# Extended data for: Intercellular lipid flow coordinates tissue-scale lipid gradients in plants

*Corresponding author. Email: Emmanuelle.bayer@u-bordeaux.fr, yvon.jaillais@ens-lyon.fr

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## Materials and Methods

### Material used for this study:

Arabidopsis thaliana accession Columbia (*Col-0*), (which is named as *WT*) and transgenic lines (all in Columbia background) were used in this study. The following lines were published before: *p35S::C4H-GFP*[1], *mctp3-2/mctp4-1*[2], *pPRD9::XVE-cals3m*[3], *pPSS1::PSS1-mCitrine in pss1-3*, *pss1-3*, , *pUBQ10::2PHEVECTIN2-mCitrine* and the cross of the two previous lines[4,5], DDRGK1-mcherry[6], p35S : DERLIN1-mscarlett[7]. Seedlings were grown vertically on half Murashige and Skoog medium (1/2 MS salts), supplemented with vitamins (2.15 g/L), sucrose (1%) and plant agar (0,8%), pH 5.7 for 7 days at 22 degrees in long day conditions cycle 16h light/8h dark.

### Cloning and plasmid construction:

*pPSS1:2xmCITRINE-NLS* was cloned using multisite gateway, using the following vectors *pPSS1/pDONRP4P1R*[4], *mCITRINEnoSTOP/pDONR221*[8], *NLS(SV40)-mCITRINE*[9] and *pB7m34GW* [10]. *pGL2::PSS1-mCHERRY, pEXP7A::PSS1- mCHERRY*, *pPEP::PSS1- mCHERRY*, *pSCR::PSS1-mCHERRY*, *pSHR::PSS1-mCHERRY*, *pSUC2::PSS1-mCHERRY, pPSS1::PSS1-mCHERRY*, *pUBQ10::PSS1-mCHERRY* were obtained by using three-fragment Gateway recombination system (Life Technologies). To do so, gateway donors vectors pDONR-P4P1R containing *GL2*, *EXP7A*, *PEP*, *SCR*, *SHR* or *SUC2* promoters[9] were used, as well as *pDONR-P4P1R* expressing *PSS1* and *UBQ10* promoters, *mCHERRY/pDONR-P2RP3* and *PSS1 cDNA/pDONR221*[4,11,12]. They were triple recombined in the final destination vector pLOK180 which expresse a red seed-coat selection marker (a gift from L. Kalmbach). The expression vectors were introduced into *Agrobacterium tumefaciens* which was then used to transform Arabidopsis heterozygous *pss1-3* expressing *pUBQ10:mCITRINE-2PH(EVECTIN2)* as in[12]. The transformed seeds were selected based on the presence of either red seed coat or BASTA resistance, depending on the selection marker. From 2 to 4 independent T3 lines with mono-insertions and suitable specific fluorescence localization were selected and analyzed for the study.

### Chemicals used for treatments:

Topfluor Phosphatidylcholine (Avanti https://avantiresearch.com/product/810281) was prepared as in[13]. Similarly, an argon gas flux was used to evaporate chloroform based Topfluor PC solution. The lipids pellet was then resuspended in liquid medium Murashige and Skoog to reach a concentration of 38 µM. Usually, 150 microliters of the stock is evaporated and resuspended in 4 milliliters of MS liquid medium. Only glass material was used: Hamilton syringe to pipette Topfluor PC and glass plates to treat the seedlings as lipids could stick to plastic. The treatment was done overnight. Carboxyfluorescein diacetate (CFDA) was prepared at a stock solution of 1mg/ml diluted in Dimethylsulfoxide (DMSO) and used for treatment at 50 µg/ml for 2 hours after dilution in MS liquid. A quick wash of 5 minutes in MS liquid was performed for both treatments. Treatment with 10 µM estradiol for induction of pPDR9::XVE-Cals3m line was performed for 12 hours.

For all the treatments, 7-day-old Arabidopsis roots have been selected and after treatment mounted on slide with propidium iodide (PI) at 10 µg/mL for observation. PI served to label cell contour and to make sure that FRAP/FLIP did not impact cell viability.

