Doctoral Dissertation (Censored)

博士論文（要約）

# Studies on the regulatory mechanism of leaf cell number and cell size focusing on *ANGUSTIFOLIA3*

（*ANGUSTIFOLIA3* に焦点を当てた葉の細胞数および細胞サイズ制御機構の解明）

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Department of Biological Sciences, Graduate School of Science,
The University of Tokyo

東京大学大学院理学系研究科

生物科学専攻

Kazune Ezaki

江崎　和音

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## Abstract

Leaves and floral organs exhibit determinate growth and their size is determined by cell number and cell size. In those organs, decreased cell number is often seen with increased cell size. This phenomenon is called compensation, since enhanced cell expansion seems to compensate deficient cell proliferation. Compensation has been found in many mutants, transgenics, and natural variants of various species. A number of examples imply the coordination system between cell proliferation and cell expansion. However, the mechanism of compensation has not been revealed in most of the cases. Since it has been suggested that compensation is mediated by heterogeneous pathways, detailed analyses of each case are required. In this study, I focused on a mutant of *angustifolia 3* (*an3*) and analyzed *an3* compensation from several aspects.

*AN3* encodes a transcriptional coregulator which plays a significant role in cell proliferation regulation. In *an3* mutant, leaf cell number is decreased to be about 30% of wild type, while cell size is increased to about 150% of wild type. Although much knowledge has been accumulated about AN3 function, its roles in cell size regulation have not been known. I utilized *extra-small sisters 2* (*xs2*) mutant, which has been identified as a suppressor of *an3* compensation, to reveal a role of *xs2* in compensated cell enlargement (CCE) in *an3*. I also observed leaf development in detail to find out factors acting in *an3* CCE. In this study, I showed several factors taking a role in *an3* CCE and found novel phenotypes of *an3* mutant.

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## Abbreviations

| | |
|---|---|
| AN | ANGUSTIFOLIA |
| ANT | AINTEGUMENTA |
| APC/C | anaphase-promoting complex/cyclosome |
| AVP | ARABIDOPSIS THALIANA V-PPASE |
| BCECF | 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester |
| CCE | compensated cell enlargement |
| CCX | CATION CALCIUM EXCHANGER |
| CDK | CYCLIN-DEPENDENT KINASE |
| CESA | CELLULOSE SYNTHASE A |
| Col-0 | Columbia-0 |
| DAS | day-after-stratification |
| DET | DE-ETIOLATED |
| DEG | differentially expressed gene |
| ECH | ENOYL-COA HYDRATASE |
| EdU | 5-ethynyl-2'-deoxyuridine |
| ER | ERECTA |
| F1 | filial 1 |
| FAA | formalin-acetic acid-alcohol |
| FAS | FASCIATA |
| FDR | false discovery rate |
| FE-SEM | field emission-scanning electron microscope |
| FM4-64 | N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide |
| GA | gibberellin |
| GFP | green fluorescent protein |
| GIF | GRF-INTERACTING FACTOR |
| GRA | GRANDIFOLIA |
| GRF | GROWTH-REGULATING FACTOR |
| ICS | isochorismate synthase, ISOCHORISMATE SYNTHASE |
| KRP | KIP-RELATED PROTEIN |

| | |
|---|---|
| miR | microRNA |
| mRNA-seq | messenger RNA sequencing |
| NAC | NO APICAL MERISTEM/ATAF/CUP-SHAPED COTYLEDON |
| NPR | NONEXPRESSER OF PR GENES, NPR1-LIKE PROTEIN |
| ox | overexpression |
| PAL | phenylalanine ammonia-lyase |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| qRT-PCR | quantitated real-time PCR |
| RbohD | RESPIRATORY BURST OXIDASE HOMOLOGUE D |
| ROS | reactive oxygen species |
| SA | salicylic acid |
| SNH | SYT N-terminal homology |
| SWI/SNF | SWITCH/SUCROSE NONFERMENTING |
| SYT | synovial translocation |
| T-DNA | transfer DNA |
| UV-B | ULTRAVIOLET-B |
| UVI | ULTRAVIOLET-B INSENSITIVE |
| V-ATPase | vacuolar-type H+-ATPase |
| VND | VASCULAR RELATED NAC-DOMAIN |
| WT | wild type |
| XS | EXTRA-SMALL SISTERS |

