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Supplemental Information

Mechanical Regulation of Auxin-Mediated Growth

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Supplemental Inventory

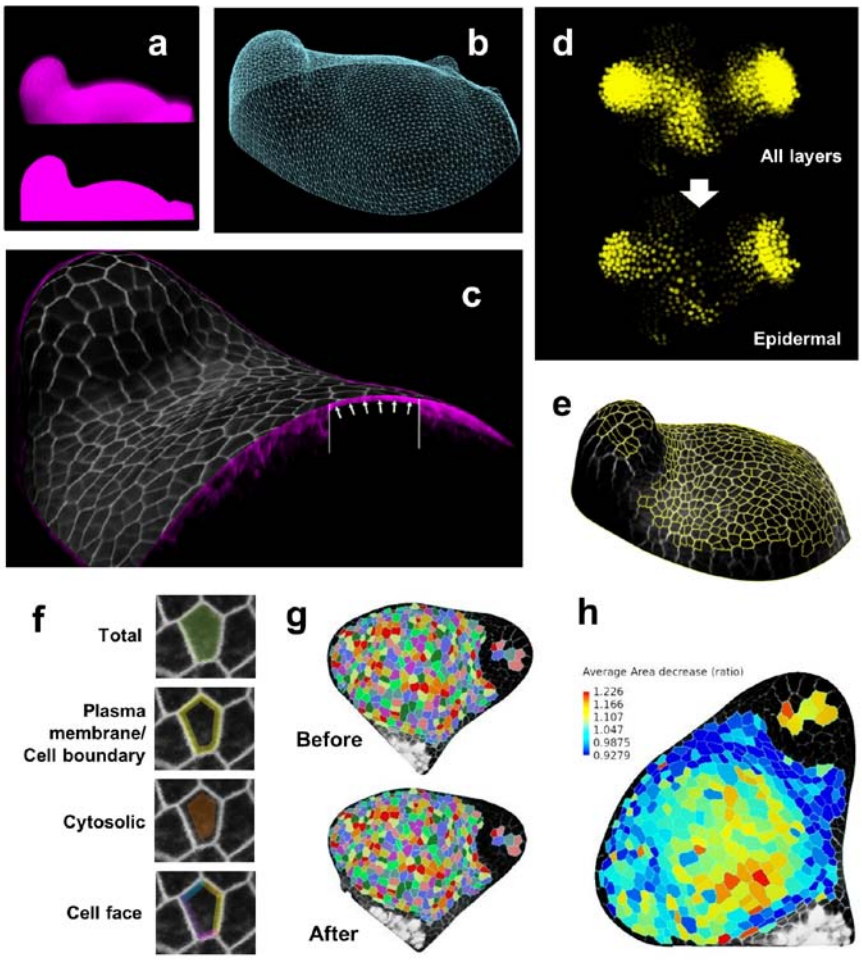


Figure S1A. MorphoGraphX Analysis of 3D Confocal Data, Related to Figure 1

The MorphoGraphX software allows image analysis on 2D curved surfaces. A typical analysis proceeds as follows: (a) Confocal image stacks are collected in two separate channels for the GFP/YFP signal and the cellular outlines (visualized by propidium iodide staining of the cell wall). The stack for the cellular outline is processed to reduce noise and produce a sharp outline of the surface. (b) The surface is then extracted as a polygonal mesh and subdivided. (c) A band of signal of uniform thickness (1-5µm from the surface) is projected onto the surface, showing the epidermal signals only (d). With MorphoGraphX images on curved surfaces can be processed using techniques normally used on flat 2D images. (e) Cell boundaries are determined using the watershed segmentation algorithm adapted to work on the curved surface mesh on which the cell wall signal is exposed. (f) After segmentation, GFP/YFP fluorescent signals are measured on a cell by cell basis, as well as geometrical properties such as cell area. The signals can be quantified in sub-cellular domains: cytosol, cell boundary (i.e. plasma membrane), and cell faces. (g) Measurements can also be compared between successive time points (e.g. before/after) by co-identification of corresponding cells. Quantification is obtained in datasheet or heat map (h) format.

