Supplementary Materials for

Cell Wall Remodeling and Vesicle Trafficking Mediate the Root Clock in Arabidopsis

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Materials and Methods

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Captions for Movie S1

Other Supplementary Materials for this manuscript includes the following:

Captions for Fiji, BASH and R codes

Captions for Datasets text files

Materials and Methods

Plant material and growth conditions

DR5::LUC and the PMEI3 and PME5 overexpression lines were described in (*1, 24*). The *agd3* mutant allele SALK_140826, *pme2* (GK-835A09), *pme3* (GK-002A10) and *pme2;pme3* were acquired from the ABRC. All plants were grown on 0.5GM medium containing 1.1 gr Murashige and Skoog basal, 1% sucrose, 1% plant agar and 5 ml MES (50 g/l, pH=5.8 with KOH) in 500 ml ddH_2O. BFA was prepared as 50 mM stock solution in DMSO and 30 µl were added to 500 ml of 0.5GM media to prepare 3 µM BFA medium; 0.5GM with 30 µl DMSO in 500 ml medium was used as control. For RNA-seq experiments we used 1.2% agar instead of 1% and supplemented the medium with 0.4 mM Luciferin from a 400 mM stock solution (in H_2O). Plants were stratified for 2 d in a 4^{o}C dark room and grown vertically for 4-10 days under long-day light conditions. PME5 or PMEI3 overexpressor (and the WT background plants WS-4/Wassilewskija ecotype) were plated and grown on 0.5GM plates as described above following daily application of 25 µl EtOH 3-4 cm from the root tip starting two days post germination.

RNA and RNA-seq library preparation

To select sections for the spatial experiment, we followed and estimated the Luciferase expression in the oscillation zone compared to background. We than performed live Lumazone time lapse imaging and measured the expression in the oscillation zone in each time point cycle. Once Luciferase expression showed a consistent increase with values that we had approximated as peak expression, the root was removed from the Lumazone box and sections were cut using a dissecting microscope with a comparison to the image in such way that each section was accurately annotated. We followed a similar procedure for the temporal experiment selecting “bp” and “ap” sections before and after peak expression, respectively. Each root region section (spatial experiment) or time point section (temporal experiment) had seven or five biological replicates, respectively. Each root section was placed in 5 µl H_2O, frozen in liquid nitrogen and ground with a Fisherbrand pellet pestle cordless motor. RNA was prepared using the RNAzol reagent according to the manufacturer’s instructions. In brief, disrupted tissue was mixed with 0.2 ml RNAzol and 80µl H_2O for 5-10 mins at room temperature and centrifuged at 12,000 g for 15 mins. Upper supernatant was transferred to a new tube, 1.25µl (0.5% of the supernatant volume) of 4-bromoanisole was added, mixed and stored at room temperature for 3-5 mins, then centrifuged at 12,000 g for 10 mins at 4^{o}C. Supernatant was transferred to a new tube, 1 µl Glycoblue was added and mixed with 300 µl isopropanol. RNA was precipitated overnight and centrifuged for 30 mins at 4^{o}C. The pellet was washed twice with 75% EtOH by releasing it from the tube wall, air-dried, and resuspended in 10 µl H_2O. RNA-seq libraries were prepared using the Smart-seq2 protocol (*11*).

Computational and statistical analysis

For the RNA-seq experiments, Illumina HiSeq 2000 reads were aligned and counted using the Additional Code S7 (whole RNA) and Additional Code S8 (Luciferase RNA read count) BASH files for the spatial experiment, and the Additional Code S9 file for the temporal experiment. These pipelines generate read counts for the TAIR10 gene list (Additional Data set

S3) of all libraries (Additional Data sets S1and S2). These two read-count files where then used for statistical analysis and plotting using R codes (Additional Code S1 and S2). To select for genes that are highly expressed in the oscillation zone-spatial, we determined a logFC>0.5 between the p region and each of the other regions (r, s, pbs and ss) using the contrast function of the edgeR package. For the temporal experiment, we required a logFC>0.5 when contrasting the bp and p time points for a gene to be considered as having increased expression during oscillation. Only GO terms with a p-value<0.01 for the first two gene lists (Table S1, A and C) and a p-value<0.05 for the third list (Table S1D) were considered (Additional Code S3 Table S2). All boxplots show five summary statistics, the median (middle bar), 25 and 50 percentile (lower and upper hinges) and the largest and smallest values, no further than 1.5 * IQR from the upper and lower hinge, respectively (two whiskers). Bar plot whiskers (Fig, 4D) represent standard error of the mean. We used the SIMPLE pipeline (*25*) to map the Ethyl methanesulfonate mutations that were identified in the forward genetic screens (Table S3). All measurements of LR and pre-branch site number were performed with n≥15. For immunohistochemistry measurements, we used our Fiji macro Additional Code S5 to select and measure the antibody-labeled cell wall area while the cross-section area was calculated manually using Fiji circle selection tool. The cell wall area of each section was integrated and divided by the root cross section area to get the ratio (related to Fig. 4A). We used the Fiji macro Additional Code S4 to calculate cell wall area and calculate raw integrated density. The ratio between these values was calculated to get the grey scale level per pixel (related to Fig. S6). To measure the immunofluorescence intensity of LM19 and LM20 antibodies in pericycle-cortex and cortex-endodermis cell wall borders near LRP and the opposite (control) sides, (Fig. 4, E to H), we drew a line along these borders and used the list option under the plot profile Fiji analyze menu. The mean value for each border was used to perform paired Wilcoxon rank sum test. For each antibody in each genotype $n_{sections}$>23 and n_{roots}=5. LRs, pre-branch site counting and area selection for immunohistochemistry were single blinded. For counting and measuring the number of pectin-marked gold particles per nm we used our Fiji macro Additional Code S6 (Fig. S7). For each genotype, n_{roots}≥4, $n_{sections}$≥9 and $n_{junctions}$≥105.