## Chapter I: General introduction

In organ development, cell proliferation and cell expansion are coordinated. Decreased cell number and increased cell size sometimes co-occur in determinate organs in plants, such as leaves and floral organs. This phenomenon is called compensation because increased cell expansion seems to compensate deficient cell proliferation. Compensation has been seen in many mutants, transgenics, as well as natural variations (Tsukaya 2002, 2003, Beemster 2003). The mechanism of compensation has been investigated and it shows that compensation occurs via heterogeneous pathways (Ferjani et al. 2010, Horiguchi and Tsukaya 2011, Hisanaga et al. 2015). Since cell number and cell size are two main determining factors of plant organ size, especially in the determinate organs, the coordination mechanism between cell proliferation and expansion is one of the significant issues.

### Compensation system for deficient cell proliferation by enhanced cell enlargement

One of the oldest reports of compensation is by Haber (1962) on a gamma-irradiated *Triticum vulgare* leaf. After heavy gamma irradiation, wheat seeds produce much smaller leaves in which cell division is almost inhibited but cell enlargement is strongly increased (Haber 1962). In addition, compensation phenomenon has been found in many mutants and transgenics of *Arabidopsis thaliana* (L.) Heynh. (hereafter Arabidopsis), *Nicotiana tabacum*, and *Oryza sativa* as described below. It is also seen in some cases of dwarf natural variants which are specialized to live in island or alpine environment (Körner 1999, Tsukaya 2002). While compensation has been found in various plant species, in most of the cases, mechanisms have not been elucidated.

During leaf and floral organ development, cell proliferation firstly occurs and cell expansion follows in many species. In Arabidopsis leaves for example, after the emergence of a leaf primordium, cell division occurs throughout the primordium for first few days. While cells in the basal part of a leaf blade continue to proliferate, cells near the leaf tip and around the midrib cease to proliferate

earlier. It makes cell proliferation zone restricted to the leaf base and blade-and-petiole junctions for a while. Finally, the cell proliferation zone disappears and the leaf rapidly expands by cell expansion (Pyke et al. 1991, Donnelly et al. 1999, Kazama et al. 2010, Andriankaja et al. 2012). Considering the order from proliferation to expansion during the leaf development, the compensatory system for deficient cell proliferation by enhanced cell expansion seems reasonable. Although the opposite pattern also has been found where cell number is increased and cell size is decreased (Usami et al. 2009), in terms of "compensation" for deficient cell proliferation, the cases where cell number is decreased and cell size is increased are focused. Here, several types of compensation are reviewed. In many cases, the cause of cell proliferation defect is clear but the mechanism of enhanced cell expansion in compensation, which is specifically called "compensated cell enlargement (CCE)," has not been known.

**Various compensation examples – Cell cycle inhibition, deficient ribosome biogenesis, and reduced cell proliferation activity**

Compensation is often seen in mutants or transgenics of cell cycle regulators, such as overexpression lines of *KIP-RELATED PROTEIN 1* (*KRP1*), *KRP2*, *KRP3*, dominant-negative mutated *CYCLIN-DEPENDENT KINASE A* (*CDKA*) or *CDKB1;1* in Arabidopsis, tobacco, and rice (Hemerly et al. 1995, Wang et al. 2000, De Veyler et al. 2001, Boudolf et al. 2004, Verkest et al. 2005, Barrôco et al. 2006, Jun et al. 2013). In addition, a mutant of anaphase-promoting complex/cyclosome (APC/C) inhibitor and mutants of 26S proteasome subunit components also show compensation (Hase et al. 2006, Kurepa et al. 2009, Sonoda et al. 2009). Since those genes control cell cycle regulators, mutated genes could interrupt the mitosis. Taken together, in above cases, cell cycle is inhibited and it leads to deficient cell proliferation. In many cases, the ploidy level is often increased suggesting that interrupted cell cycle leads to early exit from mitosis and endoreduplication onset. Since increased

ploidy level is sometimes show a correlation with cell size (Melaragno et al. 1993, Roeder et al. 2010), it may have a partial role in CCE. However, petals or rice leaves also exhibit increased cell size (Barrôco et al. 2006, Kurepa et al. 2009, Tsukaya 2013) in which endoreduplication is limited or does not occur (Barow and Meister 2003, Hase et al., 2005). It suggests that another factor is also required. Other than one case of *KRP2*ox, which will be described below, CCE-inducing factors have not been revealed in cell cycle-inhibited cases.

