Title
Importance of cell division angle, position of cell proliferative area, and localization of AN3 in lateral organ morphology

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Abstract

Leaf meristem is a cell proliferative zone present in the lateral organ primordia, and it contributes to the expansion of lateral organ lamina. In this study, we investigated how the proliferative zone affects the final morphology of the lateral organs. We examined how cell proliferative zones differ in the primordia of polar–auxin transport inhibitor (PATI)-treated leaves and floral organs from normal foliage leaf primordia of Arabidopsis thaliana with focus on the spatial accumulation pattern of mRNA and protein of ANGUSTIFOLIA3 (AN3), a key element for leaf meristem positioning. As a result, we revealed that organ shape change by PATI treatment could not be attributed to changes in leaf-meristem positioning, size of the leaf meristem, or the expression pattern of AN3. Instead, it was attributed to altered cell division angles in the leaf meristem. In contrast, different shapes between sepals and petals compared with foliage leaves were observed to be correlated with both altered meristem position associated with altered AN3 expression patterns and different distributions of cell division angles. These results strongly indicate that lateral organ shapes are regulated via two aspects: position of meristem and cell division angles; the former is mainly governed by the AN3 expression pattern.

Key words

AN3, cell division, lateral organ, leaf meristem, morphology, polar-auxin-inhibitor

Introduction

The shape of the leaf plays an important role in determining its photosynthetic function. Moreover, the shape of floral organs, which are evolutionarily derived from
leaves, is also important for reproductive success. Therefore, the shape of these lateral
organs varies among species to maximize their survival ability in their natural habitats.
To understand these variations, it is important to understand their developmental
properties.

The center of morphogenesis in plants is the meristem, where active cell division
occurs. The shoot apical meristem (SAM) is essential for the production of new above-
ground organs. In terms of lateral organs, especially for leaf primordia, the leaf meristem
supplies cells to the leaf blade; thus, researchers have investigated the nature of the
meristem to understand the morphogenesis of lateral organs (e.g. Esau, 1977; Donnelly
et al., 1999; Kazama et al., 2010; Ichihashi et al., 2011).

In many angiosperms, such as Arabidopsis thaliana, the leaf meristem is at the
base of each leaf (Tsukaya, 2014). Cell proliferation initially occurs throughout the leaf
primordium but is restricted to its basal regions as the leaf primordium grows further
(Donnelly et al., 1999; Kazama et al., 2010). The control on the restriction of cell
proliferation zone is not completely understood, but a transcriptional coactivator called
ANGUSTIFOLIA3 (AN3)/GRF-INTERACTING FACTOR1(GIF1) is considered to
positively control cell proliferation in leaf primordia; the spatial patterns of AN3/GIF1
accumulation match well the cell proliferation zone, suggesting that AN3/GIF1 may act
as an important determinant in positioning the leaf meristem(Horiguchi et al., 2005;
Kawade et al., 2017) Cell division angles in the AN3-expressing region, except for areas
along the margin and vasculature, were observed to be randomized (Yin & Tsukaya, 2016).
AN3 encodes a protein that is homologous to the human synovial sarcoma translocation
protein (Horiguchi et al., 2005; Nelissen et al., 2015), and during leaf development, it is
expressed at the base of leaf primordia. AN3 protein moves (Kawade et al., 2013) and
forms a gradient along the proximal-distal axis on the leaf, with the leaf base having the highest concentration of AN3 protein (Kawade et al.) and thus is involved in the positioning of the leaf meristem. However, how AN3 expression is controlled within the leaf meristem is still unknown.

AN3 is also involved in the morphogenesis of each floral organ (Lee et al., 2009a, 2014). A petal in the an3 mutant has a smaller number of cells and a narrower shape than that of the wild type (Lee et al., 2009b), as seen in the foliage leaves, suggesting a common role of AN3 in leaf and floral organ primordia. However, cell proliferation is active in the distal part of the petal primordia and not in the basal part, as in the leaf primordia (Disch et al., 2006; Anastasiou et al., 2007). Thus, if AN3 has the same role in the positional determination of the meristematic zone in petal primordia, AN3 proteins are expected to accumulate apically and not basally in petal primordia; however, no previous studies have examined it. Marginal/apical positioning of meristematic zone in petals may be an ancestral character that is directly comparable to apical positioning of SAM (Boyce, 2007). Although the basal positioning of the leaf meristem is common in angiosperms, marginal/apical positioning in leaf primordia is also known in some ferns and gymnosperms. Therefore, a comparison of leaf primordia with floral organ primordia of the same species, with special emphasis on AN3 expression, may contribute towards understanding the roles and evolutionary history of differences in the positioning of cell proliferation activity in the primordia of these lateral organs.

