

Supplementary Information

V-ATPase-mediated acidification of the TGN/EE is required for exocytosis and recycling in *Arabidopsis*

Supplementary methods

Transmission Electron Microscopy (TEM). For TEM analysis of wild type (Col-0) and *det3*, root tips were chemically fixed with 2.5 % glutaraldehyde for 2 h, 1% osmium tetroxide for 90 min and 1% aqueous uranyl acetate for 90 min. Thereafter samples were dehydrated in a graded series of ethanol, and finally embedded in epoxy resin. Ultrathin sections were stained with 1% aqueous uranyl acetate and lead citrate.

For TEM analysis of concanamycinA (ConcA)-treated root tips, seedlings were treated for 24 h with 1 μ M ConcA (Santa Cruz Bioscience). Samples were high pressure frozen and freeze substituted as reported¹. Ultrathin sections of 80–90 nm were obtained with using a “Ultracut S” (Leica) ultra-microtome. Sections were mounted on 100 hexagonal-mesh copper grids covered with formvar film (0,6% polyvinyl formaldehyde in chloroform) and observed with a “JEM-1400” (JEOL, Japan) electron microscope operating at 80 kV. Images were taken with a TVIPS FastScan F214 digital camera (Gauting).

Live-cell imaging. The laser-scanning confocal images (i.e. BRI1-GFP, BRI1-YFP, FM4-64 and AFCS) were acquired with a $\times 60$ water immersion lens (NA1.2) mounted on a laser scanning confocal microscopy (Olympus Fluo View 1000), at digital zoom 3. The imaging position of etiolated hypocotyls was close to the apical hook and that of roots was kept consistently in the same region of the meristematic zone of the root tip, 10 to 15 cells above the quiescent center. For BFA body size and BFA body number analyses, four images of root epidermal cells each with a 2.5- μ m Z axis distance, were taken and stacked together before quantification. GFP fluorescence was excited with a 488 nm laser line and detected between 500 nm and 550 nm. For the heat shock induction assay, pHS:BRI1-YFP/Col-0 and

pHS:BRI1-YFP/*det3* were incubated at 37°C on 1/2 MS agar plates for 1 h before chasing the fluorescence signal at room temperature. Fluorescence of the yellow fluorescence protein (YFP) was excited with a 515 nm laser line and detected between 530 nm and 600 nm. For quantification, fixed ROIs were selected to measure the fluorescent intensity of both plasma membrane and intracellular space or background with ImageJ. The relative plasma membrane fluorescence was calculated by dividing the plasma membrane intensity by the intracellular or background intensity. For FM4-64, fluorescence was excited with a 514 nm laser line and detected between 592-759 nm. AFCS was visualized with 635 nm laser excitation and a spectral detection bandwidth of 655–755 nm.

For images generated with a spinning-disk confocal microscope (i.e. GFP-CesA3, and VHA-a1-RFP), seedlings expressing the fluorescently tagged marker protein were imaged on a confocal microscope equipped with a Yokogawa spinning disk CSU-X1/A1 head fitted to a Nikon Ti-E inverted microscope under a 100/1.4 NA oil immersion objective, QUANTEM:512SC CCD camera (Roper Scientific) and $\times 1.2$ lens between the spinning disk unit and the camera. Excitation switching and shuttering were done by a multi-channel AOTF device. Emission filtering was accomplished with band pass filters (530/50 nm for GFP, 640/50 nm for RFP, and 525/50 nm for YFP; Chroma Technology). GFP and RFP were excited at 491 nm and 561 nm, respectively, by solid-state lasers with typical exposure times of 600-800 ms. Emission was collected through band-pass filters (Chroma Technologies): 525/50 nm (GFP), 535/30 nm (YFP) and 630/75 nm (RFP). Seedlings were mounted between a 76 \times 26 mm cover glass over which a 1 mm thick 1% agar pad was placed, affixed on a 15 mm round cover glass.

SYP61-pHusion and ST-pHusion, after 1 h of treatment with brefeldinA (BFA) or with DMSO in the dark, were imaged with Leica SP5 II confocal laser-scanning microscope. Cells in the elongation zone of the root of 6-day old seedlings were documented with a HCX PL APO λ blue 63.0 \times 1.20 UV water immersion objective and excited with a VIS-Argon laser at 488 nm. GFP emission was detected at 500-550 nm with a Hybrid Detector (HyD) operating in "Standard" mode.

Colocalization analysis. For SYP61-CFP VHA-a1-mRFP and VHA-a1-GFP VHA-a1-mRFP, five images of 10 independent seedling roots were obtained with a HCX PL APO λ blue 63.0 \times 1.20 UV water immersion objective and HyD in "Standard" mode on a Leica SP5 II confocal laser-scanning microscope setup. To determine the maximum correlations of the experimental setup, 30 images were taken of 10 μ M TetraSpeck™ fluorescent microspheres (Molecular Probes/Invitrogen). A resolution of 1024 \times 1024 pixels and a zoom of 2.5 \times was chosen to adjust a voxel size of 96 nm and was maintained for each sample. Cyan fluorescence protein (CFP) was excited at 458 nm and emission detected between, 460-530 nm, RFP excited at 561 nm and detected at 610-670 nm, GFP excited with 488 nm laser line and emission detected at 490-545 nm. All images were processed with ImageJ by means of Gaussian Blur Filter with a sigma radius of 1.00. Pearson's and Spearman's correlation coefficients, as well as scatterplots were calculated with the PSC Colocalization plugin² and a threshold level of 10.

CesA, Golgi, and TGN/EE motility analyses. During all experiments, cell viability was verified by monitoring cytoplasmic streaming. All image processing was done with ImageJ. For analyses involving signal intensity measurements, only linear adjustments to pixel values were made. For other images, background signals were reduced with the 'Subtract Background' tool (rolling ball radius of 20–30 pixels) in ImageJ. Image drift was corrected with the ImageJ plugins: StackReg45 and MultiStackReg (<http://www.stanford.edu/~bbusse/work/downloads.html>). Postacquisition frame averaging (http://valelab.ucsf.edu/~nico/IJplugins/Running_ZProjector.html) was applied to time series. Hypocotyls of 3-day-old etiolated seedlings were analyzed and movies were acquired from single frames of either a 5-min time series with intervals of 5-sec (CesAs), or a 1-min time series with intervals of 1 sec (Golgi or TGN/EE). Images were processed with ImageJ. Motility of GFP-CesA3 at the plasma membrane, or of GFP-CesA3-containing Golgi bodies were calculated from kymographs created in ImageJ as described^{3,4}. The "subtract background" tool (rolling ball radius 30 to 50 pixels) and StackReg plugin⁵ were used for correction of background focus drifts.

AFCS and FM4-64 uptake analyses. AFCS uptake was done as previously described^{6,7}. Briefly, five 5-day-old seedlings were dipped in a 200- μ l droplet of ligand. Seedlings were pulsed for 20 min and chased for 3 min or 40 min in 1/2 MS medium after three washes. Pictures were taken with an Olympus Fluo View 1000. The BRI1-GFP and AFCS signal colocalizations were quantified with the Olympus Fluo View software. FM4-64 uptake was done on 5-day-old light grown seedlings as described previously⁸. Plants were incubated in liquid 1/2 MS medium with 2 μ M FM4-64 for 5 min, 10min , 30 min and 1 h, 2 h, 4 h. Seedlings were washed six times in 1/2 MS medium before imaging. Quantification of relative intracellular/PM fluorescence intensity was carried out on unsaturated images with ImageJ using similar method for heat shock induction assay mentioned above.

Real-time quantitative (q) PCR. Total RNA was extracted from 20 5-day-old wild type, BRI1-GFP/*det3*, and BRI1-GFP seedlings with the RNeasy kit (Qiagen). For pHS:BRI1-YFP and pHS:BRI1-YFP *det3*, 20 5-day-old seedlings were induced at 37°C for 1 h followed by 1-h recovery at room temperature before RNA extraction. For the cDNA preparation (iScript cDNA synthesis kit), 1 μ g total RNA was used according to the manufacturer's protocol (Bio-Rad) and diluted 10 times before PCR amplification. Real-time quantitative PCR (qPCR) analysis was done with SYBR green I Master kit (Roche Diagnostics) on a LightCycler 480 (Roche Diagnostics). Expression of *BRI1* and *YFP* was normalized to the expression of the *CAP-BINDING PROTEIN 20 (CBP20)* gene.

