Non-cell-autonomously coordinated organ size regulation in leaf development

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SUMMARY

The way in which the number and size of cells in an organ are determined poses a central challenge in our understanding of organ size control. Compensation is an unresolved phenomenon, whereby a decrease in cell proliferation below some threshold level triggers enhanced postmitotic cell expansion in leaf primordia. It suggests an interaction between these cellular processes during organogenesis and provides clues relevant to an understanding of organ size regulation. Although much attention has been given to compensation, it remains unclear how the cellular processes are coordinated. Here, we used a loss-of-function mutation in the transcriptional coactivator gene ANGUSTIFOLIA3 (AN3), which causes typical compensation in Arabidopsis thaliana. We established Cre/lox systems to generate leaves chimeric for AN3 expression and investigated whether compensation occurs in a cell-autonomous or non-cell-autonomous manner. We found that an3-dependent compensation is a non-cell-autonomous process, and that an3 cells seem to generate and transmit an intercellular signal that enhances postmitotic cell expansion. The range of signalling was restricted to within one-half of a leaf partitioned by the midrib. Additionally, we also demonstrated that overexpression of the cyclin-dependent kinase inhibitor gene KIP-RELATED PROTEIN2 resulted in cell-autonomous compensation. Together, our results revealed two previously unknown pathways that coordinate cell proliferation and postmitotic cell expansion for organ size control in plants.

KEY WORDS: AN3, Compensation, Leaf chimera, KRP2, Organ size, Arabidopsis thaliana

INTRODUCTION

Organ size is determined by the number and size of constituent cells and is genetically regulated in a highly reproducible manner. Cell proliferation and postmitotic cell expansion are hence coordinated, yet the mechanisms behind these processes remain poorly understood.

In plants, spatiotemporal regulation of cell proliferation and postmitotic cell expansion is seen during leaf development. Cell proliferation is active throughout the developing leaf primordium, but it ceases from the distal to proximal region (Donnelly et al., 1999; Ichihashi et al., 2010; Kazama et al., 2010; Nath et al., 2003; White, 2006). There is a proximal-distal gradient of cell proliferation activity, whereby proliferation in the proximal region and postmitotic expansion in the distal region occur simultaneously in the same leaf primordium. During leaf development, a defect in cell proliferation often triggers enhanced cell expansion, a phenomenon that was first described nearly half a century ago in γ-irradiated wheat (Haber, 1962). More recently, similar phenomena have been reported using various mutants and transgenic strains of Arabidopsis thaliana (hereafter Arabidopsis) (Micol, 2009; Tsukaya, 2008). These phenomena have been collectively termed ‘compensation’ (Beemster et al., 2003; Tsukaya, 2002; Tsukaya, 2008). Compensation seems to occur only in determinate organs, such as leaves (Ferjani et al., 2007). It suggests an interaction of cell proliferation and expansion, and provides clues that are relevant to an understanding of organ size control (Tsukaya, 2008).

The loss-of-function mutant angustifolia3 (an3) and the overexpressor of the cyclin-dependent kinase inhibitor gene KIP-RELATED PROTEIN2 (KRP2 o/x) show features typical of compensation (De Veylder et al., 2001; Ferjani et al., 2007; Hemerly et al., 1995; Horiguchi et al., 2005). In the leaves of an3 and KRP2 o/x, cell number is decreased by more than 70% and 90% and cell size is increased by 50% and 100%, respectively, as compared with the wild type (WT) (De Veylder et al., 2001; Ferjani et al., 2007; Horiguchi et al., 2005). AN3 (which is also known as GFK-INTERACTING FACTOR1) encodes a transcriptional coactivator (Kim and Kende, 2004). KRP2, which is identical to Arabidopsis Cdc-2a-interacting protein ICK2, binds and inhibits A-type cyclin-dependent kinase, thereby restricting cell cycle progression (De Veylder et al., 2001; Lui et al., 2000; Verkest et al., 2005).

