

Report

ANGUSTIFOLIA3 Signaling Coordinates Proliferation between Clonally Distinct Cells in Leaves

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Summary

Coordinated proliferation between clonally distinct cells via inter-cell-layer signaling largely determines the size and shape of plant organs [1–4]. Nonetheless, the signaling mechanism underlying this coordination in leaves remains elusive because of a lack of understanding of the signaling molecule (or molecules) involved. *ANGUSTIFOLIA3* (*AN3*, also called *GRF-INTERACTING FACTOR1*) encodes a putative transcriptional coactivator with homology to human synovial sarcoma translocation protein [5–7]. *AN3* transcripts accumulate in mesophyll cells but are not detectable in leaf epidermal cells [8]. However, we found here that in addition to mesophyll cells [5, 6], epidermal cells of *an3* leaves show defective proliferation. This spatial difference between the accumulation pattern of *AN3* transcripts and *an3* leaf phenotype is explained by *AN3* protein movement across cell layers. *AN3* moves into epidermal cells after being synthesized within mesophyll cells and helps control epidermal cell proliferation. Interference with *AN3* movement results in abnormal leaf size and shape, indicating that *AN3* signaling is indispensable for normal leaf development. *AN3* movement does not require type II chaperonin activity, which is needed for movement of some mobile proteins [9]. Taking these findings together, we present a novel model emphasizing the role of mesophyll cells as a signaling source coordinating proliferation between clonally independent leaf cells.

Results

an3 Mutant Exhibits Proliferation Defects in Both Leaf Epidermal and Mesophyll Cells

To investigate the *an3* mutant phenotype in detail, we analyzed epidermal and subepidermal cell number in *an3-4* leaves. Compared to wild-type (WT) leaves, *an3-4* leaves had 67% fewer adaxial epidermal cells (Figure 1A) and 68% fewer palisade mesophyll cells in the subepidermal layer (Figure 1B).

These results indicate that *AN3* is involved in the control of proliferation of both epidermal and mesophyll cells in leaves.

AN3 Protein Moves into Epidermal Cells after Being Synthesized in Mesophyll Cells

To investigate whether *AN3* protein moves from mesophyll into epidermal cells in leaves, we analyzed *an3-4* transgenic lines in which a chimeric gene encoding *AN3* fused with green fluorescent protein (*AN3-GFP*) was expressed by the *AN3* promoter (*an3-4/pAN3::AN3-GFP*). The *AN3-GFP* signal was evident in both epidermal and mesophyll cells of *an3-4/pAN3::AN3-GFP* leaf primordia (Figure 1C). As a control for *AN3* mobility, we established and analyzed *an3-4* transgenic lines expressing *AN3* tandemly fused with triple GFPs (*AN3-3×GFP*) by the *AN3* promoter (*an3-4/pAN3::AN3-3×GFP*). This *AN3-3×GFP* protein is unable to move between cells [10] but could rescue epidermal and mesophyll cell proliferation when constitutively expressed by the *cauliflower mosaic virus 35S* (35S) promoter (see Figures S1A–S1E available online). In leaf primordia of *an3-4/pAN3::AN3-3×GFP*, the *AN3-3×GFP* signal was limited to only within the mesophyll cells; it was undetectable in epidermal cells (Figure 1D), indicating that the promoter activity of *AN3* is absent in leaf epidermal cells. We confirmed this result by using an *AN3* promoter- β -glucuronidase (*GUS*) reporter line (*an3-4/pAN3::GUS*) (Figures 1E, 1F, S1F, and S1G). These data show that *AN3* protein moves from mesophyll into epidermal cells in leaf primordia.

