mutants, except for *oli1 an3* (Fig. 7). These results showed that the *OLI2* to *OLI7* and *AN3* loci have independent functions in cell proliferation during leaf organogenesis, since the allele of the *an3* mutation used was a null allele, *an3-3* (Horiguchi et al. 2005). Considering that these *oli an3* double mutants exhibited enhanced compensation with varied decreases in cell number, this

also suggested that once cell number was decreased below the threshold level, further decreases in cell size cause proportional increases in cell size. On the other hand, the *oli1 an3* double mutant had a similar phenotype in the number and cell size compared to *an3* single mutants (Fig. 7A), suggesting that the *OLI1* and *AN3* loci play a role in the same pathway to promote cell proliferation, at least in leaf primordia.

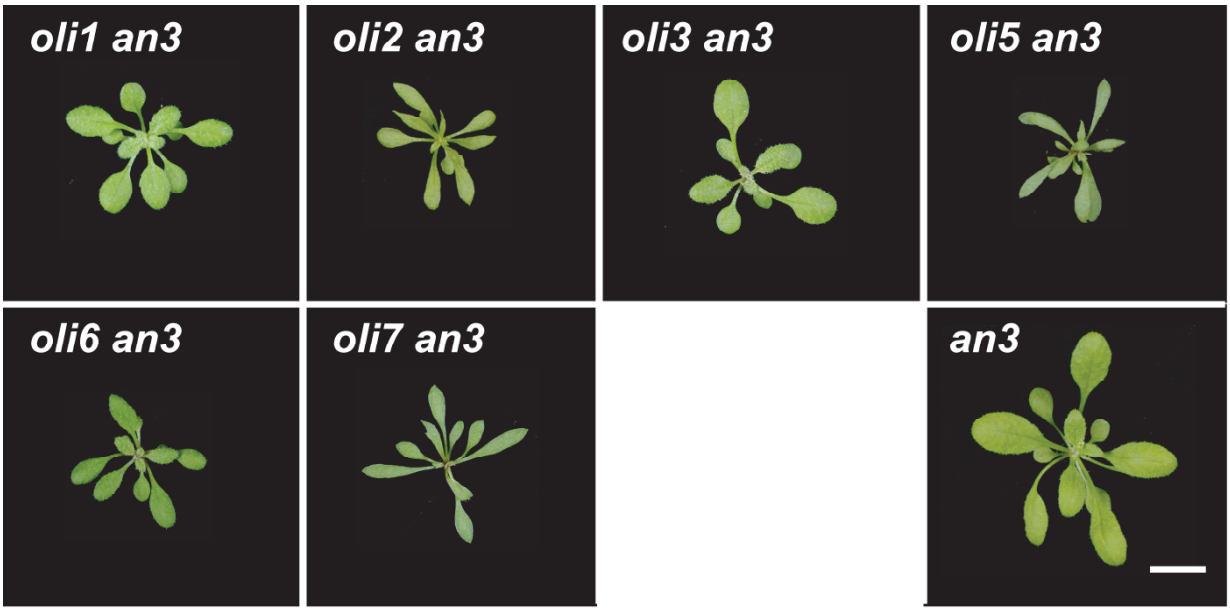

Figure 6
Rosette phenotypes of *oli an3* double mutants.
Plants grown for 3 weeks with 16-h-light/8-h-dark fluorescent illumination cycle at 22ºC are shown. Bar = 10 mm.

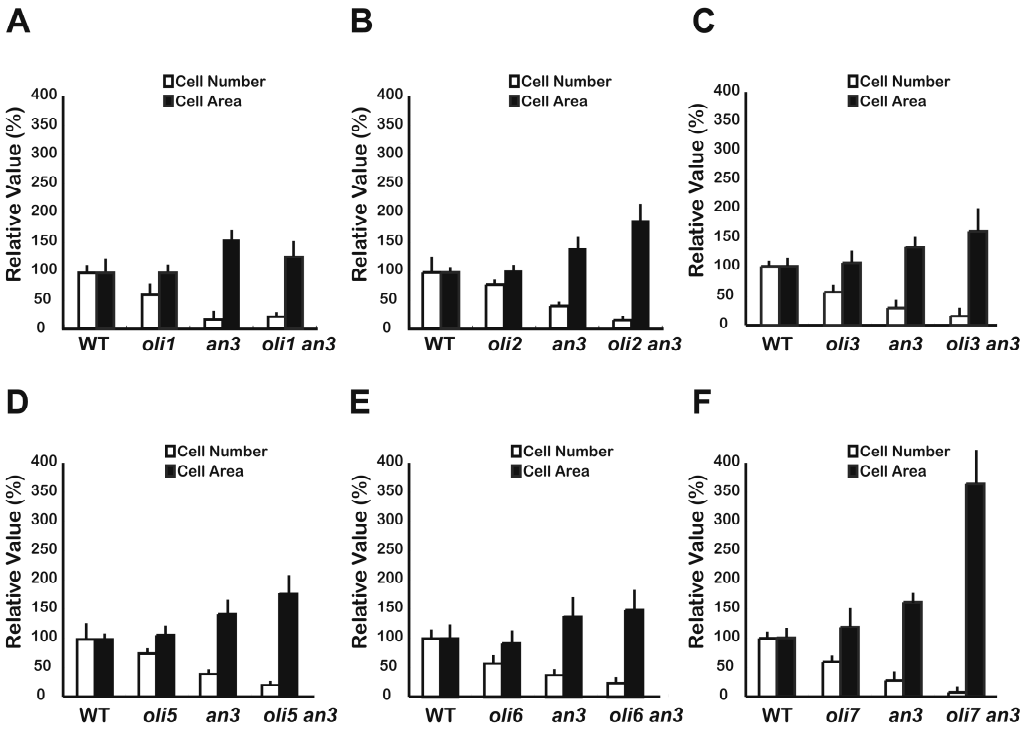

Figure 7

Number and size of palisade cells in the first leaves from 3-week-old plants.