Protein extraction and western blot analysis. For BES1 dephosphorylation analysis and BRI1-GFP detection, total proteins were extracted as described previously⁸. Five-day-old *Arabidopsis* seedlings were homogenized in liquid nitrogen and extraction buffer consisting of 20 mM Tris-HCl, 150 mM NaCl, 1% SDS, 100 mM DTT, and protease inhibitor cocktail (Complete Mini EDTA Free, Roche). BES1 was detected with rabbit polyclonal anti-BES1 antibodies 10 (1:1000)⁹ and horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (1:10000; NA934, GE-Healthcare). GFP was detected with HRP-conjugated anti-GFP

antibodies (1:40000, Miltenyi Biotech) and tubulin with mouse monoclonal anti-tubulin antibodies (1:20000, Sigma-Aldrich) and HRP-conjugated sheep anti-mouse antibodies (1:10000, GE-Healthcare). Positive signals were visualized with ECL plus (GE-Healthcare). Western blots were scanned and the percentage of dephosphorylated BES1 was calculated with the ImageJ Gel analyze feature. BRI1-GFP protein levels were quantified with ImageJ and normalized to the detected levels of tubulin.

FRAP analysis. For BRI1-GFP, FRAP experiments were done with an Olympus Fluo View 1000 laser-scanning confocal microscope equipped with an argon ion laser. Five-day-old *Arabidopsis* root epidermal cells were observed under a $\times 60$ oil immersion objective, zoom $\times 3$, 512×512 pixels, and imaged (pre- and post-bleaching) with 11% of the 488-nm line ray and detected in the range between 500 nm and 550 nm. A circular ROI of 35 μm in diameter was selected for each bleaching with 100 % of the 488 nm line ray for 90 sec. Images were recorded before and 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min after bleaching. Fluorescence intensities of bleached and unbleached plasma membranes in the same root were measured with ImageJ. To eliminate the contribution of lateral diffusion during FRAP, we measured cells in which the BRI1-GFP fluorescence was completely bleached. Plasma membrane fluorescence before bleaching was set as 100 % and immediately after bleaching as 0 %. The percentage of plasma membrane fluorescence intensity after recovery was normalized to the fluorescence value of the unbleached plasma membrane from the same image.

For the GFP-CesA, photobleaching was performed by a FRAP system (iLas; Roper Scientific) integrated into the spinning-disk setup above as described⁴. Focused laser light was scanned on the image plane by a pair of galvanometer-driven mirrors inside the FRAP unit. Scanner positions were calibrated to image coordinates by an automated procedure. YFP photobleaching was done with a 491-nm laser (5 ms per scan point of 4 pixels in diameter)

Chemical treatments. Chemicals stocks were prepared in dimethyl sulfoxide (DMSO) at the following concentrations: 6 mM ConcA (Sigma-Aldrich), 50 mM BFA (Life Technologies),

50 mM CHX (Sigma-Aldrich) and 10 mM BL (Fuji Chemical Industries). For short-term treatments, 5-day-old *Arabidopsis* seedlings were incubated in 1/2 MS liquid medium with the desired concentration for the indicated time periods. For long-term treatments, seeds were germinated and grown on agar plates containing chemicals at the concentrations indicated in the figures. Etiolated seedlings were grown on plates containing 1/2 MS medium and 1% sucrose either without or with ConCA (100 nM) and on DMSO as control. For short-term drug treatments, etiolated seedlings were incubated with inhibitors at the indicated concentrations and times in 12-cell-well culture plates.

Cell wall biochemical analyses. Cell wall monosaccharides were assayed after hydrolysis with 2 M trifluoroacetic acid (TFA) as alditol acetate derivatives essentially as described¹⁰. Six-day-old etiolated seedlings were used for the assay. Isolated cell wall material was hydrolyzed with 2 M TFA to generate monosaccharides. The monosaccharides were derivatized to their corresponding alditol acetates followed by subsequent analysis and quantification with the Agilent 6890N GC system coupled with an Agilent 5973N mass selective detector as described¹¹. *Myo*-Inositol was added as an internal standard.

The cellulose content was determined according to a modified protocol¹² in a 96-well microtiter plate at 640 nm with Glc equivalents as standard. The hexose content was determined with the anthrone assay¹³.

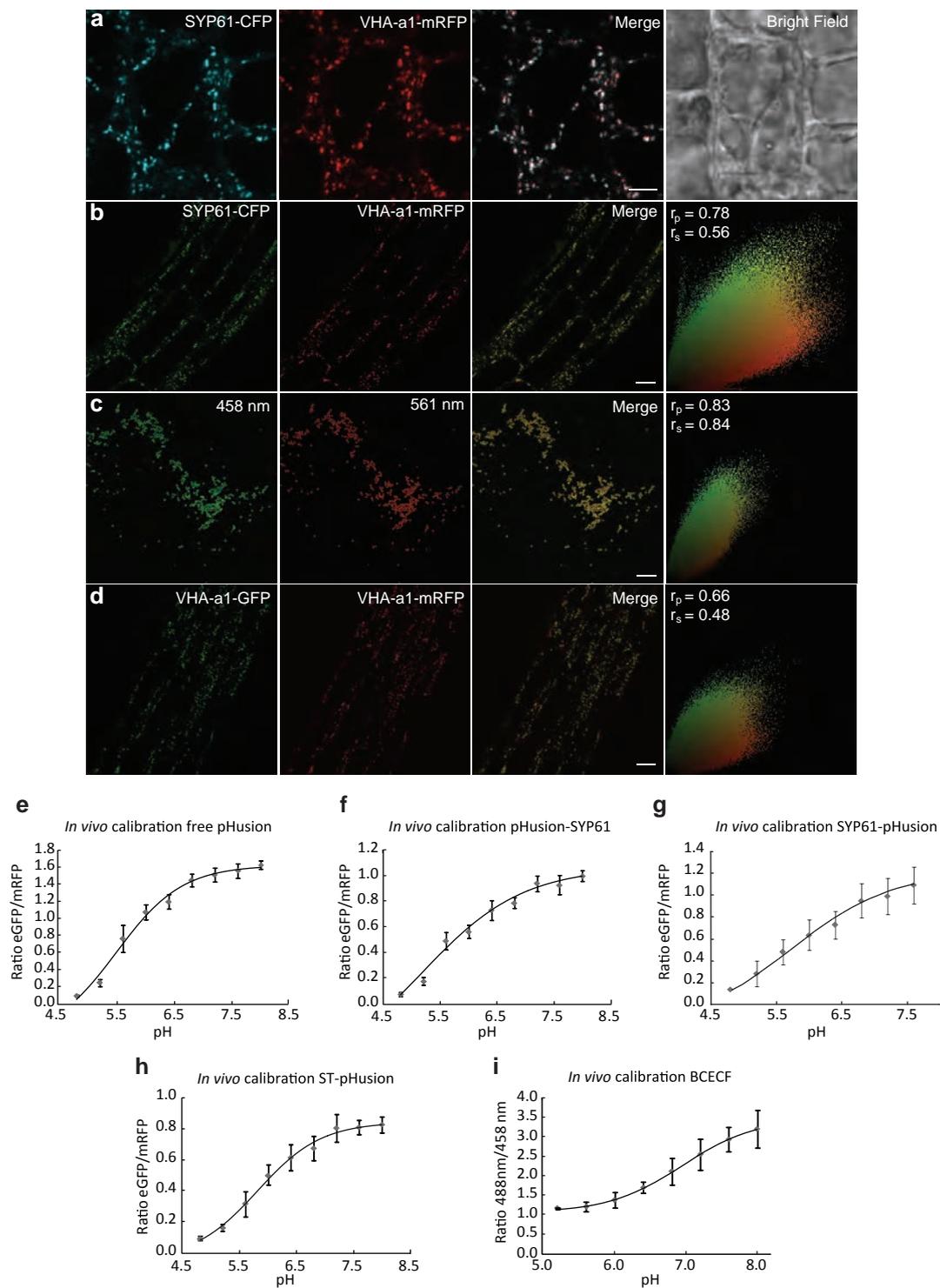
Statistical analysis. *P* values were calculated with a two-tailed Student's *t* -test with the Excel software. In other cases, data were compared with Kolmogorov-Smirnov tests and/or two-tailed Wilcoxon rank tests. In the case of Kolmogorov-Smirnov tests, outliers were identified with the Tukey outlier test.