In the shoot apical meristem, activity of the *CLAVATA1* (*CLV1*) promoter is absent in epidermal cells [11]. Similarly, in leaf primordia, *CLV1* promoter activity is also absent in epidermal cells and specific to subepidermal cells (T. Takahashi, personal communication; Figure 1G). Leaf cells in which the *CLV1* promoter is active distribute in a patchy manner: some cells show strong activity of the *CLV1* promoter when compared to 35S promoter, as determined by *GUS*-staining intensity (Figures S1H–S1K). Using the *CLV1* promoter, we next investigated *an3-4* transgenic lines expressing *AN3-GFP* or *AN3-3×GFP* specifically in subepidermal cells (*an3-4/pCLV1::AN3-GFP* or *an3-4/pCLV1::AN3-3×GFP*, respectively). In leaf primordia of *an3-4/pCLV1::AN3-3×GFP*, the *AN3-3×GFP* signal was limited to only within subepidermal cells (Figures 1H and 1I). In contrast, the *AN3-GFP* signal was clearly detected in both epidermal and subepidermal cells in the leaf primordia of *an3-4/pCLV1::AN3-GFP* (Figures 1J and 1K). These results further support our conclusion that *AN3* protein moves into epidermal cells after being synthesized in mesophyll cells.

Inter-Cell-Layer Movement of *AN3* Protein Is Bidirectional in Leaves but Less Obvious in Roots

To determine whether inter-cell-layer movement of *AN3* protein occurs in the opposite direction, from epidermal into mesophyll cells, we established and analyzed *an3-4* transgenic lines in which *AN3-GFP* was expressed by the epidermal cell-specific *PROTODERMAL FACTOR1* (*PDF1*) promoter (*an3-4/pPDF1::AN3-GFP*) [12] (Figures 2A, S1L, and S1M). The *AN3-GFP* signal was observed in both epidermal and subepidermal cells in the leaf primordia of *an3-4/pPDF1::AN3-GFP* (Figures

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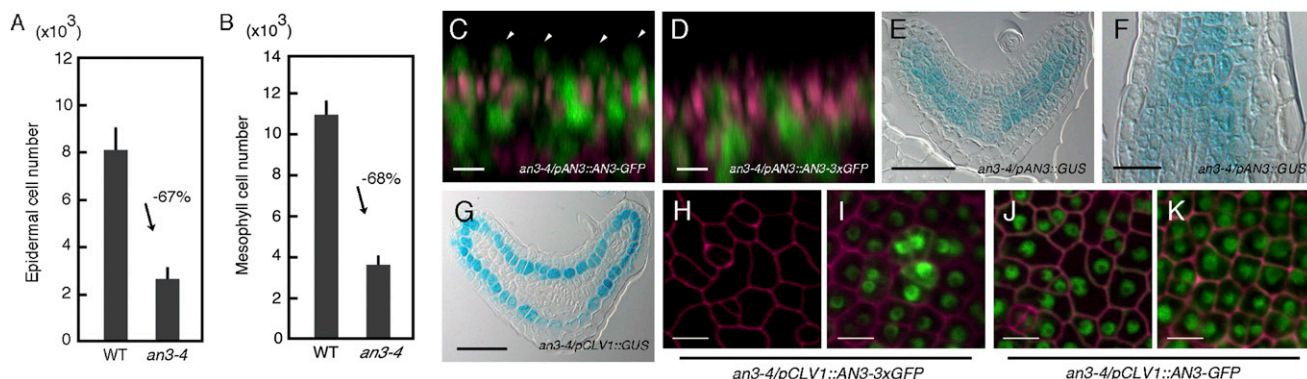


Figure 1. AN3 Protein Moves into Epidermal Cells from Mesophyll Cells

(A and B) The numbers of adaxial epidermal cells (A) and palisade mesophyll cells in the subepidermal layer (B) in the first leaves of 21-day-old WT and *an3-4* lines. The mean \pm SD from eight individual leaves is shown. Arrows indicate the difference between WT and *an3-4*.

(C and D) Optical cross-sections of the first leaf primordia in 6-day-old *an3-4/pAN3::AN3-GFP* (C) and *an3-4/pAN3::AN3-3xGFP* (D) obtained by confocal microscopy. GFP fluorescence and chlorophyll autofluorescence (as a marker of mesophyll cells) are shown in green and magenta, respectively. Arrowheads indicate GFP fluorescence in epidermal cell layers where chloroplast autofluorescence is absent.