Fixation, dehydration, infiltration and labeling

3-5 days post germination seedlings were fixed for 2.5 h in fixative composed of 1.6% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in 25 mM sodium phosphate, pH=7.1. We performed the entire process in Falcon cell strainers placed inside polypropylene Falcon tube caps to avoid chemical interaction with the resin. Fixed roots were rinsed with sodium phosphate buffer, pH=7.1 twice for 15 min each, and with water twice for 15 min each. Samples were dehydrated at room temperature through a graded ethanol series (20, 35, 50, 62, 75, 85, 95, 100, 100, 100% [v/v], (with the last 100% step including 0.5% [w/v] filtered Fast Green FCF) for 30 min at each step. Samples were Infiltrated with a graded series of cold LR White embedding resin (Ted Pella) at 4ºC (33% and 66% resin in 100% ethanol, 24 h each, followed by three changes of 100% resin, for 24 h each). Samples were cured in size 3 gelatin capsules (Fisher, 50-248-74), 100% resin, for 48 h at 60ºC. For immunofluorescence, 2 μm sections were prepared using a Reichert-Jung Ultracut E microtome and labeled as previously described (*31*) and visualized using a Zeiss confocal microscope. Immunogold labeling was performed as described (*37, 38*). LM15, LM19 and LM20 immunofluorescence secondary antibody reactions were performed with Alexa Fluor® 488 AffiniPure F(ab')$_2$ Fragment Goat Anti-Rat IgG (H+L) whereas 2F4 was labeled with Alexa Fluor® 488 AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgG (H+L). Xyloglucanase treatment

was performed as describe for the pectate lyase treatment (*34*) using 20 min boiling at 99°C as the inactive form. For the immunogold reaction, we used Rabbit anti-Rat IgG (H+L) Secondary Antibody, HRP. For more details on reagents please see Table S5.

Cloning

The 5144 bp *CLE44* promoter was cloned using primer pair 1+2 (Table S4) and cloned into pCAM1300Hyg-R4R3 with the Luciferase gene (*1*) and 3AT terminator using three-way Gateway. The pCLE44::GFP fusion was generated by combining pCLE44 promoter with a H2B nuclear localized GFP coding regions in a three-way Gateway reaction. The *GNOM* promoter, genomic region and 3' UTR were cloned into entry vectors using primer pairs 3+4, 5+6 and 6+7 (Table S4), respectively and assembled using multi-site Gateway reaction to form an expression clone. The GNOM over-expression clone was assembled according to the same protocol using the same *GNOM* genomic clone, estradiol inducible G1090 promoter (*26*) and the 3AT terminator.

Imaging

pCLE44::GFP (Fig. S1B) was imaged with a Zeizz 880 confocal microscope.
Immunofluorescence sections were imaged using Zeiss 880 or 510 confocal microscope.
Luciferase fusions were imaged using the Lumazone platform (*1*) with 7 min exposure time. For visualization of immunogold particles we used FEI Tecnai G² Twin TEM with an Eagle - 4k x 4k camera.

