Dissection of Enhanced Cell Expansion Processes in Leaves Triggered by a Defect in Cell Proliferation, with Reference to Roles of Endoreduplication

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Leaf development relies on cell proliferation, post-mitotic cell expansion and the coordination of these processes. In several Arabidopsis thaliana mutants impaired in cell proliferation, such as angustifolia3 (an3), leaf cells are larger than normal at their maturity. This phenomenon, which we call compensated cell enlargement, suggests the presence of such coordination in leaf development. To dissect genetically the cell expansion system(s) underlying this compensation seen in the an3 mutant, we isolated and utilized 10 extra-small sisters (xs) mutant lines that show decreased cell size but normal cell numbers in leaves. In the xs single mutants, the palisade cell sizes in mature leaves are about 20–50% smaller than those of wild-type cells. Phenotypes of the palisade cell sizes in all combinations of xs an3 double mutants fall into three classes. In the first class, the compensated cell enlargement 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 with 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 a part of the network that regulates cell expansion, which is required for normal leaf expansion.

Keywords: Angustifolia3 — Cell expansion — Compensated cell enlargement — Extra-small sisters — Leaf — Ploidy.

Abbreviations: an3, angustifolia3; rh1, root hairless1; xs, extra-small sisters

Introduction

The determination of the sizes of plant cells and how this mechanism contributes to the determination of organ size has been a matter of debate. In leaves, it is clear that the mature leaf size corresponds to the final number of cells multiplied by the average size of cells in a leaf, since the leaf is a determinate organ. However, cell size is not always correlated with leaf size in plants impaired in cell proliferation. For example, γ-irradiated wheat grains exhibit no cell division after germination, but develop leaves with significantly larger cells (Haber 1962). Likewise, the inhibition of cyclin-dependent kinase in Nicotiana tabacum and Arabidopsis thaliana (hereafter, Arabidopsis) results in the development of leaves that contain significantly fewer, but larger cells, as compared with the corresponding wild-type plants (Hemerly et al. 1995, Wang et al. 2000, De Veylder et al. 2001). Furthermore, a number of Arabidopsis mutants showing such phenotypes have been reported (Ito et al. 2000, Mizukami and Fischer 2000, Ullah et al. 2001, Autran et al. 2002, Clay and Nelson 2005, Horiguchi et al. 2005). These studies have suggested that cell expansion is affected by cell proliferation activity during leaf development. We call this phenomenon 'compensated cell enlargement', and consider it as an important key in the understanding of how cell proliferation and cell expansion are coordinated during leaf development (Tsukaya 2002, Beemster et al. 2003, Tsukaya 2003, Horiguchi et al. 2005, Tsukaya 2006).

In this study, we focused on the mechanisms of compensated cell enlargement observed in the mutant angustifolia3 (an3), also known as grf-interacting factor1 (gif1; Kim and Kende 2004), since an3 is one of the best characterized compensation-exhibiting mutants (Horiguchi et al. 2005). The an3 mutants have palisade cells that amount to 70% fewer, but are 50% larger, than in wild-type plants (Horiguchi et al. 2005). AN3 encodes a homolog of the human transcription coactivator 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). AN3 is thought to be a positive regulator of cell proliferation in leaves (Horiguchi et al. 2005). Compensated cell enlargement can be theoretically separated into two distinct processes: the induction process,
which involves the reduction of cell proliferation activity during leaf development; and the response process, during which cell expansion is enhanced. In the present study, we tried genetic dissection of the response process in \textit{an3}. It was clear that cell expansion-defective mutants would be convenient for this purpose. Although we had previously reported the isolation of a large number of small-leaf mutants, most of the mutants we characterized had defects in both cell proliferation and cell expansion (Horiguchi et al. 2006a, Horiguchi et al. 2006b). The same was also true for well-known dwarf mutants, such as \textit{constitutive triple response1} and \textit{dwarf1}, although they had been wrongly reported to have a specific defect in cell size (Nakaya et al. 2002, Horiguchi et al. 2006b). Since mutants defective in both cell expansion and cell proliferation are unfavorable for the genetic dissection of the compensated cell enlargement, cell expansion-specific mutants are indispensable for genetic analysis of the response stage. Therefore, in this study, we isolated and utilized 10 mutants specifically impaired in cell expansion, named \textit{extra-small sisters} (xs).

On the other hand, it is possible that compensated cell enlargement might depend on an increase in endopolyploidy level since positive correlations between cell size and endopolyploidy level have often been reported (e.g. Melaragno et al. 1993). During leaf development, the cell cycle often switches into endocycles coinciding with differentiation (Verkest et al. 2005a). To address this issue, we used flow cytometry in order to examine the involvement of endoreduplication in relation to the compensated cell enlargement in \textit{an3}. Based on the results of the above two analyses, we discuss the genetic relationships of the cell expansion pathways in normal leaf development and compensated cell enlargement.