The venation pattern is an observed difference between the leaves of angiosperms, ferns, and gymnosperms (Boyce, 2007). In the lateral organs of ferns and gymnosperms harboring meristems along the apical margin, the leaf vein shows a bifurcated pattern and is open at the end. In contrast, lobed and closed patterns are
common in eudicots including *A. thaliana*, and parallel patterns are common in monocots (Dengler & Kang, 2001). Thus, the venation pattern may be important in regulating leaf organogenesis. Mutants with an abnormal venation pattern mostly show altered leaf shapes in *A. thaliana* (Candela *et al.*, 1999). Based on this correlation, an interaction between spatial control of the leaf cell proliferation zone and leaf venation pattern has been suggested (Boyce, 2007).

In vein development in the leaf, biosynthesis and transportation of the plant hormone auxin (indole-3-acetic acid, IAA) plays an important role (Cheng *et al.*, 2006). For example, *GNOM* and *PIN-FORMED* (*PIN*) genes that control polar auxin transport (PAT) (Verna *et al.*, 2019) regulate the vein formation during leaf development. Members of the PIN family have been extensively studied as major factors in PAT, the most famous being PIN1 (Okada *et al.*, 1991). Mutations in many PIN genes induce defects in leaf vein patterns, with wide and bifurcated midveins and altered leaf blade shapes (Sawchuk *et al.*, 2013).

Many PAT inhibitors (PATIs) have been used to examine the role of PAT in plant organogenesis, including 2,3,5-triiodobenzoic acid (TIBA) and N-1-naphthylphthalamic acid (NPA). Through indirect evidence, they bind to the same auxin efflux carriers to inhibit their activity (Teale & Palme, 2018). When plants are treated with PATI, their leaves exhibit abnormal leaf venation patterns, including very thick midveins and marginal veins, similar to that of *pin1* mutants (Sieburth, 1999). In addition, leaf shape becomes rounder and shorter than that of control plants. However, to date, no study has examined the effect of PATI on the cell proliferation pattern in leaf primordia.

To completely understand the morphogenesis process of lateral organs, it is necessary to understand the role of the lateral organ meristem on the final organ shape.
and factors that affect the properties of meristems. In this study, we utilized PATI-treated leaves and floral organs as leaves with altered venation patterns or modified leaves and investigated the spatial position of cell proliferative area, cell division angle, and possible factors that control the properties of lateral organ meristems, using AN3 as a key clue.

Materials and Methods

Plant growth

For analysis of the leaf primordia, *A. thaliana* Col-0 (wild type [WT]), or those carrying *CYCLINB1;1(CYCB1;1)::GUS, an3-4, an3-4/pAtAN3::AN3-GREEN FLUORESCNT PROTEIN (GFP), an3-4/pAtAN3::AN3-3xGFP, gl-s92f, or gl-s92f/an3 were grown on sterile growth medium that contained 0.5 × Murashige and Skoog medium (MS, Wako, Osaka, Japan), 1% (w/v) sucrose (Nacalai Tesque, Kyoto, Japan), and 0.8% (w/v) agar (Nacalai Tesque, Kyoto, Japan) (Wako, Osaka, Japan) adjusted to pH 5.8 (KOH). Approximately, 1 M PATI (TIBA and NPA) stocks were dissolved in dimethyl sulfoxide and added to the medium to a final concentration of 10 µM. The medium composition was based on that described by Sieburth (1999).

Seeds were sterilized by immersing in a solution of 2% (v/v) Plant Preservative Mixture™ (Plant Cell Technology, Washington, D.C., USA), 0.1% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan), and 50 mg/L MgSO₄ for 6 h or a solution of 10% (v/v) sodium hypochlorite (Nacalai Tesque, Kyoto, Japan) and 1% (v/v) Triton X-100 for 5 min and twice with sterile water prior to plating. The plates were incubated at 24°C under constant illumination.

For analyses of the floral organ primordia, *A. thaliana* Col-0 and *an3-4/pAtAN3::AN3-1xGFP* (Kawade et al., 2013) were sown on rockwool (Toyobo, Osaka,
Japan) and grown under white fluorescent light conditions (ca. 40 μmol m⁻² s⁻¹) at 22–
23°C supplied with water containing 1 g/L powder Hyponex (Hyponex, Osaka, Japan).

GUS experiments

Detection of β-glucuronidase (GUS) activity was carried out using 5-bromo-4-
chloro-3-indolyl-β-D-glucuronide (X-Gluc) as a substrate. Plant tissue was first placed in
90% (v/v) acetone on ice for 10 min, washed with sodium phosphate buffer (pH 7.0), and
then placed in X-Gluc buffer solution (0.5 mg/mL X-Gluc, 100 mM NaPO₄ (pH 7.0), 5
mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 10 mM EDTA, 0.1% (v/v) Triton X-100) under
vacuum for 15 min or more and then placed in the dark at room temperature (ca. 20°C).