Studies on an3-dependent compensation (Ferjani et al., 2007; Fujikura et al., 2007a; Fujikura et al., 2009) have indicated that the size of mitotic cells is normal but that enhanced expansion occurs in postmitotic cells in the an3 mutant; furthermore, there is a threshold decrease in cell number or cell proliferation activity that induces compensation. Moreover, an3-dependent compensation is completely suppressed in a group of extra-small sisters (xs) mutants that are specifically defective in postmitotic cell expansion. These data suggest that an3-dependent compensation is not simply the result of a defect in cell proliferation; it probably involves an interaction between cell proliferation and postmitotic cell expansion.

We consider that compensation occurs in three successive steps. The induction step involves a defect in cell proliferation. The intermediary step links cell proliferation with postmitotic cell expansion through an unknown signal. This signal regulates the...
response step, which results in the intense expansion of postmitotic cells. In this study, we focused on the intermediary step because how the defect in cell proliferation is linked with postmitotic cell expansion is totally unknown. We assumed a cell-autonomous or non-cell-autonomous mode of action in the intermediary step (Fujikura et al., 2007b). Here, we induced leaf chimeras for AN3 or KRP2 expression to investigate the mechanism of compensation. Based on our results, we discuss the mechanisms that coordinate cell proliferation and postmitotic cell expansion for organ size control.

MATERIALS AND METHODS

Plant materials and growth conditions

The WT accession used in this study was Columbia-0. Plants were grown on rock wool at 22°C under a 16-hours light/8-hours dark cycle. Light intensity, at ~50 μmol m⁻² s⁻¹, was provided by white fluorescent lamps. Plants were watered daily with 0.5 g/l Hypoxon solution (Hyponex). For in vitro culture, surface-sterilised seeds were sown on Murashige and Skoog medium (Murashige and Skoog, 1962) containing Gamborg's B5 vitamins (Gamborg et al., 1968), 3% (w/v) sucrose and 0.5% (w/v) gellan gum (pH 5.8).

Transgenic plants for clonal analysis

Vectors for clonal analysis were constructed as described below. A chimeric gene encoding AN3 tagged with triple green fluorescent protein (3xGFP) at its C-terminus was produced as follows. The WT accession used in this study was Columbia-0. Plants were grown at 37°C for 60 minutes at 1 day after sowing (DAS). We selected a particular (Fig. 1A) (referred to as HSP::Cre; AN3-act) or repressed (Fig. 1B) in the an3-4 background were crossed with an3-4/HSP::Cre. Transgenic lines harbouring 35S::lox::GUS::lox::AN3-GFP-β-glucuronidase (GUS) affected normal leaf development (see Fig. S1 in the supplementary material). We confirmed that AN3-3xGFP had a comparable cell proliferation-promoting activity to native AN3, and that our AN3 activation and deletion systems were properly established for the analyses described below (see Figs S2 and S3 in the supplementary material).

RESULTS

Strategy for clonal analysis using AN3 chimeric leaves

We established heat shock (HS)-dependent AN3 activation and deletion systems (Fig. 1) by applying a Cre/lox-mediated recombination system (Y. Ichihashi, G.H., T. Takahashi and H.T., unpublished) to yield pGEM AN3-3xGFP. A chimeric gene encoding 3xGFP that was C-terminally fused with KRP2 (3xGFP-KRP2) was generated as follows. The KRP2 cDNA flanked by an Xhol and a SalI site was amplified by PCR using oligonucleotides 5′-CTCGAGTTAATCCCGCTCCAC-3′ and 5′-ATCTGATGATTTCCTTAT-3′ and inserted into the pGEM T-Easy vector. A 3xGFP was excised by Xhol and SalI and inserted into the SalI site of the pGEM T-Easy vector (Promega). Then, an internal Xhol site of the AN3 cDNA was replaced by a synonymous mutation using PCR-mediated site-directed mutagenesis with oligonucleotides 5′-TTGAGAGGACGCAGCCGAGGAAA-3′ and 5′-TTCCTCGAGCTTGTACCACATC-3′ and cloned into the pGEM T-Easy vector. The resultant cDNA fragment was excised by Xhol and SalI and inserted into the Xhol site of pGEM 3xGFP that contained 3xGFP with the termination codon (Y. Ichihashi, H.G., T. Takahashi and H.T., unpublished) to yield pGEM AN3-3xGFP.