Data from *oli1* to *oli7* and *oli an3* double mutants are shown. The results are mean ± s.d. expressed relative to wild-type values (n = 8 for cell number and n ≥ 240 from 8 leaves for cell size).

3.4 Discussion

To understand the relationship between cell number in leaves and the induction of compensation, I examined the effects of variations in the decrease of leaf cells on cell size by using several *oli* mutants that were specifically impaired in cell proliferation. The key point of my genetic strategy using *oli* mutants was that it was possible to specifically control the number of leaf cells at various levels. This allowed me to clearly analyze the effect of decreased cell number on the final cell size in leaves. Through several analyses in this chapter, the most important finding was that the *oli* double mutants exhibited compensation, whereas each parental single mutant did not (Fig. 5). This finding strongly supports the idea that the induction of compensation requires a reduction in cell number below certain threshold levels (Fig. 5). This threshold-dependent model accounts for the intriguing phenotypes reported in other mutants that have a defect in cell proliferation. For example, although *AN3/AtGIF1* and *AtGRF5* genes play a role in the same genetic pathway to promote cell proliferation in leaf primordia, loss-of-function of *AN3* but not *atgrf5* induces compensation (Horiguchi et al., 2005; Kim and Kende, 2004). The same relationship is observed in two genes that function in the auxin-regulated cell proliferation pathway, namely *ANT* and *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*: Hu et al., 2003; Mizukami and Fischer, 2000). In these instances, loss-of function of *AN3* and *ANT*, *AtGRF5* and *ARGOS* causes significant and moderate decreases cell number in leaves, respectively. These results strongly support the “threshold model”. On the other

hand, absence of compensation in mutant lines showing *oli* phenotype, including *atgrf5* and *ARGOS* knock-down line, can be explained without hypothesizing the threshold: in these mutants, cell expansion may also be defectiv. However, at least in *an3* and *ant*, this possibility is ruled out since over-expression of *AN3* and *ANT* promotes cell proliferation without affecting final cell size in leaves (Horiguchi et al., 2005; Mizukami and Fischer, 2000). To find how general the threshold-dependent induction of compensation is, isolation and further molecular genetic analyses of *OLI* genes would provide key information.

Another important finding was that the *oli an3* double mutants showed further decreases in cell number and further increases in cell size, except for *oli1 an3* (Fig. 6 and 7). These results indicate that cell enlargement is enhanced proportionally with the reduction in cell number once compensation is induced. Taken together, I concluded that whether compensation is induced depends on a hypothetical threshold level of cell numbers, but once compensation is induced, the magnitude of cell enlargement is proportionally enhanced by the further decrease in the number of cells. At first glance, this switching seems curious. Hypotheses to explain the switching between these two modes of dependency of cell-size control on the number of cells will be discussed in Chapter 5, after examining possible mechanisms of compensation based on the data shown in Chapter 4.

On the other hand, from analyses of phenotypes of *oli an3* double mutants, *OLI2* to *OLI7* genes were found to function in cell proliferation in a different pathway from *AN3*, at least in leaves since we used a null allele as the *an3*

mutation (Fig. 7B to F). Among these, it is noteworthy that in the *oli7 an3* double mutant (Fig. 7F), the number of palisade cells was extremely decreased to only 7.1% of the wild-type value, and the size of palisade cells was dramatically increased to 364.2% of the wild-type value. If *OLI7* and *AN3* loci have independent functions in cell proliferation control, the *oli7 an3* double mutant is expected to have an additive phenotype of both parents (*oli7*: 59.7% for cell number and 112.3% for cell size, *an3*: 30.1% and 160.8%, respectively), namely, 18.0% in cell number and 180.6% in cell size of the wild type. However, contrary to this expectation, *oli7 an3* had dramatically fewer and larger cells, which were less than half and two-fold larger than predicted values, respectively. Thus, the aberrant cell enlargement observed in *oli7 an3* may be due to synergistic effects between *oli7* and *an3* mutations.

Based on the above two findings, I will discuss possible mechanisms of compensation. One straightforward interpretation of this phenomenon comes from the well-known fact that delayed entry into mitosis leads to the production of larger daughter cells. Many studies on yeast and animal cells have indicated that cell growth is continuous during the cell cycle, and thus mutations that delay the cell cycle would simply lead to the production of larger daughter cells (e.g., Johnston et al., 1977; reviewed by Jorgensen and Tyers, 2004). Based on this idea, one would speculate that the compensation observed in plants may result from the uncoupling of cell growth from cell division. However, the situation in plants differs in several points from those in yeast and animals. The crucial difference is that post-mitotic cell growth in plants is driven by the uptake of water in vacuoles and the vast majority of a plant cell is occupied by a huge

central vacuole. In a computer model of cell cycling and post-mitotic cell expansion, increasing cell size during cell cycling only contributes to the final size at the end of post-mitotic cell expansion (Beemster et al., 2006). In addition, if compensation results from an uncoupling of cell growth and cell division, cell size should be larger in proportion to the reduction in cell number. Above all, the finding of a ‘threshold‘ reduction level in the number of cells required for induction of compensation completely ruled out this possibility. Therefore, I do not believe that compensation results from the uncoupling of cell division from cell growth in proliferating cells, as is thought to occur in yeast and animal systems. Rather, my study clearly showed a new type of interaction between cell proliferation to post-mitotic cell expansion during leaf development, although its molecular components are completely unknown. Whatever the case, cloning and expression analyses of the *OLI* genes, which is currently underway, should provide some clues to solve this enigma. The present study has revealed important factors underlying the induction of compensation: the induction of compensation requires a decrease in cell number below a threshold, and compensation is not a phenomenon that results from the uncoupling of cell division and cell growth. Further analysis based on the above findings will help to better understand the framework of the coordination of cell number and cell size in leaf development.

In this chapter, I have discussed the induction process of compensation; in the following chapter, I deal with the mechanisms of the response process of compensation, by analyzing how aberrant cell enlargement occurs when compensation is induced.