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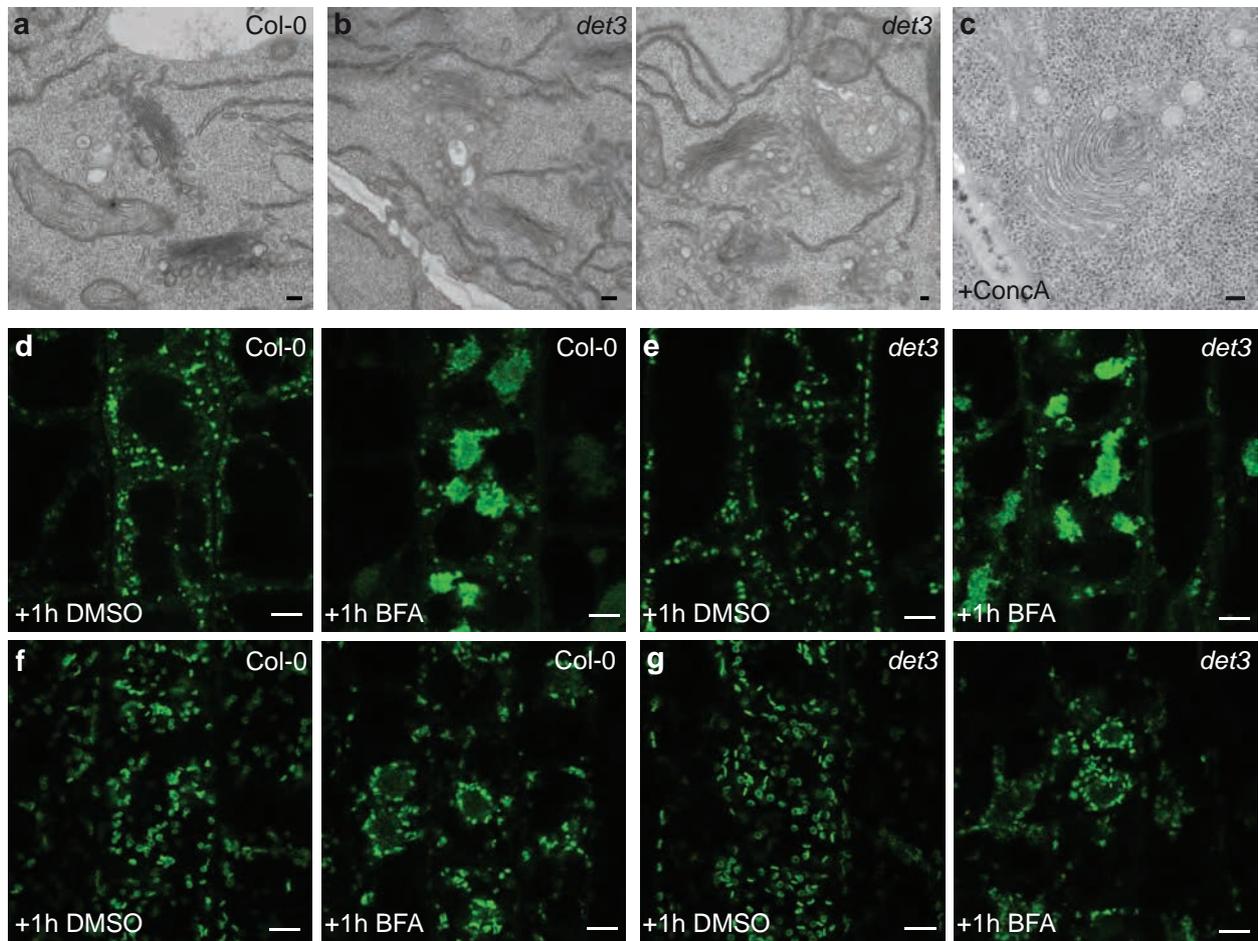
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Supplemental Table 1. Primers used in this study

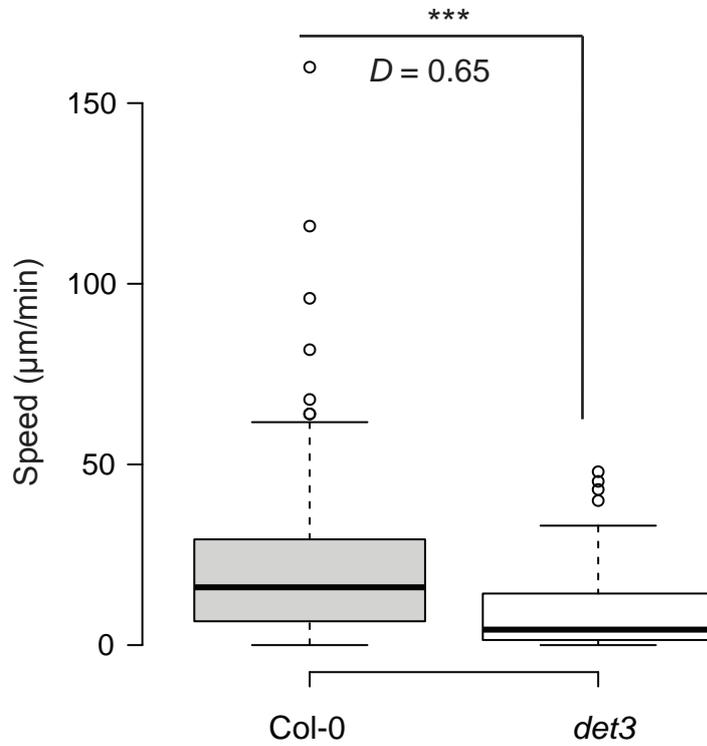
Primer	Sequence 5'-3'
Cloning	
P16TKan- <i>KpnI</i> -fwd	GAGGGTACCTGGAACCATCTTTTGGGTTTCCTTTC
P16Tkan- <i>Kpn</i> I-rev	GATGGTACCCACGCCGTCGTAGATGAGAAACC C
ST- <i>AatII</i> -fwd	CGGTGACGTCATGATTCATACCAACTTGAAGAAA
ST[noSTOP]- <i>PvuI</i> -rev	TGTCGATCGCATGGCCACTTTCTCCTGGC
SYP61- <i>BamHI</i> -fwd	AACGGATCCAACAATGTCTTCAGCTCAAGATCC
SYP61[noSTOP]- <i>HindIII</i> -rev	GTTAAGCTTGGTCAAGAAGACAAGAACGAATAG GATG
SYP61- <i>HindIII</i> -fwd	AACAAGCTTATGTCTTCAGCTCAAGATCCATTCT ACAT
SYP61- <i>Sall</i> -rev	GTTGTGCGACTTAGGTCAAGAAGACAAGAACGAA TAGGATG
pHusion- <i>HindIII</i> -fwd	AACAAGCTTATGGCCTCCTCCGAGGACG
pHusion- <i>PvuI</i> -fwd	TGTCGATCGATGGCCTCCTCCGAGGACGT
pHusion- <i>Sall</i> -rev	GTTGTGCGACTTACTTGTACAGCTCGTCCATGCC
pHusion- <i>BamHI</i> -fwd	AACGGATCCAACAATGGCCTCCTCCGAGGACG
pHusion[noSTOP]- <i>HindIII</i> -rev	GTTAAGCTTCTTGTACAGCTCGTCCATGCCGTGA GT
qPCR	
BRI1-fwd	CCGTGTACTTTCGATGGCGTTA
BRI1-rev	GAGAGACAGGAGAGACGAGGAC
YFP-fwd	GAAGCGCGATCACATGGT
YFP-rev	CCATGCCGAGAGTGATCC
CBP20-fwd	CTTCTTTCACCCCGCATAGACC
CBP20-rev	AGCTTTGCTTGCTCCTTGAACA