After GUS detection, plant tissues were rinsed in 70% (v/v) ethanol and fixed in
ethanol: acetic acid (6:1) solution. After chlorophyll was removed, the tissue was
preserved in 70% EtOH in the dark. Plant tissues were mounted on slides with chloral
hydrate solution (50 g chloral hydrate, 5 g glycerol, 12.5 mL distilled water) (Tsuge et al.,
1996) and observed under a microscope after the tissue became transparent enough.

AN3-GFP observations

Leaf primordia (5 days after sowing stage: DAS) and flower primordia of A.
thaliana an3-4/pAN3::AN3-GFP and an3-4/pAN3::AN3-3xGFP lines were fixed in 4%
(v/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) with 0.05% (v/v)
Triton X-100 by immersing them in the fixation mixture, deairing them for 10 min (for
leaf primordia) or 15 min (twice, for flower primordia) and placed at 4°C overnight.
The samples were then washed in PBS (10 min, twice) and stored in PBS at room
temperature for leaf primordia and at 4°C for flower primordia until observation.
The samples were then dissected using a sharp razor under the microscope. Leaf primordia were mounted on slides with PBS and observed under a confocal microscope (FV3000; Olympus, Tokyo, Japan) with a GFP filter for leaf primordia and an upright fluorescent microscope (DM4000; Leica Microsystems GmbH, Wetzlar, Germany) for floral organ primordia.

**Data analysis on the position of leaf meristem and the arrest front**

This method was derived from Kazama et al. (2010) and Ikeuchi et al. (2011). This method is used to determine the position of the leaf meristem. First, an image of a leaf with a GUS expression pattern was prepared. The outer region of leaf was cropped, and the image was rotated so that the leaf base was on the left side of the image. Then, the blue region was extracted, and a binary image was created using ImageJ (https://imagej.nih.gov/ij/). The number of white pixels was counted for each column, and the arrest front was determined based on the definition of the point at which the ratio of white pixels was half that of the maximum and farthest from the blade base. The distance from the leaf base of each arrest front point was plotted for each condition in a box plot. Statistical analysis was performed using the R software.

Similarly, the arrest front position was determined as following; first, a series of z-stack images were stacked using ImageJ software. Stacked images with the outer side of the leaf were cropped and rotated so that the leaf base was on the left side of the image. The region with GFP fluorescence was extracted, and from this image, a binary image was created. The number of black pixels was counted in each column. The arrest front was determined based on the definition of the point at which the ratio of black pixels was half that of the maximum and farthest from the blade base. The distance from the leaf
base of each arrest front point was divided by the leaf length because of the size difference between lines. The obtained data were plotted for each condition in a box plot. Statistical analysis was performed using the R software.

**Observation of Aniline Blue signal**

This method was derived from previous studies for the detection of newly formed cell walls (Kuwabara & Nagata, 2006; Kuwabara et al., 2011). Leaf primordia (7 DAS) of *A. thaliana gl1(glabra1)-s92f* and *gl1-s92f/an3-4* mutants and Col-0 flower petals and sepals were first fixed in a mixture of ethanol and acetic acid (4:1, v/v) for 30 min and then rinsed in 100% ethanol. Then, the samples were immersed in a mixture of ethanol and 100 mM phosphate buffer (pH 9.0; 1:1, v/v) for 30 min and then in 100 mM phosphate buffer (pH 9.0) for 10 min. Finally, the samples were immersed in a 0.02% (w/v) solution of aniline blue in 100 mM phosphate buffer (pH 9.0) for at least 7 days and up to 30 days at 4°C. Leaf primordia were mounted on slides with the staining solution and observed under a confocal microscope (FV10C-PSU; Olympus, Tokyo, Japan) under UV excitation with a DAPI (4’,6-diamidino-2-phenylindole) filter. The data were analyzed by taking each angle of the septum wall. Calculations were performed using Microsoft® Excel.

**Observation of EdU-marked cells**

We used the Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fischer Scientific, Waltham, MA, USA) to visualize the cells in S phase. We dissected the inflorescence of *A. thaliana* into several pieces and soaked the flower clusters into 10 µM 5-ethynyl-2’-deoxyuridine (EdU) solution in 1/2 MS medium with 1% sucrose for 3 h.
under ~45 µmol m$^{-2}$ s$^{-1}$ white fluorescent light. The samples were washed with PBS containing 0.1% Triton X-100 and fixed with FAA (37% [v/v] formaldehyde, 5% [v/v] acetic acid, 50% [v/v] ethanol) and stored at 4°C. Subsequent fluorescent labeling with Alexa Fluor 488 or 555 (Thermo Fischer Scientific, Waltman, MA, USA) was conducted according to the manufacturer’s instructions. Floral organs were mounted on slides, and fluorescent dye signals conjugated to EdU were observed under fluorescent microscope (DM4000; Leica Microsystems GmbH, Wetzlar, Germany).