Some mutants of ribosome proteins or factors involved in ribosome processing also exhibit compensation (Lijsebettens et al. 1994, Ito et al. 2000, Fujikura et al. 2009, Yuan et al. 2010, Horiguchi et al. 2011b). In animals, perturbed ribosome biogenesis causes a stress response called ribosomal stress response which leads to cell cycle arrest, senescence, or apoptosis (Olausson et al. 2012). In plants, similar stress response is induced in ribosome-related mutants and they exhibit characteristic phenotypes such as narrow and pointed leaf shape (Byrne 2009, Horiguchi et al. 2011b, 2012, Tsukaya et al. 2013, Ohbayashi and Sugiyama 2018). In those mutants, cell number is decreased to various degrees. On the other hand, cell size seems to be increased only when cell number is severely decreased (Horiguchi et al. 2011b). It implies a threshold system which senses decreased cell number or proliferation ability and induces CCE. Although a key mediator of ribosomal stress response in plants has been revealed (Ohbayashi et al. 2017), regulators of CCE and the putative threshold mechanism have not been clarified.

A mutant of APETALA2-like transcription factor *AINTEGUMENTA* (*ANT*), which positively regulates cell proliferation (Krizek 1999), also shows compensation in petals and leaves (Mizukami and Fisher 2000). In *ANT* overexpression line, while cell number is increased, cell size is comparable to that of wild type (Mizukami and Fisher 2000). It suggests that the opposite induction of smaller cells does not occur. Although it is clear that the mutation causes cell proliferation defect, the trigger of CCE has not been revealed.

**Three classifications of compensation in Arabidopsis**

Although various mutants and transgenics show compensation in different manners, some common pathways may exist. In Arabidopsis compensation-exhibiting mutants, classification has been attempted based on the mode of CCE (Ferjani et al. 2007, 2013a). There are three classes differing in the increase rate of cell expansion and a timing of CCE. In class I, cell expansion rate is increased in post-mitotic phase. In class II, cell expansion duration is extended. In class III, CCE occurs from cell proliferation phase with high cell expansion rate (Ferjani et al. 2007). Suppressor mutants have been found for each class and they do not suppress CCE of the other classes (Ferjani et al. 2013, Katano et al. 2016, Fujikura et al. 2020), suggesting that these compensation phenomena are mediated by different mechanisms.

Class I includes *angustifolia 3* (*an3*), *fugu 2*/*fasciata 1* (*fas1*), and *erecta* (*er*) (Ferjani et al. 2007). In *fas1*, which has a deficit in a large subunit of Chromatin Assembly Factor 1 (Kaya et al. 2001), DNA damage response is activated and it leads to delayed cell cycle and earlier endocycle entry, resulting in fewer and larger cells (Hisanaga et al. 2013). On the other hand, CCE in *an3* and *er* cannot be explained by enhanced endoreduplication (Ferjani et al. 2007). A mutant named *extra-small sisters 2* (*xs2*) was reported to suppress all class I CCE (Fujikura et al. 2020).

Class II is apparent in cotyledons and weakened in foliage leaves. Similar compensation phenotypes have been found in several mutants such as *fugu5*/*arabidopsis thaliana v-ppase 1*, glyoxylate enzyme mutants, and gluconeogenesis-defective mutant. It has been suggested that sucrose deficiency, which is caused by perturbed gluconeogenesis, triggers cell proliferation defect and CCE in class II compensation (Ferjani et al. 2011, Takahashi et al. 2017). In addition, a mutant of *ENOYL-COA HYDRATASE 2* (*ECH2*) specifically suppresses CCE regardless of cell number (Katano et al. 2016, Takahashi et al. 2017). ECH2 catalyzes the conversion of indole-3-butyric acid to IAA (Strader et al. 2010), suggesting that class II CCE may be related to auxin response and/or homeostasis. Since

auxin has a role in cell wall loosening (reviewed by Majda and Robert 2018), it is possible that auxin alters cell wall extensibility leading to larger cell size.