Chapter 4

Response Process of Compensation

4.1 Summary

In this Chapter, to genetically dissect the cell-expansion system(s) underlying the compensation seen in the *an3* mutant, I isolated and utilized ten *extra-small sisters* (*xs*) mutant lines that show a decreased cell size but a normal cell number in leaves. All the *xs* single mutants have palisade cells about 20 to 50% smaller than wild type. I produced double mutants between *xs* and *an3* to examine the effect of *xs* mutations on abnormal cell expansion observed in *an3*. As a result, phenotypes of the palisade cell sizes in all combinations of *xs an3* double mutants fall into three classes. In the first class, the compensation was significantly suppressed. Conversely, in the second class, the defective cell expansion conferred by the *xs* mutations was significantly suppressed by the *an3* mutation. The residual *xs* mutations had effects additive to those of the *an3* mutation on cell expansion. The endopolyploidy levels in the first class of mutants were decreased, unaffected, or increased, as compared to those in wild-type, suggesting that the abnormally enhanced cell expansion observed in *an3* could be mediated, at least in part, by ploidy-independent mechanisms. Altogether, these results clearly showed that a defect in cell proliferation in leaf primordia enhances the activity of a part of the cell expansion pathways, which is required for normal leaf expansion.

4.2 Introduction

In this chapter, I attempted genetic dissection of the response process of compensation observed in *an3*. If compensation is a result of hyper-activation of a cell expansion pathway which functions during normal leaf development, some cell expansion-specific mutations would suppress such an abnormal cell expansion observed in *an3*. Thus, it was expected that cell-expansion-defective mutants would be convenient for this purpose. Therefore, in this study, I isolated and utilized ten mutants specifically impaired in cell expansion with normal leaf cell number. Then I named these mutants *extra-small sisters* (*xs*). To find *xs* mutants that are able to suppress compensation, I produced double mutants between *xs* mutants and *an3*. In parallel analysis, I also determined the levels of endopolyploidy in these mutants since it often has a positive correlation with cell size (*e.g.*, Melaragno et al., 1993). Based on the results of the above two analyses, I discuss the genetic relationships of the cell-expansion pathways in normal leaf development and compensation.

4.3 Results

4.3.1 The *xs* mutants have smaller leaves than the wild type due to reduced cell size

To genetically dissect compensation in *an3*, it was necessary to identify cell-

expansion-defective mutants with normal or near-normal cell proliferation in leaves. From our vast (147 lines of mutant) collection of leaf size and shape mutants with defects in the number and size of palisade cells (Horiguchi et al., 2006a, b; Chapter I), I found that sixteen lines of mutants have specific defect in cell expansion in leaves. From these candidates, I have ruled out mutants which have a significant change in leaf shape or have pleiotropic features. As a consequence, I selected ten lines, each with a specific defect in palisade cell expansion but with normal palisade cell numbers. I named these mutants *extra-small sisters* (*xs*). Based on the segregation patterns of backcrossed F2 populations and allelism tests among *xs* mutants, all the *xs* mutations were found to be all non-allelic, single recessive mutations (data not shown).

Figure 1 shows the rosette phenotypes of the *xs* mutant plants. Most of the *xs* mutants have small-rosette phenotypes with proportional or minor changes in shape: *xs2* and *xs3* have spatulate leaves and *xs4* and *xs8* have rounded leaves. To confirm that the small rosette phenotype was caused by a reduction in the cell size rather than reduced numbers of cells, *xs* mutant leaves were subjected to histological analysis (Fig. 2). The sizes of palisade cells observed from a paradermal view ranged from 54.4 ± 11.1% (*xs1*) to 77.5 ± 14.4% (*xs9*) of the wild-type values. In contrast, the palisade cell numbers in the *xs* mutants were not significantly reduced: the greatest effect was found in *xs6*, in which the leaf cell number was only 80.1 ± 8.8% that of the wild type. These results indicate that the *xs* genes play a specific or preferential role in cell expansion processes rather than cell proliferation, at least in palisade tissues. Therefore, these *xs* mutants were judged to be suitable for the genetic

dissection of compensation in *an3*.

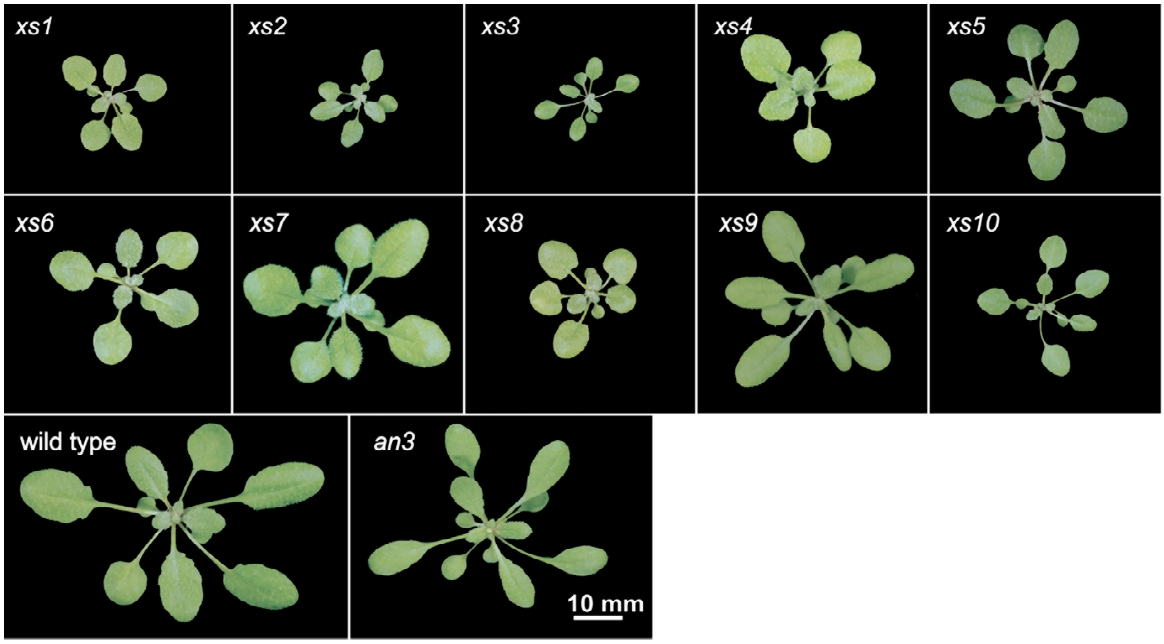

Figure 1
Rosette phenotypes of the *xs* and *an3* mutants.
Plants were grown for three weeks under a 16-h-light/8-h-dark fluorescent illumination cycle at 22°C. Bar: 10 mm.