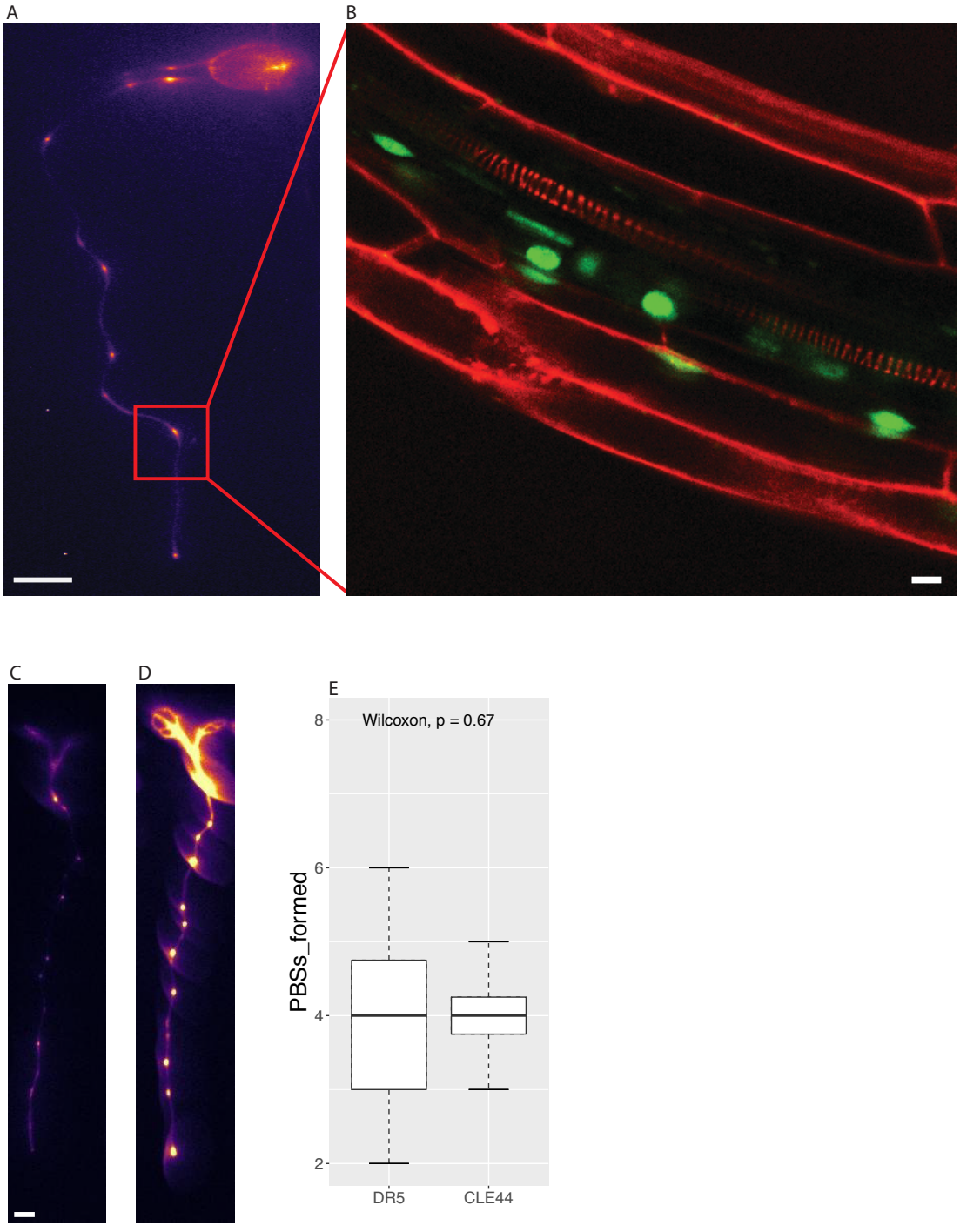

Fig. S1. DR5 and pCLE44 mark presumptive PBSs and LRP

(A) Lumazone imaging of seedling harboring both DR5::LUC and pCLE44::GFP. Youngest pre-branch site is marked by red square. Scalebar=0.2 cm. **(B)** same seedling as in (A). The region of the pre-branch site marked in (A) was bordered by proximal and distal lines and scored for pCLE44::GFP-marked LRP using the z-stack function of a confocal microscope. Scalebar=10 μm. **(C** and **D)** DR5::LUC (C) and pCLE44::LUC (D) seedlings 4 days post

germination showing PBSs along the main root. **(E)** Number of PBSs formed in plant groups shown in panel (C and D) in a 24 h time period n≥15, scalebar=0.1 cm. See also Movie 1.

