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Organ size regulation in plants: insights from compensation

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The regulation of organ size in higher organisms is a fundamental issue in developmental biology. In flowering plants, a phenomenon called "compensation" has been observed where a cell proliferation defect in developing leaf primordia triggers excessive cell expansion. As a result, final leaf size is not significantly reduced compared to that expected from the reduction in leaf cell numbers. Recent genetic studies have revealed several key features of the compensation phenomenon. Compensation is induced either cell autonomously or non-cell autonomously depending on the trigger that impairs cell proliferation; a certain type of compensation is induced only when cell proliferation is impaired beyond a threshold level. Excessive cell expansion is achieved by either an increased cell expansion rate or a prolonged period of cell expansion via genetic pathways that are also required for normal cell expansion. These results indicate that cell proliferation and cell expansion are coordinated through multiple pathways during leaf size determination. Further classification of compensation pathways and their characterization at the molecular level will provide a deeper understanding of organ size regulation.

Keywords: *angustifolia3*, cell expansion, cell proliferation, compensation, *extra-small sisters*, *fugu*, *oligocellula*, organ size

Organ size regulation is an essential process for the optimal growth and appropriate function of multicellular organisms. The size of aerial lateral organs in plants, i.e., leaves and floral organs, is determined by the number and size of constituent cells since the growth of these organs is determinate. Therefore, quantitative recognition of size-related parameters and developmental decisions is required to control the kinetic aspects of cell proliferation and cell expansion. The molecular mechanisms underlying cell proliferation and postmitotic cell expansion have been investigated extensively, mainly through the characterization of the cell cycle and endocycle in which multiple rounds of DNA replication occur without cell division (Inzé and De Veylder, 2006; Breuer et al., 2010). In addition to these cell cycle regulators, a number of genes have been identified over the last decade that regulate the size of lateral organs through the modulation of cell proliferation and/or cell expansion (Gonzalez et al., 2009; Krizek, 2009; Micol, 2009). Many of these genes encode regulatory factors that act at the transcriptional (Mizukami and Fischer, 2000; Kim and Kende, 2004; Horiguchi et al., 2005, 2009; White, 2006; Usami et al., 2009; Ichihashi et al., 2010) or posttranscriptional level (Disch et al., 2006; Li et al., 2008; Usami et al., 2009), and some are involved in cell–cell communication (Strabala et al., 2006; Anastasiou et al., 2007; Eriksson et al., 2010). In addition to such cell level regulation, cell proliferation and postmitotic cell expansion must be coordinated to establish an organ of the appropriate size. Although the mechanisms underlying such coordination are still largely unknown, recent molecular genetic studies have begun to provide some insight. In this review, we focus on the phenomenon termed "compensation," wherein a decrease in cell number in a leaf caused by a genetic defect leads to an enhanced cell expansion. This phenomenon serves as a key to understanding organ size regulation (Tsukaya, 2002, 2008; Beemster et al., 2003). When considering the mechanisms of organ size, two contrasting theories exist. Organismal perspective is based on the idea that organ size is determined independently from constituent cells (Kaplan, 2001). On the other hand, cell perspective employs an idea that cell is a basic unit that determines organ size. We employ neo-cell perspective that leaf size determination is mediated by cell–cell communication (Tsukaya, 2002). Indeed recently we demonstrated that cell–cell communication is a key mechanism behind compensation (Kawade et al., 2010).

The earliest report describing compensation was by Haber (1962) in a study characterizing leaf development using gamma-irradiated wheat grains. Cell division is severely compromised and overall leaf growth is significantly reduced in developing seedlings following gamma irradiation. Despite this, however, leaf morphogenesis occurs and cells in the leaf show larger expansion than control cells; thus, the reduction in cell proliferation triggers excessive cell expansion (Haber, 1962). In the modern molecular genetics era, compensation has been observed in transgenic plants in which the cell cycle was inhibited through manipulation of core cell cycle regulators such as CDKA;1 (Hemerly et al., 1995) and KIP-related protein2 (KRP2; De Veylder et al., 2001), and in mutants defective in positive regulators of cell proliferation, such as *AINTEGUMENTA* (*ANT*; Mizukami and Fischer, 2000) and *ANGUSTIFOLIA3*/*GRF-INTERACTING FACTOR1* (*AN3*/*GIF1*; Kim and Kende, 2004; Horiguchi et al., 2005). Many more instances have since been reported, mainly from *Arabidopsis thaliana* (**Table 1**) and other plant species such as *Oryza sativa* (Barrôco et al., 2006) and *Antirrhinum majus* (Delgado-Benarroch et al., 2009).