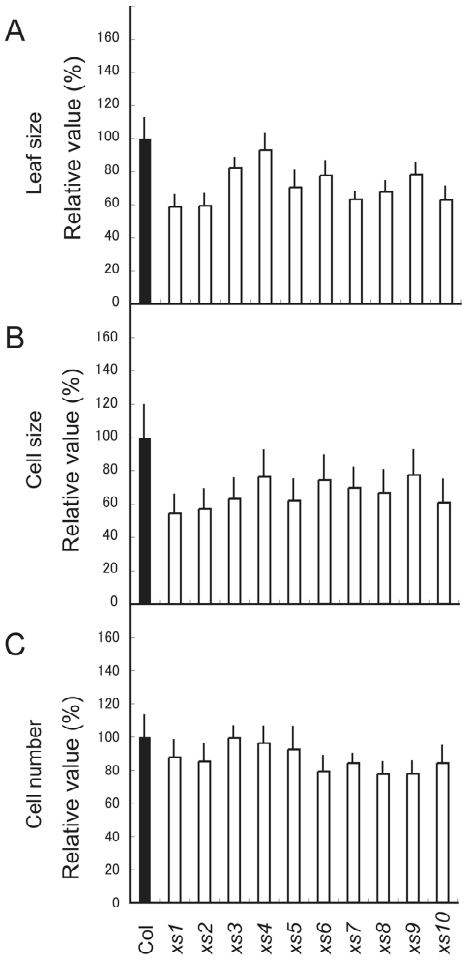

Figure 2

Leaf sizes and the sizes and number of palisade tissue cells.

First leaves from three-week-old plants were observed by microscopic analysis. (A) Leaf sizes of the *xs* mutants and wild type (n = 8). (B) Sizes of palisade cells observed from a paradermal view (n ≥ 240 cells from eight leaves). (C) Total number of palisade cells in the subepidermal layer of mature first leaves (n ≥ 8). The results are expressed relative to wild-type values ± s.d.

I also examined the epidermal cell size in the *xs* mutants. Distributions of the adaxial epidermal cell area in the leaves are presented in Figure 3 as box plots that show the median, minimum, and maximum values, and the 25th to 75th percentiles for each group. Leaf epidermal cell sizes were smaller in the *xs2*, *xs3*, *xs5*, *xs7*, *xs8*, and *xs10* mutants than in wild-type plants, whereas in other *xs* mutants (*xs1*, *xs4*, *xs6*, and *xs9*) there was no clear reduction in the epidermal cell size (Fig. 3). Next, I examined whether the *xs* mutations show cell-expansion phenotypes in other organs (Fig. 4). The root lengths of *xs2*, *xs3*, *xs7*, and *xs10* were shorter than those of wild-type plants (Fig. 4A). Consistently, the root epidermal cells in these mutants were also shorter than those in wild-type plants (Fig. 4B). When hypocotyls were grown in darkness, only *xs10* hypocotyls exhibited elongation defects, with shorter epidermal cells than those of wild-type plants (Fig. 4C and D).

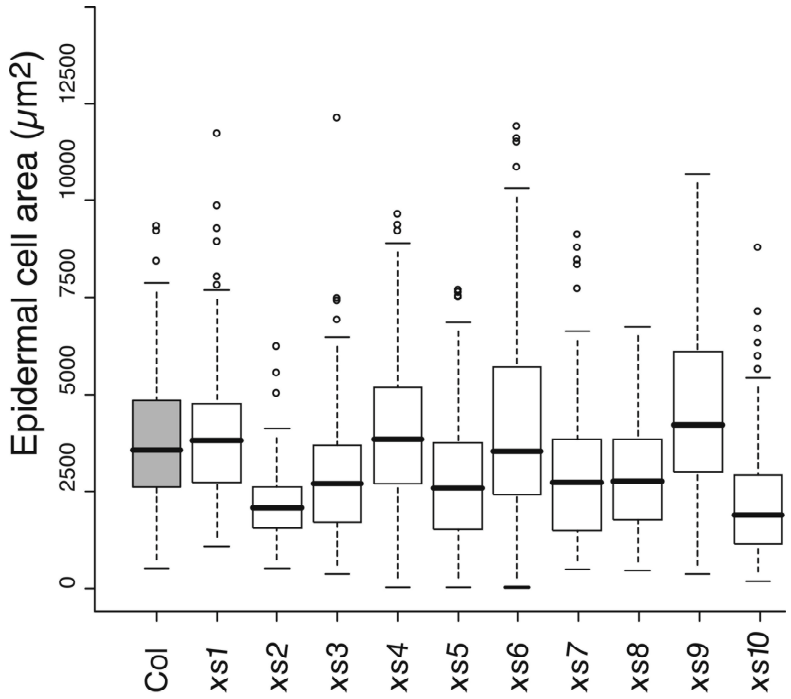

Figure 3.
Sizes of leaf adaxial epidermal cells in the *xs* mutants.
Box plots of epidermal cell size. The box includes observations from the 25th to the 75th percentile, and the horizontal line within the box represents the median value. Lines outside the box represent the 10th and 90th percentiles, and the circles represent outlying values. The sizes of epidermal cells were determined in three-week-old first leaves (n = 170, from seven leaves).