**Confocal microscopy:**

**Spinning disc microscopy**. All the confocal imaging experiments besides the colocalization experiments presented in Fig. 1a and Extended Data Fig. 3a were performed on an inverted Zeiss microscope (AxioObserver Z1, Carl Zeiss Group, http://www.zeiss.com/) equipped with a spinning disk module (CSU-W1-T3, Yokogawa) and a Prime 95B camera (Photometrics, https://www.photometrics.com/). We used mainly a 40x C-Apochromat objective (numerical aperture 1.1, water immersion) and a 63X C-Apochromat objective (numerical aperture 1.4, oil immersion) to do close up when needed (Fig. 1a). Topfluor, GFP, CFDA were excited with a 488nm laser and the ensuing fluorescence emission was filtered via a 525/50 nm BrightLine single-band bandpass filter. mCitrine was excited with a 515 nm laser and the resulting fluorescence emission was directed through a 578/105 nm BrightLine single-band bandpass filter. mcherry, mscarlett, propidium iodide were excited with 561 nm laser and the fluorescence emission was filtered using a 609/54 nm BrightLine single-band band-pass filter (Semrock, http://www.semrock.com/). For FRAP and FLIP experiments, iLas2 FRAP module (Roper scientific, http://www.biovis.com/ilas.htm) was used.

**Airyscan laser scanning microscopy.** Colocalization experiments presented in Fig. 1a and Extended Data Fig 3a were performed using an inverted LSM 980 Axio Observer Z1/7 (Carl Zeiss group, http://www.zeiss.com/) equiped with an AiryScan 2 module, GaAsP-PMT detectors and a C Plan-Apochromat 40X objective (numerical aperture 1.3, oil immersion). To prevent overlap between the two emission signals during image acquisition, channels were acquired sequentially. The fluorescence of the two markers was plotted along a line with Fiji. All imaging experiments were repeated at least three times independently (unless indicated otherwise).

**Fluorescence recovery after photobleaching (FRAP) and quantification.** 7-day-old Arabidopsis roots have been imaged. One and half of the epidermal cells surrounded per root were bleached at 70% of the laser power (488nm). 6 pictures pre-bleached were acquired and 100 pictures post-bleached every second. The XY drift was corrected with the Stackreg Fiji plugin (https://bigwww.epfl.ch/thevenaz/stackreg/). The fluorescence intensity : mean gray values were then measured across the time using Fiji https://imagej.net/software/fiji/ on a squared ROI drawn on the perinuclear ER membrane of the bleached cell for Topfluor PC, C4H-GFP or the nucleus for CFDA. Similar region in neighboring cells localized further of the bleached regionwere measured as control. The noise fluorescence, measured outside the root sample was subtracted manually for each time point. The relative fluorescence percentage for each time point was calculated as following: ((mean gray values bleached cell - mean gray values MIN)/(mean gray values control cell - mean gray values MIN)). The MIN value represents the lowest mean gray value. The maximum value was then set to 100 and the results normalized accordingly. The results were then plotted with R Studio. The mobile fraction was represented at 100 seconds. The boxplot and the statistics were done with jasp https://jasp-stats.org/.

**Fluorescence loss in photobleaching (FLIP) and quantification.** A small ROI (~1/10 of the root epidermal cell) was photobleached every second at 70% of the laser power (488nm). The integrity of the cell was controlled using propidium iodide, which labels the cell wall and leaks into the cell when membrane is damaged. Images were acquired every second during 200 seconds. 6 pictures were acquired pre-bleach. The XY drift was corrected with Stackreg plugin in Fiji. The

fluorescence intensity: mean gray values was then measured across the time using Fiji https://imagej.net/software/fiji/ on a ROI drawn on the bleached cell, its apical, basal and lateral cell as well as a control cell. The noise fluorescence was measured outside the root sample. The relative fluorescence percentage for each time point was calculated as following: ((mean gray values bleached cell or adjacent cells, control cell - mean gray value noise)/(mean gray MAX value - mean gray value noise))*100. The MAX value represents the highest mean gray value which is generally a pre-bleached value. The results were then plotted with R Studio for each cell. The mobile fraction was represented at 100 seconds. The boxplot and the statistics were done with jasp https://jasp-stats.org/.