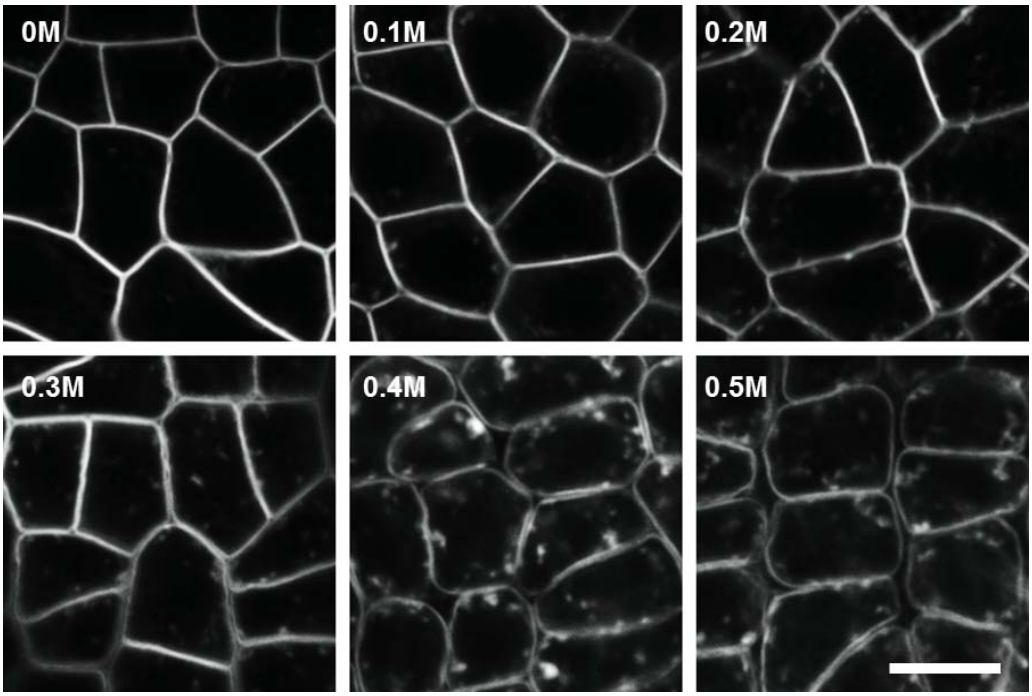


Figure S1B. Determination of the Iso-Osmotic Concentration of the Cytoplasm, Related to Figure 1

Cells on the flank of shoot apex were stained with the membrane dye FM4-64 and then subjected to 1hr incubation in 0/0.1/0.2/0.3/0.4/0.5M mannitol. Separation of the plasma membranes was detected in 0.3M and higher concentrations. Plasmolysis indicates that the turgor pressure became 0. Representative images are shown. Scale bar: 10μm.

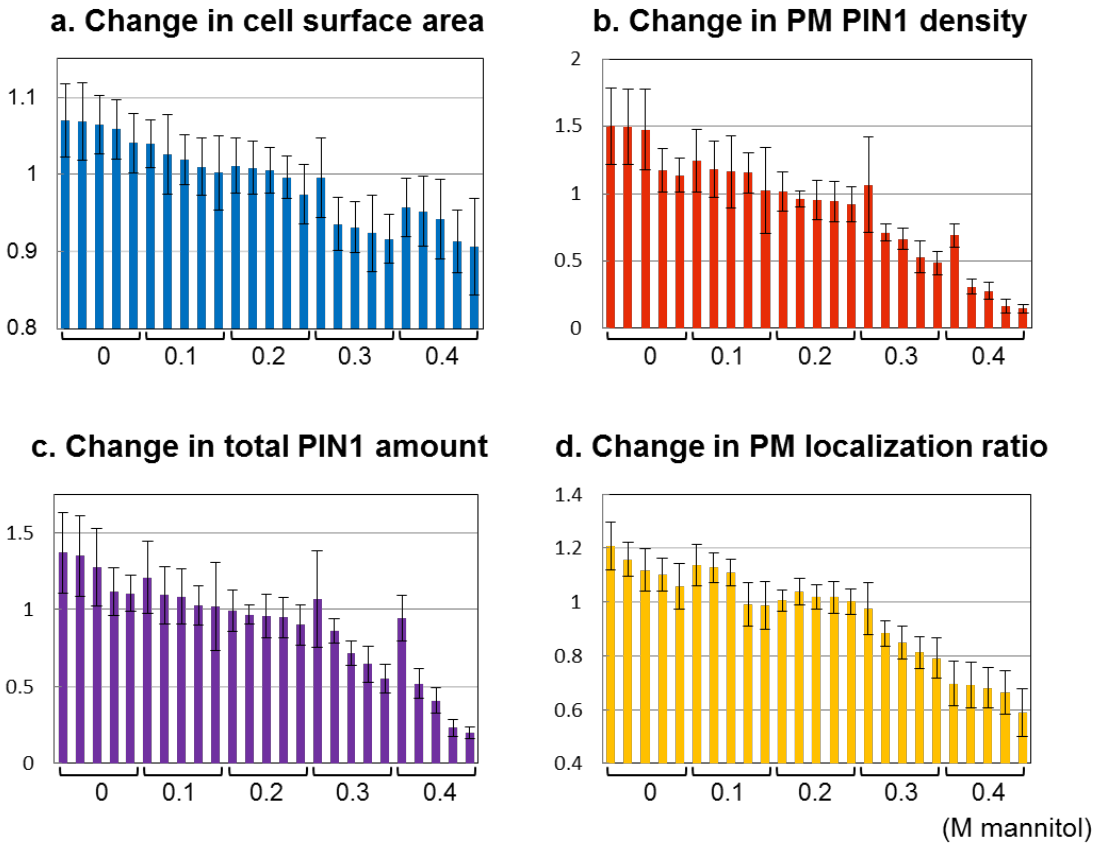


Figure S1C. Osmotic Effects on Cell Size and PIN1 Amount and Intracellular Localization, Related to Figure 1

Average and standard deviation of the osmotic effects changes (after/before ratios) on (a) cell surface area, (b) average plasma membrane (PM) PIN1 density, (c) combined PIN1 amount on the PM and in the cytosol, and (d) PM localization efficiency (i.e. PM localized/total PIN1). Five samples representing more than two biological replicates were shown for each treatment with 0/0.1/0.2/0.3/0.4M mannitol. MorphoGraphX was used to measure the changes in areas and signal intensities.