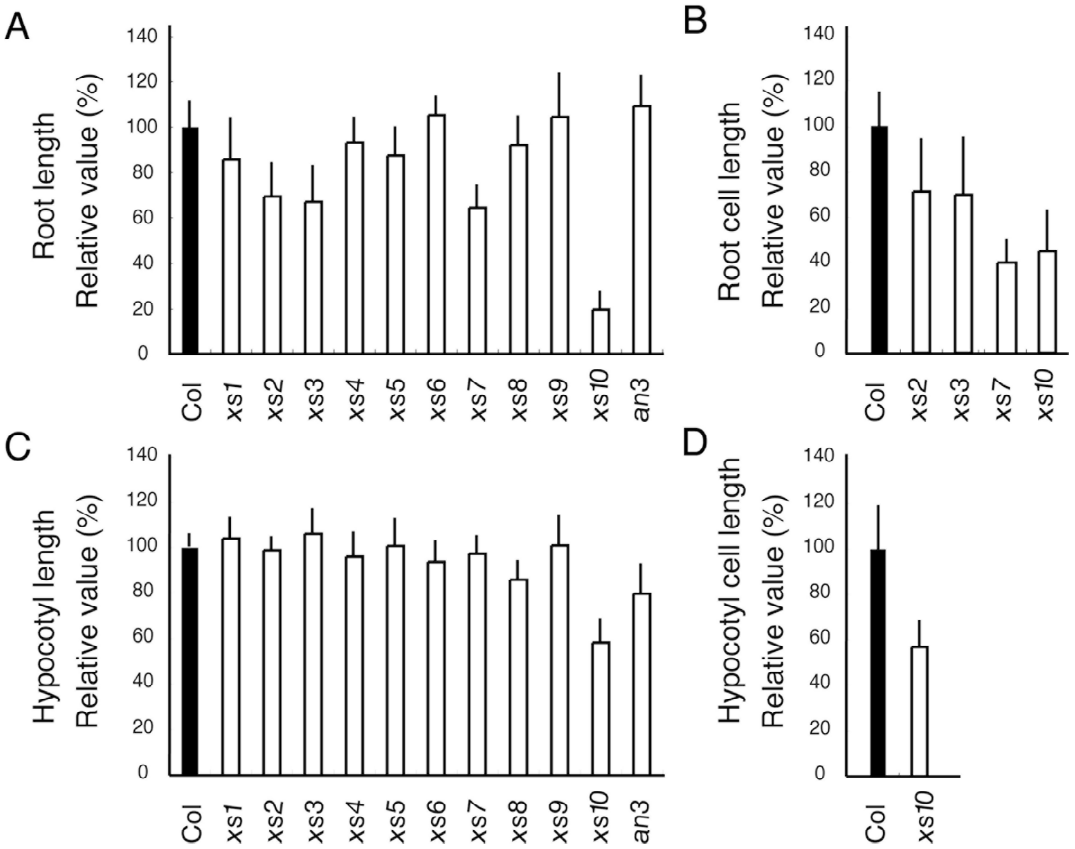

Figure 4

Lengths of roots and dark-grown hypocotyls in the *xs* mutants.

Wild type and *xs* mutant seedlings were collected eight days after sowing, and the lengths of the primary roots and hypocotyls were measured. (A) Root lengths of the wild type and the *xs* mutants (n = 12). (B) Lengths of root epidermal cells in the *xs* mutants that showed reduced root elongation in (A). The lengths of fully differentiated root epidermal cells were measured (n = 80, from twelve roots). (C) Hypocotyl lengths in dark-grown seedlings (n = 12). (D) Lengths of hypocotyl epidermal cells in dark-grown seedlings. The lengths of hypocotyl epidermal cells in the middle of the hypocotyls were measured (n = 50, from ten hypocotyls). The results are expressed relative to wild-type values ± s.d.

4.3.2 Identification of *xs* mutants that suppress compensation

As mentioned above, compensation can be divided into induction and response processes. If aberrant cell enlargement observed in compensation is resulted from general enhancement of cell expansion activities as a whole, any *xs* mutants would suppress this abnormal cell enlargement during compensation. On the other hand, if compensation is dependent on a particular cell expansion pathways, only a subset of *xs* mutations would suppress compensation. The *xs* phenotype is relatively rare but in this study I succeeded for the isolation of ten independent *xs* mutations. The availability of a variety of *xs* mutations offers an opportunity to test above possibilities for the first time. To address this, I produced double mutants between the *xs* mutants and *an3* (Fig. 5), a mutant that exhibits typical compensation (Horiguchi et al., 2005), and determined the leaf size and the number and size of palisade cells in these plants (Fig. 6).

As a result, I found that the mutants can be categorized into three classes (Fig.6). The first class, containing *xs1*, *xs2*, *xs4*, and *xs5*, was the small-cell class. The palisade cell size of these double mutants was similar to that of each *xs* parent, and their cell numbers were similar to those in *an3*. The second class was the large-cell class, containing *xs6*. Both the cell size and the cell number of double mutants of *xs6* were similar to those of *an3*. The last class was the additive class, containing *xs3*, *xs7*, *xs8*, *xs9*, and *xs10*. The cell size of these double mutants was intermediate in relation to those of both parents. These results suggest that the defective genes of mutants belonging to the small-cell

class play a critical role in the downstream steps in cell expansion in compensation. In contrast, the *xs6* mutant is not able to activate certain cell expansion processes, which is recovered in the *xs6 an3* double mutants owing to the compensation. Hence, I concluded that compensation occurs through the activation of a part of cell-expansion systems that also act in normal leaf development.