***PSS1* expression quantification:**
For the transcriptional line *pPSS1::mCitrine-NLS-mCitrine* expression pattern quantification, ROIs were drawn on nuclei and the mean gray values were quantified with fiji. For the translational line *pPSS1::PSS1-mCitrine pss1* expression quantification, ROIs were drawn on the perinuclear ER membrane and the mean gray values were quantified with fiji. From 8 to 10 cells were quantified for each root zone in each root. Violin plots displaying averages and standard deviations were done with jasp. All experiments were repeated three times independently.

**Ratio Plasma membrane/cytosol:**
To quantify the plasma membrane/cytosol ratio on the phosphatidylserine biosensor mCITRINE-2xPH(EVCT2), a ratio was made in between the fluorescence intensity/mean gray value measured in two elliptical ROIs from the plasma membrane region (one at the apical/basal sides of the membrane and one at the lateral membrane side) and two elliptical ROIs in the cytosol. Numbers of cells measured are indicated on the graph. All experiments were repeated three times independently.

**Callose immunolabeling:**
The immunolocalization procedure was performed as previously published protocol[14] with 5-days-old Col 0 *Arabidopsis thaliana* seedlings vertically grown on ½ MS agar. Immunolabelling was performed using the Immunorobot InSituPro VSI from Protigene as described in[3] with an anti-Beta1_3D-Glucan antibody (Australia Biosupplies) diluted to 1/500 in microtubule stabilization buffer (MTSB), supplemented with 5% (v/v) neutral donkey serum and donkey anti-rabbit Alexa fluor 594 antibody (Abcam) diluted to 1/500 in MTSB containing 5% (v/v) neutral donkey serum. Statistical analyses were performed in GraphPad Prism (GraphPad Software).

**Serial block face scanning electron microscopy (SBF-SEM):**
Serial block-face scanning electron microscopy (SBF-SEM) was performed as described in[15]. Briefly, 5-day-old Col-0 *Arabidopsis thaliana* seedlings vertically grown on ½ MS agar were chemically fixed, post-stained, and embedded in 812 Epoxy resin (Agar Scientific). Image acquisition was carried out using a 3View2XP system (Gatan) in a GeminiSEM 300 microscope (Zeiss), operated under high vacuum at 1.8 kV accelerating voltage, in normal mode with a 20 mm aperture. The focal charge compensator was set to 90%. Section thickness was 70 nm, with a pixel size of 5 nm and a dwell time of 2 ms per pixel. Image alignment and contrast normalization were performed using *Microscopy Image Browser*. Data analysis was conducted in *3Dmod* (IMOD). The number of plasmodesmata per cell-to-cell interface was estimated based on plasmodesmata density and the total cell wall surface area. Plasmodesmata density was determined by counting