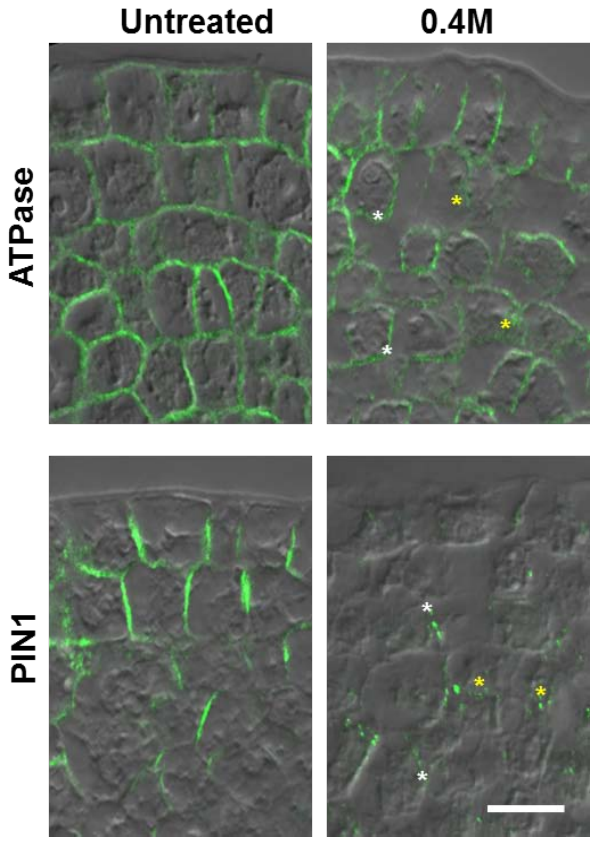


Figure S2. Subcellular Effects of Hyper-Osmotic Internalization of PIN1, Related to Figure 3

Immunolocalization of H^+-ATPase and PIN1 proteins in the central region of the wild-type tomato shoot apical meristem (longitudinal section), untreated or treated with 0.4M mannitol for 2 hours. Protein internalization after the hyper-osmotic treatment show distinct patterns. ATPase remain evenly on the plasma membrane (white *), and shimmer of the signal in the cytoplasm (yellow *). PIN1 signal is much reduced overall, but bright spots remain on the plasma membrane (white *); the only signal detected inside the cell were weak dots around the nucleus (yellow *). Scale bar: 10µm.

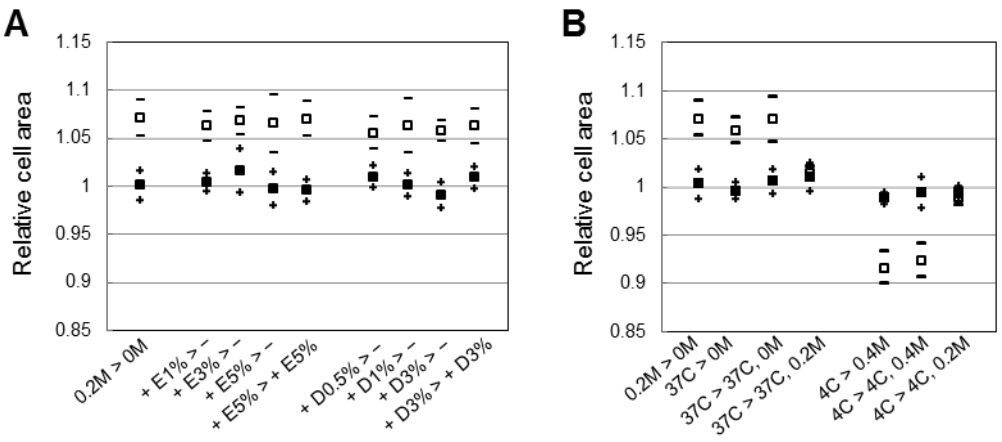


Figure S3. Effects of Membrane Modulations on Mechanical Strain, Related to Figure 5

Membrane modulation treatments with ethanol/DMSO (A) or temperature (B) did not significantly affect the mechanical strain of the tissue or the osmotically-induced changes in the mechanical strain. Tissue stain was examined by measuring changes in longitudinal area of 3-5 epidermal cells (in after to before ratio). Cell area change after the first treatment (■: average; +: standard deviation, SD) and after the second treatment (□: average; -: SD). n=5-7 samples.

In (A), the first treatment was in 0.2M mannitol with or without ethanol or DMSO (E or D, concentration in %), and the second treatment was in 0M mannitol treatment with or without 5% E or 3% D.

In (B), the first treatment was in 0.2M mannitol at room temperature (RT), 37C or 4C, and the second treatment was in 0, 0.2, or 0.4M mannitol at RT, 37C, or 4C.