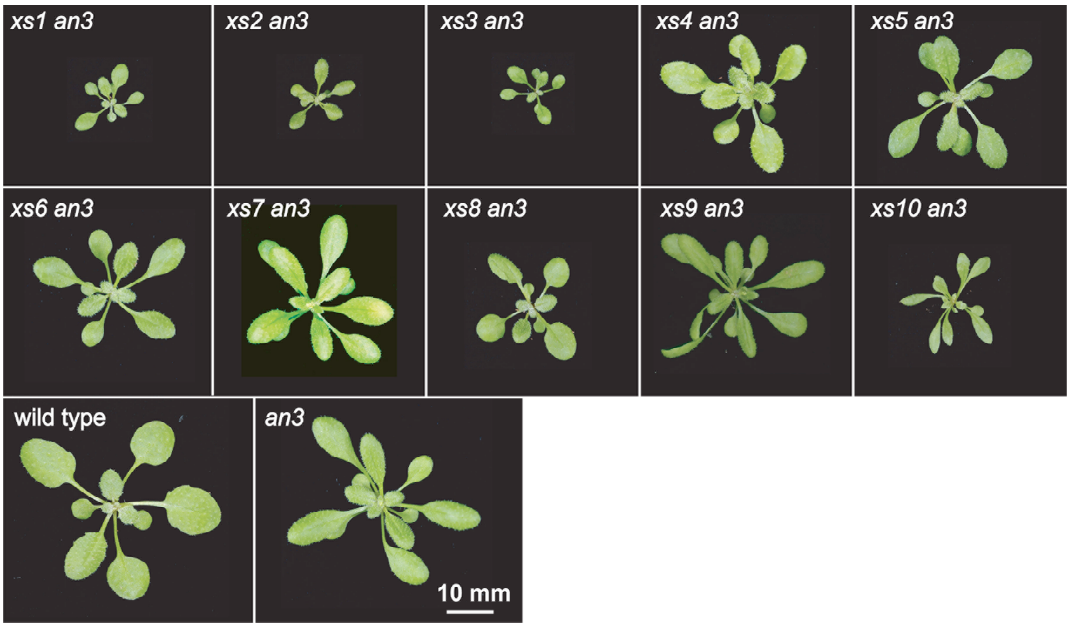

Figure 5
Rosette phenotypes of *xs an3* double mutants.
Plants were grown for three weeks under a 16-h-light/8-h-dark fluorescent illumination cycle at 22°C. Bar: 10 mm.

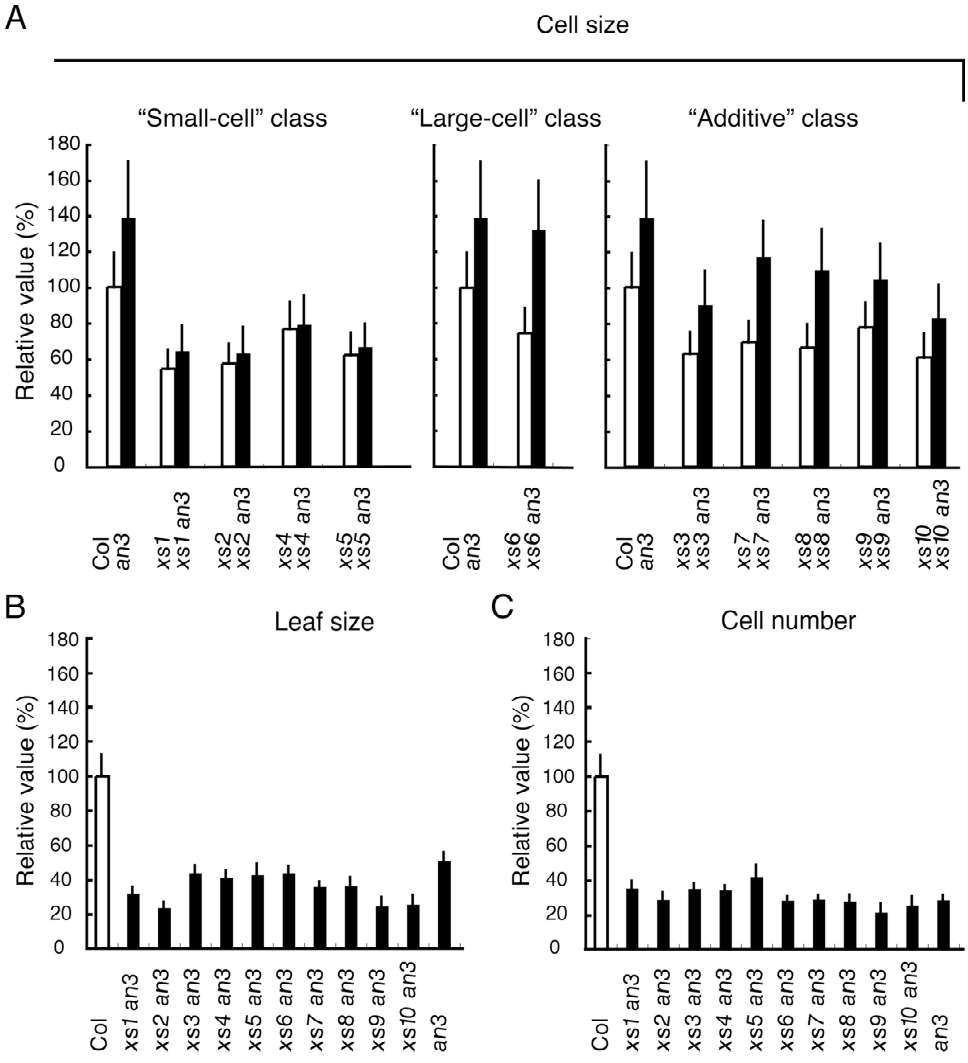

Figure 6

Characterization of *xs an3* double mutants.

First leaves from three-week-old plants were observed by microscopic analysis. (A) Sizes of palisade tissue cells of *xs an3* double mutants (n = 8). The *xs an3* double mutants were sorted into three classes: small-cell, large-cell, and additive types. (B) Leaf sizes of wild type, *xs an3* double mutants, and *an3* (n = 8). (C) Total numbers of palisade cells in the subepidermal layer of mature first leaves of the wild type, *xs an3* double mutants, and *an3* (n = 8). The results are expressed relative to wild-type values ± s.d.