Results

Change in the length of cell proliferation zone

In the PATI-treated plants, the leaves were shorter and rounder than those of the control plants (Fig. 1D-F, Fig. 2). The leaf vein pattern also differed in PATI-treated leaves (Fig. 1D-F, Fig. 2); namely, the midvein was widened and bifurcated, the lateral veins ran parallel to each other, and the veins adjacent to leaf margins were also widened. These observations are consistent with those of previous reports (Mattsson et al., 1999; Sieburth, 1999; Sawchuk et al., 2013).

To investigate the effect of inhibition of PAT on the leaf meristem, the cell proliferation zone was observed in the leaf primordia of PATI-treated plants. The CYCB1;1::GUS line is used to visualize dividing G2-M phase cells (Donnelly et al., 1999). The first and second rosette leaves of 6-DAS seedlings for control plants and 7-day-old seedlings for PATI-treated plants were used for this experiment, considering the growth retardation observed in the PATI-treated plants.

In the PATI-treated plants, the cell proliferation zone remained in the proximal position, similar to the control plants (Fig. 1A-C). The images were processed to further
examine the positioning of the cell proliferation zone (Fig. 3). We observed that both the length of the cell proliferation zone from the leaf base and the total ratio of cell proliferation zone to the total leaf length were found to be increased in PATI-treated leaves (Fig. 4).

**AN3 mRNA and AN3 protein localization in leaf primordia**

To investigate how the length of the cell proliferation zone increased, we examined the localization of *AN3*, a positive regulator of cell proliferation. As AN3 protein can move between cells, two lines, *an3-4/pAtAN3::AN3-GFP* and *an3-4/pAtAN3::AN3-3xGFP*, were used for the observation. The former can detect actual protein localization, whereas the latter is used to monitor mRNA localization because it represents accumulation of the protein without cell-to-cell movement ability (Kawade *et al.*, 2013, 2017). The first and second rosette leaves of 5 DAS seedlings were used for this experiment.

We observed that the overall localization of AN3 under PATI treatment did not change from that of the control; it remained in the proximal region (Fig. 5). This confirms the observation in the cell proliferation zone described above. The *AN3*-expressing regions were also determined using the same image processing method used for the analysis of cell proliferation zone. As a result, the *AN3*-mRNA expressing regions were slightly longer in the PATI-treated leaves than in control; in NPA-treated leaves, significant differences were detected compared with that of the control. However, protein localization did not show a statistical difference between TIBA- and NPA-treated plants (Fig. 6). Therefore, even though changes in *AN3* mRNA expression pattern may influence the increase in length in the cell proliferation regions, the spatial gradient of AN3 protein
along the longitudinal axis of leaf primordia did not change. Thus, the influence of PATI treatment on the AN3 protein accumulation pattern is rather limited.

In contrast, when \textit{an3-4/pAtAN3::AN3-3xGFP} were treated with PATI, we recognized that \textit{AN3} localization was missing in the vasculature region. This missing localization was also observed in control conditions, but in PATI-treated leaves, the vasculature was very thick, and therefore, the absence was recognizable (Fig. 1D-F, Fig. 5).

**Cell division angles in leaves**

Based on the changes in the size or pattern of the cell proliferation zone, the observed leaf-shape change by PATI treatment could not be explained. Analysis of cell division angles was performed to further investigate how it affects organ shape under PATI treatment. We used the \textit{gl1-s92f} mutant and \textit{gl1-s92f/an3-4} double mutants for this experiment. We used \textit{gl1-s92f} mutants that lacked trichomes as a control, because trichomes obstruct cell division plane observation using aniline blue staining. The angles were determined against the proximal-distal axis, starting from the leaf base and parallel to the midvein for the first and second rosette leaves of 7 DAS seedlings (Fig. 8; Fig. S1).

As a result, a variety of cell division angle patterns were observed (Fig. 7A, B, Fig. 8). In the control plants, the cell division angle occurred around 130°–140° (Fig. 7A). In PATI-treated leaves, this peak was less evident, and cell division was likely to occur in all directions (Fig. 7A). This may have contributed to changes in leaf shape, with rounder and shorter leaves, when treated with PATI (Fig. 1D-F, Fig. 2).

Cell division angle investigation was also performed in the \textit{an3-4} mutant plants. In the \textit{an3-4} mutant without PATI, the peak of cell division angle was around 140–150°,
which differed from that of the control condition (Fig. 7A, B), suggesting that AN3 might shift the cell division angle to smaller angles, that is, a shift from proximo-distal to medio-lateral direction. In the an3 mutant treated with PATI, however, the distribution of cell division angle became similar to that of WT plants treated with PATI (Fig. 7A, B). This suggests that the randomizing effect of PATI on cell division angle is superior to that of the an3 genotype of the plant biasing cell division angle to the proximo-distal axis. In addition, there was a specific pattern change in the cell division angle of each PATI type (TIBA and NPA).