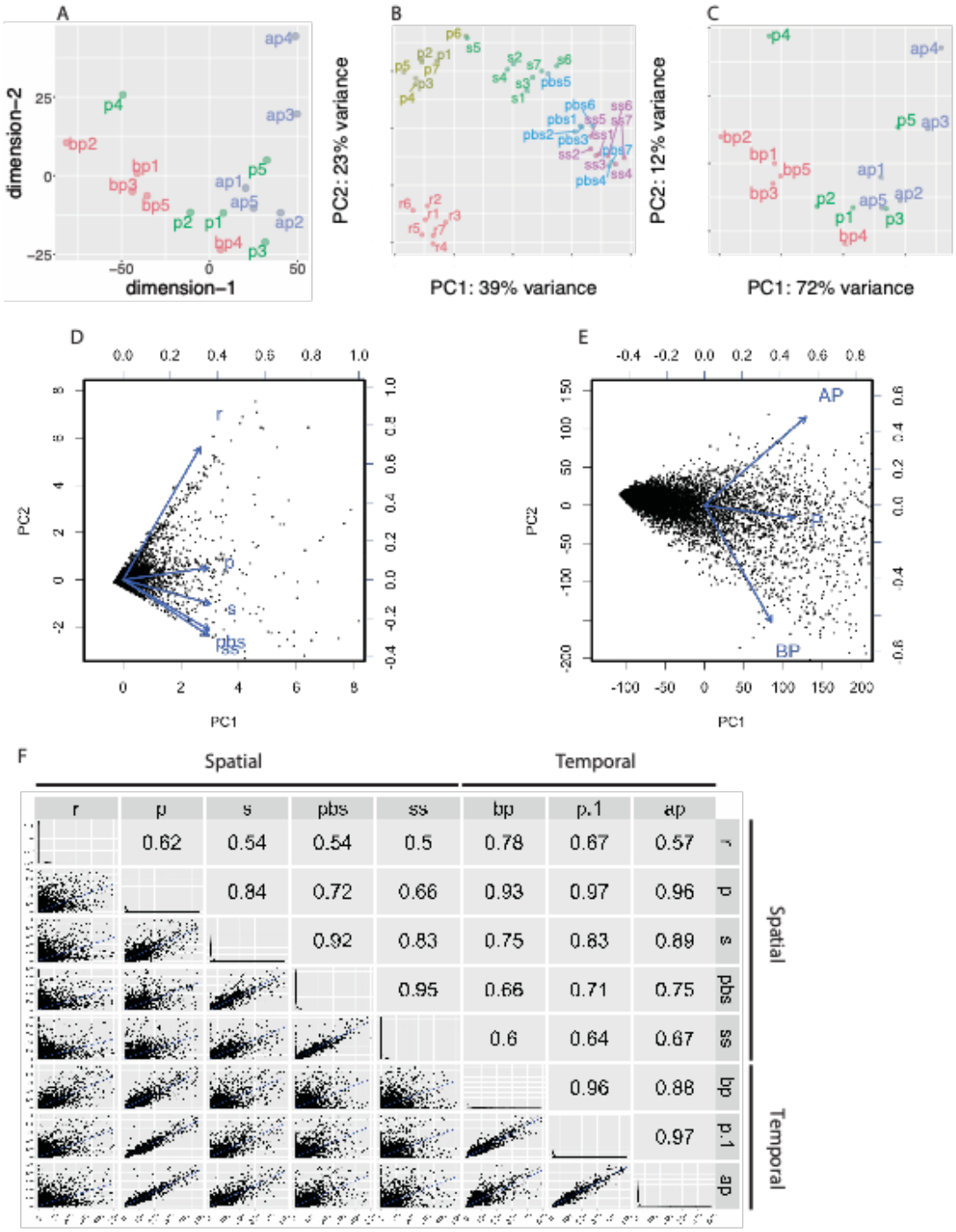

Fig. S2. Statistical analysis of RNA-seq experiments confirms data quality
(A) Multidimensional scaling analysis of the RNA-seq temporal experiment. **(B** and **C)** PCA analysis of the spatial (A) and temporal (B) experiments. Root section names correspond to those in Fig. 1, A and B, respectively. **(D** and **E)** Biplot analysis of the spatial (D) and temporal (E) experiments. Each point represents a single gene's coordinates projected onto the first two principal components. Each arrow represents a vector of the mean value of the indicated section. Note that the vectors are arranged (top to bottom, clockwise) in the same order as the sections along the root. Small angles between vectors, e.g., the one between pbs and ss (D), reflect high correlation between the regions while near-orthogonal vectors such as r and ss reflect uncorrelated relationships between samples. The biplots support the same trend shown in the MDS and PCA analyses. **(F)** Correlation analysis between all eight samples of the spatial and temporal experiments (p.1 represents the p section of the temporal experiment). Correlation coefficients (R^2) are

shown in the upper triangle of the plot. Note that p and p.1, which represent the oscillation zone in the spatial and temporal experiments, respectively, have the highest R^2 value as expected.

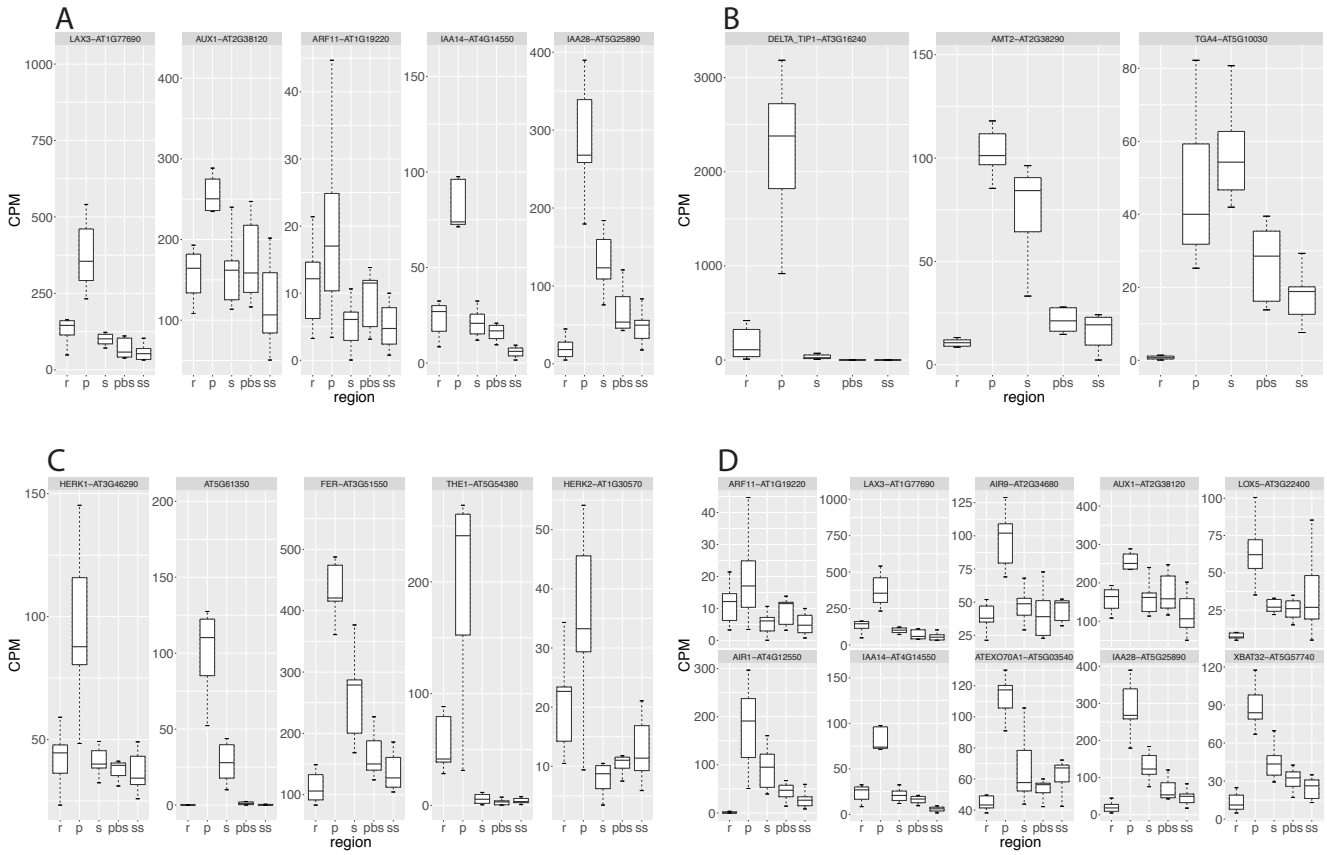

Fig. S3. Expression of selected genes in the spatial experiment

(A) Expression pattern of five auxin transport and signal transduction related genes that are also involved in LR initiation. **(B)** Expression pattern of three nitrogen-related genes that are also involved in LR initiation. **(C)** Expression pattern of five crRLKs. **(D)** Expression pattern of the 10 genes in the LR GO term that had significant oscillation zone expression in the spatial experiment.