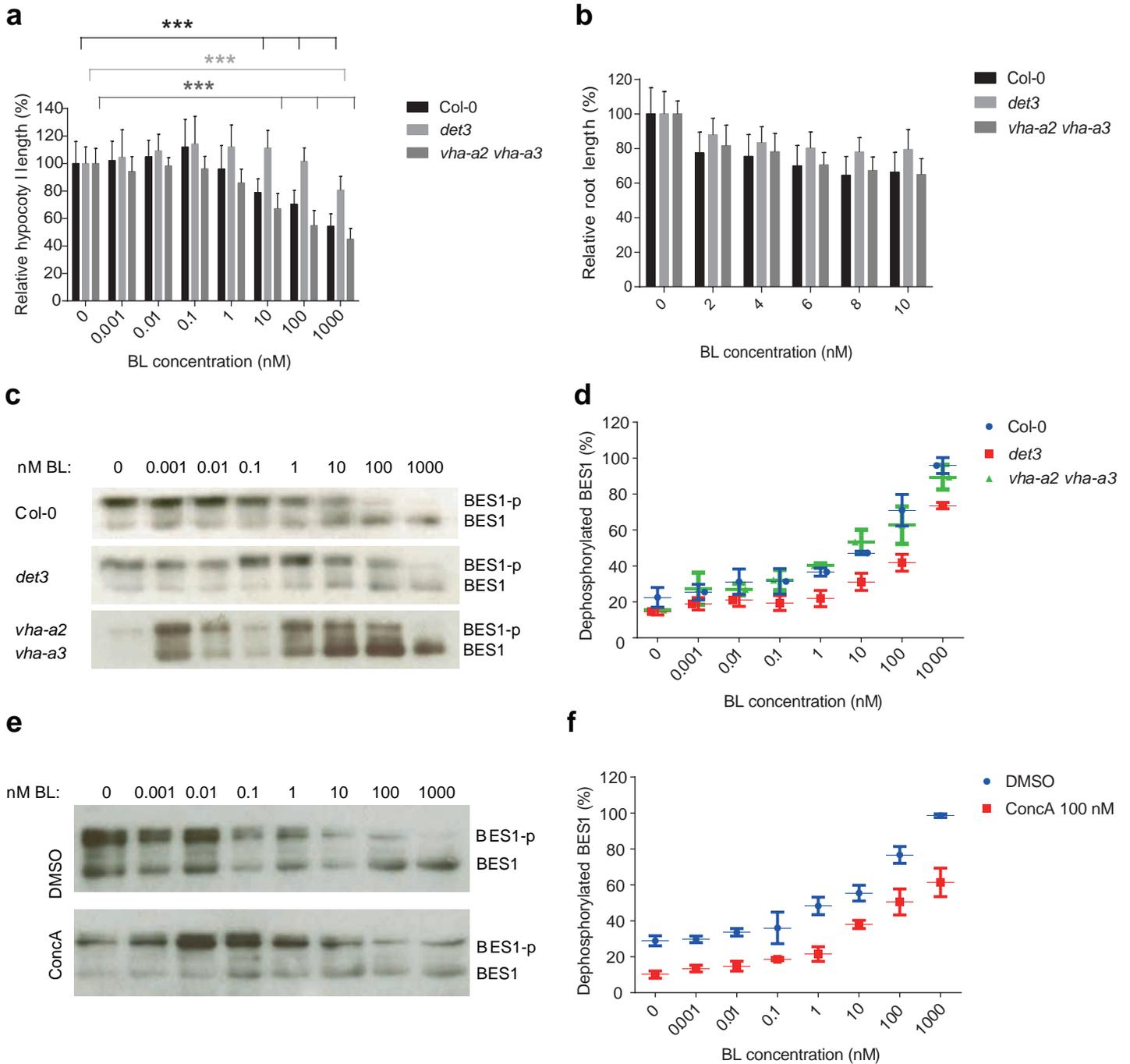
Supplementary Fig. 1. SYP61-CFP co-localizes with VHA-a1-mRFP. **a**, confocal laser-scanning microscopy images of root cells of 6-day-old *Arabidopsis* seedlings expressing SYP61-CFP and VHA-a1-mRFP. Scale bar, 5 μ m. **b-d**, Colocalization analysis of SYP61-CFP and VHA-a1-mRFP in **b**, TetraSpeck™ beads (1 μ m size) in **c**, and VHA-a1-GFP and VHA-a1-mRFP in **d** with the PSC Colocalization plugin of ImageJ by on five images of 10 different seedlings or 30 images of TetraSpeck™ beads. Pearson's (r_p) and Spearman's (r_s) correlation coefficients and scatterplots are displayed on the right. Co-localization ranges from +1 for positive- and -1 for negative correlation. Scale bars, 10 μ m. **e-g**, Calibration curves of free pHusion, SYP61-pHusion and pHusion-SYP61. Root cells of 6-day-old wild type (Col-0) seedlings ($n=10-15$) were measured for every pH value. **h**, *In vivo* calibration curve of ST-pHusion in Col-0 background. Values were measured in root cells of 6 days old seedlings. ($n=15$). **i**, *In vivo* calibration of BCECF-loaded Col-0 vacuoles. Epidermal and cortex cells of 6-day-old seedlings ($n=15$) were measured for every pH value. Error bars indicate S.D.



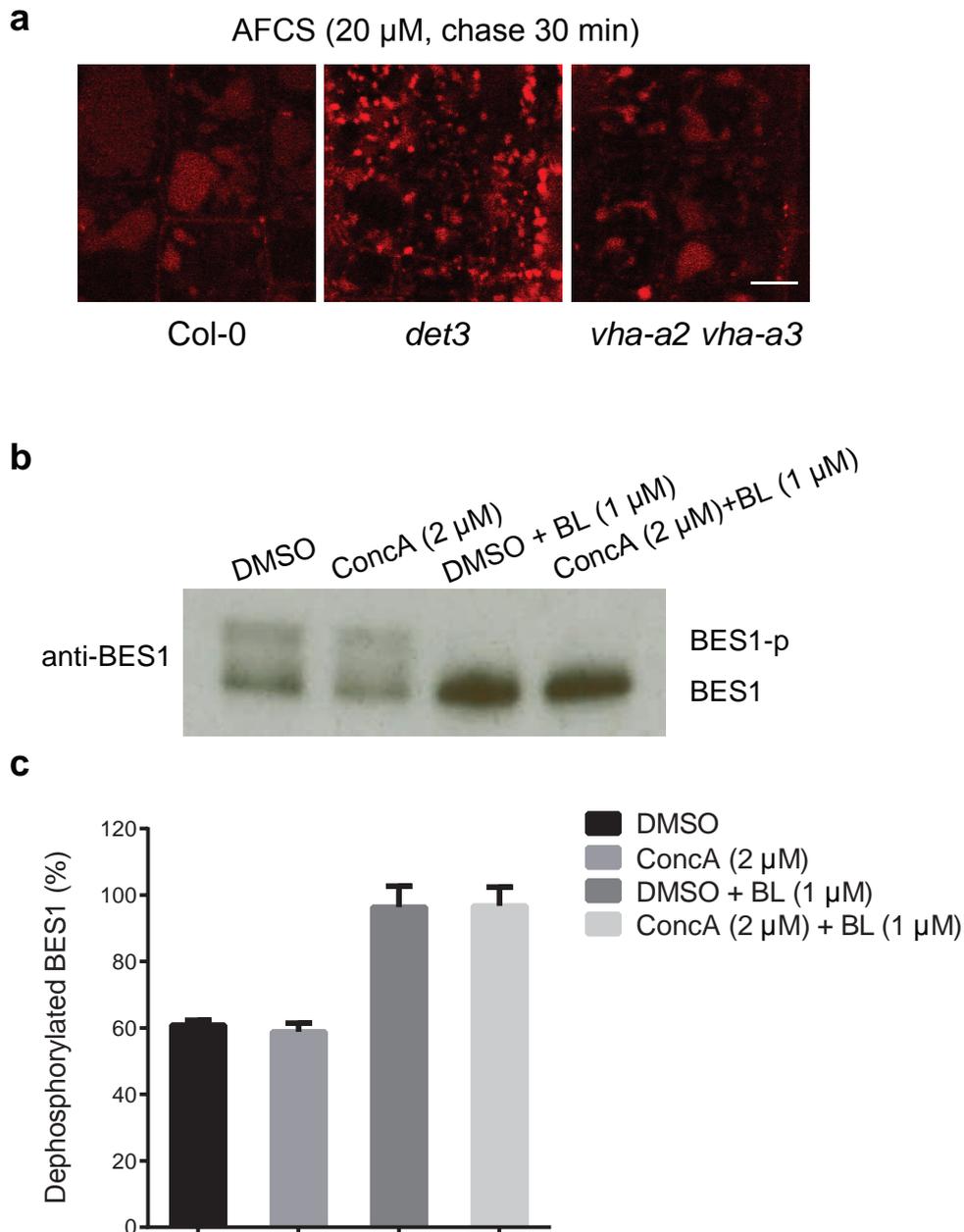
Supplementary Fig. 2. Golgi-TGN/EE morphology of *det3* and SYP61-pHusion and ST-pHusion distributions in *det3* resembles that of the wild type. **a**, TEM record of a representative wild type (Col-0) Golgi stack and TGN/EE in *Arabidopsis* root tips. Scale bar, 200 nm. **b**, Ultrastructure of *det3* Golgi and TGN/EE is not altered as compared with the wild type. Scale bar, 200 nm. **c**, ConcanamycinA (ConcA)-treated wild type root tips display a TGN/EE-Golgi hybrid compartment with clustered TGN/EE and cup-shaped Golgi stacks. Scale bar, 100 nm. **d**, Confocal microscopy images of wild type roots expressing p16:SYP61-pHusion. Whole seedlings were treated with 50 μ M BrefeldinA (BFA) or equal amounts of DMSO for 1 h in the dark. **e**, Confocal microscopy of p16:SYP61-pHusion in *det3* roots treated for 1 h with 50 μ M of BFA in the dark. **f**, Col-0 seedlings stably expressing p16:ST-pHusion treated for 1 h with 50 μ M of BFA in the dark and imaged by confocal microscopy. **g**, p16:ST-pHusion expressing *det3* seedlings were similarly as in **f** and imaged by confocal microscopy. Scale bars, 5 μ m (**d-g**).