Establishment of transgenic lines has been described previously (Clough and Bent, 1998; Fujikura et al., 2009). At least two independent single-insertion lines were established for each construct.

Generation of leaves chimeric for AN3 and KRP2 expression

The genetic strategy for clonal analysis by Cre/lox-mediated recombination was based on previously described methods (Serralbo et al., 2006; Sieburth et al., 1998). Transgenic lines harbouring 35S::lox::GUS::lox::AN3-GFP-β-glucuronidase (GUS) and 35S::lox::AN3-GFP (AN3-del) in an an3-4 background were crossed with an3-4/HSP::Cre. Transgenic lines harbouring 35S::lox::GUS::lox::AN3-GFP (AN3-act) or 35S::lox::AN3-GFP (AN3-del) in a WT background were crossed with HSP::Cre. F1 progeny, with both constructs (referred to as an3-4/HSP::Cre; AN3-act) or repressed (Fig. 1B) in the an3-4 background were crossed with HSP::Cre. F1 progeny, with both constructs (referred to as an3-4/HSP::Cre; AN3-act, an3-4/HSP::Cre; AN3-del, HSP::Cre; KRP2-act and HSP::Cre; KRP2-del in this study) were used for clonal analyses. For generation of sectorial or spotted leaves chimeric for AN3 expression, heat shock (HS) at 37°C was carried out for 60 minutes with 1- or 4-day-old seedlings, respectively. For generation of sectorial or spotted leaves chimeric for KRP2 expression, HS at 37°C was carried out for 90 minutes with 2- or 6-day-old seedlings, respectively.

Microscopy

For morphological and cellular analyses, the first leaves dissected from 21-day-old plants were briefly centrifuged before observation, as described previously (Horiguchi et al., 2006). Whole leaves were observed under a stereoscopic microscope (MZ16a; Leica Microsystems) and palisade cells in the subepidermal layer were examined using a Nomarski differential interference contrast microscope (DMRX/E; Leica Microsystems). The average leaf area (n=8 leaves) and cell size (n=160 cells from at least eight leaves) were measured according to Fujikura et al. (Fujikura et al., 2009). The total cell number of palisade cells in the subepidermal layer was estimated according to Horiguchi et al. (Horiguchi et al., 2005). Excitation of GFP was performed by standard methods.

an3-dependent compensation acts in a non-cell-autonomous manner

Chimeric leaves were generated in an3-4 harbouring single HSP::Cre and 35S::lox::GUS::lox::AN3-3xGFP (AN3-act) copies (Fig. 1A) (referred to as an3-4/HSP::Cre; AN3-act) by HS at 37°C for 60 minutes at 1 day after sowing (DAS). We selected a particular type of leaf chimaera that contained a longitudinal AN3 overexpressor (o/x) sector (Fig. 2A). These plants had asymmetric leaf blades because of the effect of this sectorial expression of AN3-3xGFP (Fig. 2A). We could clearly observe the boundary between GFP-positive...
and GFP-negative cells, suggesting that intercellular AN3-3xGFP movement was unlikely (see Fig. S4 in the supplementary material). Following HS, the AN3 o/x genotype extended through various cell layers (including epidermis, palisade tissue and/or spongy tissues; data not shown). We measured cell size in the regions where the cells in all layers had either the AN3 o/x or the an3-4 genotype (Fig. 2A, squares). We excluded the sector boundaries from our analyses because in these regions we found aberrantly small cells (see Fig. S4 in the supplementary material).