\section*{Results}

\textit{The xs mutants have smaller leaves than the wild type due to reduced cell size}

To dissect compensated cell enlargement in \textit{an3} genetically, it was necessary to identify cell expansion-defective mutants with normal or near-normal cell proliferation in leaves, since compensated cell enlargement occurs along with reduced cell proliferation. From our vast collection of leaf size and shape mutants with defects in the number and size of palisade cells (Horiguchi et al. 2006a, Horiguchi et al. 2006b), we selected 10 lines, each with a specific defect in palisade cell expansion but with normal palisade cell numbers. We named these mutants \textit{extra-small sisters} (xs). Based on the segregation patterns of backcrossed F\textsubscript{2} populations and allelism tests among \textit{xs} mutants, all of the \textit{xs} mutations were found to be non-allelic, single recessive mutations (data not shown).

Fig. 1 shows the rosette phenotypes of the \textit{xs} mutant plants. Most of the \textit{xs} mutants clearly produce smaller
most of the xs mutants have small rosette phenotypes with proportional or minor changes: 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: xs6 had the fewest palisade cell number among the xs mutants examined and, even in this case, it was 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 compensated cell enlargement in an3.

We also examined the epidermal cell size in the xs mutants. Distributions of the adaxial epidermal cell area in the leaves are presented in Fig. 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 lower 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, we 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, D).

Identification of xs mutants that suppress compensated cell enlargement

On the genetic mechanisms of compensated cell enlargement, we first examined whether the massive cell enlargement observed in compensated cell enlargement depends on the activation of cell expansion systems that are also required for normal leaf development, or not. To address this, we produced double mutants between the xs mutants and an3 (Fig. 5), a mutant that exhibits typical compensated cell enlargement (Horiguchi et al. 2005), and determined the leaf size and the number and size of palisade cells in these plants (Fig. 6). In an3, the number and size of palisade cells were about 30 and 140% of wild-type values, respectively (Fig. 6). If the affected genes in the xs mutants play a major role in compensated cell enlargement, the cell size of the xs an3 double mutants would be the same as in the parental xs mutants.

As a result, we 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 compensated cell enlargement. 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 compensated cell enlargement. Hence, we concluded that compensated cell enlargement occurs through the activation of cell expansion systems that also act in normal leaf development.

**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 compensated cell enlargement occurs in a ploidy-dependent or -independent manner, we 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 (xs4 and xs10), in comparison with wild-type plants (Fig. 7). In the large-cell class, the ploidy levels were unaffected (xs6) (Fig. 7).

![Fig. 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 3-week-old first leaves (n=170, from seven leaves).](https://academic.oup.com/pcp/article-abstract/48/2/278/2329754)

![Fig. 4 Lengths of roots and dark-grown hypocotyls in the xs mutants. Wild-type and xs mutant seedlings were collected 8 d 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 12 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 10 hypocotyls). The results are expressed relative to wild-type values ± SD.](https://academic.oup.com/pcp/article-abstract/48/2/278/2329754)
suggesting again that the compensated cell enlargement is independent of the status of endoreduplication.

Apart from compensated cell enlargement, 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 that 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 with 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.

**Discussion**

*Normal cell expansion pathways are enhanced during compensated cell enlargement*

To understand the mechanisms that link cell proliferation and cell expansion during leaf organogenesis, we genetically dissected the cell expansion systems underlying the compensation syndrome. The most important finding in this study was that the small-cell class of xs mutations (xs1, xs2, xs4 and xs5) strongly suppressed the compensated cell enlargement triggered by the an3 mutation (Fig. 6A). This finding demonstrates that compensated cell enlargement is mediated, in part, by the hyperactivation of a cell expansion system that also functions during normal leaf development. On the other hand, an alternative interpretation is that the cell enlargement observed in the an3 mutant was not a result of hyperactivation of the cell expansion system and could have resulted from the extended period of cell expansion. However, our preliminary experiments suggested that the period of cell expansion in an3 is similar to that of the wild-type plant, but the rate of cell expansion in an3 is clearly increased in comparison with that of the wild-type plant (A. Ferjani et al. unpublished results). Hence, we concluded that the compensated cell enlargement is a result of hyperactivation of cell expansion pathways that require XS genes in the small-cell class. In other words, there should be some linking mechanism(s) between cell proliferation systems and a part of cell expansion systems. The isolation of these mutant loci, which is underway in our laboratory, should provide clues leading to an understanding of the regulation of compensated cell enlargement. This result also supports our previous idea that compensated cell enlargement is not a simple uncoupling between cell cycling and
Fig. 6 Characterization of xs an3 double mutants. First leaves from 3-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 ± SD.