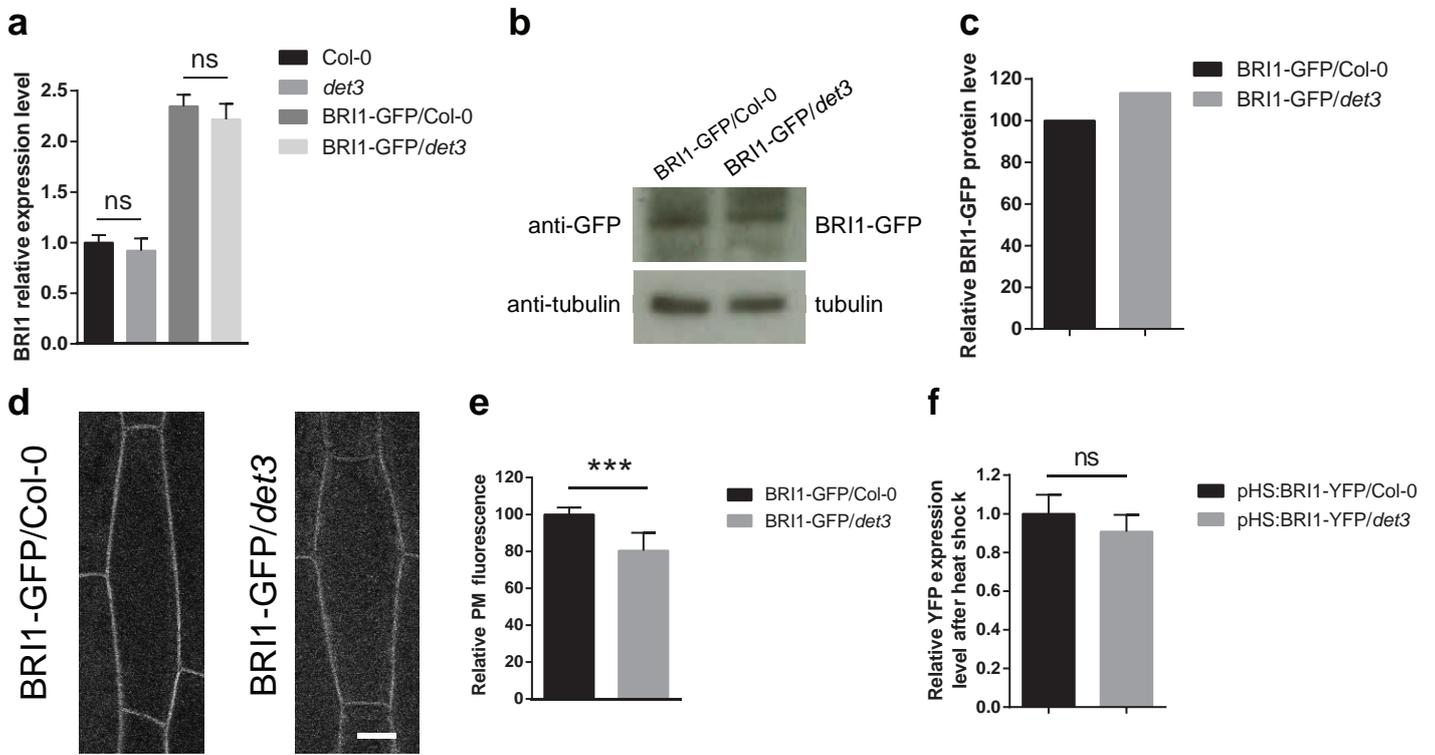
Supplementary Fig. 3. Golgi motility is reduced in *det3* root epidermal cells. Speed of Golgi in wild type (Col-0) and *det3* root epidermal cells from 7-day-old light-grown seedlings depicted as box plots. Motility of the Golgi in *det3* cells was significantly lower than that of the wild type. Estimates are done on cells from six independent seedlings. Golgi/seedling ($n > 25$). *** P -value < 0.001 ; D value from Kolmogorov-Smirnov tests.



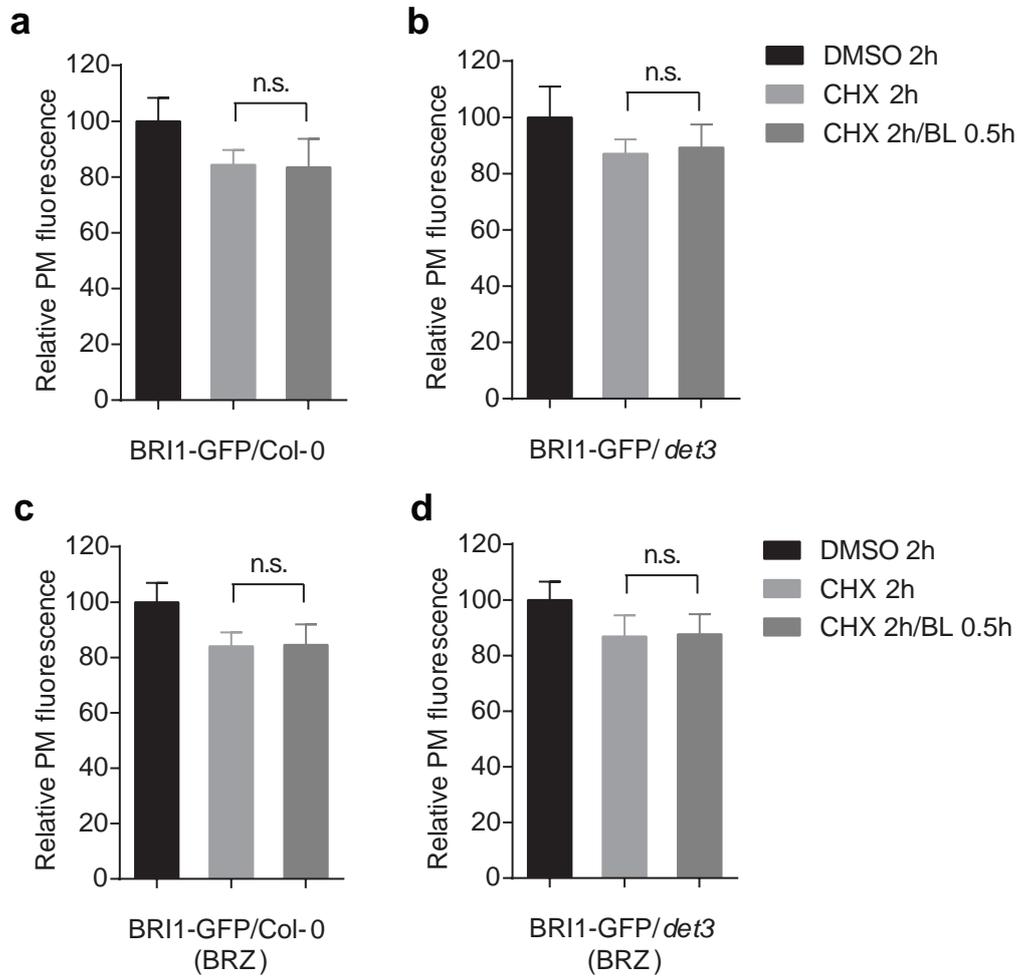
Supplementary Fig. 4. *det3* mutant is insensitive to BRs. **a**, Relative hypocotyl length of 5-day-old seedlings ($n > 25$) of the wild type (Col-0), *det3*, and *vha-a2 vha-a3* mutants grown in the dark on agar medium supplemented with different concentration of brassinolide (BL). *P* values (*t*-test), *** $P < 0.001$ relative to the respective control. Error bars indicate S.D. **b**, Relative root length of 7-day-old light grown seedlings ($n > 25$) of Col-0, *det3*, and *vha-a2 vha-a3* on agar medium supplemented with different concentration of BL. **c**, Western blot analysis of Col-0, *det3* and *vha-a2 vha-a3* seedlings grown as described in **b** using an anti-BES1 antibody. **d**, Percentage of dephosphorylated BES1 relative to the total BES1 detected in **c**. **e**, Western blot analysis of Col-0 seedlings grown either on DMSO or ConcanamycinA (ConcA) (100 nM) for 5 days with and anti-BES1 antibody. **f**, Percentage of dephosphorylated BES1 relative to the total BES1 detected in **e**.