In these leaf chimeras, we found that AN3 o/x cells showed full compensation that was comparable to an3-4 cells in the leaf chimaera of the same line and also in non-chimeric an3-4 leaves (Fig. 2B-H). This result suggests that an3-dependent compensation is a non-cell-autonomous process, and that an3-4 cells have a dominant role in intercellular cell size regulation in these chimeric leaves.

The shape of the clones in our leaf chimeras might have affected cell expansion physically. Additionally, cell size might to be predetermined depending on the genotype of the leaf primordia before HS. In these cases, the presence of this intercellular signalling can be questioned. Thus, we investigated chimeric leaves containing clones of another shape induced by an AN3 deletion system in which AN3-3xGFP was removed by HS (Fig. 1B). We determined the HS conditions required for induction of chimeric leaves in which small clusters of AN3 o/x cells were randomly distributed amongst a background of those with the an3-4 genotype (Fig. 2I). Chimeric leaves were generated in the an3-4 harbouring single HSP::Cre and 35S::lox:AN3-3xGFP::lox::GUS (AN3-del) copies (referred to as an3-4/HSP::Cre; AN3-del) by HS at 37°C for 90 minutes at 4 DAS. We found that even AN3 o/x cells in the chimeric leaves showed full compensation (Fig. 2J-P). By contrast, compensation was absent in non-HS AN3 o/x cells in the same line (Fig. 2P). This result demonstrated that final cell size was not predetermined in very early leaf primordium. Furthermore, the shapes of the clones had no effect on cell size. These data indicate that an3-dependent compensation is induced non-cell-autonomously.

**Intercellular signalling in an3-dependent compensation is restricted to within one-half of a leaf partitioned by a midrib**

Most of our leaf chimeras contained a longitudinal AN3 o/x sector; in some cases, the AN3 o/x cells were separated from the an3-4 cells by a midrib of AN3 o/x cells (Fig. 3A). The sizes of the AN3 o/x and an3-4 cells in these chimeric leaves were similar to those of the WT and an3-4 mutant, respectively, regardless of the distance from the sector boundaries (Fig. 3B,C). This finding indicates that intercellular signalling in an3-dependent compensation in these chimeric leaves was restricted to within one-half of a leaf blade partitioned by the midrib. In agreement with this idea, AN3 o/x cells that co-existed with the an3-4 cells on one side of the midrib showed compensation, whereas AN3 o/x cells on the other side of the midrib without an3-4 cells did not (see Fig. S5 in the supplementary material).
We induced chimeric leaves containing a KRP2 system in order to account for the effect of clone distribution on cell size (Fig. 4F). We found that the sizes of o/x and WT cells in these chimeric leaves were similar to those of the constitutive KRP2 system (Fig. 4A). The leaf morphology of the chimera was similar to that of the constitutive KRP2 line (Fig. 4A; see Fig. S6 in the supplementary material). The experimental design for KRP2 overexpression was similar to that described for an3. We analysed the leaf phenotypes of the an3 o/x sector by HS treatment (37°C for 60 minutes at 2 DAS) of the transgenic line harbouring single HSP::Cre and 35S::lox::GUS::lox::3xGFP-KRP2 (KRP2-act) copies in a WT background (referred to as HSP::Cre; KRP2-act) (Fig. 4G,H). The KRP2 o/x cells showed compensation, whereas the WT cells did not (Fig. 4I-K). In contrast to an3 leaf chimeras, enhanced cell expansion was consistently observed in KRP2 o/x cells, even along the sector boundaries (Fig. 4I-K). These results demonstrated that compensation induced by KRP2 overexpression occurred cell-autonomously.