The position of cell proliferative area in floral organs

Although floral organs such as sepals, petals, stamens, and carpels are homeotic to leaves, their shapes are absolutely different. Even between sepals and petals, both of which are planar organs, there are distinct differences in shape in A. thaliana. In the distal part, the sepal is narrower, and the petal is wider. In this aspect, foliage leaves are narrower in the distal part, similar to sepals.

We observed cell proliferation patterns in floral organ primordia to investigate whether these patterns might influence the difference in the final organ shape. To visualize dividing cells in floral organs, we used EdU. In a sepal, cell division was observed in the basal part of the organ primordia through the observed developmental stages (Fig. 10), which resembled to that in leaves. In contrast, cell division in the petal primordia was observed in whole organ when the organ was around 100–150 μm in length, and the distal and marginal regions when the organ was around 400 μm in length (Fig. 10), which marked a clear difference from that of leaves and sepals. A similar tendency was also observed in the aniline-blue-stained samples (Fig. 9).
AN3 protein localization in floral organs

As AN3 is a key regulator of the leaf meristem position, and it promotes the cell division of petals and sepals, we suspected that AN3 protein accumulation patterns may be different between them, which have different positions of the cell proliferative area. AN3-GFP signals were only observed in the basal part of sepal organ primordia, whereas the signals were observed in the distal region through the observed developmental stages in petal organ primordia. Moreover, in terms of petal primordia, sparse signals were also observed in the central region and the basal part in the later stage 9 (Fig. 10), where EdU signals were rarely observed.

Phenotype of an3 mutant in sepal

It is reported that an3 mutants have narrower petals and lesser number of cells than that of the WT (Horiguchi et al., 2005; Lee et al., 2009a); the sepal phenotype has not been investigated. In order to investigate whether AN3 maintains leaf meristem in sepal primordia, which was suggested by the AN3 protein accumulation pattern and proliferative area, we compared the phenotype of an3-4 and the WT in the sepals (Fig. 11A–B). The area of the organ was smaller in an3 than in the WT (Fig. 11C), and we also observed that the an3 mutant had less complex veins as compared to that of the WT, which was evaluated based on the number of secondary and higher veins in the m-shaped or two n-shaped primary veins (Fig. 11C, D).
Cell division angles in floral organs

To observe the influence of cell division angle on the final shape of floral organs, cell division angle analysis was conducted in the sepals and petals. Flowers at stages 8–10 were used in this experiment to observe active cell proliferation (Alvarez-Buylla et al., 2010).

The cell division angle in floral organs peaked at approximately 60°–90° and 140°–180° (Fig. 8). This twin peak pattern is different from the cell division angle in leaves, which only had one peak (Fig. 7A, B). Because sepals and leaves had similar localization of the cell proliferation zone, we expected that the distribution of cell-division angles would be similar, but on the contrary, the overall tendency of cell division in sepals was similar to that of petals. This suggests that the pattern of cell division angles is not associated with the localization of the cell proliferation zone but with organ identity.

The cell divisions with the 60°–90° peak, which were divisions in the mediolateral direction of the primordia, were identified mostly in the central regions of the petal primordia. In contrast, the divisions that corresponded to the 140°–180° peak, which were divisions in the proximo-distal direction, were identified mostly in the marginal regions of the petal primordia (Fig. 9).

Discussion

In this study, we examined how cell proliferative zones differ in the primordia of PATI-treated leaves and floral organs from normal foliage leaf primordia of *A. thaliana* with focus on the spatial expression pattern of *AN3*, a key element for leaf meristem positioning (Kawade et al., 2010). As a result, we revealed that organ shape change by PATI treatment cannot be attributed to changes in leaf-meristem positioning, size of the
leaf meristem, or the expression pattern of \textit{AN3} and is rather attributed to altered cell division angles in the leaf meristem. In contrast, different shapes of sepals and petals compared with foliage leaves were found to be correlated with both altered meristem position associated with altered \textit{AN3} expression patterns and different distributions of cell division angles. These results strongly indicate that lateral organ shapes are regulated via two aspects: position of meristem and cell division angles; the former is mainly governed by the \textit{AN3} expression pattern. In the following sections, several aspects of the above findings are discussed.

The position of leaf meristem in PATI-treated plants

When \textit{A. thaliana} plants were treated with PATI, the cell proliferation zone in leaf primordia did not show any changes in terms of localization. However, both the length of the cell proliferation zone in leaf primordia and the ratio of the cell proliferation zone occupying the primordia were observed to be increased (Fig. 4). As previously reported (Sieburth, 1999), PATI-treated leaves were rounder and shorter, whereas a longer proliferation zone would be expected to produce a longer leaf. Instead, changes in the cell division angles could be attributed to the altered leaf shape.