Fig. 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 3-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.
cell growth (Tsukaya 2002, Tsukaya 2003, Tsukaya 2005, Beemster et al. 2006, Tsukaya 2006). 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 common target gene(s) exist that are regulated by a cell expansion process involving the XS6 gene and also by compensated cell expansion. The activity of such gene products would be lower in xs6 than in wild-type plants. Despite this, in the xs6 an3 background, compensated cell enlargement over-rides the effects of the xs6 mutation on the activity of common targets. Therefore, cell expansion pathways can be distinguished according to their epistasis to compensated cell enlargement. 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. Our results with the xs and an3 mutants strongly suggest that cell expansion pathways also form networks of which a subset, represented by the XS genes of small-cell class, is linked to the cell proliferation pathway. 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.

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. It is believed that 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 (Autron et al. 2002, Verkest et al. 2005b). However, no such correlation was found in an3 or most of the xs mutants: a positive correlation among cell size, cell number and ploidy levels was found in only two of the 10 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 topoisoenserase 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 (data not shown) 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 for 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 AtCDTI1a locus. AtCDTI1a and b encode licensing factors of DNA replication (Castellano et al. 2004). Without appropriate CDTI function, DNA replication proceeds in an unscheduled manner, causing impaired cell expansion for unknown reasons (Raynaud et al. 2005). We have carried out a rough mapping of the XS5 locus and placed it on the top portion of chromosome 2 (data not shown), corresponding to neither the AtCDTI1a nor the AtCDTI1b locus. No other recessive loci that cause atcdti1-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 compensated cell enlargement.

The seven other xs mutants, xs1, xs3, xs4 and xs6–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 that 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 understand better 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 facts behind the control of leaf cell enlargement: compensated cell enlargement 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 networks exist for the control of leaf cell expansion. Further analyses based on the above findings will supply new clues leading to an understanding of the global mechanisms in the control of leaf organogenesis at the organ level.

Materials and Methods

Plant materials

The wild-type accession of *A. thaliana* used in this study was Col-0. A null allele of *an3-3* (Horiguchi et al. 2005) was used as a representative of mutants showing compensated cell enlargement. The *xs* mutants were identified from the M2 progeny of a fast-neutron-mutagenized population as small-leaf mutants in the Col-0 background and were backcrossed three times to Col-0. The plants were grown for 3 weeks under a 16 h light/8 h dark fluorescent illumination cycle (approximately 48 μmol m⁻² s⁻¹) at 22 °C as described previously (Horiguchi et al. 2005), and the first set of leaves was used for microscopic observation.

To produce double mutants, the *an3* and *xs* mutants were crossed and mutants exhibiting phenotypes of either parent were collected from F₂ progeny. In the next generation, putative double mutants that segregated with phenotypes similar to those of either parent were selected. The putative double mutants were crossed to the other parental mutant. The phenotypes of the F₁ plants were confirmed regardless of whether the parental phenotypes were observed to determine the genotypes of the putative double mutants.

To determine root and hypocotyl lengths, seeds were sown on Murashige and Skoog medium supplemented with 1% (w/v) sucrose and Gamborg’s B5 vitamins (Gamborg et al. 1968), and solidified with 0.4% (w/v) Gellan gum (Murashige and Skoog 1962). The seeds were then incubated at 4 °C in darkness for 3 d. After the cold treatment, the seedlings were grown for 8 d with continuous fluorescent illumination (approximately 26 μmol m⁻² s⁻¹) at 22 °C. The seedlings were grown on vertically placed plates to measure the root length. For hypocotyl measurements, seedlings were grown on plates in darkness for 8 d.

Microscopic observations

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 4 °C for 12 h, cleared with a chloral hydrate solution as described (Tsuge et al. 1996), and observed under microscopes. The samples were then photographed under the microscopes and the cell size and leaf size were determined using Image J (http://rsb.info.nih.gov/ij/; NIH, MD, USA). For each leaf, >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, we created a replica image of the adaxial epidermis using the dried-gel method (Horiguchi et al. 2006b) and determined the pavement cell size using Image J.

Ploidy measurements

To measure the nuclear DNA content, the first set of leaves from 21-day-old seedlings was used. The ploidy level was determined using flow cytometry and EPICS XL (Beckman Coulter Inc., CA, USA), as described (Kozuka et al. 2005).

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