In class III, *KRP2*ox shows CCE from proliferation phase. It is caused by increased activity of vacuolar-type $H^+$-ATPase (V-ATPase) (Ferjani et al. 2013) and suppressed by a mutant *de-etiolated 3* which has a defect in a subunit C of the V-ATPase (Shumacher et al. 1998, Fukao and Ferjani 2011, Fukao et al. 2011, Ferjani et al. 2013). Thus, early vacuolar development appears to cause CCE in class III compensation. It should be noted that in some *KRP2*ox lines showing severe phenotypes, ploidy level is unchanged. It indicates that endoreduplication is not related to CCE in this case (De Veylder et al. 2001, Ferjani et al. 2007). Although the mechanism is not known how the V-ATPase activity is activated, it might be possible that it is shared in several cell cycle-inhibited cases.

As shown in the above-mentioned studies, compensation found in various species would be heterogeneous phenomena. On the other hand, several compensations share the same feature suggesting that several common pathways may also exist, as seen in class II compensation. In this study, compensation in the *an3* mutant (*an3* compensation, afterward) was focused. Since AN3 has a significant role in plant development and its functions have been widely studied as mentioned below, it could be a model system to examine the compensation phenomenon.

**AN3 roles in the organ development**

AN3, also known as GRF-INTERACTING FACTOR 1 (GIF1), is a member of GIF family which consists of AN3, GIF2, and GIF3 in Arabidopsis. They work redundantly in various tissues and AN3 has a main role among them (Kim and Kende 2004, Lee et al. 2009, Lee et al. 2014, 2018, Ercoli et al. 2018). GIFs are homologs of human synovial translocation (SYT) protein also known as synovial sarcoma associated protein (SS18) and they conserve SYT N-terminal homology (SNH) domain (Clark et al. 1994, Thaete et al. 1999, Kim and Kende 2004, de Bruijn and Kessel 2006). SYT interacts

with BRAHMA and BRAHMA HOMOLOG 1, which are components of SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complex, thorough SNH domain and work as a transcriptional coactivator (Nagai et al. 2001, Perani et al. 2003, Middeljans et al. 2012). Similarity to SYT, AN3 itself lacks DNA binding domain and it recruits SWI/SNF chromatin remodeling complex to regulate the expression of target genes (Vercruyssen et al. 2014, Debernardi et al. 2014, Nelissen et al. 2015). In the plant lineage, AN3 may have obtained a new role as a transcriptional corepressor in addition to one as a transcriptional coactivator (Nelissen et al. 2015, Ercoli et al. 2018).

GROWTH-REGULATING FACTORs (GRFs) are plant specific transcription factors and some of them interact with AN3 (Kim and Kende 2004, Horiguchi et al. 2005, Vercruyssen et al. 2014, Debernardi et al. 2014). Most of the GRFs enhance cell division activity. For example, overexpression of *GRF2*, *GRF3*, or *GRF5* leads to a larger leaf because of increased cell number (Horiguchi et al. 2005, Gonzalez et al. 2010, Rodriguez et al. 2010, Debernardi et al. 2014, reviewed by Omidbakhshfard et al. 2015, Kim 2019). In maize leaf development, different GRFs are interacted with AN3 in cell proliferation and cell expansion phases and it is suggested that AN3 plays a significant role in regulating the transition from proliferation to expansion by changing the interactors (Nelissen et al. 2015).

Relationship between AN3 and ANT has been also suggested. In flower development, *AN3*, *GRF8*, and *KLUH*/*CYTOCHROME P450 FAMILY 78 SUBFAMILY A POLYPEPTIDE 5* have been suggested as potential targets of ANT (Krizek et al. 2020). In addition, the expression levels of *AN3* and *ANT* are decreased in *ant* and *an3* mutant, respectively (Jun et al. 2019). These indicates that significant regulators of cell proliferation may regulate each other to mediate cell proliferation activity.