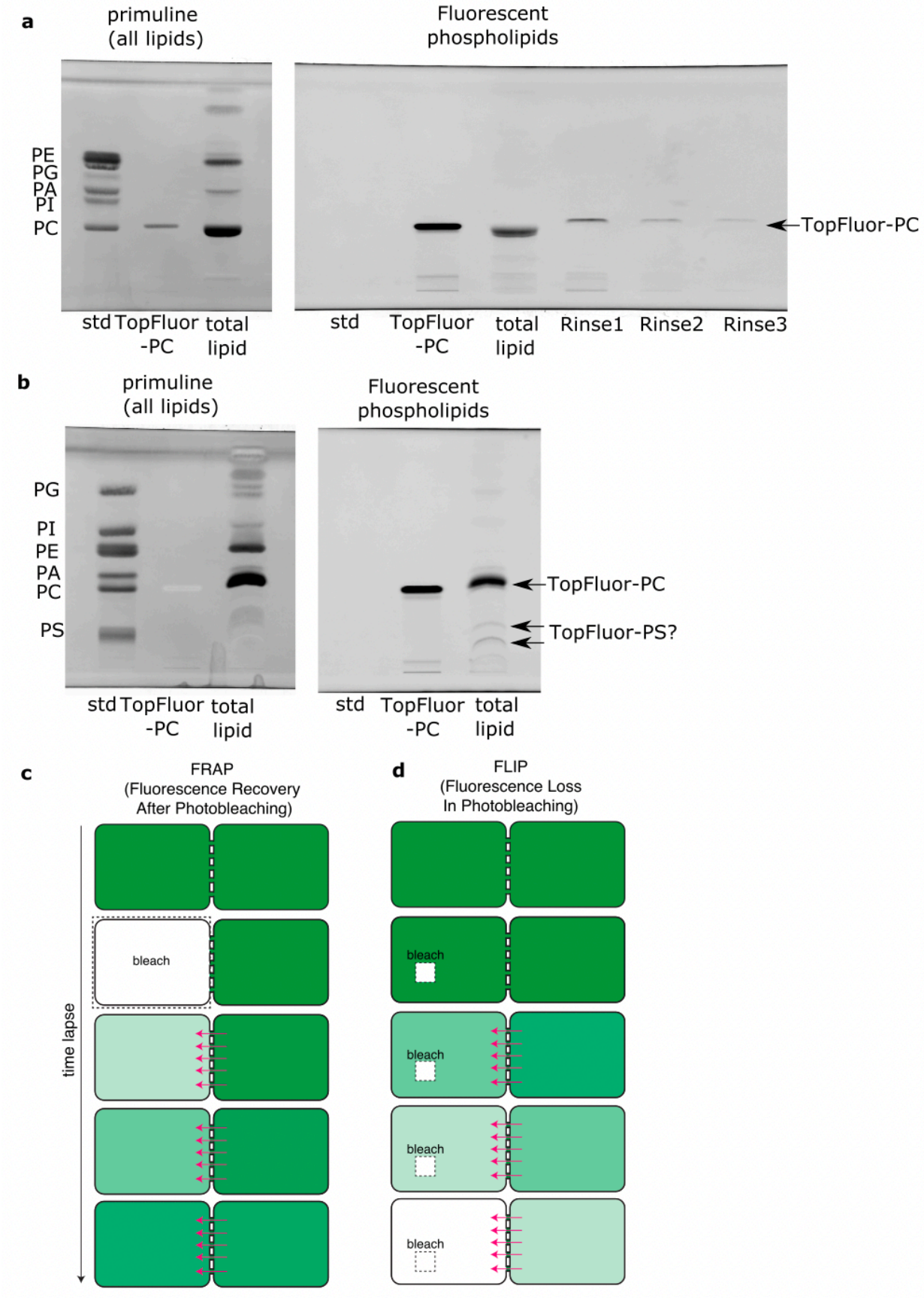
plasmodesmata in one out of every four sections of a single SBF-SEM stack. Cell wall surface measurements were obtained through segmentation in IMOD on the same interval. The total cell wall surface area was derived from confocal z-stacks of propidium iodide-stained roots. Cell walls were segmented using *Imaris*, and the surface area was quantified with *ImageJ.*