4.3.3 Endopolyploidy levels in *xs* mutant leaves

Endoreduplication coincides with the differentiation of a cell, and the level of ploidy often correlates with the final cell size (e.g., Melaragno et al., 1993). To address the issue of whether the aberrant cell expansion observed in compensation occurs in a ploidy-dependent or -independent manner, I determined the ploidy levels in the first leaves of *an3* and *xs* mutants by flow cytometry. The ploidy levels in *an3* were relatively normal (Fig. 7), suggesting that the abnormal cell expansion in *an3* is regulated in a ploidy-independent manner. In contrast, in the small-cell class of *xs* mutants, the ploidy levels were increased (*xs5*), decreased (*xs2*), or unaffected (*xs1* and *xs4*), in comparison with wild-type plants (Fig. 7). In the large-cell class, the ploidy levels were unaffected (*xs6*) (Fig. 7), suggesting again that the compensation is independent of the status of endoreduplication.

Apart from compensation, these *xs* mutants can be sorted into three classes according to the changes in the ploidy levels (Fig. 7). In the first class, including *xs2* and *xs10*, the ploidy level is lower than in the wild type. In these mutants, the level of 8C nuclei is lower, with a concomitant increase in the level of 2C nuclei, when compared to the wild-type ploidy distribution. Therefore, in these mutants, endoreduplication is impaired to some extent. The second class includes *xs5* which has an elevated ploidy level. In contrast, *xs1*, *xs3*, *xs4*, *xs6*, *xs7*, *xs8*, and *xs9* could be classified in a normal ploidy group, suggesting that these *XS* genes regulate cell expansion in a ploidy-independent manner. Alternatively, these *XS* genes might act downstream of endoreduplication.

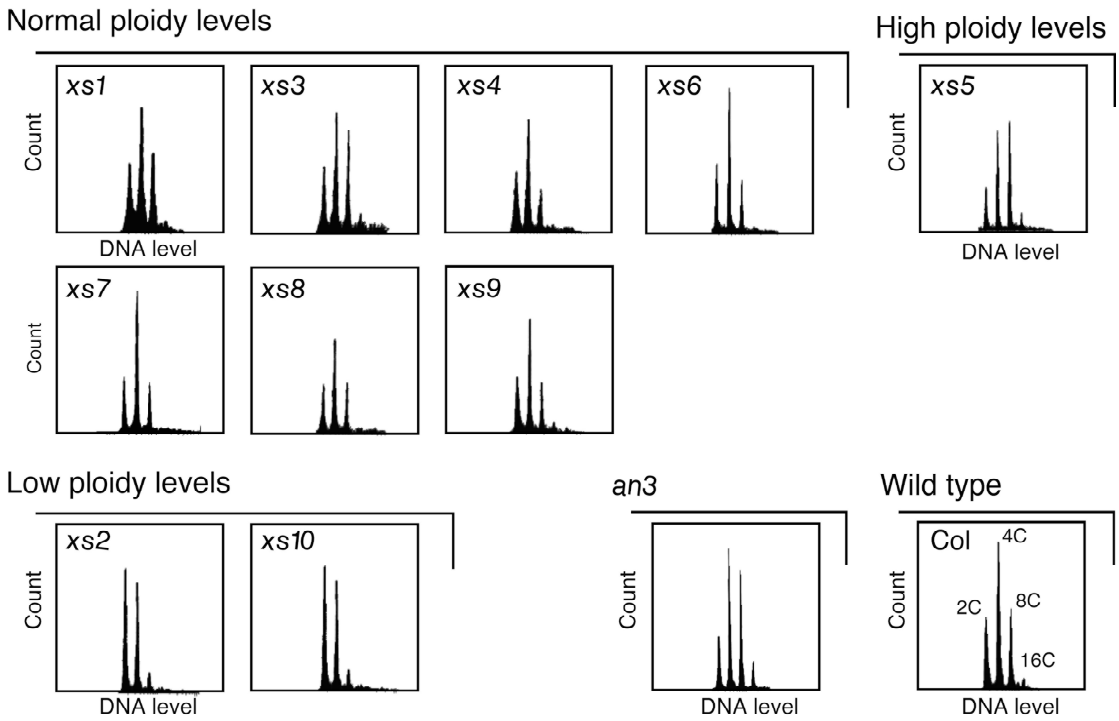

Figure 7
Ploidy levels of the *an3* and *xs* mutants.
The *xs* mutants were classified into three groups, with normal, low, and high ploidy levels. Flow cytometric analysis was performed using three-week-old first leaves. Note that the *an3* mutant shows a relatively normal profile in the ploidy level. Typical ploidy distribution patterns from three independent trials are presented.

4.4 Discussion

4.4.1 Some of cell-expansion pathways are enhanced during compensation

To understand the mechanisms that link cell proliferation and cell expansion during leaf organogenesis, I genetically dissected the cell-expansion systems underlying the compensation syndrome. The most important finding obtained from the experiments shown in Chapter 4 was that the small-cell class of *xs* mutants (*xs1*, *xs2*, *xs4*, and *xs5)* suppresses the abnormal cell enlargement triggered by the *an3* mutation (Fig. 6A). This finding strongly suggests that compensation depends on these *XS* genes that function in normal cell expansion. It also shows that a reduction in cell proliferation by loss-of-function of the *AN3* somehow up-regulates these *XS* functions. In other words, there should be some linking mechanism(s) between cell-proliferation systems and a part of cell-expansion systems. The isolation of responsible genes for these mutant loci, which is underway, should provide clues leading to an understanding of the regulation of compensation. This result should supply an important clue for understanding not only the mechanisms of compensation but also the entire system of cell expansion control in leaf organogenesis.

Another important finding was that the *an3* mutation can suppress cell expansion defects in *xs6*. This result could be explained if we hypothesize common target gene(s) that is regulated by a cell expansion process involving the *XS6* genes and also by compensation, cell-expansion pathways can be

distinguished according to their epistasis to compensation. Furthermore, the *xs* mutants in the additive class suggest the presence of cell expansion pathways that act in parallel with compensated cell expansion.

It is widely accepted that developmental processes are governed by a gene regulation network. My results obtained by the genetic analyses of the *xs* and *an3* mutants strongly suggest that cell-expansion pathways also form networks and a subset of which represented by the *XS* genes of small-cell class is linked with the cell-proliferation pathway which is regulated by *AN3*. How cell proliferation and cell expansion are linked offers an inviting issue for future studies, and could be addressed through detailed molecular analyses of the *xs* and *an3* mutants.