Another important feature of AN3 is the intercellular movement. In leaf primordia, *AN3* is expressed in mesophyll cells and synthesized protein moves from mesophyll to epidermal cells (Kawade et al. 2013). It also makes a gradation in the proximal-distal direction where the protein level

is high in the proximal part and low in the distal (Kawade e al. 2017). If AN3 movement is perturbed, cell proliferation activity is limited resulting in less cell number compared to wild type (Kawade et al. 2013, 2017). The protein movement and tissue-specific expression are also seen in rice leaves, though the protein movement direction is opposite (Shimano et al. 2018). Furthermore, protein movement is also suggested in floral organs (Kinoshita et al. unpublished). Thus, the ability of intercellular movement is conserved among several tissues and species and it would be important to coordinate proliferation activity between tissues.

While AN3 functions in cell proliferation during organ development have been revealed as summarized above, *an3* compensation mechanism has not been revealed.

**Compensation in *an3* mutant**

In *an3* mutant, leaf cell number decreases to about 30% of wild type and cell size is increased to about 150% (Horiguchi et al. 2005). In kinematic analyses, it has been suggested that *an3* mutant ceases cell proliferation earlier and starts cell expansion earlier compared to wild type (Ferjani et al. 2007, Lee et al. 2009). In addition, the cell expansion rate in post-mitotic phase was increased in *an3* (Ferjani et al. 2007, Lee et al. 2009). The timing when the cell expansion rate becomes the highest was similar or only slightly earlier in *an3* mutant compared with that of wild type (Ferjani et al. 2007, Nozaki et al. 2020), suggesting that similar length of the cell expansion phase. It has not been known how cell expansion rate is increased in *an3*.

In *AN3* RNAi lines, CCE was induced when cell number is severely decreased, suggesting a threshold in reduction of cell number (Fujikura et al. 2009). As a further investigation, *an3* mutant was crossed with *grandifolia1-D* (*gra1-D*) mutant to increase cell number. In *gra1-D* mutant, a part of the chromosome is duplicated and cell number is more than double of wild type. It is due to longer cell proliferation duration (Horiguchi et al. 2009). In the *an3 gra1-D* double mutant, cell number in a

mature leaf was comparable to that of wild type, whereas cell size was increased as much as *an3* cells. It suggests that the cell proliferation activity more likely affects CCE induction, not the final cell number (Horiguchi et al. unpublished). This result can be interpreted that cell proliferation rate in *an3 gra1-D* was low enough to induce CCE regardless of longer cell proliferation to produce as many cells as in wild type.

Analyses utilizing *AN3*-chimeric leaf system, in which *AN3* can be induced partially in a leaf, revealed that if *an3* cells and *AN3*-expressing cells coexist in a leaf, both cells exhibit CCE. It indicates that *an3* CCE-inducing factor(s) works non-cell-autonomously (Kawade et al. 2010). It should be noted that it is, however, not seen in epidermal cells. In epidermis, only *an3* cells exhibit CCE in *AN3*-chimeric leaves (Nozaki et al. 2020). These results suggest that *an3* CCE behavior is different depending on tissues, though the detailed mechanisms have not been revealed yet.

Based on these previous studies, *an3* compensation mechanism has been proposed as follows. In *an3* mutant, cell proliferation activity is severely reduced because of the absence of AN3, which is a cell proliferation promoting factor. Reduced cell proliferation triggers CCE post-mitotically and non-cell autonomously in palisade tissue cells (Horiguchi et al. 2005, Ferjani et al. 2007, Fujikura et al. 2007b, Kawade et al. 2010, Hisanaga et al. 2015). However, CCE-inducing factors and detailed CCE processes have not been uncovered to date.

In this study, I attempted to find out the factors which is involved in *an3* CCE to reveal the mechanism of *an3* compensation. To this end, I used *xs2*/*ccx4* mutant, which has been identified as *an3* compensation suppressor, to examine the role of XS2 in *an3* CCE (Chapter II). In addition, I investigated processes of *an3* CCE in detail to uncover factors which contribute cell size increase (Chapter III).