In this study, it was observed that even when PAT was inhibited, there was no change in the basal expression pattern of \textit{AN3} (Fig. 5). The \textit{AN3} mRNA expression zone was slightly expanded to the distal direction in the expression zone ratio, although the actual length was not significantly different (Fig. 6). Indeed, the final \textit{AN3} protein distribution remained unchanged under PATI treatment (Fig. 6). Moreover, the results for NPA and TIBA showed the different trend; while TIBA increased the mRNA localization zone and protein localization of AN3, NPA increased \textit{AN3} mRNA localization zone but
did not affect protein localization of AN3. In either case, AN3 mRNA expression and AN3 protein diffusion were not regulated by auxin.

We also observed that AN3 was not expressed in leaf veins (Fig. 5). This trend was seen in PATI-treated leaves as well as in control conditions; however, it was not evident because the veins in control conditions were much thinner than those of PATI-treated leaves (Fig. 1D-F). This may happen if AN3 expression is shut down in differentiated vascular cells, which may imply that the vasculature differentiation by auxin is superior to cell proliferation maintenance by AN3.

**Cell division angle and leaf shape**

A longer proliferation zone is expected to make a longer leaf; however, in PATI treatment, it produced shorter leaves. The cell division is also an important factor in both leaf development and leaf vein architecture (Kang et al., 2007). In this study, analysis of the cell division angle revealed that the pattern differed between PATI-treated plants (Fig. 7A). This difference in the pattern could be a cause for the differences in leaf shape. Namely, in comparison with control leaf primordia that had major division angles in 130°–140°, PATI-treated leaf primordia had dispersed division angles in many directions, forming a round and short leaf, which matched the phenotype (Fig. 1D-F, Fig. 3). We observed the an3 mutant tended to divide around 140°–150°, which partially explains the narrow leaf phenotype of an3 mutants, as cell division along the proximal-distal axis was increased.
Determining cell division angle in leaves

In this study, there were two components that changed the cell division angle: PAT and AN3. When PAT was inhibited, the peak in the cell division angle distribution became less evident (Fig. 7A). As auxin flow controls vascular cell polarity (Linh et al., 2018), and plays a role in cell division angle (Yin & Tsukaya, 2016), it is possible that the cells divide in the direction of auxin flow. In contrast, both NPA and TIBA affect actin dynamics (Teale & Palme, 2018; Zou et al., 2019) that can affect cytoskeletal regulation of cytokinesis, and may be one of the underlying reasons for the change in cell division angle.

Additionally, in the an3 mutant, cell division along the proximal-distal axis accounted for a larger proportion of cells than that in the WT (Fig. 7B). The increase in cell division along the proximal-distal axis may be due to changes in the overall direction of cell division during leaf development. In a previous report, the phase of cell division was divided into two phases: the first has more divisions along the proximal-distal axis than the second phase (Horiguchi et al., 2011). Furthermore, in an3 mutants, the transition from the first to the second phase does not occur before the termination of cell division activity (Horiguchi et al., 2011). The shift in the peak of the cell division angle observed in the an3 mutant reflects this failure in shifting to the second phase of cell proliferation, which confirms the results of previous studies. Therefore, AN3 functions in the transition to second phase of cell division, and as a result, cells divide along the proximal-distal axis in the absence of AN3. More precisely, AN3 might promote the shift of the cell division angle from the proximo-distal to medio-lateral direction.

In addition, in an3 mutants treated with PATI, the cell division angles were similar to those of WT treated with PATI (Fig. 7A, B). This suggests that the randomizing
effect of PATI on cell division angle is superior to that of AN3 and that the loss of PAT results in cell division in random directions irrespective of the presence or absence of AN3.

**Position of meristematic tissue determines the final floral organ shape**

The final organ shape is determined by various factors, such as acceleration and deceleration of proliferation, oriented cell division and expansion, and the meristem position (Tsukaya, 2018). In this study, we showed that the position of the cell proliferative area was completely different between sepals and petals, which are morphologically different. The petal of *A. thaliana* with a modest fan shape has a proliferative region in the distal part, which is similar to ferns with fan-shaped morphology that is rare in angiosperm leaves, coinciding with leaf meristem at the apical margin (Boyce, 2007; Tsukaya, 2014, 2018). This suggests that the morphological differences between sepals and petals could be partly explained by the meristematic position in each organ.