**KRP2 has no direct function in enhanced cell expansion in postmitotic cells**

Compensation in KRP2 o/x was found to be regulated in a cell-autonomous manner, in contrast to that described for an3. This raises the possibility that KRP2 might have a direct effect on the enhancement of cell expansion in postmitotic cells. Studies have reported that KRP2 is expressed more abundantly in expanding and endoreduplicating tissue than in the actively proliferating tissues in the WT (Ormenese et al., 2004; Verkest et al., 2005). Thus, we investigated the effect of KRP2 overexpression in postmitotic cells. We analysed the HSP::Cre; KRP2-act line in which KRP2 overexpression was induced at 4, 7 and 10 DAS by HS at 37°C for 90 minutes. In these plants, GFP signal was detected in most cells in the first leaves (data not shown). Our previous data indicated that cell proliferation terminates at ~11 DAS in the first leaves of the WT (Ferjani et al., 2007). If KRP2 directly enhances the expansion of postmitotic cells, cells in this line should exhibit compensation in response to HS even after exiting the mitotic cell cycle. When KRP2 overexpression was induced at 4 DAS, it affected leaf morphology (Fig. 5A), and compensation was observed throughout the leaves (Fig. 5B). Induction of KRP2 overexpression at 7 DAS resulted in induction of compensation in the proximal region of the first leaves, but the size of the cells in the distal region was similar to that of non-HS cells of the same line (Fig. 5B). These results might be explained by a proximal-distal gradient of cell proliferation activity because at 7 DAS, postmitotic cells were observed in the distal region of the first leaves, whereas actively proliferating cells were still seen proximally (Ferjani et al., 2007). Importantly, no enhanced cell expansion was observed when KRP2 overexpression was induced at 10 DAS (Fig. 5B). These data demonstrate that to induce compensation KRP2 must be overexpressed in cells at the stage of active proliferation.

**an3 has an additive effect on cell proliferation in KRP2 o/x**

To investigate the genetic relationship between an3 and KRP2 o/x in leaf development, we analysed the leaf phenotypes of the an3 mutant overexpressing KRP2. The an3-4 mutation decreased the cell number in the first leaves by 73%, as compared with the WT. The first leaves of an3 KRP2 o/x showed a similar decrease in cell number (70%), compared with KRP2 o/x (Fig. 6A). This result indicated that the an3-4 mutation had an additive effect on cell proliferation in KRP2 o/x. The size of the cells in an3 KRP2 o/x was increased, compared with those in an3 and KRP2 o/x (Fig. 6B-D,G), indicating that enhanced cell expansion in an3 is further promoted by compensation induced by KRP2 overexpression. The leaf area of an3 KRP2 o/x was decreased compared with that of WT, an3-4 and KRP2 o/x, owing to the decrease in the number of cells (Fig. 6C,H).

**Cell-autonomous action of compensation induced by KRP2 overexpression**

The results reported here describe a non-cell-autonomous mechanism that coordinates cell proliferation and postmitotic cell expansion during leaf development. To determine whether this is a common property of the coordination mechanism, we investigated whether compensation induced by KRP2 overexpression is mediated in a cell-autonomous or non-cell-autonomous manner using a KRP2 deletion system (Fig. 4A; see Fig. S6 in the supplementary material). The experimental design for KRP2 clonal analysis was similar to that described for an3.

We induced leaves chimeric for KRP2 expression by HS treatment (37°C for 60 minutes at 6 DAS) using the KRP2 deletion system (Fig. 4A). The leaf morphology of the chimera was similar to that of the constitutive KRP2 o/x (Fig. 4B; see Fig. S6 in the supplementary material), but there was a co-existence of GFP-positive (KRP2 o/x) and GFP-negative (WT) cells (Fig. 4C,D). We found that the sizes of KRP2 o/x and WT cells in these chimeric leaves were similar to those of the constitutive KRP2 o/x line and the WT, respectively (Fig. 4E). These data indicated that compensation induced by KRP2 overexpression is regulated in a cell-autonomous manner.

We analysed chimeric leaves induced by a KRP2 activation system in order to account for the effect of clone distribution on cell size (Fig. 4F). We found that the sizes of o/x and WT cells in these chimeric leaves were similar to those of the constitutive KRP2 o/x line and the WT, respectively (Fig. 4E). These data indicated that compensation induced by KRP2 overexpression is regulated in a cell-autonomous manner.
DISCUSSION

In this study, we demonstrated that two qualitatively different modes (i.e. non-cell-autonomous and cell-autonomous modes in an3 and KRP2 o/x, respectively) are involved in the coordination of cell proliferation and postmitotic cell expansion in leaves. Genetic analysis of an3-4 KRP2 o/x indicated that the an3 mutation and KRP2 overexpression trigger compensation through different mechanisms.

