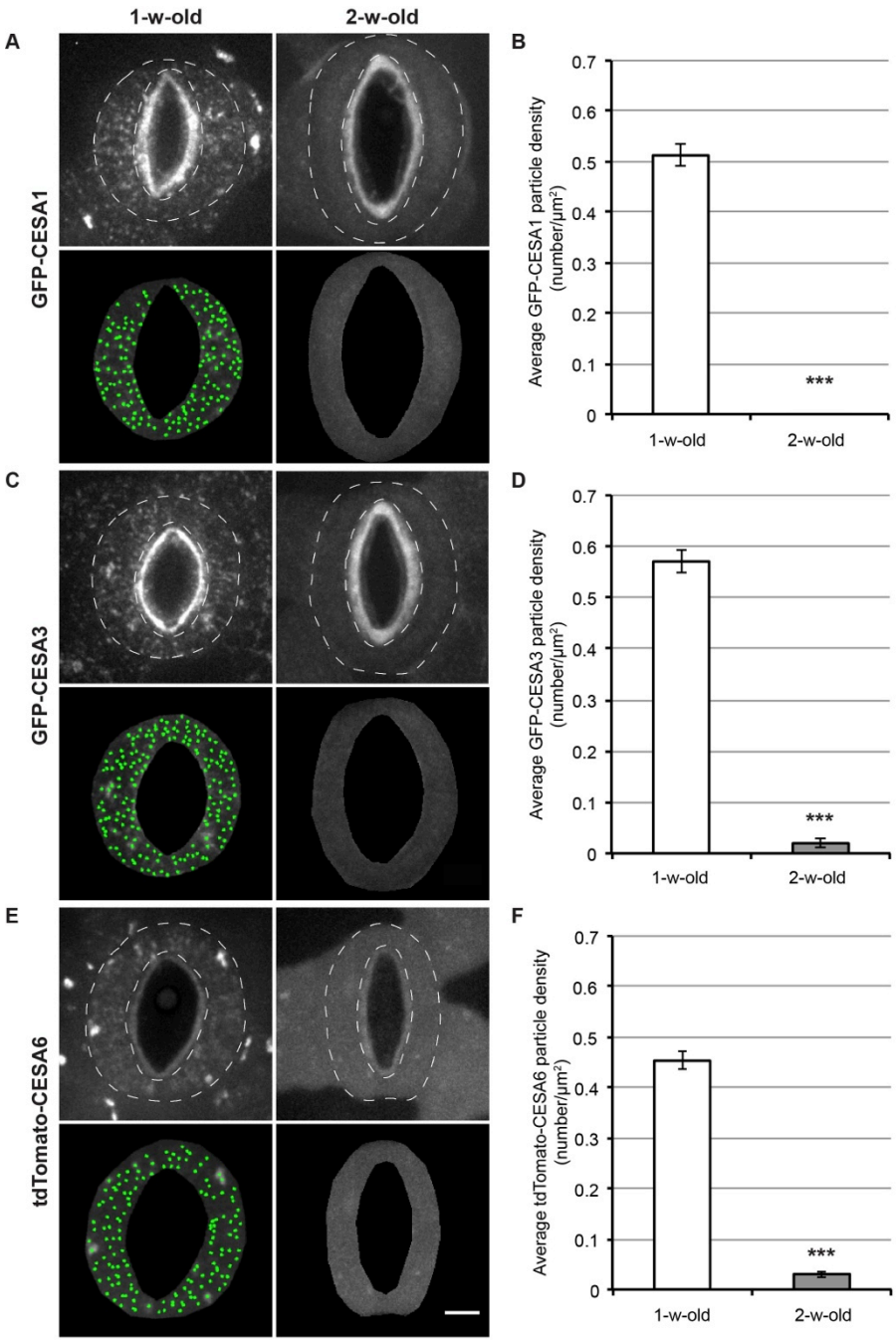
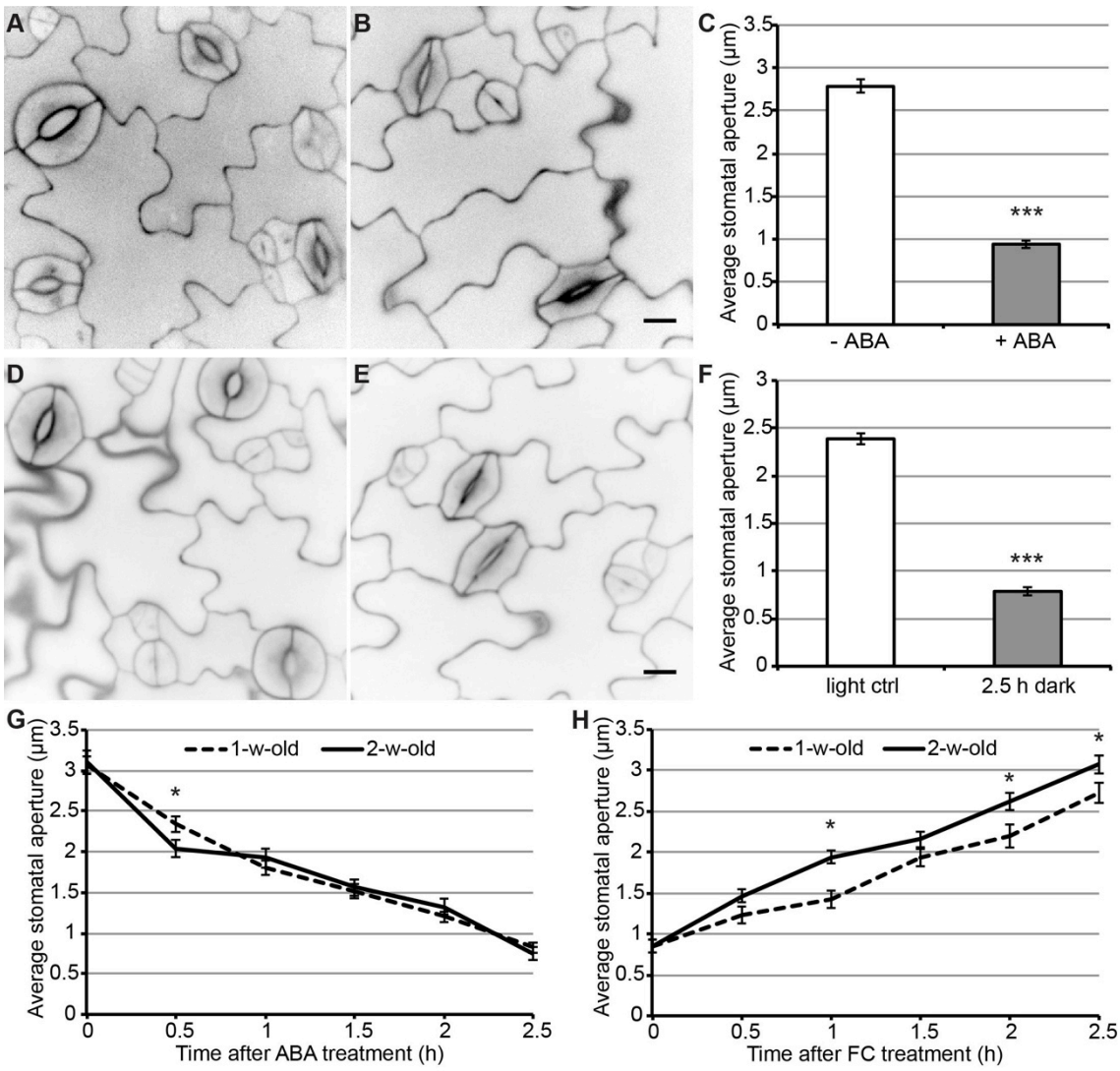
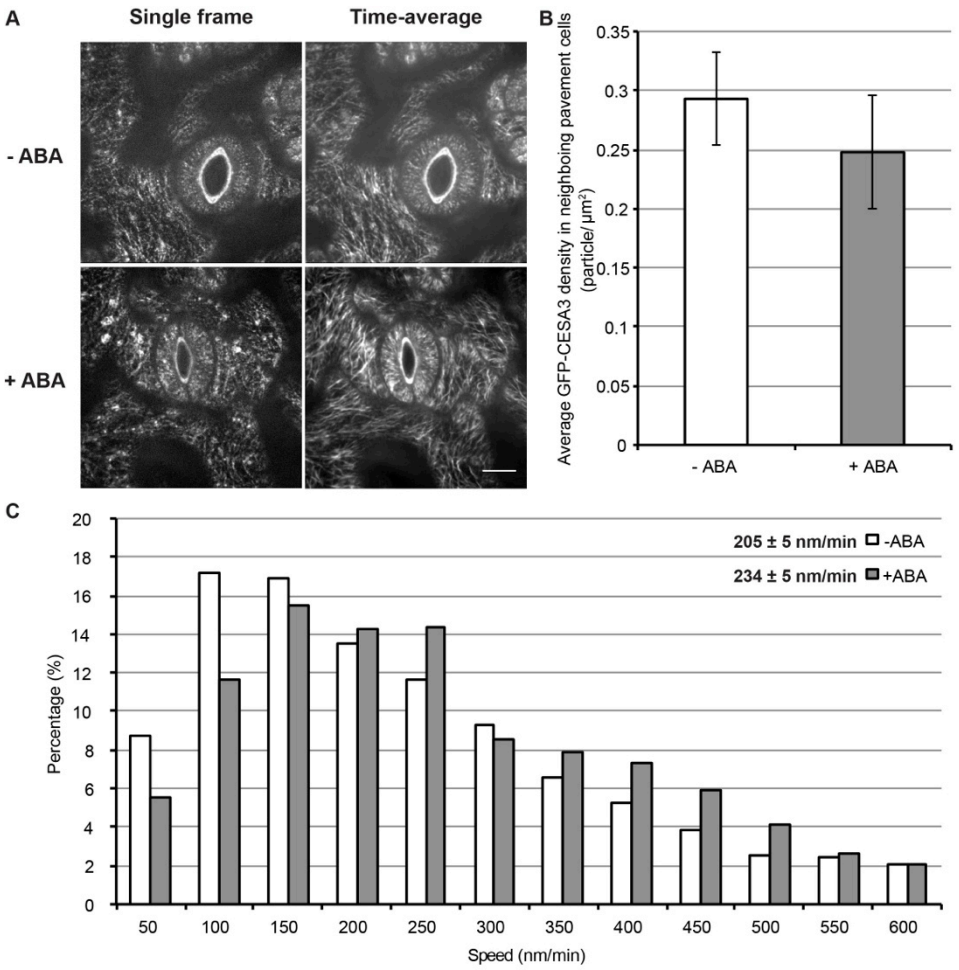