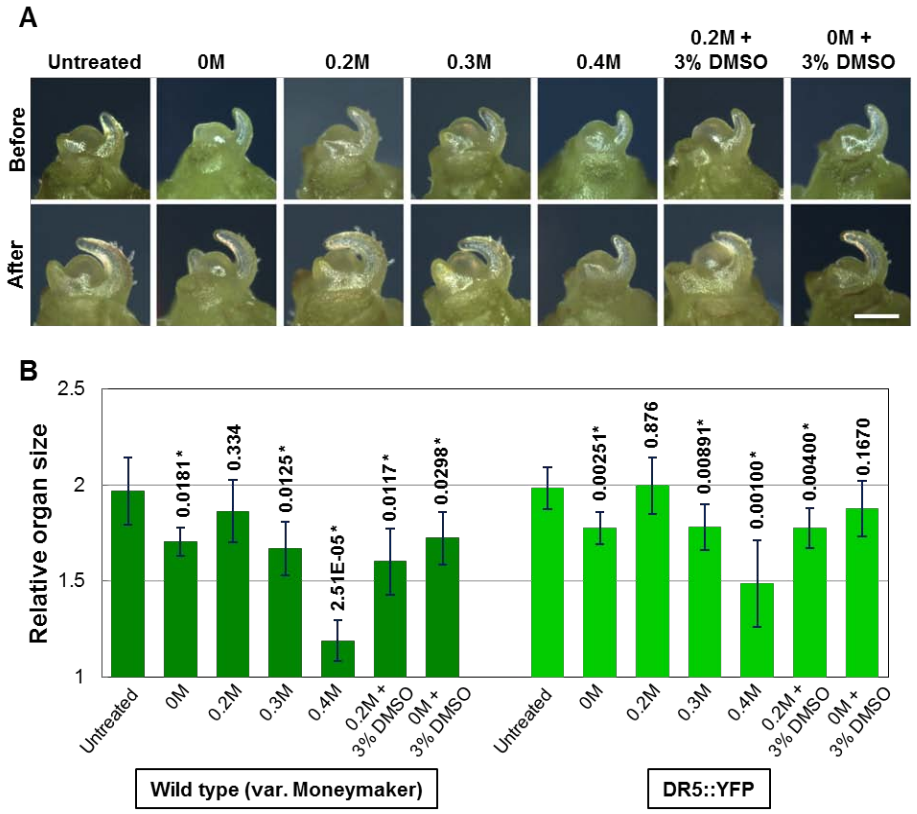


Figure S4. Effects of Osmotic and/or DMSO Treatment on Growth of Young Primordia, Related to Figure 7

Dissected wild type (var. Moneymaker) or DR5::YFP shoot apices were treated for 2hrs with 0/0.2/0.3/0.4M mannitol or for 30min with 0/0.2M mannitol containing 3% DMSO, and then cultured for 20hrs. The size change of the The P3 primordium was monitored for growth measurement (A). Change (after/before ratio) in the profile area was calculated (B). Average and standard deviations were shown for each treatment type, along with the p value from the Student t-test, in which results from each treatment was compared with the untreated samples (marked with * if they are different from the untreated samples at 95% confidence level). Scale bar: 100μm.

Supplemental Experimental Procedures

Plant Materials

The tomato transgenic line transformed with the AtPIN1p::*PIN1-GFP* construct has been characterized, and it has been verified that the GFP is expressed and localized in the same domain as the endogenous PIN1 protein [26]. DR5::*VENUS*X6 was a gift from Naomi Ori (Hebrew University, Israel) [47]. The tomato cultivar Moneymaker (Wyss, Zuchwil, Switzerland) was referred as the wild-type. Plants were grown on soil under long day (16hrs 60uE/m^2s light), in 65-80% humidity, at 20-22°C, for 14-21days. Five or more samples representing at least two biological replicates (i.e. independent batches) were analyzed in each treatment. Except for the 20hr growth assay, we pre-stained the tissue with 0.1% propidium iodide (SIGMA, St. Louis, USA) for 10-15min to monitor the viability of the cells.

Image Collection and Analysis

GFP or YFP signal was quantified using ImageJ (http://rsbweb.nih.gov/ij) or the newly developed software MorphoGraphX (http://www.MorphoGraphX.org). For both methods, 3D stacks of optical sections were collected using a confocal microscope (Leica TCS SP5) with a long-distance water immersion lens (HCX APO L UV-I 63.0 x 0.90 W) and argon laser emitting at the wavelength of 488nm (Leica Microsystems, Wetzlar, Germany). XYZ stacks were collected in three channels: #1: GFP/YFP (500-540/505-545 nm), #2: PI (615-660 nm), and #3: autofluorescence (665-800 nm). Other specifications of the scanning setting were: pinhole size 1AE, 1X zoom, 30% laser power, 30-50% laser output, and scanning speed of 200 or 400Hz.

Because many of the treatments changed the optical penetrance of the scanning media (e.g. mannitol solutions) and/or the tissue itself (e.g. the water/mannitol treatments, cold treatment, etc.), we normalized the GFP/YFP signal with the autofluorecence from the same cells in the epidermal layer on the same optical sections. The changes in autofluorescence were calculated between the averages of 50 plastid-like spots in the epidermal cells per sample (measured pixel-by-pixel gray scale-level with ImageJ). Although PI signal was also detectable in the autofluorescence channel, the plastid spots were seen only in the autofluorescence channel, not in the PI channel, and thus the treatments' effects on PI signal did not interfere with the normalization. In addition to correcting treatment-dependent visibility differences, the normalization was also effective in lowering the noise and variability depending on the batches, image analysis methods (MorphoGraphX or ImageJ), and/or photo-bleaching; normalization resulted in smaller deviations of the final data, compared to the raw PIN1 or autofluorescence data. Some of the treatments may have affected the autofluorescence, rather than or as well as PIN1. All the normalization ratios obtained for the experiments reported in this study are shown below.