Our findings from clone analysis provide important insights into the coordination mechanisms that operate between cell proliferation and postmitotic cell expansion. We showed that even AN3 o/x cells exhibited full compensation when they co-existed with an3-4 cells in a chimeric leaf. This result suggests that an intercellular signalling molecule (‘factor X’) that enhances postmitotic cell expansion is abundantly produced in the an3-4 cells and transmitted from cell to cell. Furthermore, we observed a distinct type of intercellular signalling from a specific type of chimeras: when AN3 expression was induced in one-half of a chimeric leaf, AN3 o/x cells did not show compensation, although an3-4 cells on the other side did. This suggests that the intercellular signalling mechanism involved in an3-dependent compensation is limited to within one-half of a leaf. The discovery of subdivided compartments in the leaf primordium will provide novel insight into the intercellular mechanisms that are responsible for the coordination of cellular processes during leaf development.

A positive correlation is often seen between cell size and the level of endoreduplication (e.g. Melaragno et al., 1993); however, the ploidy level of leaf cells in the an3 mutant is relatively normal (Fujikura et al., 2007a). This suggests that factor X modulates an endopolyploidy-independent cell expansion pathway. Previous analyses have shown that enhancement of cell expansion in the an3 mutant is due to an increase in the rate, not the period, of endopolyploidy-independent cell expansion (Ferjani et al., 2007). In addition, a subset of XS genes involved in normal cell expansion has a role in the an3-dependent compensation. The xs1, xs2, xs4 and xs5 mutant lines show decreased cell size, but normal cell numbers, in leaves. In xx an3 double mutants, an3-dependent compensation is fully suppressed (Fujikura et al., 2007a). Thus, factor X seems to

Fig. 4. Cell-autonomous regulation of compensation in KRP2 o/x. (A) Structure of the DNA construct for the KRP2 deletion system. HS treatment induced the expression of Cre recombinase, which removed the 3xGFP-KRP2 region. As a consequence of recombination, WT cells were generated and are visually distinguishable from KRP2 o/x cells by the loss of the GFP signal. (B) A chimeric Arabidopsis leaf in which small cell clusters with the KRP2 o/x genotype were randomly distributed in a WT background induced by the KRP2 deletion system. (C, D) Bright-field (C) and GFP fluorescence (D) images of palisade cells in the chimeric leaves observed from a paradermal view. GFP fluorescence (green) marks the nuclei of KRP2-overexpressing cells (arrowhead). Typically sized KRP2 o/x and WT cells are outlined in red and blue, respectively. (E) The size of the palisade cells in the WT, constitutive KRP2 o/x, and in leaf chimera with KRP2 o/x and WT genotypes. The mean ± s.d. from eight individual leaves is indicated. (F) Structure of the DNA construct for the KRP2 activation system. HS treatment induced the expression of Cre recombinase, which removed the GUS region. As a result of recombination, KRP2 o/x cells were generated in which the nucleus is marked with a GFP signal. (G, H) A chimeric leaf containing a longitudinal KRP2 o/x sector induced by the KRP2 activation system (H). WT (grey) and KRP2 o/x (green) clones are distributed as illustrated (J). (I) The size of the palisade cells in the WT, KRP2-act homozygote, and in leaf chimera with KRP2 o/x and WT genotypes. The regions where cell size was measured (a–d) are indicated in H. The size of the palisade cells in non-HS HSP::Cre; KRP2-act leaves is shown as a control. The mean ± s.d. from eight individual leaves is indicated. (J, K) Bright-field (I) and GFP fluorescence (K) images of palisade cells at the boundary between the WT and KRP2 o/x sectors in chimeric leaves, as observed from a paradermal view. GFP fluorescence marks the nucleus of KRP2-overexpressing cells (arrowheads). Typically sized KRP2 o/x and WT cells are outlined in red and blue, respectively. Scale bars: 5 mm in B, G; 50 μm in C–J.
accelerate the normal cell expansion pathway in which the \textit{XS1}, \textit{XS2}, \textit{XS4} and \textit{XS5} genes might be involved. Alternatively, one of them might encode factor X itself. The cloning of these genes and functional analyses are now in progress in our laboratory.