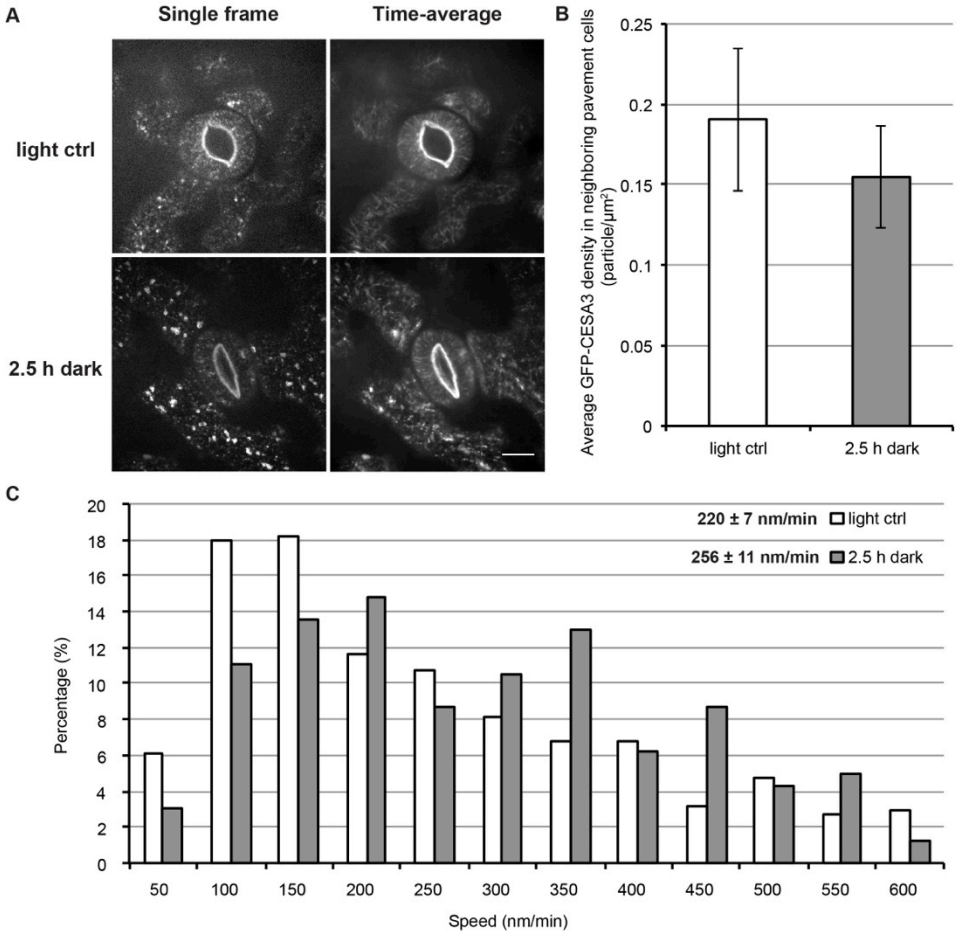
Supplemental Figure S1. Distributions of FP-CESA particles in guard cells from cotyledons at different ages. **A**, **C** and **E**, maximum projections of guard cell pairs on the abaxial side of cotyledons from 1-week-old or 2-week-old seedlings grown in soil expressing GFP-CESA1 (**A**), GFP-CESA3 (**C**), and tdTomato-CESA6 (**E**), respectively. ROIs are defined as the areas between the two dashed ovals and FP-CESA particles detected are in green. Scale bar in E is 5 μm. **B**, **D** and **F**, average density of GFP-CESA1 (**B**), GFP-CESA3 (**D**) and tdTomato-CESA6 (**F**) particles in guard cell pairs from 1-week-old or 2-week-old seedlings. Asterisks indicate significant difference between ages (n > 10 guard cell pairs from at least 4 seedlings per genotype at each age; *** P < 0.001, Student's t-test).