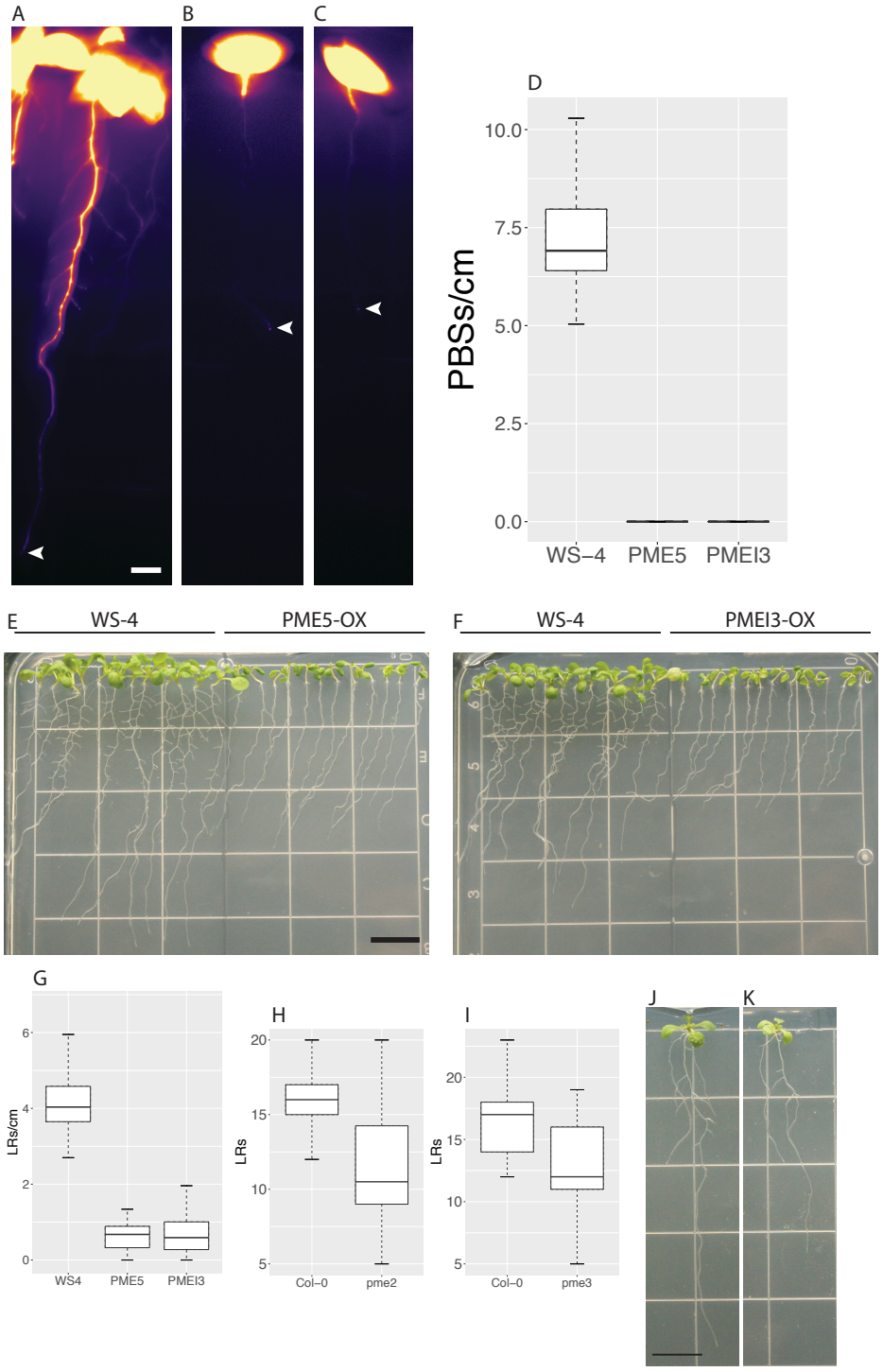

Fig. S4. Destabilization of esterified and de-esterified pectin reduces PBS and LR formation
(A to **C)** WT DR5::LUC expressing seedlings (WS-4; (A)), PME5 overexpression (B) and PMEI3 overexpression (C) under EtOH inducible conditions. Arrowhead marks root tips. Scalebar=0.2 cm. **(D)** Quantification of DR5::LUC PBS number in WT (WS-4), PME5 overexpression and PMEI3 overexpression. p-value<10^{-8} (Wilcoxon Rank Sum test). **(E** and **F)** Seedling phenotype of PME5 overexpression (E) and PMEI3 overexpression (F) compared to WS-4 WT.

Scalebar=1 cm. **(G)** Quantification of LRs and LRP shown in (E and F). p-value<10^{-10} (Wilcoxon Rank Sum test). **(H** and **I)** Quantification of LRs in *pme2* (F) and *pme3* (G). p-value=0.0002 (F) and p-value=0.01 (G) (Wilcoxon Rank Sum test). **(J** and **K)** Representative phenotype of Col-0 (J) and *pme2;pme3* (K). Scalebar=1 cm.