Supplementary Fig. 5. Defective endocytic trafficking in *det3* does not cause BR insensitivity. **a**, Pulse-chase AFCS uptake experiments in wild type (*Col-0*), *det3* and *vha-a2 vha-a3* double mutant. Scale bar, 5 μ m. **b**, Western blot analysis of BES1 in total proteins extracted after the indicated treatments. Five-day-old wild type seedlings were treated with DMSO (1 %) for 2 h, ConcA (2 μ M) for 2 h, DMSO (1 %) for 1 h followed by BL (1 μ M) for 1 h, and ConcA (2 μ M) for 1 h followed by BL (1 μ M) plus ConcA (2 μ M) for 1 h. **c**, Percentages of dephosphorylated BES1 relative to the total BES1 calculated according to the western blot in **b**. AFCS, Alexa Fluor 647-castasterone. ConcA, ConcanamycinA.

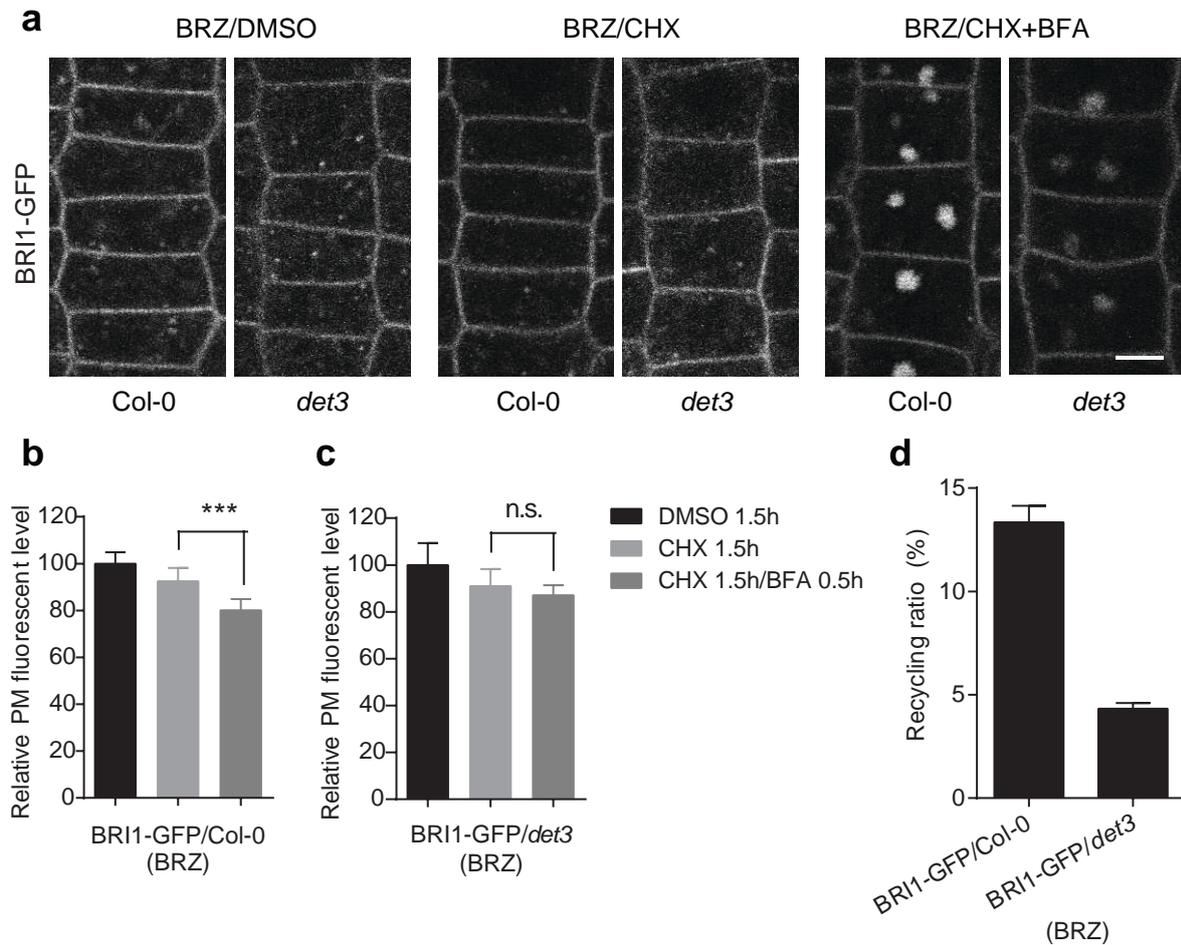


Supplementary Fig. 6. *DET3* mutation does not alter the transcription and protein levels of *BRI1*. **a**, *BRI1* transcription analysis by qPCR ($n=3$). No significant differences (t -test) were observed between the wild type Col-0 and *det3*, and between BRI1-GFP/Col-0 and BRI1-GFP/*det3*. Error bars indicate S.D. The *CAP-BINDING PROTEIN 20* (*CBP20*) gene was used as internal control. **b**, and **c**, Western blot detection of BRI1-GFP protein in BRI1-GFP/Col-0 and BRI1-GFP/*det3* by anti-GFP antibodies, respectively. BRI1-GFP protein level was normalized to the tubulin level. **d**, Confocal images of 5-day-old dark-grown BRI1-GFP/Col-0 and BRI1-GFP/*det3* hypocotyl cells. Scale bar, 10 μ m. **e**, Quantification of relative plasma membrane (PM) fluorescence intensity of BRI1-GFP/Col-0 and BRI1-GFP/*det3* (30 cells close to the apical hook of six plants were measured). P values (t -test), *** $P < 0.001$ relative to the respective control. Plasma membrane fluorescence was normalized to background fluorescence for each measurement. **f**, *BRI1* transcript analysis by qPCR ($n= 3$) after 1 h heat shock (HS) induction at 37°C. The yellow fluorescent protein (YFP) fused to *BRI1* did not differ in pHS:BRI1-YFP/Col-0 and pHS:BRI1-YFP/*det3* The *CBP20* gene was used as internal control. No significant differences were observed (t -test). Error bars indicate S.D.