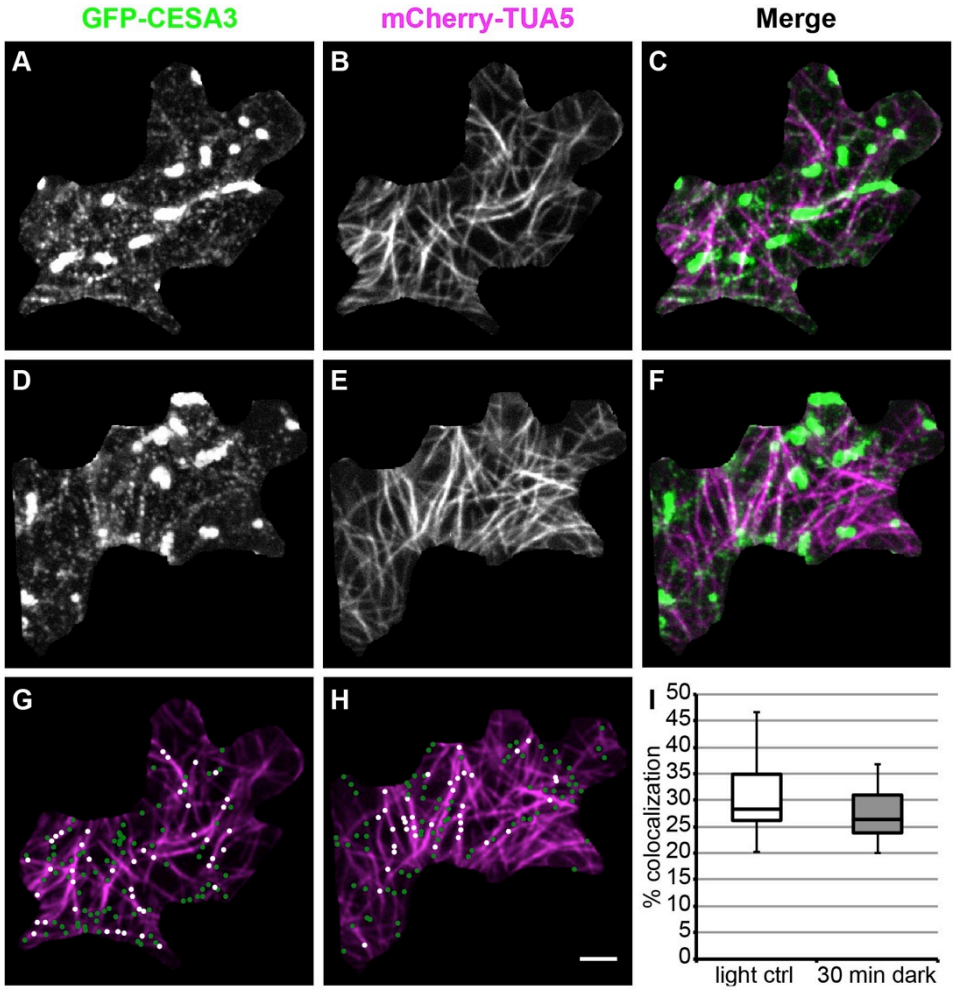
Supplemental Figure S2. Stomata in young seedlings respond to stimuli that induce stomatal opening or closure. **A**, **B**, **D** and **E**, representative snapshot images of stomata from 6-d-old seedlings in the absence (**A**) or presence (**B**) of 50 µM ABA for 2.5 h, or under light control (**D**) or after dark treatment for 2.5 h (**E**). Cell outlines were visualized by staining with 100 µg/ml PI. Scale bar is 10 µm. **C** and **F**, measurement of stomatal apertures with error bars representing standard errors. Asterisks indicate significant difference in average stomatal aperture between the two treatments (n > 200 guard cell pairs from 18 seedlings per treatment, three independent experiments; *** P < 0.001, Student's t-test). **G** and **H**, time-course stomatal responses to 1 µM FC-induced opening (**G**) or 50 µM ABA-induced closure (**H**) in 1-week-old versus 2-week-old Col-0 seedlings. Error bars are standard errors. Asterisks indicate significant difference between ages at each time point examined (n > 100 stomata per genotype per time point from three independent experiments; * P < 0.05, Student's t-test).



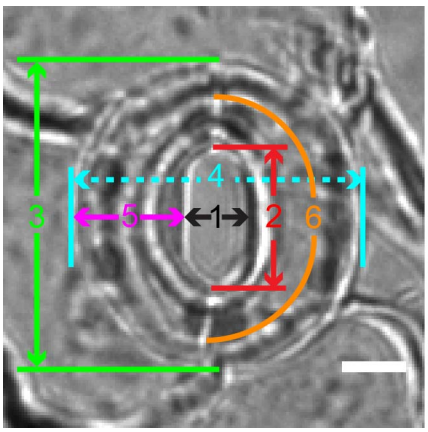
Supplemental Figure S3. GFP-CESA3 particles in neighboring pavement cells have faster movement after 50 μM ABA treatment for 2.5 h. **A**, distribution of GFP-CESA3 particles and tracks in neighboring pavement cells of 6-d-old seedlings in the absence or presence of 50 μM ABA, respectively. Single frame images are on the left and time average projections of 31 frames (5 min duration and 10 s interval) are on the right. Scale bar is 10 μm. **B**, average density measurement of GFP-CESA3 particles at plasma membrane. Error bars are standard errors (n = 11 guard cell pairs from at least 6 seedlings in each treatment, three independent experiments; P = 0.47, Student's t-test). **C**, histogram of GFP-CESA3 particle speed distributions (n > 720 particles out of 11 neighboring pavement cells from at least 6 seedlings per treatment, three independent experiments; P < 0.02, Student's t-test).