ImageJ was used to measure intensity of signal per pixel to quantify the strength of autofluorescence. In some experiments (indicated in the description of each experiment), PIN1 plasma-membrane (PM) density was also measured directly with ImageJ. XYZ stacks of the longitudinal sections of AtPIN1p::PIN1-GFP shoot apices were collected (in #1-3 channels; Line Average = 8; Z interval = 1µm), and the same cells on the corresponding Z planes were identified in each scan. The #1 and #3 channels were then converted to PNG, RGB format. From the #1 channel image, GFP signal was measured at 60 points along the side of PM to which PIN1 was polarized, in the same 5-10 cells of the L1/epidermal layer near the meristem median. From the #3 channel image, autofluorescence at 50 organelles were measured from 10-15 cells from the L1/epidermal layer. The averages of the 50 measurements were then used to calculate change (after-to-before ratio or A/B ratio) in the PM PIN1 density, which was then normalized by the change in autofluorescence (Auto):

$$(\text{PIN1 density change})^{\text{after/before}} = (\text{PIN1 density}^{\text{after}}/\text{PIN1 density}^{\text{before}}) \div (\text{Auto}^{\text{after}}/\text{Auto}^{\text{before}})$$

Normalization Ratios ($Auto^{after}$ /$Auto^{before}$) Obtained in Each Experiment

