Supplemental Data

Supplemental Materials and Methods

Plant Growth Conditions

Plant material and growth conditions. All *Arabidopsis thaliana* plants were of the ecotype Columbia 0 (Col-0). Plants were grown on soil under short day conditions (16 h light /16 h dark, 100 µE m^{-2} s^{-1} of Cool White Neon lamps) at 22°C and 75% relative humidity. Seeds were surface-sterilized by vapor-phase sterilization (Clough and Bent, 1998) and plated on half-strength MS medium (Murashige and Skoog, 1962) (Duchefa, http://www.duchefa-biochemie.com/) supplemented with 0.1% sucrose (w/v), 0.05% 2-(N-morpholino)ethanesulfonic acid (MES) (w/v), pH 5.8, and 0.8% (w/v) plant agar (Duchefa, http://www.duchefa-biochemie.com/). After stratification at 4°C in the dark for 2 days, plates were transferred to the growth chamber under long day conditions (16 h light/8 h dark, 100 µE m^{-2} s^{-1} of Cool White Neon lamps) at 22°C. The plates were kept vertically and seedlings were used for imaging 6-7 days after germination.

Wide-field fluorescence microscopy. For wide-field Ca^{2+} imaging analyses in seedling roots of pUBQ10-R-GECO1, pCaMV35S-YC-Nano 65, and pUBQ10-pH-GFP lines, an inverted fluorescence Nikon microscope (Ti-E; http://www.nikon.com/) with a CFI Plan Apo VC 20X (N.A 0.75) was used. Excitation light was produced by a fluorescent lamp (Prior Lumen 200 PRO; Prior Scientific; http://www.prior.com) set to 20% with 561 nm (540/25 nm) for the R-GECO1, and 440 nm (436/20 nm) for YC-Nano 65. R-GECO1 fluorescence emissions was collected at 505–530 nm and at 576–626 nm respectively. For the analysis of the YC-Nano 65 line, the FRET CFP/YFP optical block A11400-03 (emission 1, 483/32 nm for ECFP; emission 2, 542/27 nm for FRET/Citrine) with a dichroic 510 nm mirror (Hamamatsu) was used. For pH-GFP imaging, the emissions were collected using a 505/530 nm bandpass filter (Chroma Technology Corp., Bellows Falls, Vermont, USA) with both excitation wavelengths (405 and 488 nm) used sequentially to illuminate the sample. Camera binning (2 x 2 or 4 x 4) and exposure times (from 50 to 400 ms) were adjusted depending on the sensor line. Images were acquired every 5 s. Filters, and the dichroic mirrors were purchased from Chroma Technology (http://www.chroma.com/). NIS-Elements (Nikon; http://www.nis-elements.com/) was used as a platform to control the microscope, illuminator, and camera. Images were analyzed using FIJI.

Seedling imaging. Six-day-old seedlings were used for root imaging. For root experiments, seedlings were kept in the growth chamber until the experiment and gently removed from the plate according to Behera et al. (2018), placed in the dedicated chambers and overlaid with cotton wool soaked in imaging solution with (5 mM KCl, 10 mM MES, 1.5 mM $CaCl_2$ pH 5.8 adjusted with Tris-

base) or without calcium (5 mM KCl, 10 mM MES, 0 mM $CaCl_2$ pH 5.8 adjusted with Tris-base). The root was continuously perfused with imaging solution using a perfusion pump (Behera et al., 2018) while the shoot was not submerged. For the experiments reported in Fig. 3 treatments were carried out by supplementing the imaging solution (1.5 mM calcium or 0-calcium) with 0.01 mM NAA (from a 10.74 mM stock solution) administered for 3 min under continuous perfusion. For the experiment reported in Fig. 4 the solution with 1.5 mM calcium was changed with the one with 0-calcium at the indicated time.

Quantitative imaging analysis. Fluorescence itensity was determined over regions of interest (ROIs), which corresponded to the transition zone of seedling root tips. R-GECO1 emissions of the analyzed ROIs were used for single fluorescence emissions analyses. cpVenus and ECFP emissions of YC-Nano 65 of the analyzed ROIs were used for the ratio (R) calculation (cpVenus/ECFP). pH-GFP 405 and 488 emissions of the analyzed ROIs were used for the ratio (R) calculations ($405_{em}/488_{em}$). Background subtraction was performed in all experiments.

$$\text{Ratio cpVenus/ECFP} = (\text{cpVenus} - \text{cpVenus Background})/(\text{ECFP} - \text{ECFP Background})$$

$$\text{Ratio } 405/488 = (405_{em} - 405_{em} \text{ Background})/(488_{em} - 488_{em} \text{ Background})$$

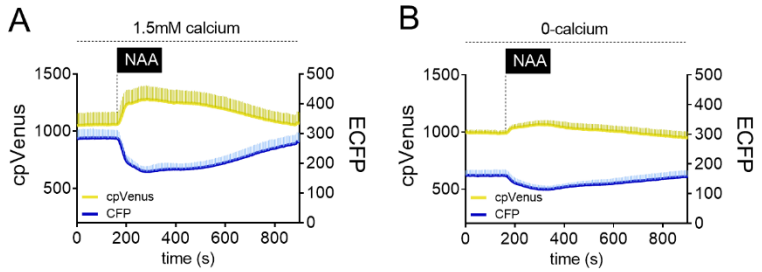
Fluorescence (F) and ratio (R) values for the R-GECO1 and YC-Nano 65 at different time points were normalized to the initial fluorescence (F_0) ($\Delta F/F_0$) or ratio (R_0) ($\Delta R/R_0$) and plotted versus time.

$$\Delta F/F_0 = (F_{\text{R-GECO1}} - F_{0\,\text{R-GECO1}})/F_{0\,\text{R-GECO1}}$$

$$AR/R_0 = (\text{Ratio}_{\text{cpvenus/ECFP}} - R_{0\,\text{cpvenus/ECFP}})/R_{0\,\text{cpvenus/ECFP}}$$

Statistical analysis. All the data are representative of n ≥ 4 experiments. Reported traces are averages of traces from all single experiments ± standard deviations (SD). Data from experiments with at least n = 5 were plotted as box-and-whisker plots using GraphPad, in which all the experimental points are plotted, and their distribution represented as a box that extends from the 25^{th} to 75^{th} percentiles. The line in the middle of the box is plotted at the median. p values were calculated with an unpaired Student's *t* test.

Supplemental Figures



Supplemental Figure S1. Single wavelength emissions as arbitrary units of fluorescence of cpVenus (yellow trace) and ECFP (light blue trace) of the YC-Nano 65 used for the ratio calculations of Fig. 3C, G. (A) Averaged cpVenus and ECFP fluorescence emissions over time in root tip cells in response to 10 µM 1-naphthylacetic acid (NAA) as indicated by the black box on the x-axis in presence of 1.5 mM $CaCl_2$ in the imaging solution. (B) Averaged cpVenus and ECFP fluorescence emissions over time in root tip cells in response to 10 µM NAA NAA as indicated by the black box on the x-axis in 0-calcium in the imaging solution. Traces are the average of n = ≥ 5. Error bars = SD.