(E–G) Cross-sections (E and G) and longitudinal section (F) of leaf primordia in 6-day-old *an3-4/pAN3::GUS* (E and F) and *an3-4/pCLV1::GUS* (G) seedlings. GUS activity was visualized as a blue stain.

(H–K) Confocal images of the first leaf primordia in 9-day-old *an3-4/pCLV1::AN3-3xGFP* (H and I) and *an3-4/pCLV1::AN3-GFP* seedlings (J and K). GFP fluorescence and propidium iodide (PI) stain are shown in green and magenta, respectively.

Scale bars represent 5 μ m (C and D), 50 μ m (E and G), 20 μ m (F), and 10 μ m (H–K). See also Figure S1.

2B and 2C). This result indicates that AN3 protein moves from epidermal into subepidermal cells.

The AN3 promoter is active in both epidermal and inner cells located near the quiescent center of the *an3-4/pAN3::GUS* root tip (Figure 2D). However, its activity was lost in epidermal cells located just above this region. To investigate whether movement of AN3 protein between cells is observed in this region, we analyzed roots in *an3-4/pAN3::AN3-GFP* and *an3-4/pAN3::AN3-3xGFP* lines. In contrast to in leaves, AN3-GFP and AN3-3xGFP signals were scarcely detected in epidermal cells, although these signals were evident in inner cells in the roots of *an3-4/pAN3::AN3-GFP* and *an3-4/pAN3::AN3-3xGFP* (Figures 2E and 2F). When we analyzed *an3-4* transgenic lines constitutively expressing AN3-GFP by the 35S promoter (*an3-4/p35S::AN3-GFP*), the intensity of the AN3-GFP signal was similar between epidermal and inner cells in roots (Figure 2G). These results indicate that the scarce AN3-GFP signal in root epidermal cells in *an3-4/pAN3::AN3-GFP* lines is due not to rapid degradation of AN3-GFP protein in root epidermal cells but rather to a restriction of inter-cell-layer AN3-GFP movement. Thus, we concluded that AN3 protein moves bidirectionally between epidermal and mesophyll cells in leaves, but that the movement is less obvious in roots.

AN3-Mediated Signaling Is Critical for Proper Leaf Size and Shape

To test whether AN3-GFP protein is involved in transcriptional control of cell proliferation in both epidermal and mesophyll cells, we examined the numbers of epidermal and mesophyll cells in *an3-4/pCLV1::AN3-GFP* leaves (Figures S3A–S3E). Both types of cell were more abundant in this transgenic line than in the WT and *an3-4* lines (Figures S3B and S3C). Consistent with this, leaves were also larger in *an3-4/pCLV1::AN3-GFP* than in WT and *an3-4* (Figures S3A and S3D). The leaf index (ratio of the length to the width of the leaf blade) was fully rescued by subepidermal cell-specific expression of AN3-GFP to a level comparable to that observed in WT leaves (Figure S3E). The expression of AN3-GFP specifically in epidermal

cells was also sufficient to complement the *an3-4* leaf phenotype (Figures S3F–S3J). The expression of GFP alone in the *an3-4* background had no effect on leaf development (Figures S3F–S3J). These results demonstrate that the AN3 protein participates in the control of proliferation of epidermal and mesophyll cells.