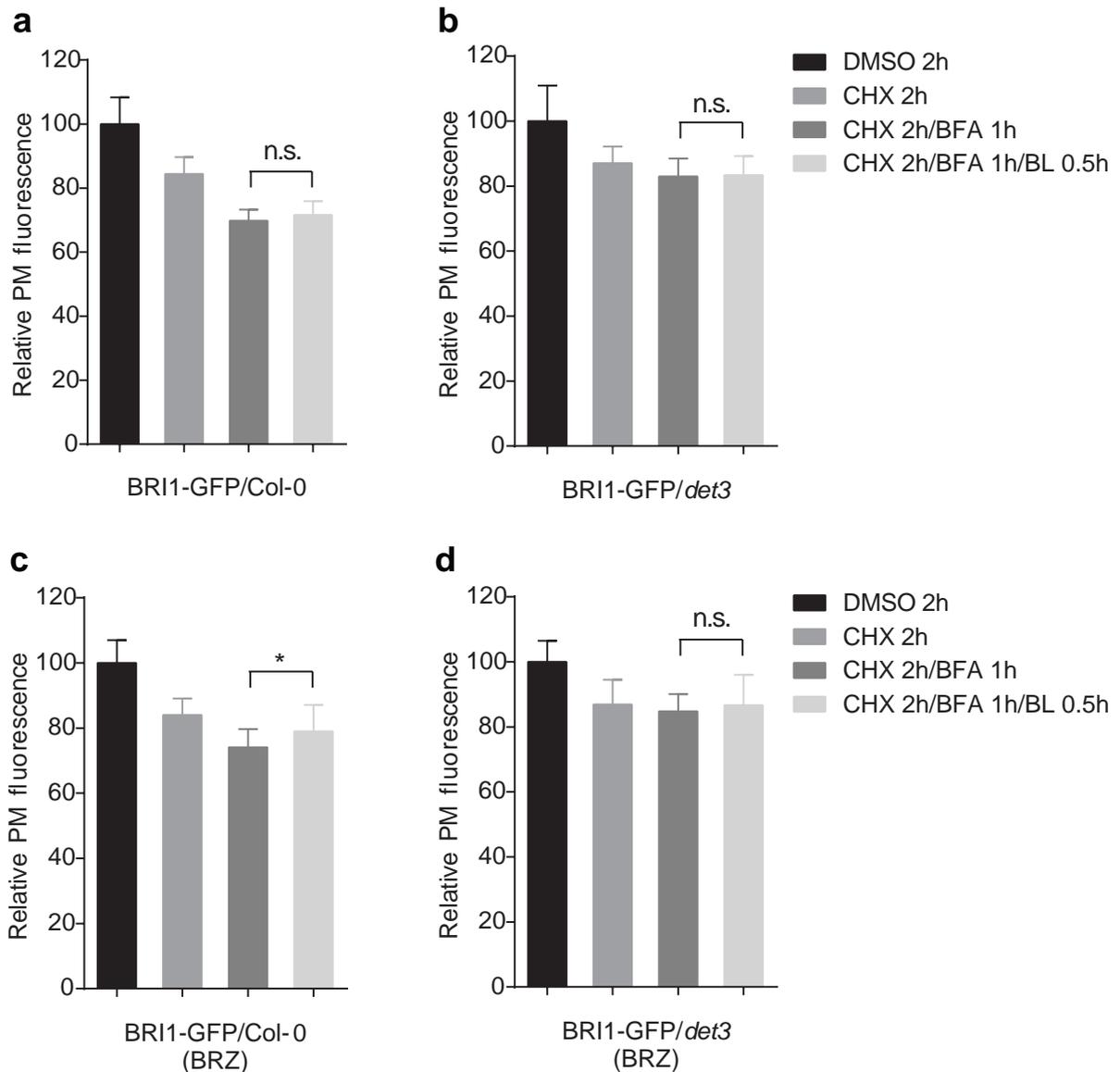


Supplementary Fig. 7. The plasma membrane pool of BRI1 is not affected by exogenous ligand application.

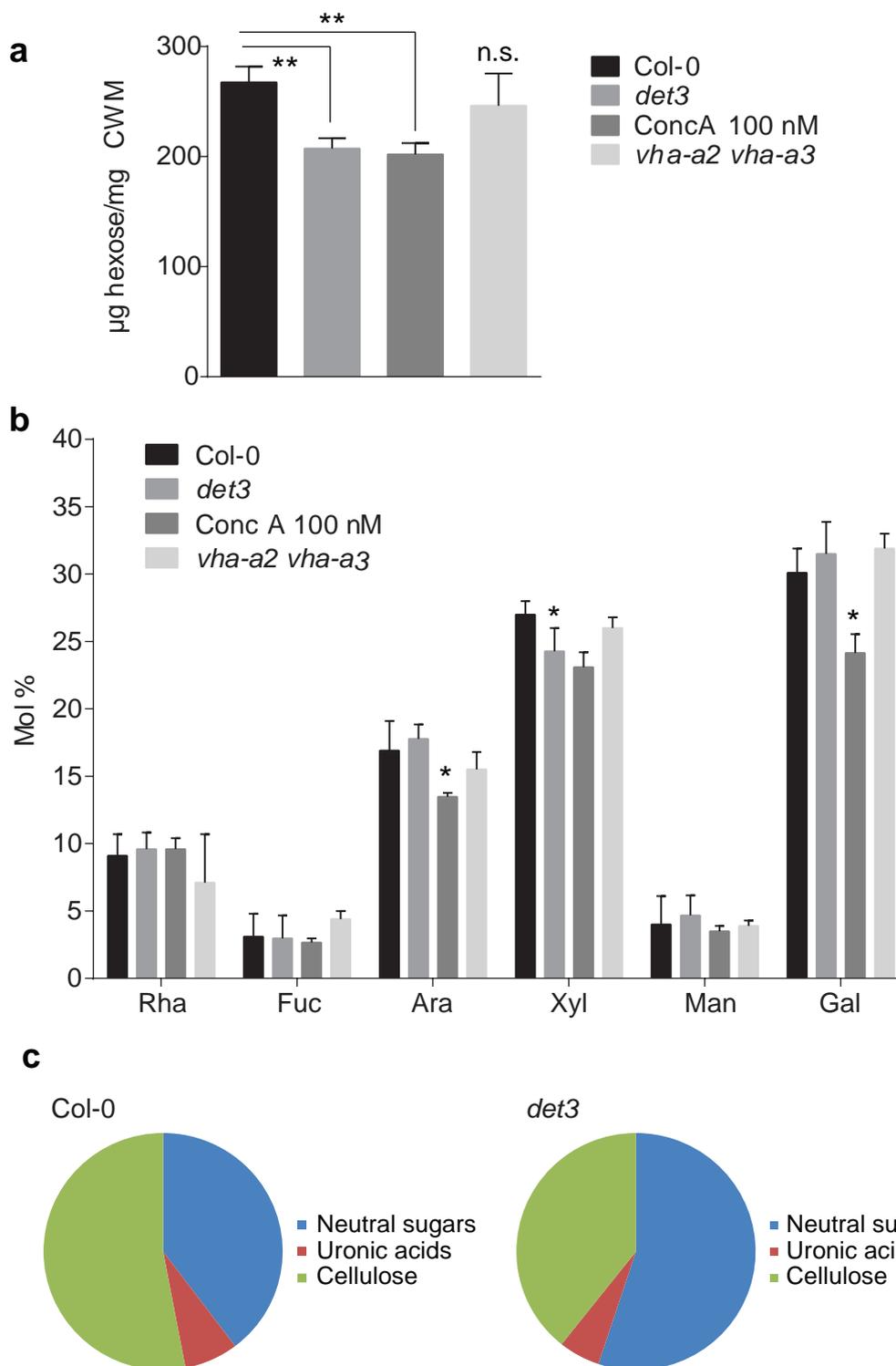
a and **b**, Five-day-old BRI1-GFP/Col-0 and BRI1-GFP/*det3* seedlings treated with DMSO (1 %) for 2 h, CHX (50 μ M) for 2 h, CHX (50 μ M) for 1.5 h followed by BL (1 μ M) / CHX (50 μ M) for 0.5 h. Relative plasma membrane BRI1-GFP fluorescence was measured and calculated with ImageJ (for each treatment, at least 15 cells from three roots were measured). **c** and **d**, Three-day-old BRI1-GFP/Col-0 and BRI1-GFP/*det3* seedlings transferred to medium supplemented with 5 μ M BRZ for 2 days before treatments. Seedlings were treated as in **a** and **b** and the relative plasma membrane BRI1-GFP signaling was calculated as in **a** and **b** (for each treatment, at least 15 cells from three roots were measured). BRZ, brassinazole. CHX, cycloheximide. BL, brassinolide. No significant differences were observed (*t*-test). Error bars indicate S.D.