Supplemental Figure S4. GFP-CESA3 particle speed is increased in neighboring pavement cells after dark treatment for 2.5 h. **A**, distribution of GFP-CESA3 particles and tracks in neighboring pavement cells of 6-d-old seedlings grown on ½ MS + 1% sucrose plates under light control or 2.5 h dark conditions, respectively. Single frame images are on the left and time average projections of 31 frames (5 min duration and 10 s interval) are on the right. Scale bar is 10 μm. **B**, average density measurement of GFP-CESA3 particles at plasma membrane. Error bars are standard errors ($n \geq 10$ guard cell pairs from at least 6 seedlings in each treatment, three independent experiments; $P = 0.51$, Student's *t*-test). **C**, histogram of GFP-CESA3 particle speed distributions (n ~200 particles out of more than 10 neighboring pavement cells from at least 6 seedlings per treatment, three independent experiments; $P < 0.01$, Student's *t*-test).

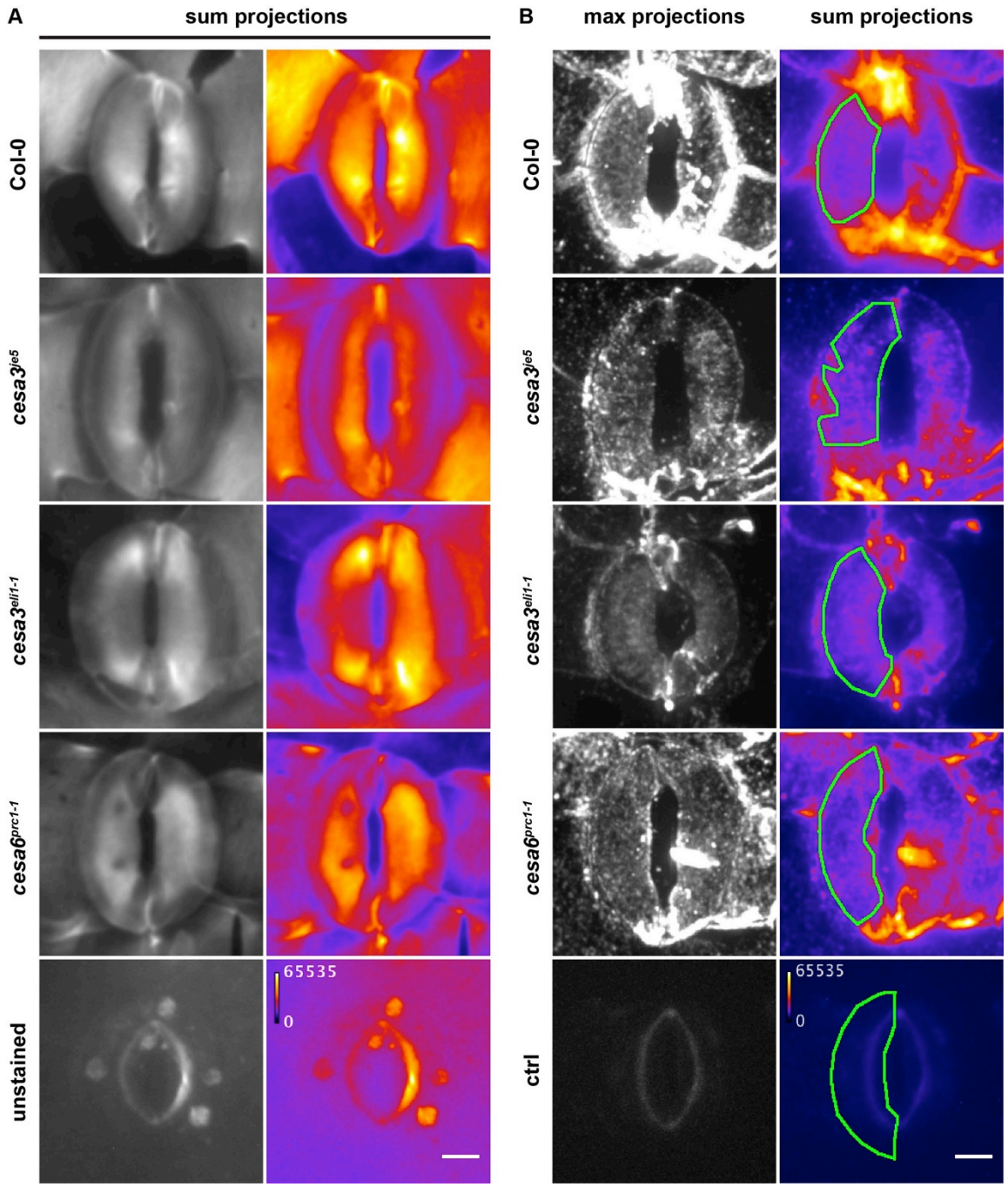


Supplemental Figure S5. Colocalization analysis of GFP-CESA3 particles and MTs in neighboring pavement cells. **A – F**, maximum projections of z-series of GFP-CESA3 particles and mCherry-TUA5-labeled MTs in pavement cells under light control condition (**A – C**) or after dark treatment for 30 min (**D – F**). In all merged images on the right (**C** and **F**), GFP-CESA3 labeling is in green and mCherry-TUA5 labeling is in magenta. **G** and **H**, representations of GFP-CESA3 particles detected in the same pavement cells as in C and F, respectively. Particles coaligned with MTs are in white and those not colocalized with MTs are in dark green. Scale bar in H is 5 µm. **I**, box plot of the percentage of GFP-CESA3 particles colocalized with MTs in neighboring pavement cells under light control or 30 min dark conditions. No significant difference was found between treatments (n > 2000 particles from ≥ 15 pavement cells for each treatment, more than three independent experiments; P = 0.12, Student's t-test).