Although predominant cell division occurs in submarginal plate meristem to widen the leaf blade area in leaf primordia (e.g., Poethig and Sussex, 1985), it was also shown that marginal meristem residing in the margin of the primordia also exists (Alvarez *et al.*, 2016). Alvarez *et al.* (2016) showed that when NGATHA and CINCINNATA-class-TCP were knocked down, indeterminate marginal growth occurred in the entire margin of the leaf blade, suggesting potential meristem activity in this area. Interestingly, marginal growth occurs only in the distal region of floral organs, including sepals and petals. Although in our experiments, active cell division occurred in the proximal part of the sepal primordia, it is possible that the potential marginal meristem in the distal part
also contributes to the elaborate final shape of the sepal (Floyd & Bowman, 2010). In terms of petals, active cell division occurs in the distal margin in the first place; therefore, the marginal meristem may contribute more than leaves or sepals. Since the activity of meristem in the distal marginal area of a blade has been discussed as an ancestral character (Floyd & Bowman, 2010), petals may retain this developmental character (Boyce, 2007).

We observed EdU signals in the entire margin of the petal primordia at least until the organ size was 400 \( \mu \text{m} \) in length, even in the proximal region. Considering the results of Alvarez et al. (2016) on leaf primordia, the nature of proliferative cells in marginal areas is different among different organs.

Determining factors for the meristematic position

Several key genes are known to positively control cell proliferation in the leaf meristem (Nakata et al., 2012; Ichihashi & Tsukaya, 2015). Among them, the AN3-protein-accumulated region matches well with the cell proliferative area in leaf primordia (Kawade et al., 2017), suggesting that AN3 is an important determinant of the leaf meristem position. Moreover, the smaller size of a petal in \( an3 \) or triple knockdown of the GIF family (Kim & Kende, 2004; Horiguchi et al., 2005; Lee et al., 2009a) suggested that AN3 is also involved in the promotion of cell proliferation in the petal. However, its functioning zone in primordia has not been well investigated. In this study, we showed that AN3-expressed region overlapped well with the cell proliferative area in both sepals and petals, as observed in leaf primordia. In addition, we first showed that sepals in \( an3 \) mutants are likely to have a smaller number of cells as petals or foliage leaves. These results strongly suggest that AN3 functions as a determinant of the meristematic position and activity in floral organs. In the past, JAGGED (JAG) was examined as a candidate
of a regulatory gene for the specific morphology in the Arabidopsis petal differed from leaves, from the following points: the loss-of-function \textit{jag} mutant has narrower and shorter petals, the \textit{JAG} over-expressor has larger petals; its mRNA is expressed distal margin (Sauret-Güeto et al. 2013). At that time point \textit{JAG} was the only one candidate ‘organizer’ that gives the petal with a pattern of growth orientations that fans out. But now \textit{AN3} became to be an additional candidate that fulfil the required, above-mentioned conditions. Indeed \textit{AN3} was identified as a direct target of \textit{JAG} (Schiessl et al. 2014). The role of \textit{AN3} as the ‘organizer’ should be examined carefully in the future.

However, in terms of petal primordia, \textit{AN3} protein signals were also observed in the less proliferative area. This might be due to a lack of associating transcriptional factors such as GROWTH REGULATING FACTOR5 (GRF5), which is necessary to promote cell proliferation in leaf primordia (Horiguchi et al., 2005), in such regions. Alternatively, because signals in the proximal part were not as strong as those in the distal region, the concentration of \textit{AN3} proteins might not be enough to promote cell division to the extent that EdU was incorporated.

The regulation of meristem position floral organ identity genes needs to be investigated (Coen and Meyerowitz, 1991). Honma and Goto (2001) and Pelaz et al. (2001) revealed that when A genes (\textit{APELATA1}) and B genes (\textit{APELATA3} and \textit{PISTILATA}) were ectopically expressed together with \textit{SEPALLATA2/3} in rosette leaves, the rosette leaves obtained petal identity, and the color and cell shape became petal-like. However, the overall organ shape has not yet been investigated in detail. From our results, the transformed organ is not fan-shaped, but has a taper off shape, which is similar to rosette leaves, cauline leaves, and sepals. This suggests that factors other than the floral
identity homeotic genes control the final organ shape. Revealing the mechanisms of AN3 expression control might shed light on this possibility.

The leaves of some gymnosperms and ferns are considered to grow from the meristem in the distal margin. The positioning of these meristems may also be determined by the spatial distribution of leaf meristem-controlling genes such as AN3/GIF1. As GIF family genes exist in most eukaryotic organisms, including the basal land plants, Marchantia polymorpha, Physcomitrium patens, and Sellaginella moellendorffii (Kim and Tsukaya, 2015), further analyses of the GIFs in gymnosperms and ferns will answer this question.

Determining cell division angle in floral organs

In this study, the cell division pattern in floral organ primordia was investigated for its possible roles in each floral organ morphology, and both the petals and sepals showed a pattern with twin peaks in the distribution of cell division angles, which was different from that of leaf primordia that had only a single peak (Fig. 8). Although both sepal and leaf primordia have a cell proliferation zone in the basal region, the cell division angle was controlled differently, which may imply that cell division angles depend on organ identity and affect their final shapes.