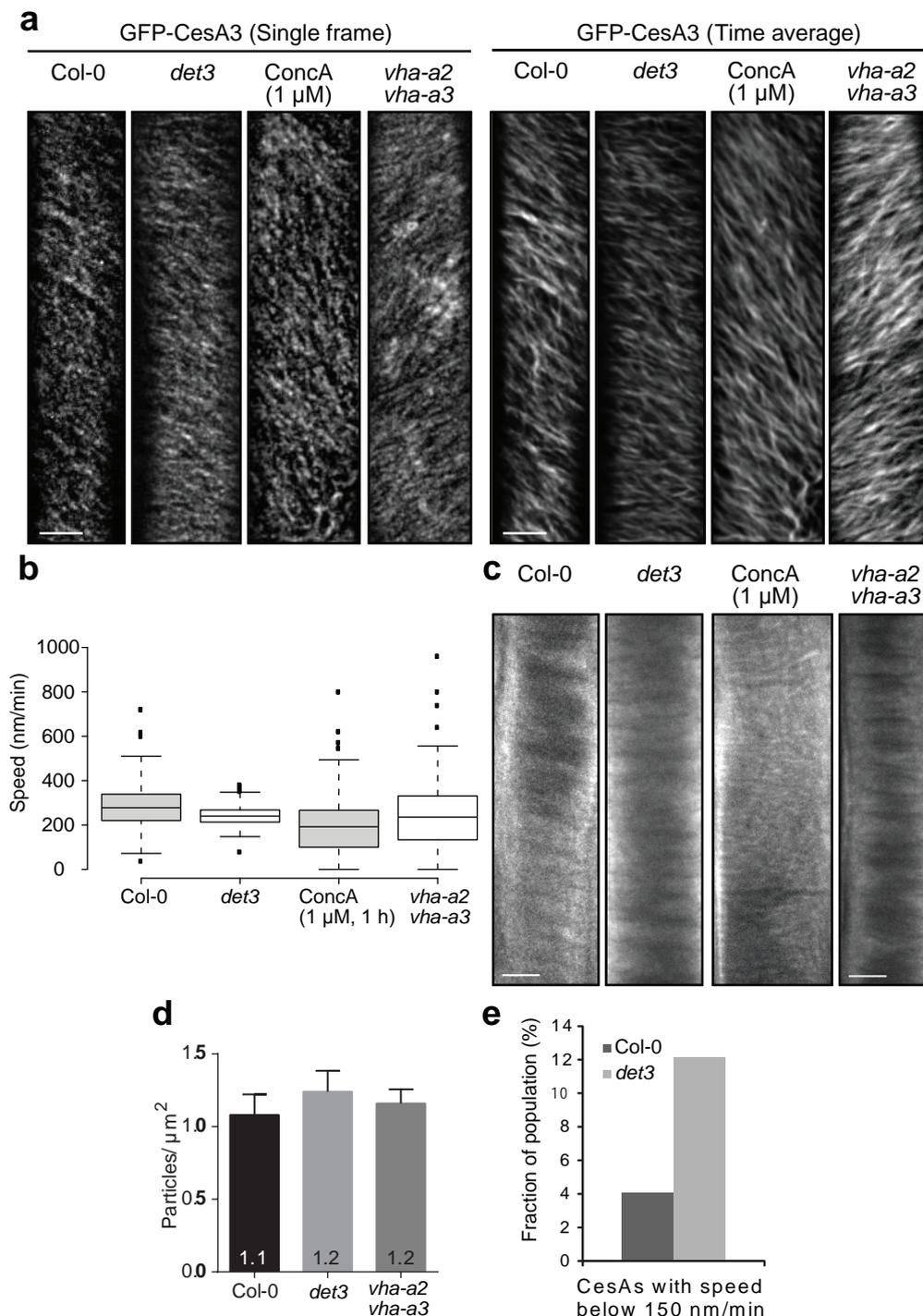
Supplementary Fig. 8. Slower recycling of BRI1-GFP in *det3* in the presence of BRZ. **a**, Three-day-old BRI1-GFP/Col-0 and BRI1-GFP/*det3* seedlings were transferred to medium with BRZ (5 μ M) for 2 days before treatments. Seedlings were treated with DMSO (1 %) for 1.5 h, CHX (50 μ M) for 1.5 h, CHX (50 μ M) for 1 h followed by BFA (50 μ M) / CHX (50 μ M) for 0.5 h. Images show BRI1-GFP fluorescence signaling of root epidermal cells after treatments. Scale bar, 5 μ m. **b** and **c**, Quantification of relative plasma membrane (PM) fluorescence intensities of BRI1-GFP/Col-0 and BRI1-GFP/*det3* root epidermal cells represented in **b** (for each treatment, at least 15 cells from three roots were measured). *P* values (*t*-test), ***, *P* < 0.001 relative to the respective control. **d**, Percentages of BRI1-GFP proteins under recycling in total plasma membrane BRI1-GFP pool for both BRI1-GFP and BRI1-GFP/*det3* (at least 15 cells from three roots were measured). Error bars indicate S.D. BRZ, brassinazole. CHX, cycloheximide. BL, brassinolide.



Supplementary Fig. 9. Plasma membrane BRI1 levels are slightly elevated in the wild type *Arabidopsis* seedlings grown on BRZ and treated with BL in the presence of BFA. **a** and **b**, Five-day-old BRI1-GFP/Col-0 and BRI1-GFP/*det3* seedlings treated with DMSO (1%) for 2 h, CHX (50 μ M) for 2 h, CHX (50 μ M) for 1 h followed by BFA (50 μ M) / CHX (50 μ M) for 1 h, CHX (50 μ M) for 1 h followed by BFA (50 μ M) / CHX (50 μ M) for 0.5 h and BL (1 μ M) / BFA (50 μ M) / CHX (50 μ M) for 0.5 h. Relative plasma membrane BRI1-GFP fluorescence was measured and calculated with ImageJ (for each treatment, at least 15 cells from three roots were measured). **c** and **d**, Three-day-old BRI1-GFP/Col-0 and BRI1-GFP/*det3* seedlings transferred to medium supplemented with BRZ (5 μ M) for 2 days before treatments. Seedlings were treated as in **a** and **b** and the plasma membrane GFP signal was measured and calculated as in **a** and **b** with ImageJ (for each treatment, at least 15 cells from three roots were measured). A slight increase in the plasma membrane GFP signal was noticed after BFA treatment in the wild type, but not in *det3* *P* values (*t*-test), *, *P* < 0.05 relative to the respective control. Error bars indicate S.D. BRZ, brassinazole. BFA, Brefeldin A. CHX, cycloheximide. BL, brassinolide.



Supplementary Fig. 10. Cell wall composition in different genotypes and after different inhibitor treatments. **a**, Cellulose levels in wild type, *det3*, wild type seedlings grown on ConcanamycinA (ConcA) (100 nM) and *vha-a2 vha-a3* seedlings (5-day-old etiolated seedlings). Data are based on three biological repeats. *P* value (*t*-test), ***P* < 0.01. **b**, Neutral sugar assays of etiolated wild type, *det3* and *vha-a2 vha-a3* seedlings and of 5-day-old etiolated seedlings grown on ConcA (100 nM). No differences between wild type, *det3*, and *vha-a2 vha-a3* seedlings were detected, and only minor changes in seedlings grown on ConcA. *P* values (*t*-test), ***P* < 0.01. Error bars indicate S.D. **c**, Contribution of different cell wall components to the cell wall in wild type (Col-0; left) and *det3* (right). The cell wall components are divided into cellulose, neutral sugars (hemicelluloses and neutral pectins), and uronic acids (charged pectins). Data are means of three biological replicates from 6-day-old etiolated hypocotyls in which the total amount of the three components was set to 100%.



Supplemental Fig. 11. Speed, orientation and density of the CesAs at the plasma membrane are not changed in *det3*. **a**, Single frames (left) and time average images (right) from GFP-CesA3 in 3-day-old wild type, *det3* and *vha-a2 vha-a3* etiolated hypocotyls, and in hypocotyls treated with ConcanamycinA (ConcA) (1 μ M, 1 h). Scale bar, 10 μ m. **b**, Quantification of CesA speed in cells as exemplified in **a** as boxplot representations. Error bars indicate S.D. No significant differences (Students *t*-test). **c**, Cellulose microfibril orientation visualized by the cellulose binding dye Pontamine Scarlet 4B. All genotypes and treatments display transversely organized microfibrils in expanding cells of 3-day-old etiolated hypocotyls. Scale bars, 10 μ m. **d**, Density of GFP-CesA foci at the plasma membrane in hypocotyl cells of 3-day-old etiolated wild type, *det3*, and *vha-a2 vha-a3* seedlings. Error bars indicate S.D. Density measurements were based on three cells from six independent seedlings. **e**, Fraction of CesA particles (number of particles per total number of particles) that migrate with a speed of ≤ 150 nm/min in wild type and *det3* mutant cells. Fraction is calculated based on raw data from velocity measurements in **b**.