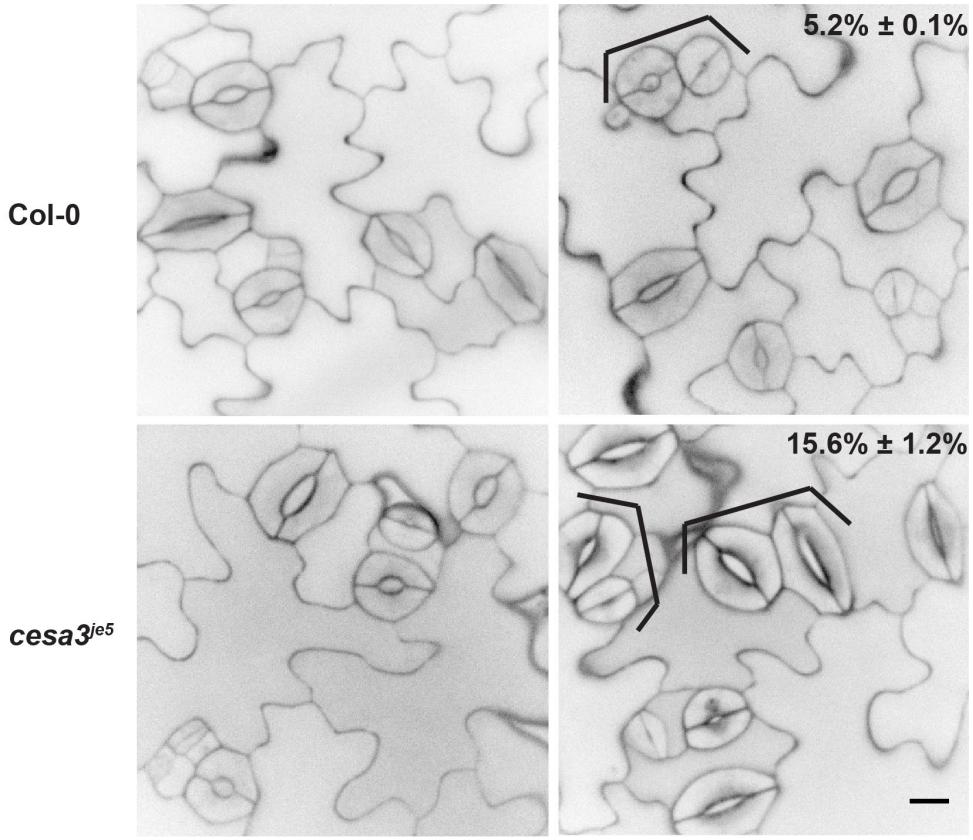


1 stomatal aperture
2 stomatal pore length
3 guard cell pair height
4 guard cell pair width
5 guard cell diameter
6 guard cell length

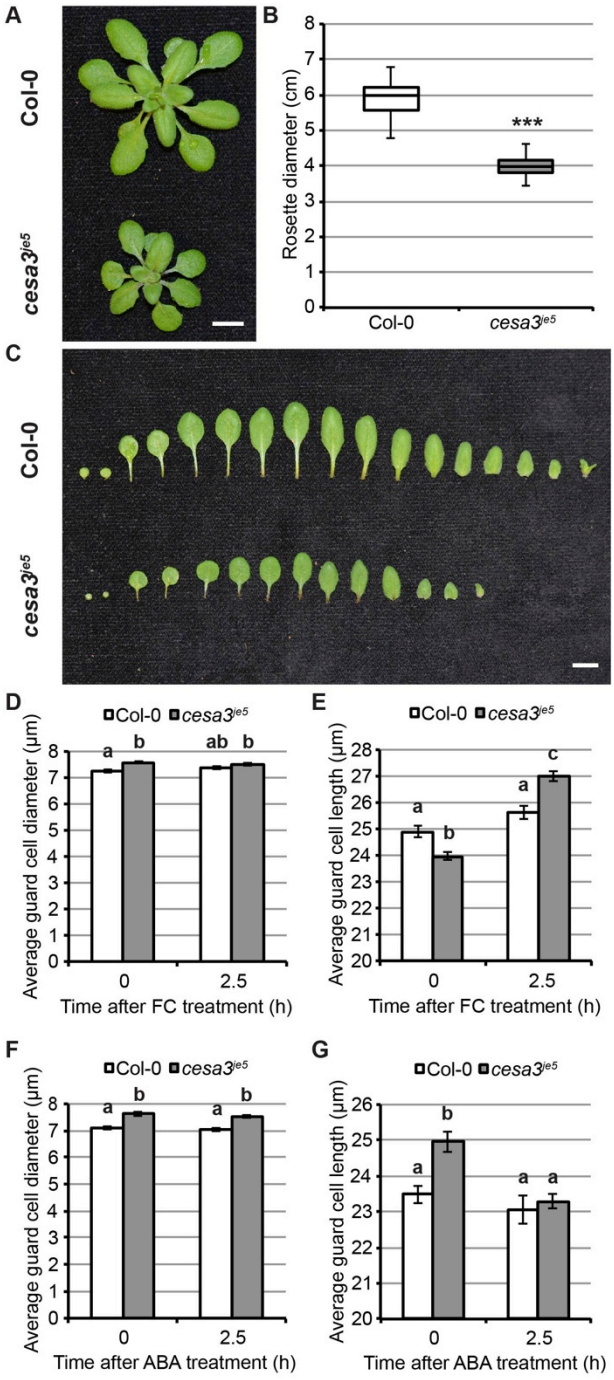
Supplemental Figure S6. Legend of measurements in a stomatal complex. Scale bar is 5 µm.