From the results of our cell-layer-specific rescue experiments, we assumed that AN3 signaling has functional importance per se for the proliferation of epidermal cells and thus plays a role in the determination of leaf size and shape. To address this idea, we examined leaf phenotypes in *an3-4/pAN3::AN3-GFP* and *an3-4/pAN3::AN3-3xGFP* leaves (Figure 3). The numbers of epidermal and mesophyll cells in *an3-4/pAN3::AN3-GFP* leaves were rescued to a level comparable to those in WT leaves (Figures 3B and 3C). Leaf size and shape in *an3-4/pAN3::AN3-GFP* were also fully restored to the WT phenotype (Figures 3A, 3D, and 3E). On the other hand, in *an3-4/pAN3::AN3-3xGFP* leaves, the number of epidermal cells was similar to that in *an3-4*, although the number of mesophyll cells was partially rescued (Figures 3B and 3C). In addition, our analysis using *an3-4/pAN3::AN3-3xGFP* leaves revealed that interference with AN3 protein movement resulted in a failure to rescue leaf size and shape (Figures 3A, 3D, and 3E). We obtained similar results when we analyzed leaves in *an3-4* transgenic lines expressing AN3-3xGFP by the *CLV1* promoter (*an3-4/pCLV1::AN3-3xGFP*) (Figures S3A–S3E). These results demonstrate that AN3 signaling itself, but not an AN3-dependent downstream factor, is critical for active proliferation of epidermal cells, and ultimately the determination of leaf size and shape.

In addition, we investigated whether AN3 protein controls the proliferation of root epidermal cells. There was no difference in the number of proliferating epidermal cells among WT, *an3-4*, *an3-4/pAN3::AN3-GFP*, and *an3-4/pAN3::AN3-3xGFP* roots (Figure S2A). The ectopic expression of AN3-GFP in root epidermal cells had no impact on the proliferation of epidermal cells in *an3-4/p35S::AN3-GFP* roots (Figure S2A). The length of primary roots was also normal in all transgenic

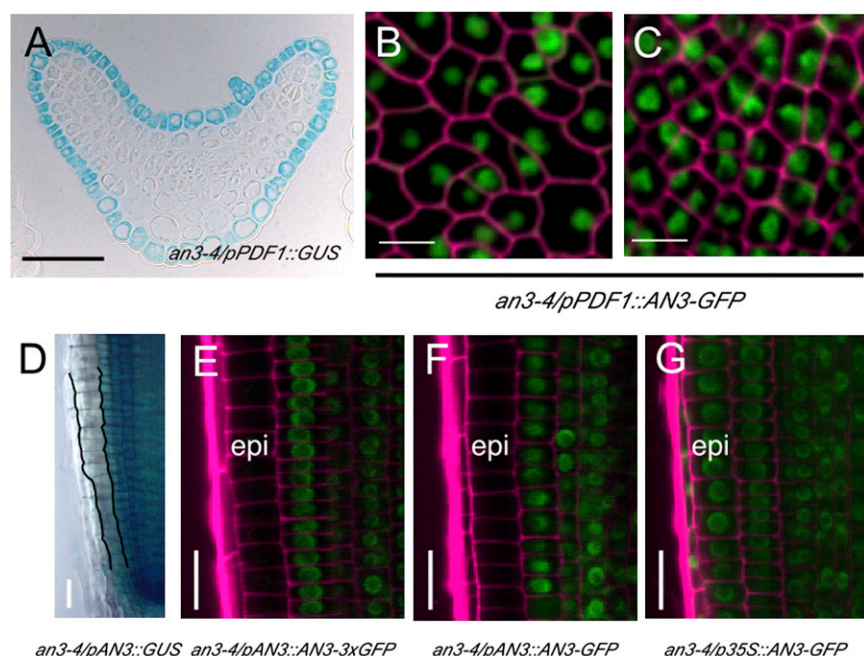


Figure 2. AN3 Protein Moves Bidirectionally between Cell Layers in Leaves, but Not in Roots

(A) Cross-section of the first leaf primordia in 6-day-old *an3-4/pPDF1::GUS*.

(B and C) Confocal images of epidermal (B) and mesophyll cells (C) in the first leaf primordia of 9-day-old *an3-4/pPDF1::AN3-GFP* seedlings. GFP fluorescence and PI stain are shown in green and magenta, respectively.

(D) GUS-stained roots in 7-day-old *an3-4/pAN3::GUS* line. Epidermal cell file is outlined in black.