DEFECTIVE CELL PROLIFERATION TRIGGERS COMPENSATION

Changes in the number and size of leaf cells in response to the alterations of core cell cycle regulator activities have a seesaw-like relationship; enhanced and reduced cell proliferation negatively and positively affects postmitotic cell expansion, respectively. This relationship has held true in several cases in which the expression

Table 1 | Examples of compensation-exhibiting mutants and transgenic plants.

Gene	Type of mutation	Reference
AE7	Loss-of-function	Yuan et al. (2010)
AN3/GIF1	Loss-of-function	Kim and Kende (2004), Horiguchi et al. (2005)
ANT	Loss-of-function	Mizukami and Fischer (2000)
CDKA;1	Dominant negative	Hemerly et al. (1995)
CYCD3	Loss-of-functions of *CYCD3;1*, *CYCD3;2*, and *CYCD3;3*	Dewitte et al. (2007)
ER	Loss-of-function	Horiguchi et al. (2006), Ferjani et al. (2007)
ETG1	Loss-of-function	Takahashi et al. (2008)
FAS1	Loss-of-function	Exner et al. (2006), Ramirez-Parra and Gutierrez (2007)
FAS2	Loss-of-function	Exner et al. (2006)
FUGU1	Recessive mutation	Ferjani et al. (2007)
FUGU2	Recessive mutation	Ferjani et al. (2007)
FUGU3	Dominant mutation	Ferjani et al. (2007)
FUGU4	Dominant mutation	Ferjani et al. (2007)
FUGU5	Recessive mutation	Ferjani et al. (2007)
GPA1	Loss-of-function	Ullah et al. (2001)
ICK1	Overexpression	Wang et al. (2000)
KRP2	Overexpression	De Veylder et al. (2001)
MAX2	Loss-of-function	Horiguchi et al. (2006)
miR396	Overexpression	Liu et al. (2009), Rodriguez et al. (2010)
OLI	Loss-of-functions of *OLI2* and *OLI5* or *OLI2* and *OLI7*	Fujikura et al. (2009)
PFL2	Loss-of-function	Ito et al. (2000)
RPS6A	Loss-of-function	Horiguchi et al. (2011)
RPS21B	Loss-of-function	Horiguchi et al. (2011)
RPS28B	Loss-of-function	Horiguchi et al. (2011)
SMP	Epimutation	Clay and Nelson (2005)
SWP	Loss-of-function	Autran et al. (2002)
UVI4/PYM	Loss-of-function	Hase et al. (2006)

levels of core cell cycle regulators were manipulated. Transition from the cell cycle to endocycle occurs during leaf development. Differentiating cells often undergo several rounds of endocycle and expansion in a manner correlated with nuclear DNA content (Melaragno et al., 1993). A precocious transition from the cell cycle to endocycle increases the number of rounds of endocycle and causes leaves to have fewer and larger cells (Boudolf et al., 2004; Verkest et al., 2005). Conversely, *CYCD3;1* and *E2Fa* overexpression prolongs cell proliferation and inhibits the endocycle, resulting in the inhibition of cell expansion that usually takes place in association with endocycling (De Veylder et al., 2002; Dewitte et al., 2003).

In these instances, the cause is clearly altered cell proliferation activity. However, in some specific cases, cell number and size are regulated at the whole-plant level. The *more and smaller cells* (*msc*) mutants show increased cell number and reduced cell size in leaves (Usami et al., 2009). The *msc* phenotype seems to be the opposite of the prototypic compensation. This suggests that an increase in cell number is able to negatively affect cell size during leaf development. However, *msc* genes are not directly involved in the regulation of cell proliferation. Rather, they are associated with heteroblasty in which various leaf traits such as leaf shape, cell number, cell size, and trichome distribution progressively change during the transition from juvenile to adult phases (Usami et al., 2009). In the wild-type, cell number increases and cell size decreases in leaves formed at higher nodes. On the other hand, in *msc* mutants, such developmental changes take place faster than in the wild-type, indicating that the *msc* phenotype is caused by accelerated heteroblasty and not increased cell proliferation (Usami et al., 2009). The *msc1-D* mutant has an miR156 resistance mutation in the *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE15* (*SPL15*) gene, while *msc2* and *msc3* are new alleles of *PAUSED* (*PSD*) and *SQUINT* (*SQN*), respectively (Usami et al., 2009).