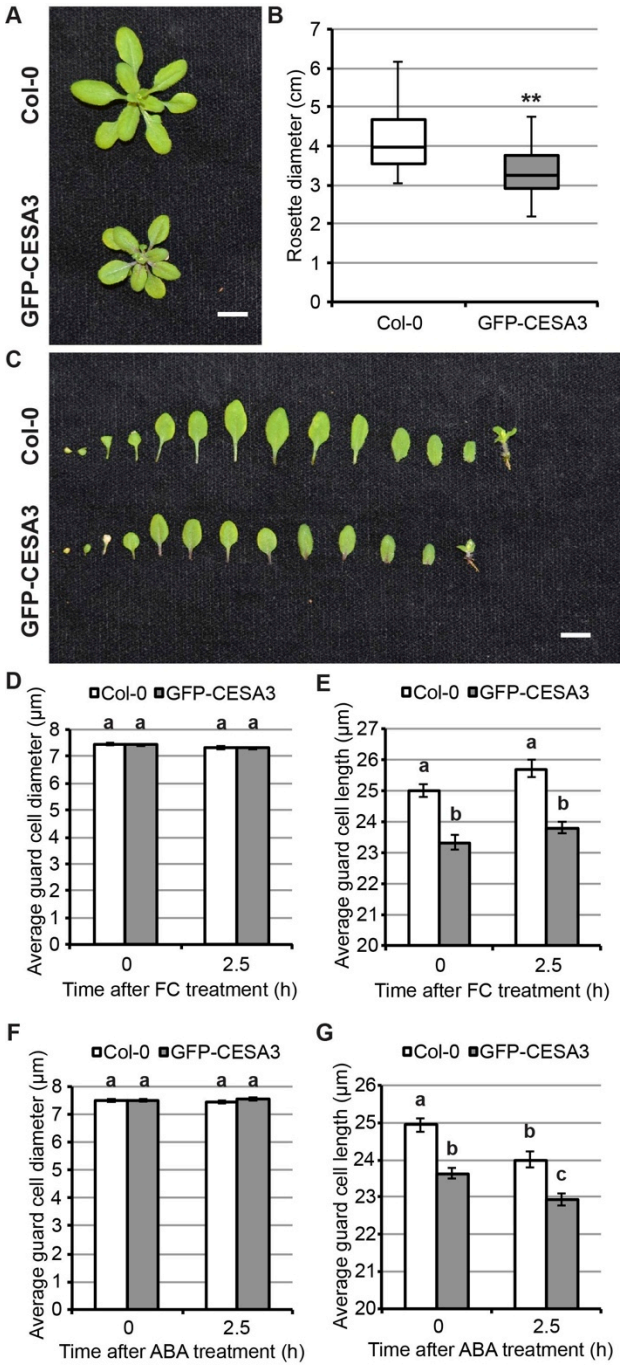
Supplemental Figure S7. Representative images of S4B staining and CBM3a immunolabeling in guard cells of Col-0, *cesa3*^{je5}, *cesa3*$^{eli1-1}$, and *cesa6*$^{prc1-1}$ plants. **A**, sum projections of S4B staining in guard cells. Images in the right-hand panel are applied with a fire look-up table. Max projection images of S4B staining are presented in **Figure 6** and **Supplemental Fig. S13**. **B**, representative images of CBM3a labeling in guard cells. Maximum projections are on the left, and sum projections are on the right with a fire look-up table applied and ROIs defined. In both A and B, the unit of color intensity scale is AFU/pixel^2 and scale bars are 5 μm.



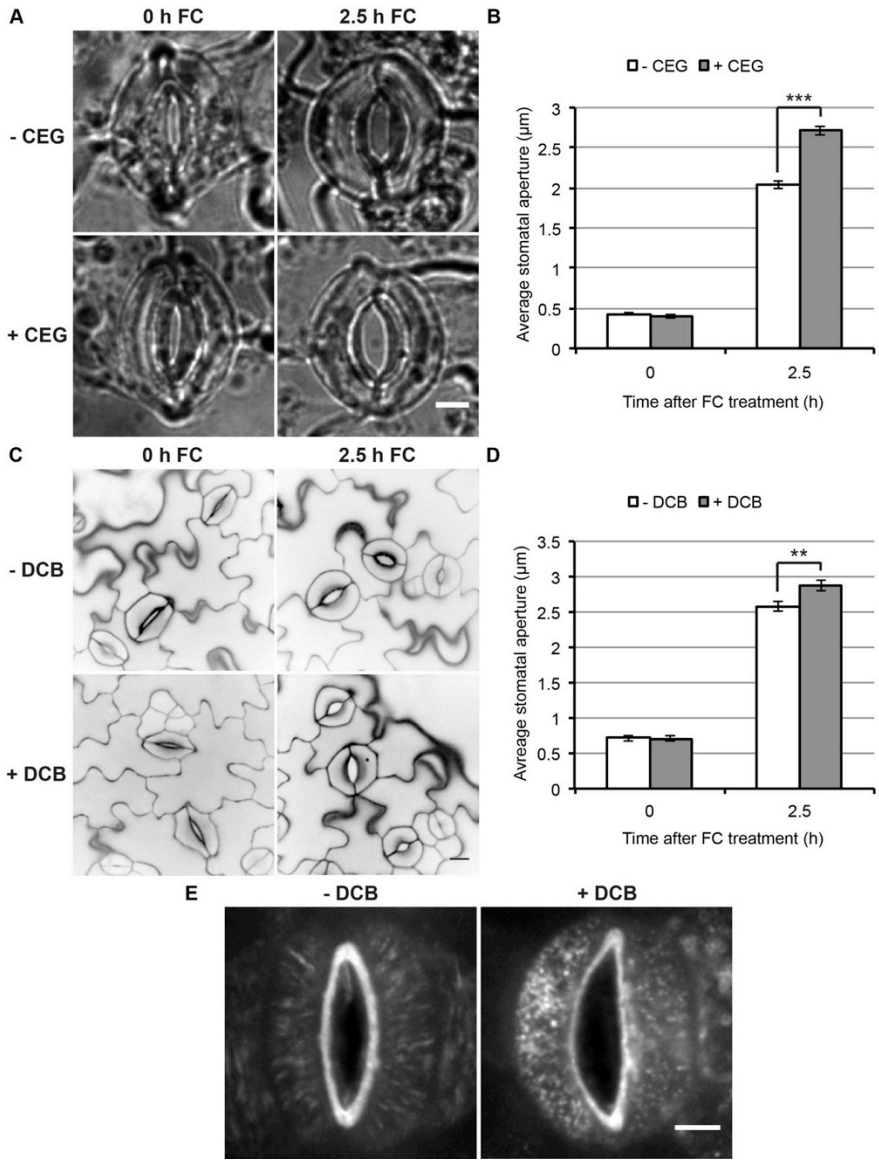
Supplemental Figure S8. Stomatal patterning phenotype in *cesa3*^{je5} mutants. Stomata on the abaxial side of cotyledons from 6-d-old seedlings were visualized by staining with 100 µg/ml PI. Brackets indicate clustered stomata that are in pairs. Numbers in the upper right-hand corner indicate the percentage of stomata that are in pairs (SEM). Scale bar is 10 µm.