## Chapter II: Characterization of *extra-small sisters 2*, *an3* compensation suppressor

### Introduction

In the large mutagenesis experiment and following selection of altered leaf size/shape mutants (Horiguchi et al. 2006a, 2006b), *extra-small sisters* (*xs*) have been isolated as cell expansion deficient mutants. Out of ten *xs* mutants, four lines including *xs2* have been identified as a suppressor of compensated cell enlargement (CCE) in *angustifolia3* (*an3*) mutant. Since those *xs* mutants specifically suppressed *an3* CCE not affecting cell number, they were expected to be good materials to explore *an3* compensation mechanism (Fujikura et al. 2007a). Recently, Fujikura et al. (2020) have found that *xs2* mutant has 8-bp deletion within *CATION CALCIUM EXCHANGER 4* (*CCX4*) which encodes a putative membrane cation transporter (Morris et al. 2008). A mutant line having T-DNA insertion in *CCX4* also shows smaller leaves as well as smaller cells, and F1 generation of *xs2* and *ccx4* mutants did not show any phenotypic differences compared to each parent, indicating that *ccx4* is a causal mutation of *xs2*. In *xs2* and *ccx4* mutants, salicylic acid (SA) level was increased 1.96- and 2.59-fold higher, respectively, compared to that in wild type. In an experiment spraying exogenous SA, cell size is reduced in wild type and more reduced in *an3* mutant, suggesting that high SA accumulation causes smaller cells in *xs2*/*ccx4* mutants and it also negatively affects CCE in *an3* compensation (Fujikura et al. 2020). Thus, further analysis on the role of *xs2*/*ccx4* in *an3* CCE was expected to give more insights about *an3* compensation.

SA is a phytohormone playing a role in biotic and abiotic stress responses as well as growth control in acclimation to environment. It is synthesized in a plastid and cytosol from chorismate, via two independent pathways: isochorismate synthase (ICS) pathway or phenylalanine ammonia-lyase (PAL) pathway. ICS pathway is thought as a main pathway and it has not been known whether these two pathways have division of roles. Genes involved in these SA-biosynthesis pathways are upregulated in response to pathogen infection (Nawrath and Métraux 1999, Wildermuth et al. 2001,

Garcion et al. 2008, Huang et al. 2010). There are two types of SA receptors, one is NONEXPRESSER OF PR GENES 1 (NPR1) working as a transcriptional coactivator and the other is NPR1-LIKE PROTEIN 3 (NPR3) and NPR4 as transcriptional corepressors (Fu et al. 2012, Wu et al. 2012, Ding et al. 2018). High level endogenous SA often leads to not only enhanced pathogen resistance but also growth defect, as seen in mutants of *CONSTITUTIVE EXPRESSION OF PR GENES 5* (Bowling et al. 1997), *ACCELERATED CELL DEATH 6* (Rate et al. 1999), and the small ubiquitin-related modifier E3 ligase, *SAP AND MIZ1 DOMAIN CONTAINING-LIGASE 1* (Catala et al. 2007, Lee et al. 2007). Those mutants exhibit several features such as small or abnormal cell shape, cell death, and defects in trichome development. These traits are suppressed by introducing *npr1* mutation or *NahG* transgene which encodes bacterial SA hydroxylase (Delaney et al. 1994, Bowling et al. 1997, Rate et al. 1999, Kirik et al. 2001, Miura et al. 2010). Some of the features induced by highly accumulated SA are associated with less endoreduplication or changed expression patterns of xyloglucan endotransglucosylase/hydrolase genes (Kirik et al. 2001, Miura et al. 2010).