(E–G) Confocal images of roots in 7-day-old *an3-4/pAN3::AN3-3xGFP* (E), *an3-4/pAN3::AN3-GFP* (F), and *an3-4/p35S::AN3-GFP* (G) lines. epi, epidermal cell layer. GFP fluorescence and PI stain are shown in green and magenta, respectively.

Scale bars represent 50 μ m (A), 10 μ m (B and C), and 20 μ m (D–G). See also Figure S2.

Discussion

A number of studies have pointed out the possible impact of inter-cell-layer signaling between clonally distinct cells

lines used in this study (Figure S2B). These results indicate that root epidermal cells normally proliferate without AN3 protein.

Inter-Cell-Layer Movement of AN3 Protein Does Not Require Chaperonin Complex Activity

Protein movement between cells is well studied in roots (e.g., [13–15]), but not in leaves. However, a recent study revealed that the type II chaperonin complex including chaperonin-containing T-complex protein subunit 8 (CCT8) is required for the inter-cell-layer movement of a subset of mobile proteins such as maize KNOTTED1 (KN1, a homolog of SHOOTMERISTEM LESS in *Arabidopsis thaliana*) and TRANSPARENT TESTA GLABROUS1 (TTG1) in leaves [9]. Thus, a candidate mechanism for inter-cell-layer movement of AN3 protein in leaves is chaperonin-mediated protein movement. To gain insight into this, we analyzed the mobility of AN3 in a chaperonin mutant background. In this experiment, we introduced a *cct8-1* mutation into transgenic lines expressing AN3-GFP by the *PDF1* or *CLV1* promoter (*cct8-1/pPDF1::AN3-GFP* or *cct8-1/pCLV1::AN3-GFP*). The AN3-GFP signal was observed in both epidermal and mesophyll cells in both the *cct8-1/pPDF1::AN3-GFP* and *cct8-1/pCLV1::AN3-GFP* lines (Figures 4A–4D). This indicates that the inter-cell-layer movement of AN3 protein does not require the type II chaperonin complex activity.

Lastly, we carried out a genetic analysis using a double mutant of *an3-4* and *cct8-1*. We found that there were 65% fewer epidermal cells in *an3-4* leaves than in WT leaves (Figures 4E and 4F). We observed an additive decrease in the number of epidermal cells when *cct8-1* was combined with *an3-4* (70% fewer epidermal cells in *an3-4 cct8-1* double-mutant leaves compared to *cct8-1* leaves) (Figures 4E and 4F). This indicates that AN3 signaling promotes proliferation of epidermal cells in a manner distinct from that for chaperonin-mediated KN1 and TTG1 movement. We did not analyze the number of mesophyll cells in the *an3-4 cct8-1* double mutant because of a severe defect in cell layer organization between palisade and spongy mesophyll cells, particularly at leaf margins (Figure S4).

on the determination of leaf size and shape [1–4]. However, the signaling mechanism and its functional importance are largely unknown, due to the lack of understanding of the molecular entity mediating the signaling process. We here identified AN3 as a signaling molecule that mediates inter-cell-layer signaling for coordinated proliferation of epidermal and mesophyll cells in leaves.

Based on our movement assay of AN3 protein and cell-layer-specific rescue experiments, we propose a novel model to explain how clonally distinct cells coordinately proliferate to determine leaf size and shape. In this model, mesophyll cells act as a signaling source producing and supplying AN3 protein to epidermal cells, thereby promoting epidermal cell proliferation. Our experiment using the immobile AN3-3xGFP protein confirmed the functional importance of AN3 signaling derived from mesophyll cells: the proliferation defect in epidermal cells caused by *an3-4* mutation was fully complemented by AN3-3xGFP when expressed in all cell layers, but not when expressed only within mesophyll cells by use of the AN3 promoter. Importantly, we demonstrated that AN3 protein itself, but not its downstream factor, is indispensable to leaf size and shape. These findings reveal an important molecular aspect of inter-cell-layer signaling that coordinates proliferation of clonally distinct cells.