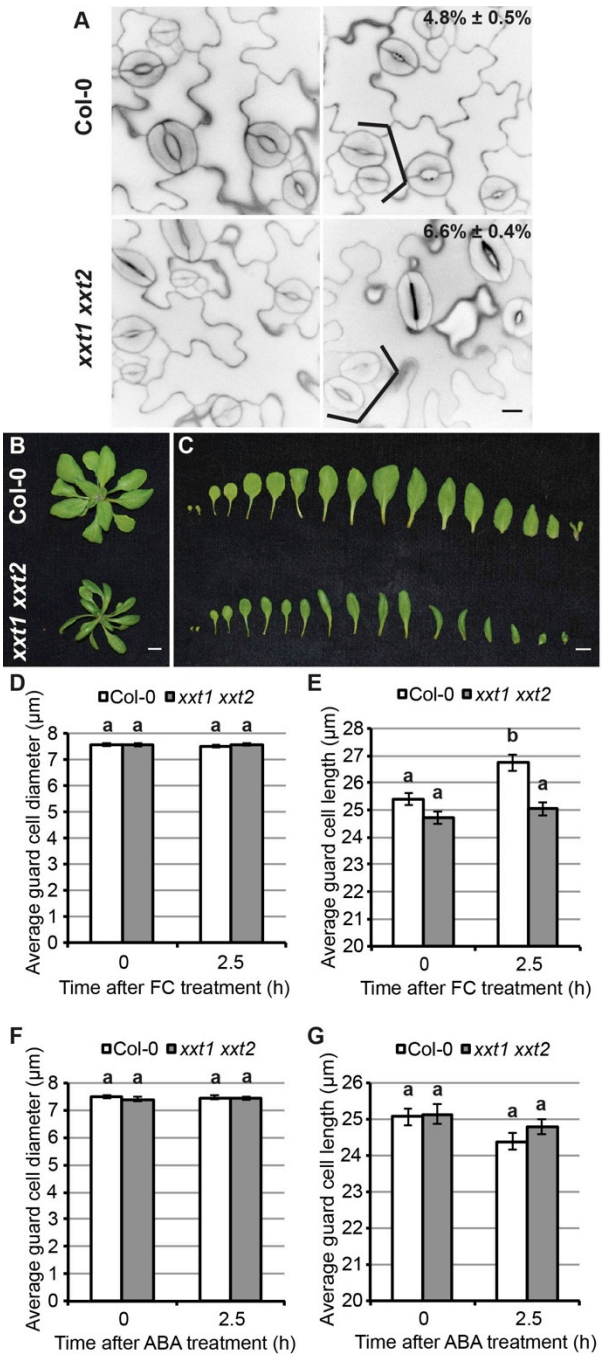
Supplemental Figure S9. Rosette leaf phenotype and measurement of guard cell diameter and length in Col-0 and *cesa3*^{je5} mutants. **A – C**, rosettes (**A**), box plot of rosette diameter measurement (**B**) and dissected individual leaves (**C**) of 3-week-old Col-0 and *cesa3*^{je5} plants grown in a 16 h light/8 h dark photoperiod. Scale bars in A and C are 1 cm. Asterisks in B indicate significant difference (n = 48 plants for each genotype from three independent experiments; *** $P < 0.001$, Student's t-test). **D** and **E**, measurements of guard cell diameter (**D**) and length (**E**) at the beginning or the end of 2.5 h FC treatment. **F** and **G**, measurements of guard cell diameter (**F**) and length (**G**) at the beginning or the end of 2.5 h ABA treatment. Error bars are standard errors and lowercase letters represent significantly different groups (n > 80 guard cell pairs per genotype per time point in each treatment, $P < 0.05$, one-way ANOVA and Tukey test).



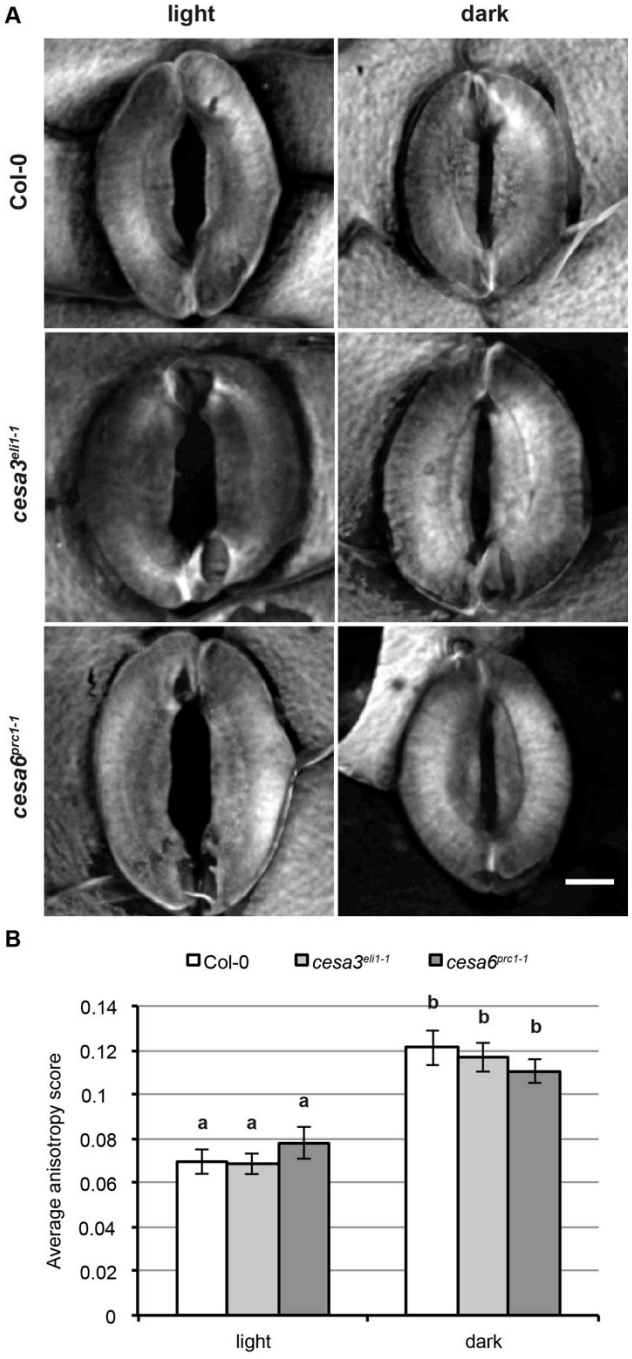
Supplemental Figure S10. Rosette leaf phenotype and measurement of guard cell diameter and length in Col-0 and GFP-CESA3 plants. **A – C**, rosettes (**A**), box plot of rosette diameter measurement (**B**) and dissected individual leaves (**C**) of 3-week-old Col-0 and GFP-CESA3 plants grown in a 16 h light/8 h dark photoperiod. Scale bars in A and C are 1 cm. Asterisks in B indicate significant difference ($n \geq 40$ plants for each genotype from three independent experiments; ** $P < 0.01$, Student's *t*-test). **D** and **E**, measurements of guard cell diameter (**D**) and length (**E**) at the beginning or the end of 2.5 h FC treatment. **F** and **G**, measurements of guard cell diameter (**F**) and length (**G**) at the beginning or the end of 2.5 h ABA treatment. Error bars are standard errors and lowercase letters represent significantly different groups ($n > 90$ guard cell pairs per genotype per time point in each treatment, $P < 0.05$, one-way ANOVA and Tukey test).