In contrast to \textit{an3}-dependent compensation, we showed that compensation in \textit{KRP2 o/x} functions in a cell-autonomous manner. \textit{KRP2} is structurally and functionally related to \textit{KRP1}, which has been shown to act non-cell-autonomously (De Veylder et al., 2001; Weinl et al., 2005). This suggests that native \textit{KRP2} might also inhibit cell proliferation non-cell-autonomously in leaf primordia. However, in our system, the overexpressed \textit{KRP2} was designed to be incapable of intercellular movement by tagging with 3xGFP, and this showed that the compensation was a cell-autonomous process. These facts indicate that a downstream pathway in the inhibition of cell cycle progression by \textit{KRP2} acts in a cell-autonomous manner.

Cells in \textit{KRP2 o/x} leaves are twice the size of those of the WT during the stage of cell proliferation (De Veylder et al., 2001; Ferjani et al., 2007). Therefore, cells overexpressing \textit{KRP2} enter the postmitotic process with more cytoplasmic components than WT cells. The cell-autonomous action of compensation in \textit{KRP2 o/x} might be a result of abundant cytoplasm. Alternatively, it has been speculated that a defect in cell proliferation might be memorised in each cell. In fission yeast, circular ribosomal DNA accumulates in direct proportion to the number of cell divisions and causes ageing (Sinclair and Guarente, 1997). The mechanism that links cell proliferation and cell expansion in a single cell remains an enigmatic, but interesting, issue.

Our experimental systems involved overexpression of \textit{KRP2}; therefore, the physiological significance of this effect should be carefully considered. In the present study, we demonstrated that induction of compensation requires the ectopic overexpression of \textit{KRP2} during the stage of active cell proliferation. This indicates that compensation is caused by a defect in mitotic cell cycling. A recent study indicated that DELLA proteins are likely to suppress cell proliferation activity in early leaf development by promoting the expression of cyclin-dependent kinase inhibitor genes, including \textit{KRP2} (Achard et al., 2009). Nevertheless, the accumulation of DELLA proteins suppresses postmitotic cell expansion without induction of compensation (Achard et al., 2009). Therefore, it would be worth considering the effect of \textit{KRP2} overexpression in actively proliferating cells on the subsequent postmitotic cell expansion in which the DELLA factors are involved.

In summary, our studies show that during normal leaf development, cell proliferation is linked with postmitotic cell expansion in a cell-autonomous and a non-cell-autonomous manner. These findings should provide novel insight into the mechanism of organ size control in plants.

Acknowledgements
We thank Prof. T. Takahashi (Okayama University, Japan) and Dr H. Ichikawa (National Institute of Agrobiological Science, Japan) for providing seeds of the \textit{HSP::Cre} lines and pSMAB704 binary vector, respectively. This work was supported by grants-in-aid for Creative Scientific Research (No. 18GS0313 to H.T.), Scientific Research on Priority Areas (No. 19060002 to H.T.), Scientific Research A (No. 17207005 to H.T. and G.H.), Exploratory Research (No. 19K13171 to H.T. and G.H.), and Scientific Research on Priority Areas (No. 16H06287 to H.T.) awarded by the Ministry of Education, Culture, Sports, Science and Technology (Japan).
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18657020 to G.H.), a fellowship from the Japan Society for the Promotion of Science (JSPS) (No. 217214 to K.K.) and the Toray Science Foundation (to H.T.). This article is freely accessible online from the date of publication.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.057117/-/DC1

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