The causal gene of *xs2* mutant, *CCX4* encodes a putative $H^+$-dependent $Na^+$, $K^+$ exchanger (Morris et al. 2008) but the detailed function in plants remain to be delineated. In the CCX family, *CCX3* is the most closely related gene to *CCX4* (Shigaki et al. 2006). *CCX3* is about 80% identical to *CCX4* in nucleotide sequences, and they share a similar structure having five and seven transmembrane domains with a long interval of hydrophilic region (Morris et al. 2008). In Arabidopsis, it is suggested that CCX3 localizes in vacuolar membrane and has a role in $Na^+$, $K^+$, and $Mn^{2+}$ homeostasis. Overexpression of *AtCCX3* in tobacco, it causes necrotic lesion in leaves with the increased level of protein oxidation. It is argued that altered cellular cation levels lead to accumulate excess reactive oxygen species (ROS) (Morris et al. 2008). As ROS and SA signaling show interactive activation (León et al. 1995, Neuenschwander et al. 1995, Rao et al.1997, Shirasu et al. 1997), SA level could be also increased in the *AtCCX3*-overexpressed tobacco. Considering the similarity of *CCX3* and *CCX4*,

defects in *CCX4* may change the cation balance in *xs2* mutant leading to excess accumulation of ROS and SA. Since information described so far fragmentarily connects *ccx4* mutation, SA accumulation, and small cell phenotype in leaves, further examinations were done in this study to examine the involvement of SA response pathway in *an3* CCE. In addition, to investigate the relationship between *xs2*/*ccx4* and *an3*, kinematic analysis of leaf development was conducted.

## Results

### II-i. *xs2*/*ccx4* showed cell death phenotype in mature leaves

It has been suggested that highly accumulated SA in *xs2*/*ccx4* mutants causes smaller cells (Fujikura et al. 2020). To examine more details, cell death phenotype was examined since it is often seen under SA overaccumulation (Vanacker et al. 2001). Mature first leaves were stained with trypan blue which dyes only dead cells. As a result, more densely stained parts were seen in *xs2*, *ccx4*, and *an3 ccx4* compared to wild type and *an3* (Fig. 1A). In microscopy observation, these stained parts were mostly seen in mesophyll cells (Fig. 1B). This mesophyll specific staining was consistent with a previous study which observed cell death phenotype in a SA overaccumulation mutant (Vanacker et al. 2001). This result supported that *xs2*/*ccx4* phenotype is strongly related to highly accumulated SA.

### II-ii.

(This part contains unpublished data.)

## Discussion

In this chapter, the relationship between *xs2*/*ccx4* and *an3* CCE was genetically investigated with special emphasis on cell enlargement process. Results obtained in this study strongly suggested that activated SA signaling is the cause of small cell phenotype in *xs2*/*ccx4* mutant. It is consistent with the previous results showing that *xs2* and *ccx4* accumulate excess amount of SA and that exogenous SA treatment decreases cell size (Fujikura et al. 2020). Cell death phenotype in *xs2*/*ccx4* mutants and previously reported lower ploidy level in *xs2* (Fujikura et al. 2007a) are also consistent with excess accumulation of SA.

(This part contains unpublished data.)

## Materials and Methods

### Plant materials and growth conditions

The wild-type accession used in this study was Columbia-0 (Col-0). Seeds of Col-0 was given by Dr. Kawade (NIBB), and those of *an3-2*, *xs2*, *ccx4-1* (SALK_113447) were from Dr. Fujikura (Kobe university). Original descriptions are: *an3-2* in Horiguchi et al. 2005 and *xs2* in Fujikura et al. 2007. *ccx4-1* are obtained from the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/). Seeds were sown on rockwool (Nittobo, Tokyo, Japan; Grodan, Roermond, Netherlands), daily watered with 0.5 g $L^{-1}$ Hyponex (Hyponex). Plants were grown under white fluorescent lamps (50 – 90 μmol $m^{-2}$ $sec^{-1}$) at 22℃, in a long-day photoperiod of 16-hour light/8-hour dark. For trypan blue staining and shoot morphology comparison, plants were grown in continuous light.

### Trypan blue staining

The protocol followed Fernández-Bautista et al. (2016). Leaves were harvested with tweezers and immersed in the fresh trypan blue staining solution (10 mg/ml trypan blue dissolved in solution containing equal amounts of lactic acid (85% w/w), phenol (TE buffered, pH 7.5 – 8.0), glycerol, and distilled water). After one-hour staining, leaves were washed with 99% EtOH, replacing EtOH several times until the leaves were bleached. After the bleaching, leaves were mounted with 60% glycerol solution for the microscopy observation (MZ16a and DM4500; Leica Microsystems).