**High Performance Thin Layer Chromatography (HPTLC):**

Col-0 *Arabidopsis thaliana* seedlings were vertically grown for 6 days on a sterile mesh placed over ½ MS agar plates. Seedlings were then incubated overnight in a 6-well plate containing liquid ½ MS medium supplemented with 40 µM TopFluor-PC (Avanti). The following day, seedlings were rinsed three times with liquid ½ MS medium. Roots were excised, and at least 100 mg of fresh root tissue was collected for a biphasic extraction of lipids, performed as described in[16]. Samples and standards (std) —including 3 mg egg yolk phosphatidylcholine (PC), 7.5 mg soybean phosphatidylinositol (PI), 5 mg egg yolk phosphatidylethanolamine (PE), 7.5 mg egg yolk lecithin phosphatidylglycerol (PG), and 8 mg egg yolk phosphatidic acid (PA) (all from Sigma-Aldrich) were deposited on HPTLC Silica Gel 60 F254 plates (20 × 10 cm, Sigma-Aldrich) using a semi-automatic sample applicator (CAMAG). High-performance thin-layer chromatography (HPTLC) was performed following the method described by[17], using a solvent mix composed of 10 mL methyl acetate, 10 mL chloroform, 10 mL isopropanol, 4 mL methanol, and 3.6 mL 0.25% KCl or described by[18] using a solvent mix composed of 4mL methyl acetate, 5mL chloroform, 4.2mL isopropanol, 1.7mL methanol, 1.3mL 0.25% KCl, 0.5mL triethylamine (phosphoLIMBO protocol). Fluorescent phospholipids were visualized using a ChemiDoc MP imaging system (Bio-Rad) at 525 nm. Total phospholipids were revealed by staining with a primuline solution (0.025 mg/mL in 80:20 [v/v] acetone/water), followed by UV excitation at 302 nm using the same imaging system.

**Statistics and quantification:**

For making graphs, plots and statistics: Jasp and R Studio have been used. Statistical test and n are indicated in the legends[5].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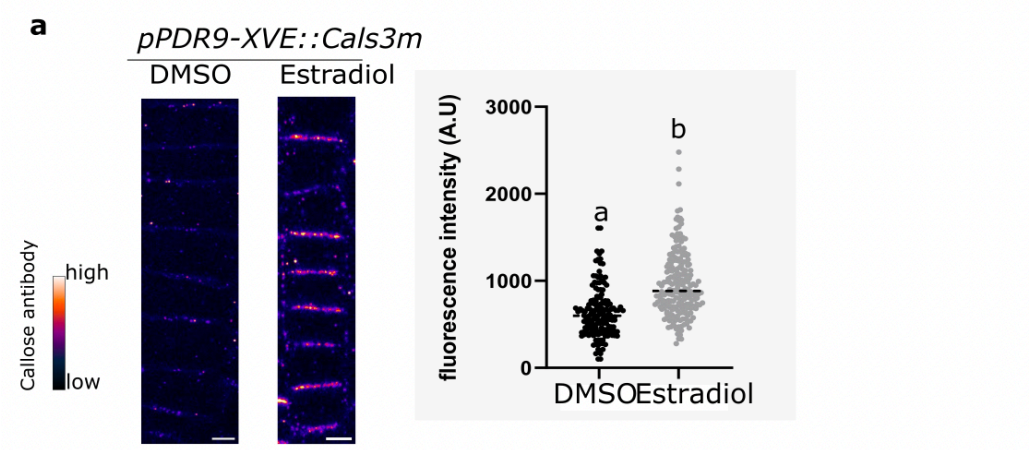
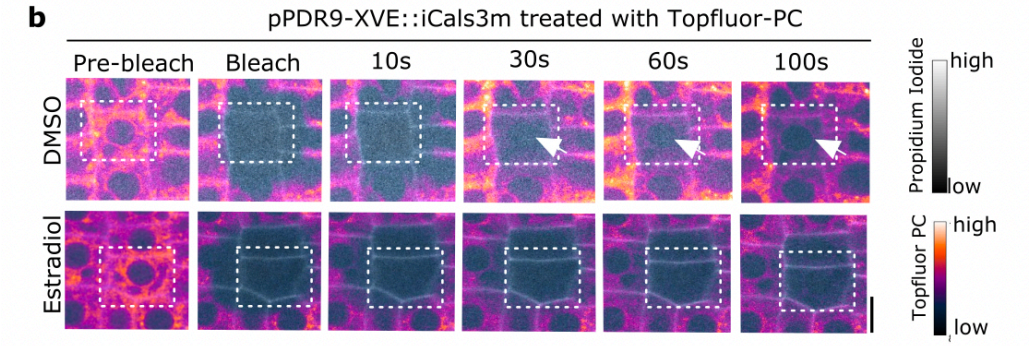
**Extended Data Fig. 1: TopFluor-PC is intact in plant extracts and may be slightly metabolized into PS.** (**a**) Full blot of the experiments shown in Fig. 1b. HPTLC of lipid extracts with Vitiello migration protocol visualized either at 302 nm (left, all lipid species being labeled by primuline), or at 525 nm (right, showing only TopFluor-labelled lipids). Std: standards (PE: Phosphatidylethanolamine, PG: Phosphatidylglycerol, PA: phosphatidic acid, PI: phosphatidylinositol, PC: Phosphatidylcholine). The lane labeled "TopFluor-PC" was loaded with pure TopFluor-PC, while the lane labeled "total lipids" was loaded with a total lipid extract for roots pretreated with TopFluor-PC and then rinsed three time. The three lanes on the right show lipid extraction in the wash fraction. Note that PG and PE migrate very similarly and cannot be fully resolved. (**b**) HPTLC of lipid extracts with PhosphoLIMBO migration protocol with inclusion of a PS standard, showing that the minor low migrating bands likely represents TopFluor-PS species. (**c-d**) schematic representation of the expected outcome from FRAP (**c**) and FLIP (**d**) experiments if two cells are connected. These techniques complement each other. FRAP quantifies the diffusion of fluorescent molecules into the bleached area, thereby showing the mobile fraction and its dynamics, but gives no information on the source of the fluorescence recovery. FLIP reveals the locations of nonmobile fluorescent molecules by quantitatively depleting the mobile population. Thus, if two cell are connected, the non-bleached cell will also lose fluorescence, quantitatively depending on its connectivity with the bleached cell.