In the above instances increase or decrease in cell number is associated with decrease or increase of cell size, respectively as if they have a seesaw-like relationship. However, certain instances of compensation, at least of the prototype one, have different characteristics than this seesaw-like relationship. In the case of *ant*- and *an3*-induced compensation, cell number is fewer, but cell size is larger compared to the wild-type (Mizukami and Fischer, 2000; Horiguchi et al., 2005). In these cases, the cause of compensation is clearly defective cell proliferation, as *ANT* and *AN3* are expressed in young leaf primordia with active cell proliferation and are gradually downregulated as the leaf matures (Horiguchi et al., 2005; Kang et al., 2007). In contrast to these loss-of-function phenotypes, their overexpression promotes cell proliferation in leaf primordial; however, this does not cause inhibition of cell expansion (Mizukami and Fischer, 2000; Horiguchi et al., 2005). These observations suggest that for *ANT* and *AN3* compensation is induced only when their loss-of-function impairs cell proliferation. It is not yet clear what mechanistic differences determine whether unidirectional or seesaw-like compensation occurs, but the next issues to be resolved will involve identification of the transcriptional targets of AN3 and ANT and how these transcriptional regulators control cell proliferation.

These observations indicate that altered cell proliferation is a trigger for compensation. Conversely, is it possible that altered postmitotic cell expansion influences cell proliferation in the same leaf primordium? There is no clear evidence in support of this possibility; among the mutants with phenotypes characterized by either fewer but larger cells or more but smaller cells, no known genes have specific functions in postmitotic cell expansion. Rather, several lines of evidence support the suggestion that altered postmitotic cell expansion does not affect cell proliferation. There are mutants with a cell expansion-specific phenotype, but a normal number of leaf cells. Both *rotunda2* (*ron2*) and *rpl4d* enhance cell expansion in leaves without any changes in cell number (Cnops et al., 2004; Horiguchi et al., 2011). Similarly, the *bigpetal* (*bpe*) mutation increases epidermal cell size without affecting cell number in petals (Szécsi et al., 2006). A number of *extra-small sisters* (*xs*) mutants show specific reductions in final cell size in leaves (Fujikura et al., 2007a). Furthermore, the levels of *ATHB16* and *ARGOS-LIKE* (*ARL*) expression are negatively and positively correlated with epidermal cell size in leaves, respectively, without affecting cell number

(Wang et al., 2003; Hu et al., 2006). These observations suggest that modulation of cell expansion in many cases does not trigger compensation through cell proliferation. Taken together, these observations indicate that some specific types of compensation are unidirectionally induced only when cell number is decreased.

HOW IS COMPENSATION TRIGGERED?

As discussed above, a decrease in cell proliferation specifically induces compensation. This raises the question of how compensation is triggered. The observations that *rotundifolia4-D* (*rot4-D*) and *growth regulating factor5* (*grf5*) decrease cell number in the leaves, but fail to induce compensation provide insight into this question (Narita et al., 2004; Horiguchi et al., 2005). The *grf5* phenotype is especially important as GRF5 is an interacting partner of AN3, and both of these molecules promote cell proliferation (Horiguchi et al., 2005). The degrees of reduction in cell number in these mutants are milder than those of *ant* or *an3* (Narita et al., 2004; Horiguchi et al., 2005), suggesting that there is a threshold level of cell number or cell proliferation activity below which compensation is triggered. This was confirmed by two experiments. In the first experiment, a series of *AN3*-silenced lines were used. The leaf cell number was reduced at various levels according to the strength of *AN3* silencing, but compensation was triggered only when cell number was substantially reduced (Fujikura et al., 2009). In the second experiment, a series of *oligocellula* (*oli*) mutants were used. The leaves of these *oli* mutants had a reduced number of normal-sized cells (Fujikura et al., 2009). In contrast, *oli2 oli5* and *oli2 oli7* double mutants had fewer leaf cells than the parental single mutants and showed compensation (Fujikura et al., 2009). These three *OLI* genes encode a yeast NOP2 homolog that is involved in ribosome biogenesis (*OLI2*) and two ribosomal protein RPL5 paralogs (*OLI5* and *OLI7*; Fujikura et al., 2009). These findings were also consistent with the observation that strong ribosomal protein-defective mutants exhibit compensation (Ito et al., 2000; Horiguchi et al., 2011). Similar observations were made using *KRP2* and miR396. KRP2 inhibits CDKA;1 activity, while miR396 targets many members of the *GRF* family (De Veylder et al., 2001; Rodriguez et al., 2010). Overexpression of these genes impairs cell proliferation and induces compensation, but only in mutants showing strong expression (Verkest et al., 2005; Rodriguez et al., 2010). The existence of a threshold for the induction of compensation suggests that there is an unknown mechanism of sensing cell proliferation activity and modulating cell expansion during differentiation.