Fig. 1B-G

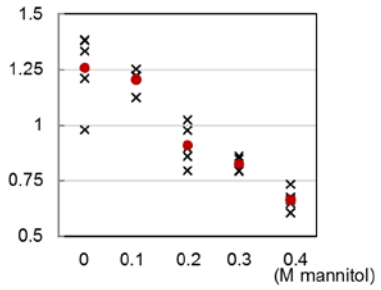


Fig. 1H

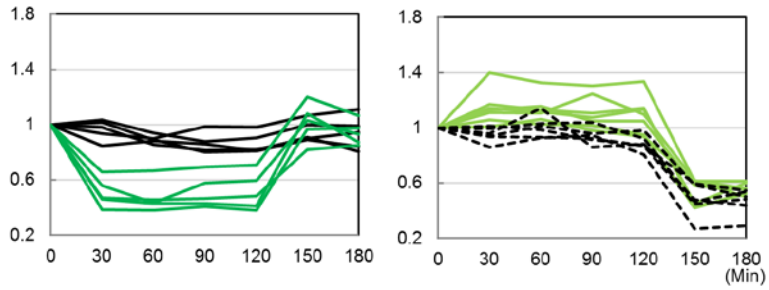


Fig. 4B

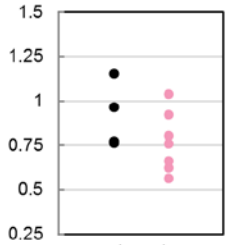


Fig. 4C

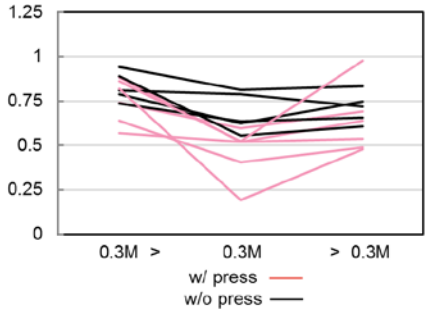


Fig. 4D

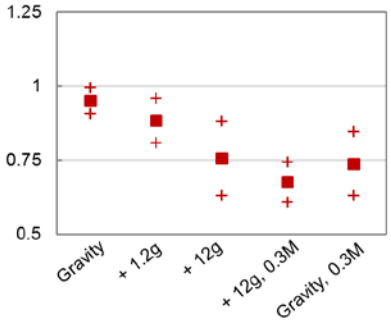


Fig. 5A

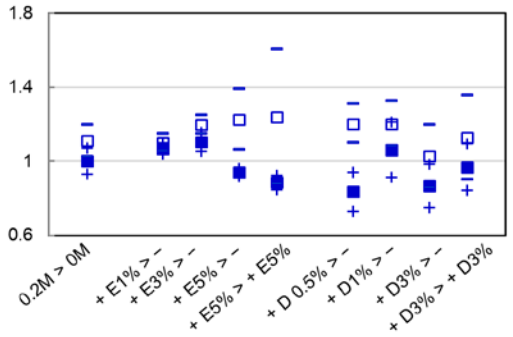


Fig. 5B

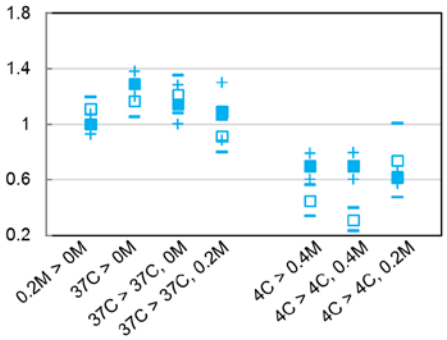


Fig. 6D

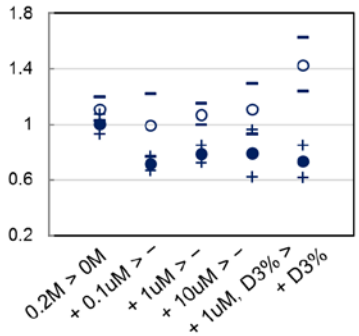


Fig. 6E

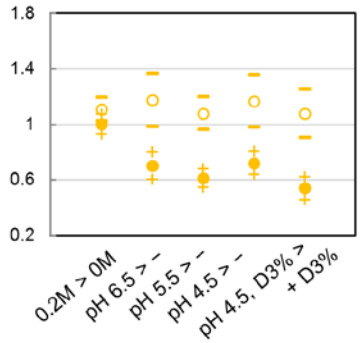
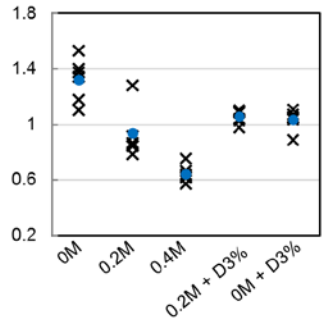
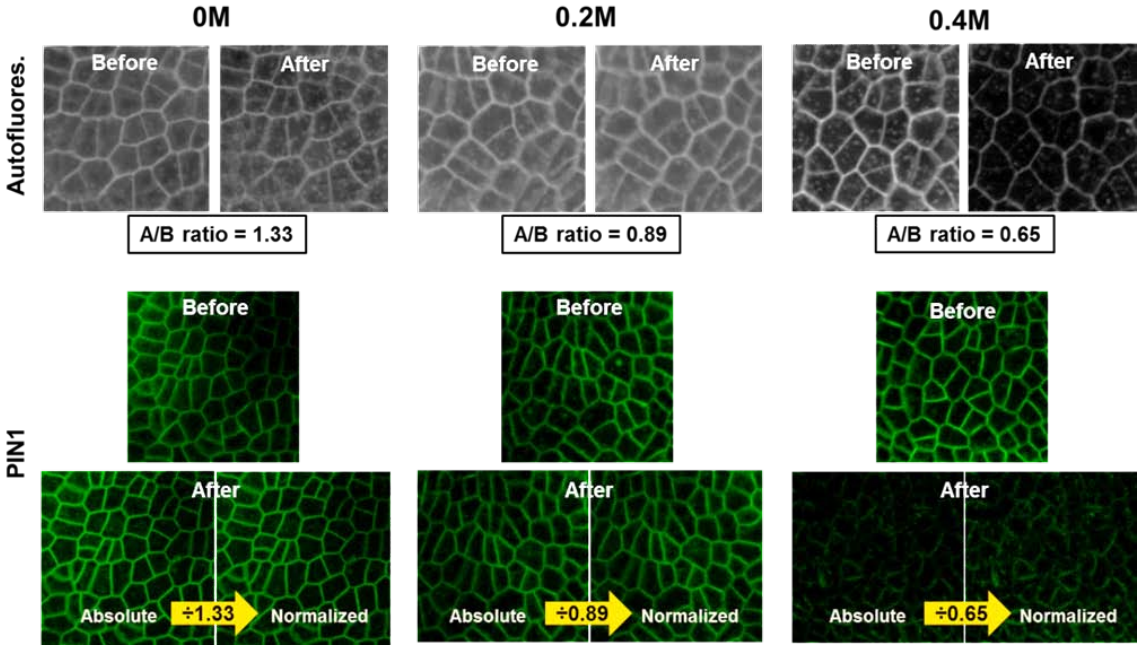


Fig. 7A



The MorphoGraphX software was developed to perform cell segmentation and quantification of fluorescent signals on curved 2D surfaces. XYZ stacks of transverse sections (at Line Average = 4) were collected at Z intervals of 0.5µm. The collection settings for the GFP/YFP and autofluorescence channels (#1 and 3) were identical during an experiment, while for the PI channel (#2) the settings were adjusted to obtain the best cell definition. The segmentation process began with the extraction of surface geometry from the #3 channel (autofluorescence channel) using the marching-cubes algorithm (Bloomenthal, 1994). After fine subdivision of the surface mesh (to 250,000-300,000 points), the paradermal signal (1-5µm from the surface) was projected onto the surface, giving a sum projection of a curved slice of the data of uniform, smaller than one-cell layer thickness near the surface. Snapshots were then taken and used to measure autofluorescence signal intensity for normalization. The #2 channel was then projected onto the same surface geometry to reveal the cell borders. The surface was segmented into cells using the watershed technique, which was adapted for use on a curved surface mesh. Corresponding cells in pre- and post-treatment scans were co-identified. Cell size changes were measured for individual cells, and tables and heat maps were created. To quantify fluorescence signals and localization, the paradermal signals (1-5µm from the surface) of stacks from the #1 channel (GFP/YFP signal) were projected onto the now segmented geometry at the normalized strength (see the examples in the figure below). Snapshots were taken to show the epidermal GFP or YFP signal. Fluorescence signal within 1µm of the cell boundary was considered PM signal, with the rest defined as internal. Tables and heat maps were then created to provide PM or total (= PM and internal) signal strength and area at cellular resolution.