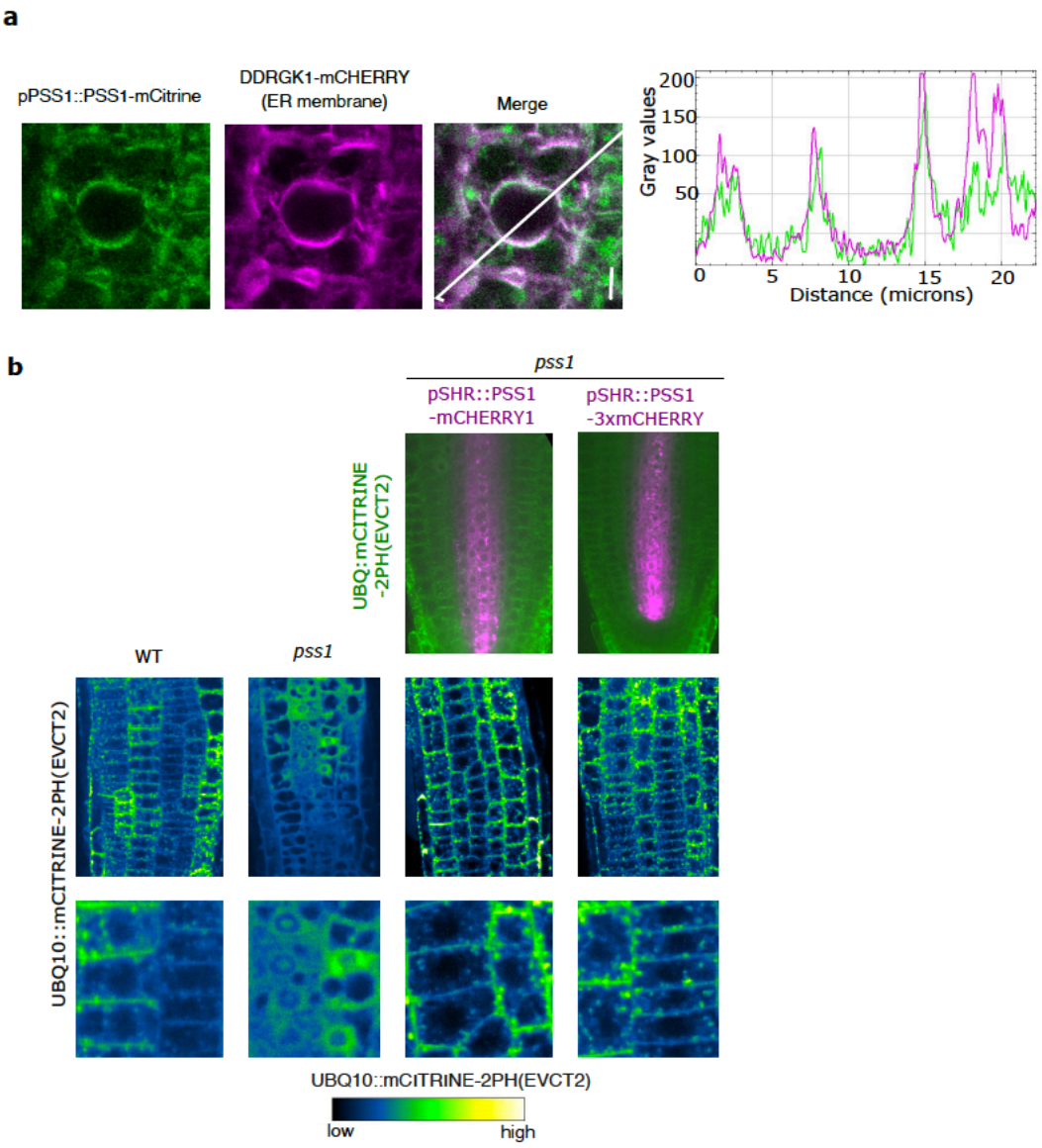


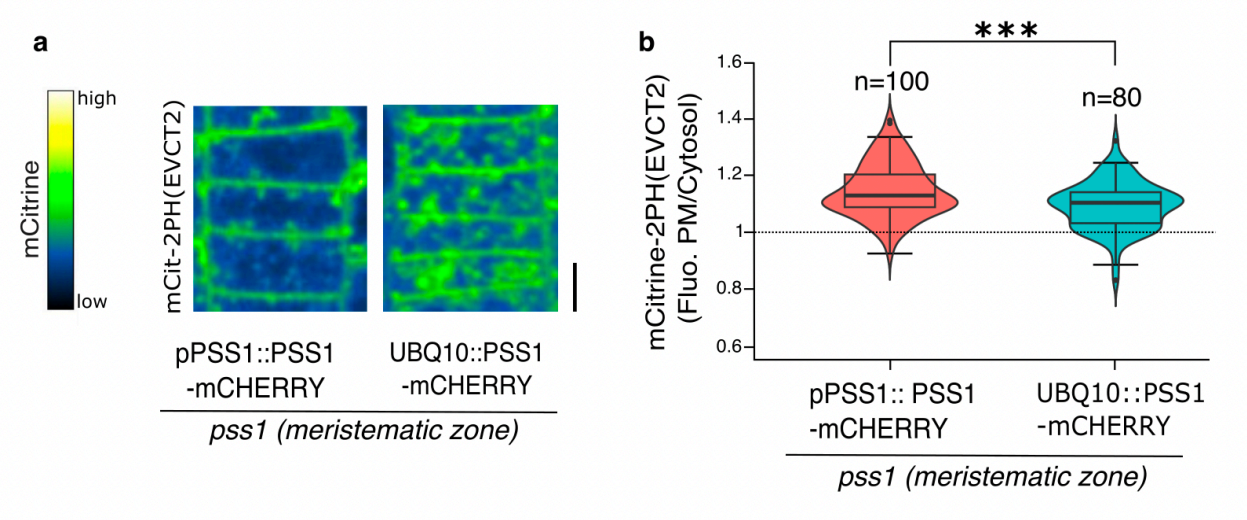

c

| | | p-value/p-tuke |
|---|---|---|
| Bleached cell | Control cell | < 0.001 *** |
| | Basal cell | < 0.001 *** |
| | Lateral cel | < 0.001 *** |
| | Apical cell | < 0.001 *** |
| Control cel | Basal cell | < 0.001 *** |
| | Lateral cel | < 0.001 *** |
| | Apical cell | < 0.001 *** |
| Basal cell | Lateral cel | 0.05 * |
| | Apical cell | 0.57 ns |
| Lateral cell | Apical cell | < 0.001 *** |