Our data on the inter-cell-layer movement of AN3 protein in leaves and roots suggest that this process is controlled in an organ-dependent manner and is associated with its functional importance for epidermal cell proliferation. Similar to AN3, the inter-cell-layer movement of KN1 is controlled in an organ-dependent manner [16]. Although this process is mediated by CCT8 [9], we showed that proliferation control of epidermal cells mediated by the inter-cell-layer movement of AN3 protein occurs normally in the *cct8-1* genetic background. Therefore, the inter-cell-layer movement of AN3 protein is developmentally regulated in a manner distinct from those described previously in leaves.

When immobile AN3-3xGFP protein was expressed by the *CLV1* promoter, a patchy distribution of the AN3-3xGFP signal

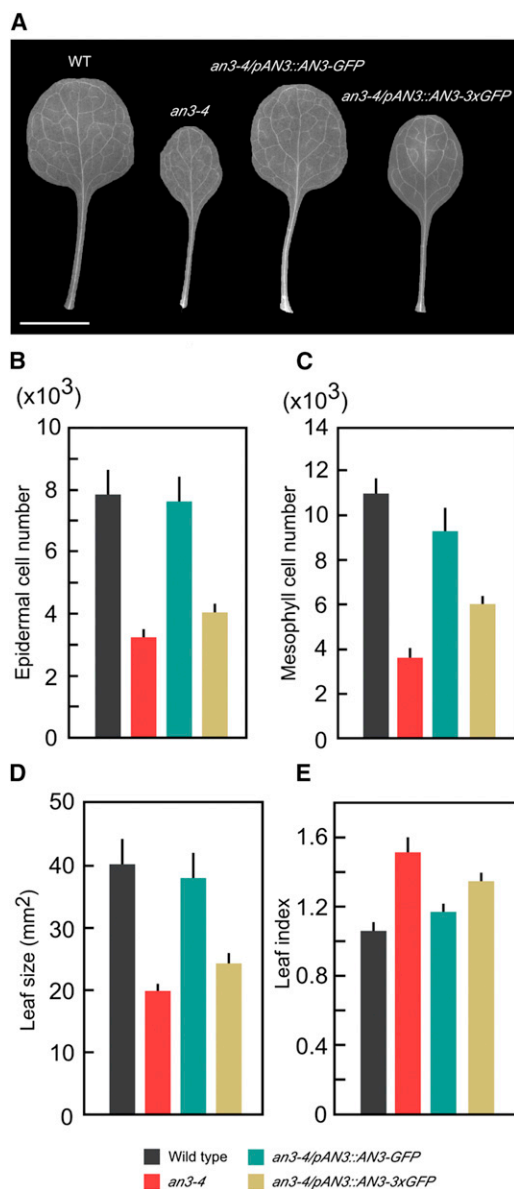


Figure 3. AN3 Signaling Is Required for Active Proliferation of Epidermal Cells and Determination of Leaf Size and Shape

(A) The first leaves of 21-day-old WT, *an3-4*, *an3-4/pAN3::AN3-GFP*, and *an3-4/pAN3::AN3-3xGFP* lines (left to right). Scale bar represents 5 mm. (B–E) Epidermal cell number (B), mesophyll cell number (C), leaf size (D), and leaf index (E) in the first leaves of 21-day-old WT, *an3-4*, *an3-4/pAN3::AN3-GFP*, and *an3-4/pAN3::AN3-3xGFP* lines. The mean \pm SD from eight individual leaves is shown. See also Figure S3.

was observed among cells (Figure 1I). In contrast, the AN3-GFP signal was uniform among *an3-4/pCLV1::AN3-GFP* cells (Figure 1K). These observations suggest that in addition to the anticlinal movement, AN3 protein moves laterally between mesophyll cells. Therefore, one reason for incomplete rescue of mesophyll cell proliferation in *an3-4/pAN3::AN3-3xGFP* and *an3-4/pCLV1::AN3-3xGFP* might be the inhibition of lateral movement of AN3 protein. In addition, as previously discussed [17, 18], we cannot exclude the possibility that biochemical interaction between epidermal and mesophyll cells plays a role, specifically that proliferation of mesophyll cells