In the petal, the cell division occurred at 60°–90° angle in the central regions, and the cell division with the 140°–180° angle was mostly in the marginal regions, whereas such a pattern was not seen in the sepals (Fig. 9). This difference may cause differences in shape between sepals and petals, and petal shape, with the distal part being wider than the proximal part, may be caused by the cell divisions that contribute to width in the marginal regions. Thus, we revealed that lateral organ shapes are regulated by two
factors: the position of the cell proliferative zone governed by the spatial expression pattern of AN3 and cell division angles. Even in one species, by changing these two factors, a variety of lateral organ shapes could be realized.

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Author Contributions
AK, MN, and HT designed the experiments; AK and MN performed the experiments and analyzed the data; AK, MN, and HT wrote the manuscript.

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Conflict of Interest
No conflict of interest.

Data Availability
Data available on request from the authors.
References


Fig. 1 Cell proliferation is visualized by GUS staining in PATI-treated CYCB1;1::GUS.

A: 6 DAS control, B: 7 DAS TIBA-treated, C: 7 DAS NPA-treated leaf primordia.

D: 12 DAS control, E: 12 DAS TIBA-treated, F: 12 DAS NPA-treated leaf primordia.

Leaf primordia of similar length were compared.

Scale bars: A: 100 µm, B, C: 200 µm, D-F: 500 µm
**Fig. 2** Leaf length/width ratio of leaf primordia at cell proliferation stage.

From left to right, data on control, TIBA-treated, and NPA-treated leaf primordia are shown, n = 4, Dunnett's test ***: \( p < 0.001 \)
**Fig. 3** Determination of cell proliferation zone.

A: Binary images of GUS-stained leaf primordia. Scale bar: 50 µm

B: The ratio of white pixels from a binary image was calculated, and the length of the cell proliferation zone (more than 50% of the white pixels) was determined. The red arrow indicates the end of the cell proliferation zone.
**Fig. 4** Length and the ratio of cell proliferation occupying leaf primordia.

A: Length of cell proliferation zone from leaf base

B: The ratio of cell proliferation zone to the total leaf length.

n = 8, Dunnett's test, ***: p < 0.001
Fig. 5 GFP localization of an3/pAtAN3::AN3-3xGFP (A-C) and an3/pAtAN3::AN3-GFP (D-F).

Control (A, D), TIBA-treated (B, E), and NPA-treated (C, F) leaf primordia are shown. White dotted lines indicate GFP-localized area, and magenta lines indicate vasculature.
Fig. 6 Ratio of AN3 mRNA and protein-localized area to the total length of leaf primordia.

From left, an3/pAtAN3::AN3-GFP Control, TIBA and NPA; an3/pAtAN3::AN3-3xGFP Control, TIBA and NPA. n = 4, Dunnett's test, *: p < 0.05. No mark implies no statistically significant difference was observed.
**Fig. 7** Cell division angles in leaf and floral organs. Four samples were investigated for each condition.

A: From left, WT, TIBA, NPA; 1221, 1158, 1061 pair of cells were analyzed, respectively.

B: From left, *an3* mutant, TIBA (*an3* mutant), NPA (*an3* mutant); 971, 703 and 604 pair of cells were analyzed, respectively.
**Fig. 8** Cell division angles in floral organs. Four samples were investigated for each condition.

From left, WT sepal, WT petal; 56 and 222 pair of cells were analyzed, respectively.
**Fig. 9** Cell division angle distribution in floral organs. The angles are mapped to the right half of the diagram, as the organs are symmetrical.

The magenta dots indicate the cell division angle in 180°–140° (the upper peak in Fig. 8), the green dots indicate 90°-60° (the lower peak in Fig. 8), and the black dots indicate the angles that are in neither peak. n = 3. A: Sepal; 46 pair of cells were analyzed. B: Petal; 159 pair of cells were analyzed.
**Fig. 10** AN3-1xGFP signals and EdU staining in sepal and petal primordia.

The younger primordia are shown in the left column. Normal transmitted light microscope images are shown on the right side of each fluorescent microscope image. The yellow line shows areas with clear AN3-1xGFP signals. Magnified views in each square of the sepal primordium are shown on the upper right.

Bar = 50 µm
**Fig. 11** Phenotype of the sepal in the *an3-4* and the wild type. (A, B) Images of the sepal in each genotype, *an3-4* and the wild type. Some cuts were made to flatten the organs. (C) The area of sepals in each genotype. (D) Two patterns of primary veins defined in this study. (E) The number of higher veins of the sepals in each genotype. *

\[ p < 0.05, \quad **p \ < \ 0.001, \quad \text{Bar} = 500 \ \mu m \]