**Extended Data Fig. 2: Callose is overproduced in induced *pPDR9::XVE-Cals3m* and blocks TopFluor-PC cell-cell diffusion. (a)** Representative pictures of callose immunolabeling in *pPDR9::XVE-Cals3m* root cells treated with DMSO or estradiol, scale bar : 5µm (left panel), and quantification of the fluorescence intensity of callose immunolocalization in the respective treatments, n=4 roots. The experiment was replicated 2 independent times. Statistical analysis was done with a t-test, p value<0.0001. (**b**) Representative pictures related to the graph in main Fig. 2h and 2i of root epidermal cells expressing *pPDR9::XVE-Cals3m* treated with TopFluor-PC and either DMSO (control) or estradiol (callose induction), each labeled with Propidium Iodide during FRAP experiments. The dotted square represents the photobleached region. The white arrowhead depicts the reappearance of the perinuclear ER membrane. Scale bar: 10 µm. **(c)** Table summarizing the p-values after multiple post hoc tests of the graph presented in Fig 2g.




**Extended Data Fig. 3: PSS1 localizes at the ER membrane and a PSS1-3xmCHERRY fusion complements the PS sensor localization in the *pss1* mutant. (a)** Colocalization of airyscan pictures of root meristematic cells expressing *pPSS1::PSS1-mCitrine* together with the ER membrane marker *DDRGK1-mcherry*. Scale bar: 1 µm (upper panel). Gray values of the two markers were plotted along the white dashed line: PSS1-mCitrine (green curve), DDRGK1-mcherry (magenta curve). **(b)** Representative pictures of the tissue specific expression patterns of *PSS1* in *pSHR::PSS1-mCHERRY/pss1, pSHR::PSS1-3xmCHERRY/pss1* expressing root cells (magenta) and expression of the PS sensor mCitrine-2PH(EVCT2) (green) (upper row), Scale bar : 40 µm; representative pictures of the PS sensor *mCitrine-2PH(EVCT2)* in the root epidermal cells of the respective pre-cited lines, WT and *pss1*, scale bar: 20 µm (middle row) and their respective close up, scale bar: 10 µm (lower row).




**Extended Data Fig. 4: Overexpression of PSS1 impacts PS distribution within the root meristematic cells. (a)** Representative pictures of the PS sensor mCitrine-2PH(EVCT2) localization in the root epidermal tissue of *pPSS1::PSS1-mCHERRY/pss1* and *pUBQ10::PSS1-mCHERRY/pss1* lines in the meristematic zone, scale bar : 5 µm. **(b)** Ratios of the PS sensor mCitrine-2PH(EVCT2) Plasma membrane/cytosol fluorescence in *pPSS1::PSS1-mCherry/ pss1* (n=100 cells) and in *pUBQ10::PSS1-mCherry/pss1*(n=80 cells) in the meristematic zone. Bars indicate the mean and SD. (Kruskal Wallis test was performed *** p value<0,001). The experiment was replicated 2 independent times.

**Supplementary Videos**

**Supplementary Video 1:** FRAP analysis of CFDA dynamics.

**Supplementary Video 2:** FRAP analysis of TopFluor-PC dynamics.

**Supplementary Video 3:** FRAP analysis of C4H-GFP dynamics.

**Supplementary Video 4:** FLIP analysis of TopFluor-PC dynamics.

**Supplementary Video 5:** FRAP analysis of TopFluor-PC dynamics upon callose induction by estradiol in *PDR9::cals3m* line.

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