The PIN1 polarity was quantified as the contrast (ratio) of PM PIN1 densities between the highest (Max) and the lowest (Min) sides within a cell. Using MorphoGraphX, GFP signal from the 3-4µm from the surface (which roughly corresponds to the middle of the outermost cell layer) was used for measurement. PM PIN1 density was calculated individually for each wall adjoining neighbor cells, and the ratio of local to Min PM PIN1 density was calculated for each side of a cell and shown as cellular heat maps. The Max/Min polarity was deduced for 300-400 cells all over the apex for each sample. Since errors in the polarity calculation favors extreme values in one tail (higher numbers), the distribution was not normal. Therefore median was obtained for each sample. Proportional change (in %) in PIN1 signal densities at Max and Min walls, as well as the ratio between the local values (Max/Min), also not normally distributed, were also calculated. We assumed the medians were normally distributed and did Student's t test between the observed Max/Min ratios and the hypothetical changes without local variations (Max/Min=1).

Images in the figures were assembled and in some cases enhanced for better clarity using Adobe Photoshop (Adobe Systems, San Jose, USA) or Microsoft Office Powerpoint (Microsoft, Redmond, USA); identical modifications were made to all images of quantitatively compared sets.

Statistical Analysis

The measurements made with ImageJ and/or MorphoGraphX were subsequently analyzed using Microsoft Office Excel (Microsoft, Redmond, USA) or R (www.r-project.org) to calculate average signal densities, ratio of PM Vs. total signals, or changes in cell area of fluorescent signals during the course of experiments. Quantifications within a sample (based on 50-400 readings, depending on the experiments) were normally distributed, except for the polarity calculations. For statistics among samples in the same data set, n was usually more than five but less than 10, and because of the tight distributions, we assumed normal distributions. Student's t test was used to test for difference between data sets.

Determination of Iso-Osmotic Points

The wild type or AtPIN1p::*PIN1-GFP* shoot apices were stained in 0.1g/L FM4-64 (Molecular Probe/Invitrogen, Eugene, USA) for 15min and then immersed in 0/0.1/0.2/0.3/0.4/0.5M mannitol for 1hr. The FM4-64 signal in the epidermal cells on the meristem flank was scanned using the confocal microscope, with excitation at 488nm and collection at 600-660nm. Images were collected with 63X long distance lens. The cytoplasmic iso-osmotic point was determined by the sign of plasmolysis (gap between the PMs of two adjacent cells). The extracellular iso-osmotic point was determined based on the (lack of) cellular strains upon 2hr osmotic treatments. The cells in the meristematic region, which do not grow at reliably detectable levels (more than 1-2%) during 2hrs, were monitored for osmotically induced deformation (expansion/shrinkage). The iso-osmotic point is close to 0.2M with mannitol, since it did not expand or shrink the cells, indicating that the solution did not alter the osmolality of the cell wall.

Osmotic Treatment

AtPIN1p::*PIN1-GFP* shoot apices were dissected, stained in 0.1% propidium iodide, and adjusted to the iso-osmotic 0.2M mannitol for 20-30min before the pre-treatment scan. The apices were then transferred to the mannitol solution and plate of indicated concentration (0-0.4M). For Fig. 1B-G and Fig. 2, the post-treatment scan was collected after 2hrs, and the data were analyzed with MorphoGraphX. For the time-course experiment (Fig. 1H), longitudinal sections were collected every 30min and analyzed with ImageJ.

Immunolocalization

Immuno-detection of endogenous PIN1 was carried out basically as described [25], except that the tissues were fixed in methanol: aceton (1:1). Wild type shoot apices were dissected and treated in the mannitol solutions of indicated concentrations for 1 or 2hrs prior to fixation. The same protocol was used for detection of the PM-localized ATPase. The primary antibody against the ATPase (a gift from Dr. Marc Boutry, Université Catholique de Louvain, Belgium; Morsomme et al., 1998) was used at 1:200 dilution. Micrographs of confocal sections superposed to Normaski light microscopy images are shown.

Membrane Dynamics Experiment

Membrane dynamics and PIN1 intracellular trans-localization were simultaneously monitored by following the membrane marker FM4-64 and PIN1-GFP signals. FM-4-64 staining protocol used was the same as in Determination of iso-osmotic point (see above), except that AtPIN1p::PIN1-GFP apices were used instead. The apices were incubated sequentially in 0.2M mannitol for 30min, 0.4M mannitol for 1hr, and in 0M mannitol for 30min. Confocal scanning of cells on the apical flank was made after 30min in 0.2M, 5min and 30min in 0.4M, and 30min in 0M. Signal was collected at 500-540nm (GFP) and 600-660nm (FM4-64) with 63X long distance lens. The gain and offset were adjusted for the best qualitative, rather than quantitative, information (i.e. spatial distributions).