4.4.2 The role of the *XS* genes in normal cell expansion and endoreduplication

The results of the analysis of the *xs* mutants also provided insight into the roles of the *XS* genes in normal cell expansion. In some cases, cell size is positively correlated with the levels of endoreduplication (*e.g.*, Melaragno et al., 1993). For example, in *CYCD3;1*-overexpressing plants, cell cycling is reinforced and, as a consequence, leaf cells fail to enter endocycles and remain very small (Dewitte et al., 2003). Conversely, a precocious transition into endocycles reduces the cell number and increases the cell size in leaves (Verkest et al., 2005b; Autran et al., 2002). However, no such correlation was found in *an3* or most of the *xs* mutants: a positive correlation among cell size and ploidy levels

was found in only two of the ten mutants, *xs2* and *xs10* (Fig. 4). In *xs2* and *xs10*, the ploidy levels are decreased but the cell numbers are normal, suggesting that these *XS* genes play a role in endoreduplication after the transition from the cell cycle into endocycles. Little is known of the factor(s) that determine the number of endocycles in leaves, with the exception of mutants in the DNA topoisomerase VI (TOPO VI) complex, such as *root hairless1* (*rhl1*), whose leaves lack nuclei higher than 16C (Sugimoto-Shirasu et al., 2005). *xs2* and *xs10* differ from *rhl1* and related mutants in the trichome and root-hair phenotypes. In addition, the chromosomal position of *xs2* (Fig. 8) does not correspond to any of the *TOPO VI* loci.

The increased ploidy level in *xs5* is a rather surprising result. In this mutant, the level of 8C nuclei was significantly increased, although the increase in the levels of 16C nuclei was less significant. This result suggests that the responsible gene in this mutant plays a negative role in endoreduplication pathways that are required normal cell expansion in developing leaves. This result also suggests that increases in ploidy levels do not always cause cell enlargement. This situation is similar to the effects of the *Arabidopsis thaliana* Cdc10-dependent transcript1 (*AtCDT1*) knockdown line. *AtCDT1a* and *b* encode licensing factors of DNA replication (Castellano et al., 2004). Without appropriate CDT1 function, DNA replication proceeds in an unscheduled manner, causing impaired cell expansion for unknown reasons (Raynaud et al., 2005). I have carried out a rough mapping of the *XS5* locus and placed it on the top portion of chromosome 2 (Fig. 8), corresponding to neither the *AtCDT1a* nor the *AtCDT1b* locus. No other recessive loci that cause *atcdt1*-like phenotypes

are known. Thus, *XS5* defines a new locus that is important in limiting DNA replication. This observation suggests that the extent of cell expansion is, in some cases, not strictly linked to ploidy levels within leaves in either normal leaf development or compensation.

The seven other *xs* mutants, *xs1*, *xs3*, *xs4*, and *xs6* to *xs9*, have normal ploidy levels in leaves. This could be interpreted in at least two ways. One possibility is that these *XS* genes promote cell expansion downstream of a ploidy-dependent pathway. A second possibility is that these genes act independently of the ploidy levels. The latter possibility has also been suggested by the observations that the overexpression of *KRP2* inhibits endoreduplication but allows excessive cell expansion (De Veylder et al., 2001). However, the cellular processes involved in the enhanced cell expansion are not known. Likewise, the key cell expansion processes enhanced after the increase in the ploidy level are almost unknown. Global expression profiling in yeast has suggested that a subset of genes is specifically up- or down-regulated according to the ploidy level (Galitski et al., 1999). Whether similar genes are involved in the systemic variation in ploidy levels in plants is unclear. In addition, substantial differences in the driving force of cell expansion in plants (such as vacuolation) suggest that the nature of ploidy-driven genes in plants, if any, could be different from those in yeast. To distinguish these possibilities, it would be informative to determine whether the cell size in the *xs* mutants increases upon polyploidization treatments. Such experiments are now underway. Irrespective of the above pathways, these *XS* genes will help to better understand the cellular processes that are important in controlling leaf

cell size, and will serve as markers to dissect the complex web of genetic regulation of leaf development.

In summary, the present study has revealed important clues behind the control of leaf cell enlargement: compensation depends on a specific enhancement of normal processes of cell expansion control; compensation is not a mere uncoupling of cell cycling and cell enlargement; and parallel pathways exist for the control of leaf cell expansion. Further analyses based on the above findings will supply new insights leading to the understanding of the global mechanisms in the control of leaf organogenesis at the organ level.

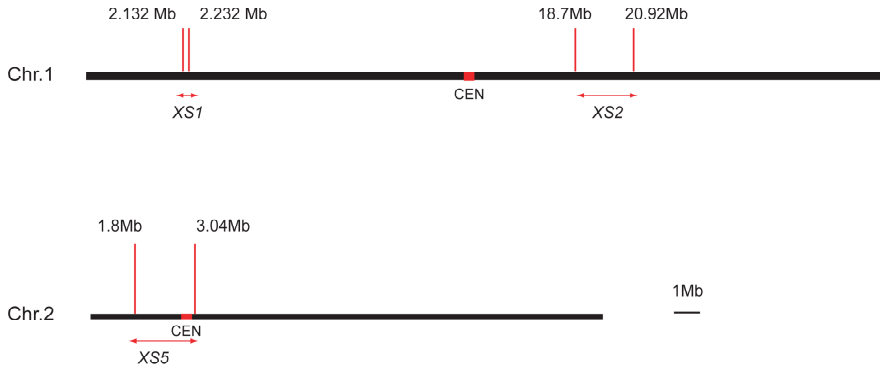

Figure 8
Map of chromosomes 1 and 2 with the positions of genetic markers used for mapping of *xs1*, *xs2* and *xs5* loci.
DNA samples from 192 homozygous mutants isolated from F2 plants of cross between *xs* mutant with Landsberg *erecta* line were used to determine the location of each locus. Bar = 1Mb

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