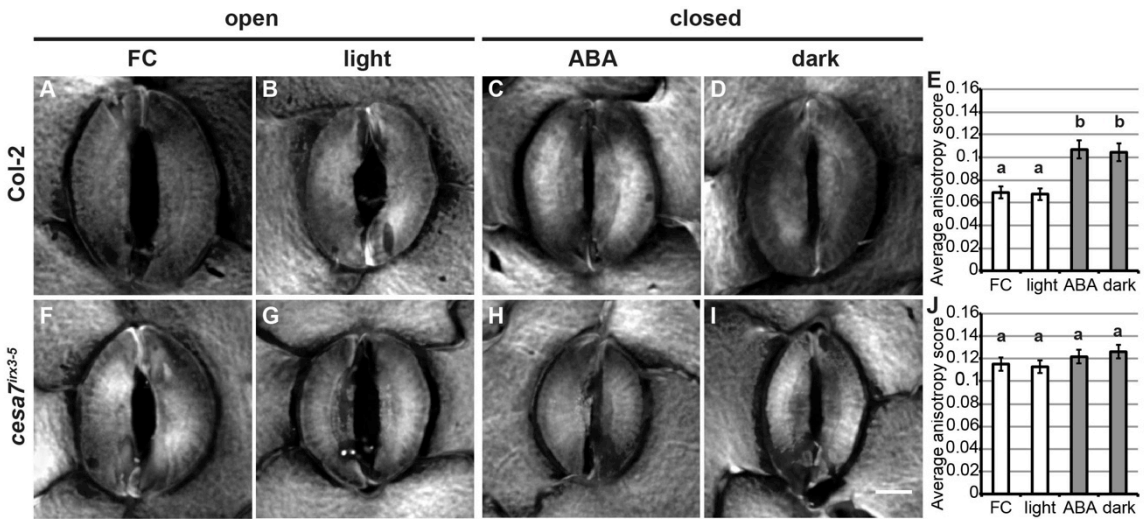
Supplemental Figure S11. Stomatal responses to FC after cellulase or DCB treatment. **A** and **B**, the effect of CEG treatment on stomatal responses to 1 µM FC-induced opening in epidermal peels from ~3-week-old Col-0 rosette leaves, with representative bright field stomatal images in A, and measurements of stomatal apertures in B. Scale bar is 5 µm. Error bars are standard errors. Asterisks indicate significant difference between treatments ($n \geq 300$ stomata per treatment per time point from more than three independent experiments; *** $P < 0.001$, Student's *t*-test). **C** and **D**, the effect of DCB treatment on stomatal responses to 1 µM FC-induced opening in 6-d-old Col-0 seedlings, with representative PI staining images in C, and measurements of stomatal apertures in D. Scale bar is 10 µm. Error bars are standard errors. Asterisks indicate significant difference between treatments ($n > 170$ stomata per treatment per time point from three independent experiments; ** $P < 0.01$, Student's *t*-test). **E**, time average projections of GFP-CESA3 movement in guard cells of 6-d-old seedlings in the absence or presence of 10 µM DCB. Scale bar is 5 µm.



Supplemental Figure S12. Stomatal patterning, rosette leaf phenotype, and measurement of guard cell diameter and length in Col-0 and *xxt1 xxt2* mutants. **A**, stomata on the abaxial side of cotyledons from 6-d-old seedlings visualized by staining with 100 µg/ml PI. Brackets indicate clustered stomata that are in pairs. Numbers in the upper right-hand corner indicate the percentage of stomata that are in pairs (SEM). Scale bar is 10 µm. **B** and **C**, rosettes (**B**) and dissected individual leaves (**C**) of 3-week-old Col-0 and *xxt1 xxt2* plants grown in a 16 h light/8 h dark photoperiod. Scale bars are 1 cm. **D** and **E**, measurements of guard cell diameter (**D**) and length (**E**) at the beginning or the end of 2.5 h FC treatment. **F** and **G**, measurements of guard cell diameter (**F**) and length (**G**) at the beginning or the end of 2.5 h ABA treatment. Error bars are standard errors and lowercase letters represent significantly different groups ($n \geq 75$ guard cell pairs per genotype per time point in each treatment, $P < 0.05$, one-way ANOVA and Tukey test).



Supplemental Figure S13. S4B-stained cellulose distribution patterns and anisotropy measurements in open versus closed stomatal guard cells induced by light or dark treatment in Col-0, *cesa3*$^{eli1-1}$, and *cesa6*$^{prc1-1}$ mutants. **A**, maximum projections of S4B staining pattern in guard cells from rosette leaves. Stomatal opening was induced by light for 2.5 h. Stomatal closure was induced by dark treatment for 2.5 h. Scale bar is 5 µm. **B**, anisotropy quantification of S4B-stained cellulose in guard cells. Error bars are standard errors and lowercase letters represent significantly different groups ($n \geq 12$ guard cell pairs per genotype per treatment; $P < 0.05$, one-way ANOVA and Tukey test; ANOVA was performed across genotypes and across treatments).



Supplemental Figure S14. S4B-stained cellulose distribution patterns and anisotropy measurements in open versus closed stomatal guard cells of Col-2 and *cesa7*$^{irx3-5}$ plants. **A – D** and **F – I**, maximum projections of S4B staining pattern in guard cells from rosette leaves. Stomatal opening was induced by 1 µM FC for 2.5 h (**A** and **F**) or light for 2.5 h (**B** and **G**). Stomatal closure was induced by 50 µM ABA for 2.5 h (**C** and **H**) or dark treatment for 2.5 h (**D** and **I**). Scale bar in I is 5 µm. **E** and **J**, anisotropy quantification of S4B-stained cellulose in guard cells within each genotype. Error bars are standard errors and lowercase letters represent significantly different groups ($n \geq 24$ guard cell pairs per genotype per treatment; $P < 0.05$, one-way ANOVA and Tukey test).

Supplemental Table S1. *Measurement of GFP-CESA3 particle density in guard cells, guard cell area, the ratio of thresholded area to guard cell area, and fluorescence intensity of mCherry-TUA5-labeled MTs in guard cells under light control condition or after dark treatment for 30 min*

Treatment	GFP-CESA3 density (particles/μm^2)	Guard cell area (μm^2)	Thresholded area/ guard cell area	Fluorescence intensity (AFU/μm^2)
light control	0.47 ± 0.03	319 ± 8	0.398 ± 0.008	115,673 ± 4061
30 min dark	0.38 ± 0.04	361 ± 21	0.421 ± 0.010	112,409 ± 2828

Measurements were performed using the same image set as in colocalization analysis of GFP-CESA3 particles and MTs (**Figure 2**). Values are presented as mean ± SE and no significant difference was found between treatments by Student's *t*-test (n ≥ 24 guard cell pairs for each treatment, more than three independent experiments). AFU, arbitrary fluorescence unit.