Pressing Experiment

AtPIN1p::*PIN1-GFP* apices were dissected, pre-stained with propidium iodide, and calibrated in 0.2M mannitol before the pre-treatment scan (for longitudinal sections). Then the meristem dome was gently pressed down by a pulled glass rod (20-50um in diameter). Both the tissue and the rod were mounted in

0.8% agarose, 0.2M mannitol. After 30min of pressing (during which the rod was re-positioned every 5-10min to maintain the pressing effect), a post-treatment scan was obtained. The GFP signal density was measured using ImageJ. In the pressing-and-osmotic experiment, the tissue was kept in 0.3M mannitol for the entire duration. After the first 30min, pressing was applied to some of the apices for 30min and subsequently removed for another 30min.

Centrifuging Experiment

Dissected and propidium iodide-stained AtPIN1p::*PIN1-GFP* apices were mounted on a 1% agarose plate made with 0.2M mannitol and scanned in 0.2M mannitol. The apices were then spun for 30min at the speed of 100 or 300rpm inside a SIGMA 6K-15 centrifuge (SIGMA Laborzentrifugen, Osterode am Harz, Germany) with a swinging bucket-type rotor. The other samples were treated and centrifuged in 0.3M mannitol. Scans were made in transverse directions, and MorphoGraphX was used to generate snapshots of normalized epidermal signal. The same 5-10 cells in all scans were used for ImageJ quantification.

Membrane Modification Experiment

Plasma membranes were modulated by membrane-interactive compounds or temperature. The dissected, propidium idodide-stained AtPIN1p::*PIN1-GFP* apices were treated with 0.2M mannitol containing ethanol (1/3/5%) or DMSO (0.5/1/3%) for 30min. The tissues were next treated with 0M mannitol for another 30min; in some samples, 5% ethanol or 3% DMSO was added for the second treatment. Longitudinal sections were collected before and after the treatments for ImageJ quantification of PIN1 signal density. The area defined by the same 3-5 cells in each scan was compared to show the mechanical strain (i.e. cell area change). Similarly, some apices were first treated with 0.2M mannitol that was kept at the room temperature, warmed to 37°C (in a 37°C incubator), or cooled to 4°C (on ice), for 30min, and next with a 0M or 0.4M mannitol solution at room temperature, 37°C, or 4°C, for another 30min.

Auxin and Acid Growth Induction

For the auxin treatment, active auxin indole-3-acetic acid (IAA) (Fluka, Buchs, Switzerland) was applied to dissected, propidium iodide-stained AtPIN1p::*PIN1-GFP* apices in 0.2M mannitol at a commonly used range of concentrations (0.1/1/10μM; diluted from 1,000X stocks in 100% ethanol) for 30min. 10μM IAA was less effective than 0.1 or 1μM, as commonly observed for exogenous auxin treatment (Cleland, 1992). The tissues were then placed in 0M mannitol for another 30min, with or without co-treatment of 3% DMSO.

For acid treatment, dissected, propidium iodide-stained AtPIN1p::*PIN1-GFP* apices were immersed into buffers at pH 4.5/5.5/6.5, which were made in 0.2M mannitol for 30min. The buffers contained 20mM MES (Fluka, Buchs, Switzerland) as described in Cleland (1992). The tissues were then treated with 0M mannitol with/without 3% DMSO for 30min.

XYZ stacks were collected in the longitudinal sections, and PM PIN1 density and cell area were quantified using ImageJ. The area defined by the same 3-5 cells in each scan was measured to calculate the cell growth.

Auxin Accumulation Assay

The osmotic treatment of DR5::*YFP* plants [47] (Fig. 4H, I) was conducted similarly as described above and analyzed with the combination of MorphoGraphX and ImageJ. The epidermal snapshots of the YFP signal were generated, and grayscale level per pixel was measured with ImageJ for 10 cells in the P1 and P2 primordia, as well as in the meristem/I1 primordium. For the DMSO treatments, apices were immersed in 0.2 or 0M mannitol containing 3% DMSO for 30min and then left in air for 1hr 30min before the after-treatment scan in 0.2M mannitol.

Growth Assay

Wild-type or DR5::*YFP* apices were dissected and treated with 0/0.2/0.3/0.4M mannitol for 2hrs or 3% DMSO in 0 or 0.2M mannitol for 30min. Then they were grown in tissue culture for 20hrs in LD condition. The P3 primordia were pictured from the side before treatment and after culturing, and the profile area of the primordia was measured with ImageJ. The change in the medial profile area was calculated to deduce growth. Average and standard deviation were calculated, and the difference from the untreated samples was tested with Student's t test.

Supplemental References

Bloomenthal. (1994). An implicit surface polygonizer. Graphics Germs 1V, Paul Heckbert (ed.), Academic Press, New York.

Morsomme, P., Dambly, S., Maudoux, O., and Boutry, M. (1998). Single point mutations distributed in 10 soluble and membrane regions of the *Nicotiana plumbaginifolia* plasma membrane PMA2 H^+-ATPase activate the enzyme and modify the structure of the C-terminal region. J. Biol. Chem. *273*, 34837-34842.

Cleland, R. (1992). Auxin-induced growth of Avena coleoptiles involves two mechanisms with different pH optima. Plant Physiol. *99*, 1556-1561.