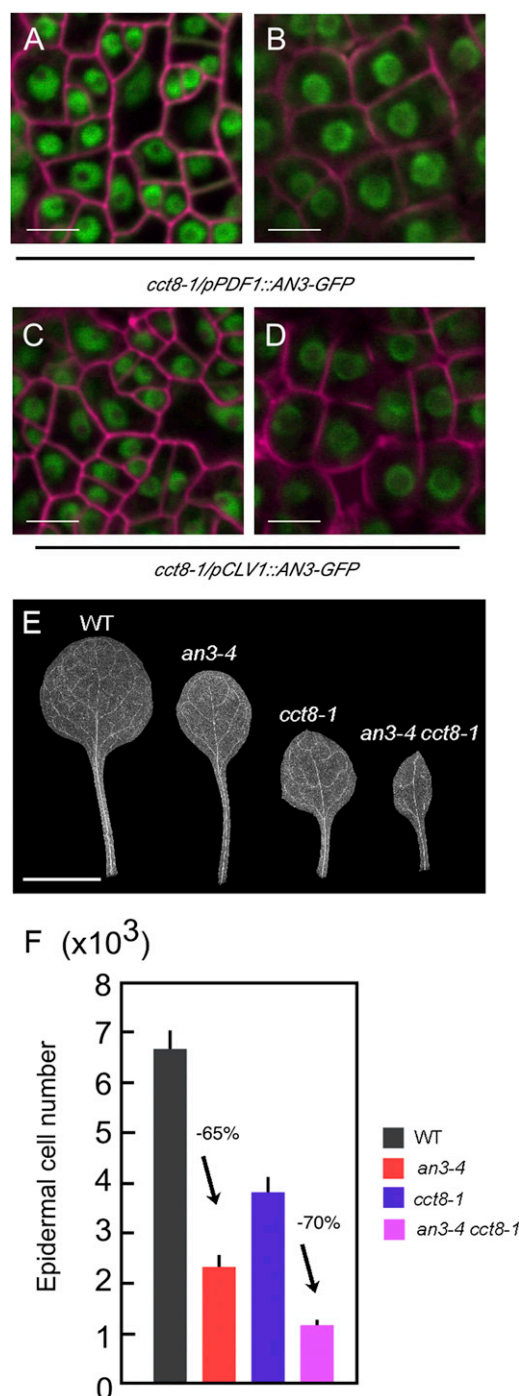


Figure 4. *an3-4 cct8-1* Double-Mutant Analysis

(A–D) Confocal images of epidermal (A and C) and mesophyll cells (B and D) in the first leaf primordia of 10-day-old *cct8-1/pPDF1::AN3-GFP* (A and B) and *an3-4/pCLV1::AN3-GFP* (C and D) seedlings. GFP fluorescence and PI stain are shown in green and magenta, respectively. Scale bar represents 10 μm . (E) The first leaves of 21-day-old WT, *an3-4*, *cct8-1*, and *an3-4 cct8-1* (left to right). Although the *cct8-1* mutant often had upwardly curled leaves, we selected flat ones in this study to ensure morphological and cellular analyses. Scale bar represents 5 mm. (F) Number of epidermal cells in the first leaves of 21-day-old WT, *an3-4*, *cct8-1*, and *an3-4 cct8-1*. The mean \pm SD from eight individual leaves is shown. Arrows indicate the difference between adjacent bars. See also Figure S4.

follows that of epidermal cells by mechanical constraint. Technical advances in the precise measurement of mechanical properties within a developing leaf are required to address this.

In summary, our data provide a novel framework in which to consider the control of leaf size and shape via inter-cell-layer signaling. Although the functional importance of epidermal cells is currently a popular topic in plant development [3, 19–21], we have shown an essential role of mesophyll cells as a source of AN3 signaling. Understanding AN3 signaling dynamics within a developing leaf represents a future challenge for understanding how leaf size and shape are determined.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.03.044>.

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