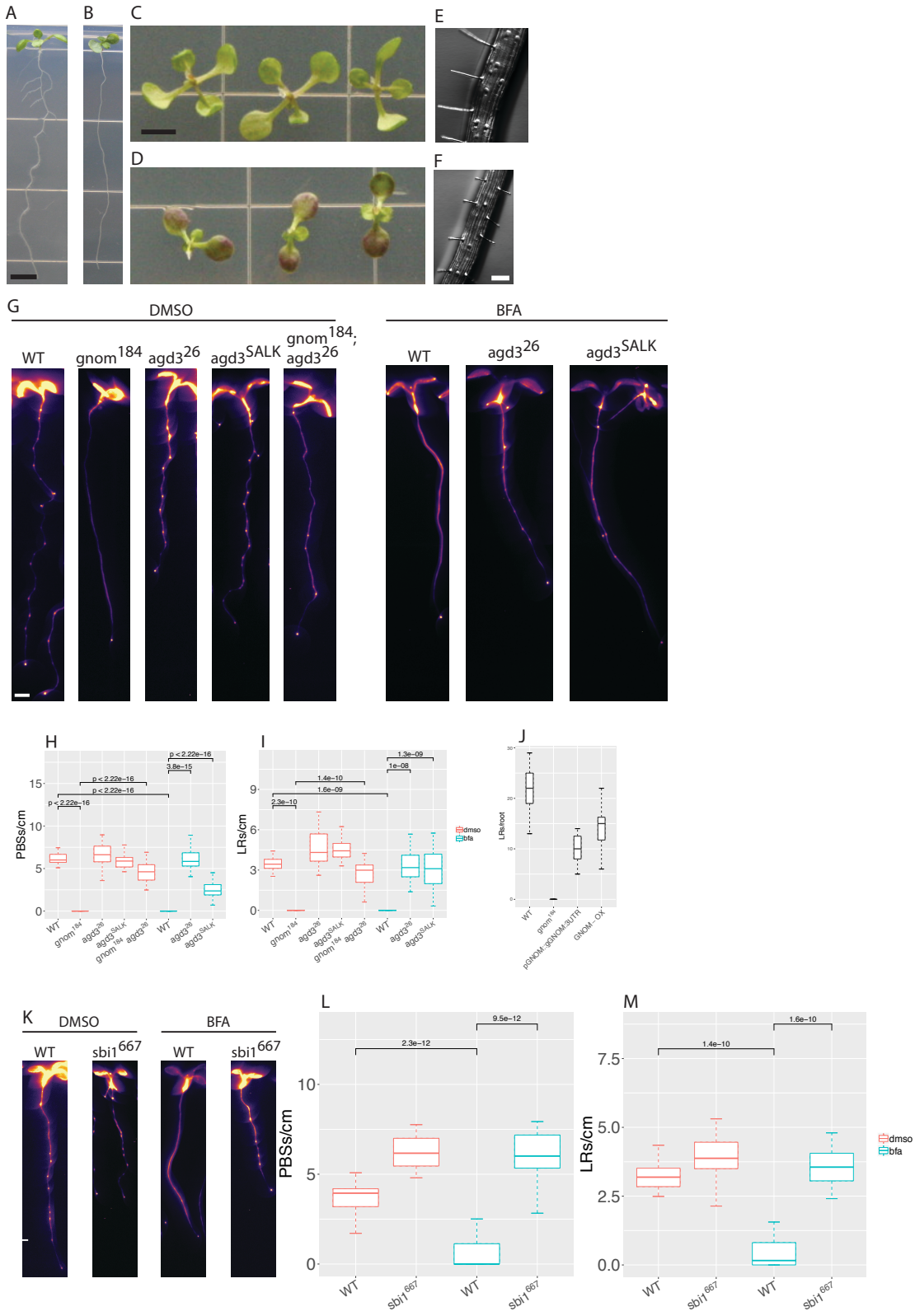
A
B
C
D
E
F
G
DMSO
BFA
WT
gnom184
agd326
agd3SALK
gnom184; agd326
WT
agd326
agd3SALK
H
PBSs/cm
p < 2.22e−16
3.8e−15
I
LRs/cm
2.3e−10
1.4e−10
1.6e−09
1e−08
1.3e−09
dmso
bfa
J
LRs/root
pGNOM::gGNOM:3UTR
GNOM−OX
K
DMSO
BFA
WT
sbi1667
L
PBSs/cm
2.3e−12
9.5e−12
M
LRs/cm
1.4e−10
1.6e−10

Fig. S5. BFA treatment, *gnom*^{184} and its suppressors affect lateral root development
(**A** and **B**) WT (Col-0 (A)) and *gnom*^{184} (B) seedling phenotypes. Scalebar=0.5 cm**. (C** and **D)** Shoot phenotype of WT (Col-0 (C)) and *gnom*^{184} (D) seedlings. Scalebar=0.5 cm. **(E** and **F)** Root hair phenotype of WT (Col-0 (E)) and *gnom*^{184} (F). Scalebar=100 μm. **(G)** DR5::LUC expression patterns of various phenotypes under control or BFA treatment. Scalebar=0.2 cm. **(H** and **I)** pre-branch site (H; DR5::LUC) and LR (I) Quantification of the genotypes and treatments depicted in (G). **(J)** Partial complementation of the *gnom*^{184} allele with its native promoter and 3' UTR region and with the over-expression, estradiol inducible G1090 promoter. **(K)** *sbi1*^{667} mutant siblings with and without BFA treatment. Scalebar=0.5 cm. **(L** and **M)** Quantification of pre-branch site (L) and LR (M) number in the *sbi*^{667} mutant. t-test p-values (H, I, L, M) are indicated above boxes.