HOW ARE PROLIFERATING AND DIFFERENTIATING CELLS LINKED?

Since proliferating cells and differentiating cells coexist in the same leaf primordium during leaf development (Donnelly et al., 1999; Kazama et al., 2010), it is possible that a defect in cell proliferation affects postmitotic cell expansion in a non-cell autonomous manner. Alternatively, mitotic cells showing unusually low cell proliferation activity may enhance their own cell expansion after exiting the mitotic cycle. Studies of genetic chimeras showed both of these suggestions to be true depending on the trigger of the defects in cell proliferation. When chimeric leaves with *an3* mutant sectors and *AN3*-overexpressing sectors were examined using the CRE-*lox*P system, compensation was seen irrespective of leaf cell genotype (**Figures 1A–D**; Kawade et al., 2010). This suggested that a mobile signal is transmitted from the *an3* sectors to neighboring cells to enhance cell expansion. Interestingly, this signal seems unable to move laterally beyond the midvein in the leaf primordia, as a longitudinal half-and-half chimera separated by the midvein exhibited compensation only in the *an3* half (**Figure 1E**). On the other hand, *KRP2*-induced compensation occurred in a cell autonomous manner when wild-type sectors and *KRP2*-overexpressing sectors coexisted in leaves (**Figures 1A,F,G**; Kawade et al., 2010). There are two possible interpretations regarding how individual cells cause compensation on overexpression of *KRP2*. Since mitotic cells overexpressing *KRP2* are already larger than wild-type (Ferjani et al., 2007), such a difference may be maintained until maturation of the leaves. The other interesting interpretation is that cells "remember" very low levels of cell proliferation activity and show enhanced cell expansion based on this "memory" when they enter the postmitotic phase of leaf development (Fujikura et al., 2007b; Kawade et al., 2010). Thus, compensation appears to be a heterogeneous phenomenon, with multiple inputs and pathways leading to enhanced cell expansion.

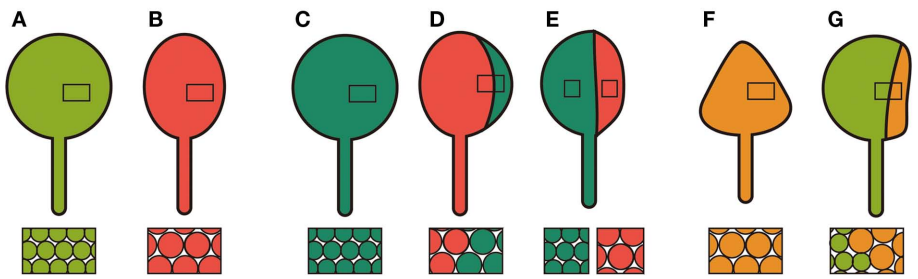

FIGURE 1 | Schematic diagram of chimeric leaves and cell size. (A) A wild-type leaf. **(B)** An *an3* leaf with cells larger than wild-type. **(C)** An *AN3*-overexpressor in the *an3* background with normal-sized cells. **(D)** A chimeric leaf consisting of *AN3*-overexpressing (deep green, right) and *an3* (red, left) cells. Cells are larger than wild-type regardless of genotype. **(E)** A half-and-half chimera. An *AN3*-overexpressing sector containing midrib (deep green, right) showed maintenance of normal cell size, while an adjacent *an3* sector (red, left) contained cells larger than those in the *AN3*-overexpressing sector. **(F)** A *KRP2*-overexpressing leaf with cells larger than wild-type. **(G)** A chimeric leaf consisting of wild-type (light green, left) and *KRP2*-overexpressing (yellow, right) sectors. Cells in the *KRP2*-overexpressing sector were larger than those in the wild-type sector.

HOW IS CELL EXPANSION ENHANCED?

When compensation is triggered, differentiating cells show enhanced cell expansion through at least two different mechanisms. One route is the endocycle. In some compensation-exhibiting mutants, such as *fasciata1* (*fas1*), *fugu2*, and *struwwelpeter* (*swp*), the ploidy distribution of leaves shifts to a higher level (Autran et al., 2002; Exner et al., 2006; Ferjani et al., 2007; Ramirez-Parra and Gutierrez, 2007). Timing of transition from mitotic cycle to endocycle can vary in different cells in the same tissue. Occurrence of endoreduplication coincides with cell expansion in epidermis (Beemster et al., 2005). This may explain correlation between ploidy level and cell size (Traas et al., 1998). In yeast, ploidy-associated changes in gene expression have been demonstrated and many of which encodes cell surface components or their regulators (Wu et al., 2010). However, it is not clear whether similar transcriptional changes occur in plants.