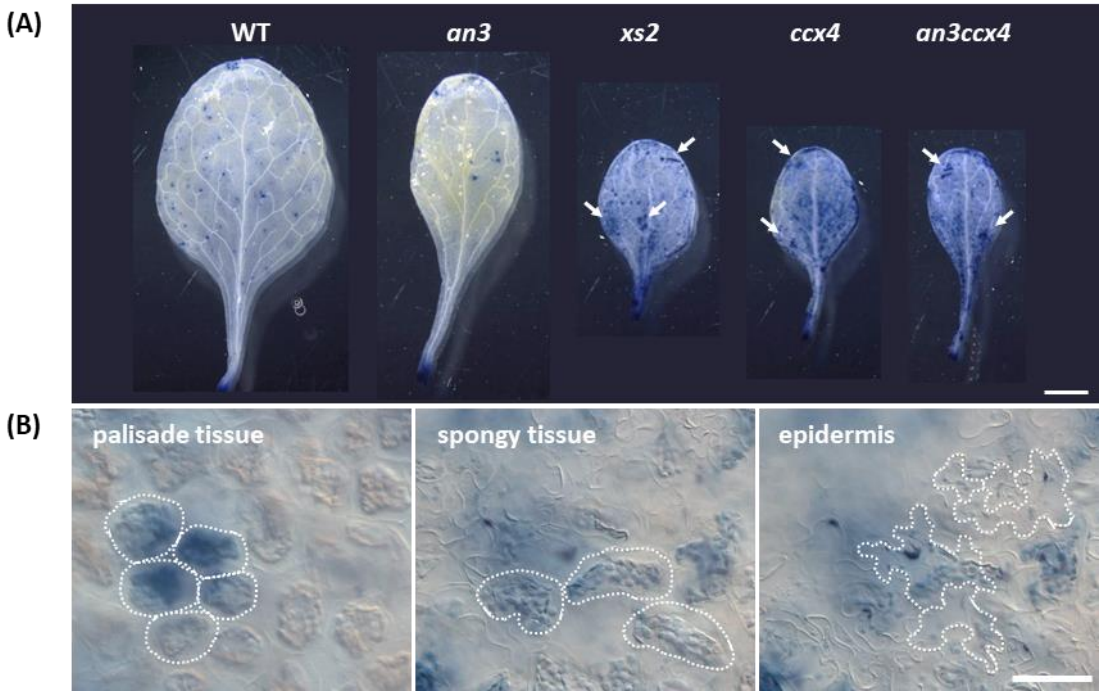


**Figure 1.** Trypan blue staining in mature first leaves.

**(A)** First leaves on 20 DAS of wild type (WT), *an3*, *xs2*, *ccx4*, and *an3 ccx4* stained by trypan blue. Leaves of *xs2*, *ccx4*, and *an3 ccx4* were more stained compared to those of WT and *an3*. Arrows indicate densely stained parts. **(B)** Cells in *xs2* leaf stained by trypan blue. Palisade tissue, spongy tissue, and abaxial epidermal cells were taken at the same position changing the z-stack position. Dotted lines exhibit representative cell shape. Bars = 1 mm (A), 50 μm (B).

## Chapter III. Examinations of compensated cell enlargement mechanism in *angustifolia3* mutant

(Chapter III will be published within five years, so that it will not be open.)

## Chapter IV: General Discussion

Compensation phenomenon in which decreased cell number co-occurs with increased cell size indicates the coordination system between cell proliferation and cell expansion in organogenesis. It has been suggested that compensation mechanism is mediated by several different pathways. Although some key factors of compensated cell enlargement (CCE) have been found in some cases, in many other cases, compensation mechanisms are still unknown. In this study, focusing on compensation in *angustifolia3* (*an3*) mutant, which has a defect in key regulator of cell proliferation, the mechanism inducing CCE was investigated.

(This part contains unpublished data.)

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