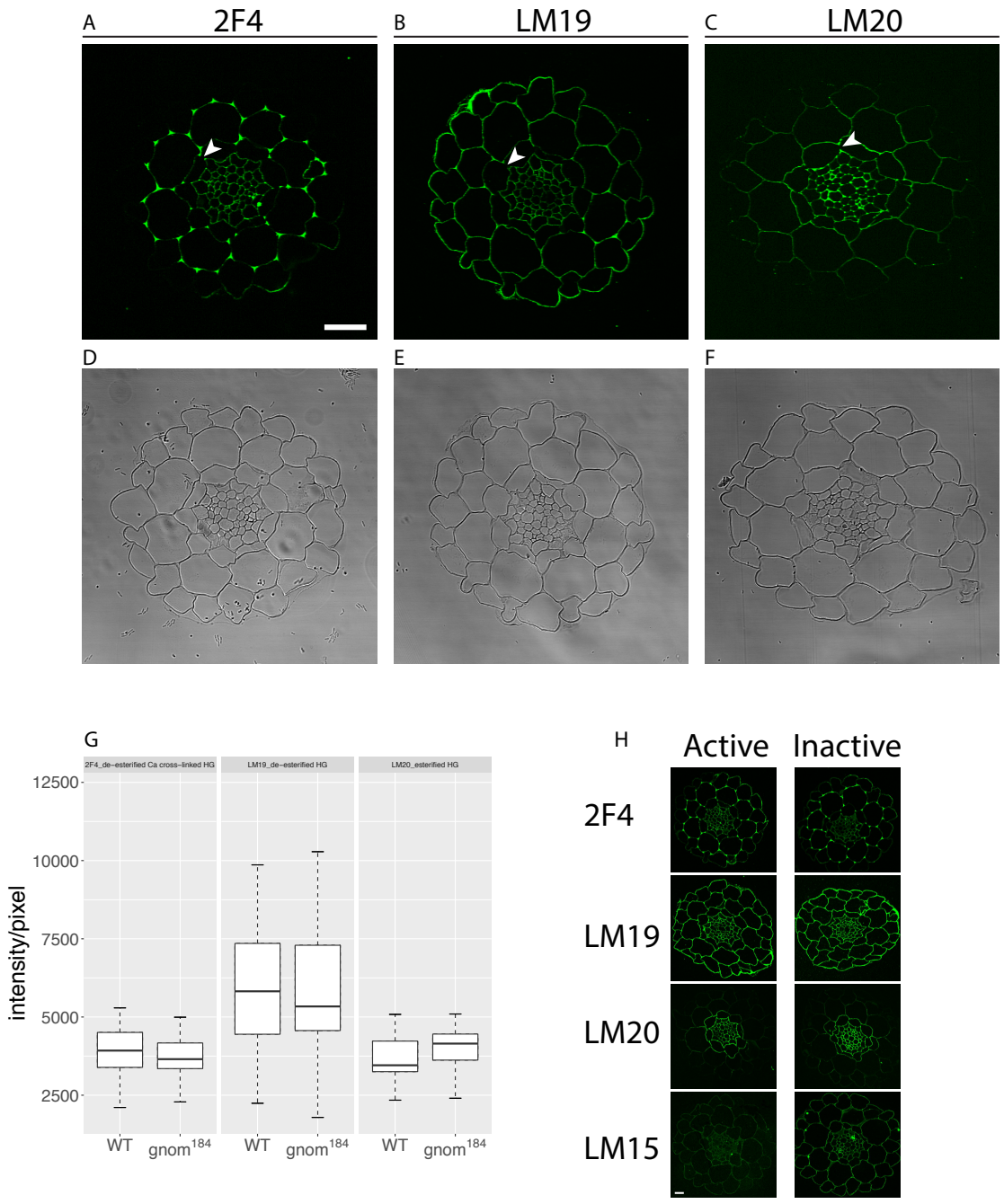

Fig. S6. *gnom*^{184} does not affect pectin density in the root

(A to **C)** Immunohistochemistry of WT root cross sections labeled with 2F4 (A), LM19 (B) and LM20 (C) antibodies. Note the lack of signal in Casparian strip region (arrowheads). **(D** to **F)** bright field images of the sections shown in (A to C). **(G)** Quantification of pixel intensity of the three antibodies shown in (A to C) in WT and *gnom*^{184} roots. Scalebar=20 μm. **(H)** Xyloglucanase treatment (left column) does not show an effect in any of the pectin antibody (first three rows) signal intensity or distribution. LM15 antibody binds xyloglucan and was used as control for xyloglucanase activity. Scalebar=10 μm.