The second route is a ploidy-independent process. This is an ambiguous classification as no molecular components involved in this process have been identified and it does not mean that different compensation-exhibiting mutants without an increased ploidy level share a common mechanism of enhanced cell expansion. Some compensation-exhibiting mutants have a relatively normal ploidy level [e.g., *erecta* (*er*); Ferjani et al., 2007] or even a reduced ploidy level (e.g., *KRP2*-overexpressors; De Veylder et al., 2001). In tetraploid *Arabidopsis*, palisade cell size observed from the paradermal viewpoint is 1.6-fold larger than that in diploid counterparts (Tsukaya, unpublished). The palisade cells in *an3* are about 1.5-fold larger than those of the wild-type, but the increase in ploidy level is not significant (Ferjani et al., 2007; Fujikura et al., 2007), suggesting that a ploidy-independent process mediates part of the enhanced cell expansion. In the case of *an3*-induced compensation, three suppressor mutations, *xs1*, *xs2*, and *xs5*, were identified (Fujikura et al., 2007a). These *xs* mutants have normal numbers of smaller leaf cells in comparison to the wild-type, suggesting that genes involved in normal cell expansion are also required during compensation (Fujikura et al., 2007a). These *xs* mutants have normal (*xs1*), reduced (*xs2*), and increased (*xs5*) ploidy levels compared with wild-type (Fujikura et al., 2007a). At present, these phenotypes are difficult to interpret. It is possible that a certain ploidy level may be required to establish compensation, but a further increase in ploidy level is not necessarily required, at least in *an3*-induced compensation. These results also suggest that in certain genetic backgrounds, ploidy-associated and ploidy-independent compensation pathways are induced simultaneously. This puzzling situation would be solved when these *XS* genes are cloned and such experiments are in progress.

A comparative study using several compensation-exhibiting mutants, including *fugu* and *an3*, also suggested that multiple physiological pathways are involved in enhanced cell expansion during compensation (Ferjani et al., 2007). Most compensation-exhibiting mutants examined, such as *fugu2* and *an3*, show faster leaf cell expansion compared to the wild-type (Ferjani et al., 2007). One exception is *fugu5* in which the duration of cell expansion is extended compared to the wild-type (Ferjani et al., 2007). Thus, induction, transmission, and response processes of compensation are linked by multiple parallel pathways and further studies are required to determine whether these pathways are interconnected.

DOES COMPENSATION HAVE PHYSIOLOGICAL AND DEVELOPMENTAL ROLES?

In many cases, compensation does not fully restore leaf area. Thus, compensation is unlikely to be a mechanism that maintains an organ at a constant size. Compensation may play a role in an environmental response. When wild-type plants are irradiated with ultraviolet B (UV-B), the number of epidermal cell decreases and compensation is induced (Wargent et al., 2009). Curiously, *uv resistance locus8* (*uvr8*), which was recently shown to be defective in a UV-B receptor (Rizzini et al., 2011), shows reduced cell number in response to UV-B irradiation but does not show enhanced cell expansion (Wargent et al., 2009). An increase in ploidy level confers UV-B tolerance in both tetraploid *Arabidopsis* and *UV-B-insensitive4* (*uvi4*) mutants (Hase et al., 2006). Furthermore, the UV-B response includes induction of the *PHR1* gene encoding photolyase through downregulation of *DP-E2F-LIKE1* (*DEL1*), which is an endocycle repressor (Radziejwoski et al., 2011). These reports suggest that compensation associated with increased ploidy level may reflect part of the UV-B resistance mechanism.

In addition to the possibility that compensation reflects an environmental response, it may also represent a normal developmental mechanism for cell expansion. For example, the linkage of cell proliferation and postmitotic cell expansion may achieve fine-tuning of organ size, inhibition of premature entry into differentiation, or rapid change from proliferating to differentiating status. Although these possibilities remain to be confirmed, the establishment of the genetic framework of compensation over the past decade will enable these issues to be tested. For identification of the molecular components involved in compensation, more precise measurements and observations of cellular behavior in relation to developmental timing during compensation are needed in future experiments. The results that will be obtained from these approaches will help to elucidate underlying molecular mechanisms to gain insight into the physiological/developmental significance of compensation.

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