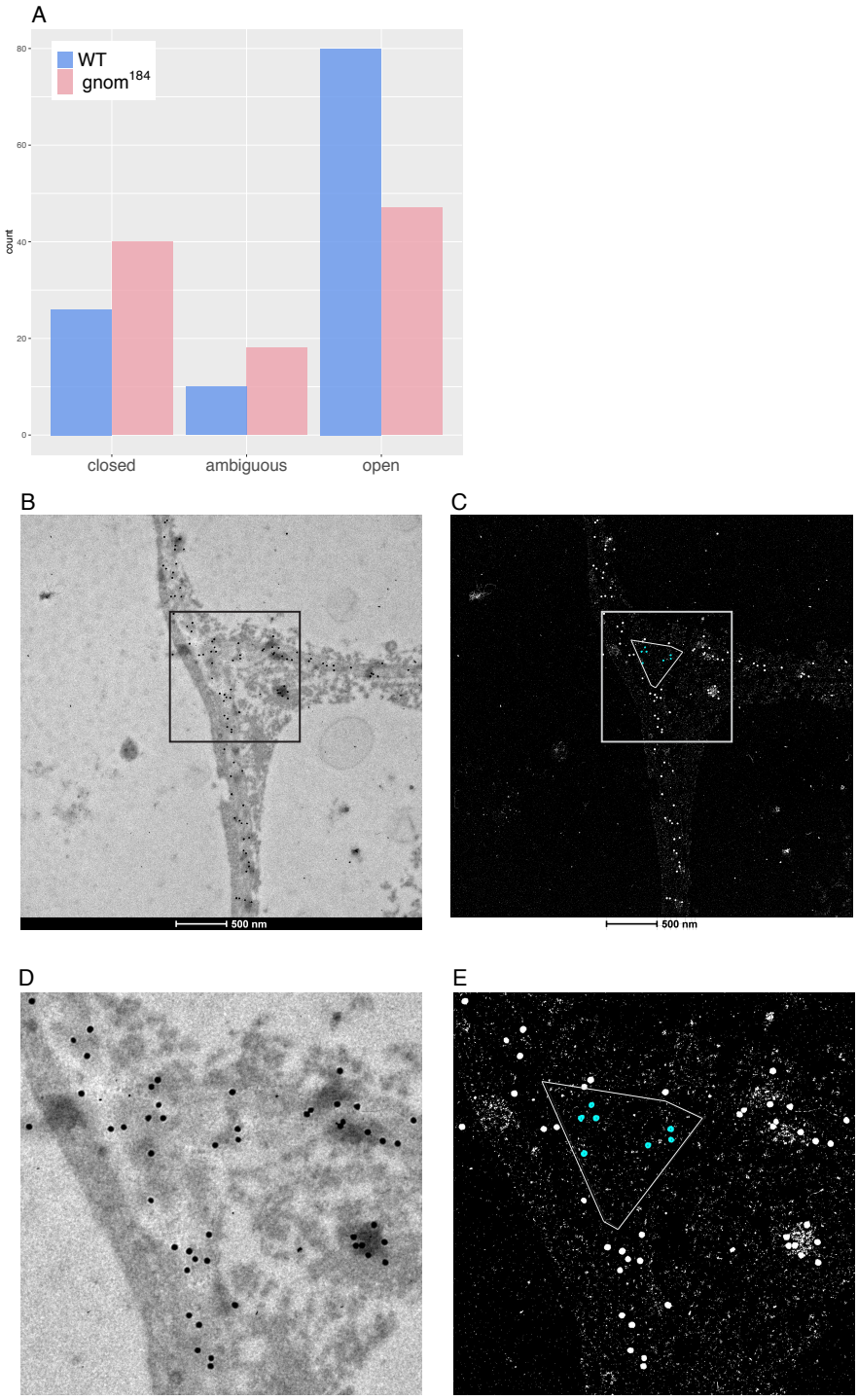

Fig. S7. TEM analysis of de-esterified HG

(A) Each pericycle-endodermis junction quantified in Fig. 4, A to C was evaluated as "open" where the gold particles form a near-triangle shape, "closed" with no clear space between the cell wall intersection or undetermined (ambiguous) in WT and *gnom*^{184}. The number of each type was plotted for each genotype. $p<0.002$ (χ^2 test). **(B** and **C)** An example of Additional Code S6 output. The program sequentially opens each original immunogold image file (B), requesting to mark a region of interest (junction) and generates a binary image with all marked gold particles it

identified outside the region of interest (C, white triangle) in white and inside the region of interest in cyan. Number of cyan marked gold particles and the area of the region of interest are scored in a separate text file. **(D** and **E)** magnification of the square marked area in (B and C), respectively.

Table S1. RNA-seq selected genes. More information is available in the first Excel tab.

Table S2. GO analysis results. More information is available in the first Excel tab.

Table S3. SIMPLE pipeline DNA-seq/EMS screens results.

Table S4. Primer list.

Table S5. List of materials and reagents.

Movie S1.
24 h time lapse of DR5::LUC (left) and pCLE44::LUC (right) seedings. Images were taken every 30 min.

Additional Data set S1.

Transcript read count of all 35 libraries present in the spatial experiment.

Additional Data set S2.

Transcript read count of all 15 libraries present in the temporal experiment.

Additional Data set S3.

TAIR10 functional transcription.

Additional Code S1.

R code for statistical analysis and plotting of the spatial experiment. Requires Additional Data sets S1 and S3 as input.

Additional Code S2.

R code for statistical analysis and plotting of the temporal experiment. Requires Additional Data sets S2 and S3 as input.

Additional Code S3.

R code for statistical analysis and plotting of GO terms. Requires gene lists generated in Additional Code S2 and S3.

Additional Code S4.

Fiji macro for segmentation antibody-marked cell wall regions and calculation of raw integrated density. Grey level per pixel value was calculated based on these two measurements (related to Fig. S6).

Additional Code S5.

Fiji macro for segmentation of antibody-marked cell wall regions and calculation of their total area. Cross-section area was calculated manually using Fiji circle selection tool. The cell wall area to cross section area value was calculated based on these measurements (related to Fig. 4A).

Additional Code S6.

Fiji macro for segmentation of gold particles, counting their number and the junction area in pericycle-endodermis marked junctions (Fig. S7).

Additional Code S7.

BASH code for aligning and counting genomic, RNA-seq reads of the spatial experiment. Requires Illumina HiSeq 2000 .fastq files. Together with Additional Code S8, generates Data set S1.

Additional Code S8.

BASH code for aligning and counting Luciferase, RNA-seq reads of the spatial experiment. Requires Illumina HiSeq 2000 .fastq files.

Additional Code S9.

BASH code for aligning and counting RNA-seq reads of the temporal experiment (genomic and Luciferase fragments). Requires Illumina HiSeq 2000 .fastq files.