Cell Reports

Supplemental Information

Single-Cell Telomere-Length Quantification Couples Telomere Length to Meristem Activity and Stem Cell Development in *Arabidopsis*

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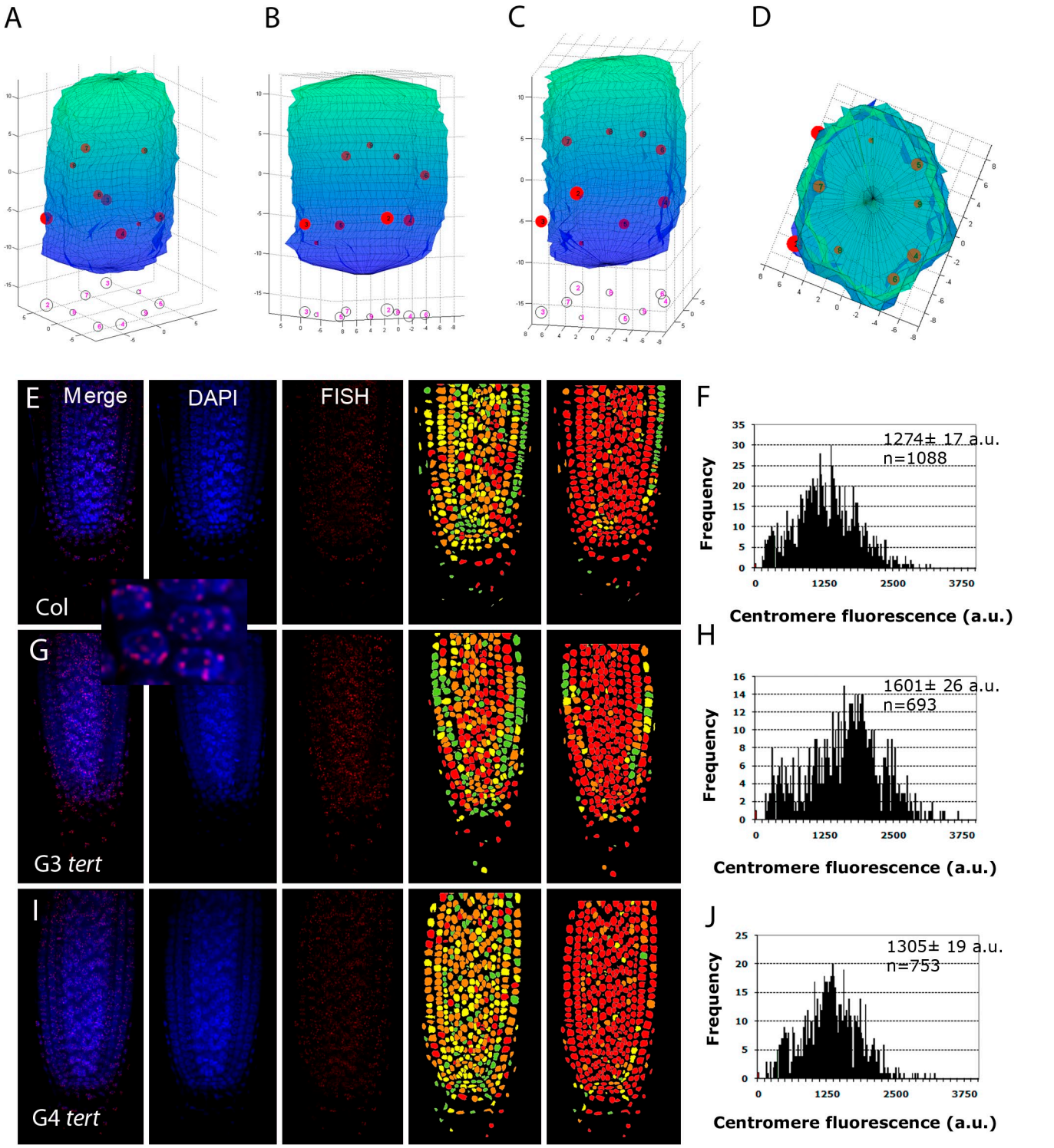

A
B
C
D
E
Merge
DAPI
FISH
Col
F
1274± 17 a.u.
n=1088
Frequency
Centromere fluorescence (a.u.)
G
G3 tert
H
1601± 26 a.u.
n=693
Frequency
Centromere fluorescence (a.u.)
I
G4 tert
J
1305± 19 a.u.
n=753
Frequency
Centromere fluorescence (a.u.)
K
% of cells within a telomere lengthper root
0-896 a.u.
896-1247 a.u
1247-2015 a.u
2015-4095 a.u
Col
G3 tert
G4 tert
L
% of cells within a telomere lengthper root
0-397 a.u.
397-575 a.u
575-1071 a.u
1071-4095 a.u
Col
G3 tert
G4 tert

Figure S1. Related to Figures 1,2. Cellular centromere distribution.

(A-D). **3D spatial localization of centromeres inside an individual cell.** (A-C) Different views of the three-dimensional model of the cell. The Z-axis corresponds to the layers of the confocal stack. The red spheres represent the centromeres detected in the layer-wise quantization process. The spheres are vertically projected onto the lower plane to provide a 2D view of the spatial distribution of the centromeres inside the cell nucleus. The diameter of the spheres is proportional to the detected size of the centromeres, whereas the spheres position along the X, Y and Z-axes corresponds to their location within the cell nucleus. (D) Zenithal view of the three-dimensional model of the cell.

(E-L). **Centromere length analysis by Q-FISH of whole-mounted roots of G3 and G4 *tert*.**

(E, G, I) Q-FISH of 6-day-old roots using a centromeric PNA probe. Centromere probe is labeled with Cy3, cell nuclei stained with DAPI and a merged image for both channels is shown (see inset). Representative centromere length pseudo-color images of WT sixday- old roots (E), G3 *tert* (G) and G4 *tert* (I) plants. Nuclei are coloured according to their average centromere fluorescence in arbitrary units.

(F, H, J) Q-FISH histograms showing centromere fluorescence frequencies in WT plants (F), G3 *tert* (H) and G4 *tert* plants (J). No statistically significant differences in centromere fluorescence were detected between WT and *tert* mutants, indicating that the differences in telomere length described in this study are not due to differences in "probe accessibility". Three independent roots were used for the analysis. n= total number of nuclei used for theanalysis. Average centromere fluorescence and SEM are indicated. (K, L) Percentage of cells showing a given fluorescence in WT, G3 *tert* and G4 *tert* plants, with percentages calculated for centromere data (K) and for telomere data (L).

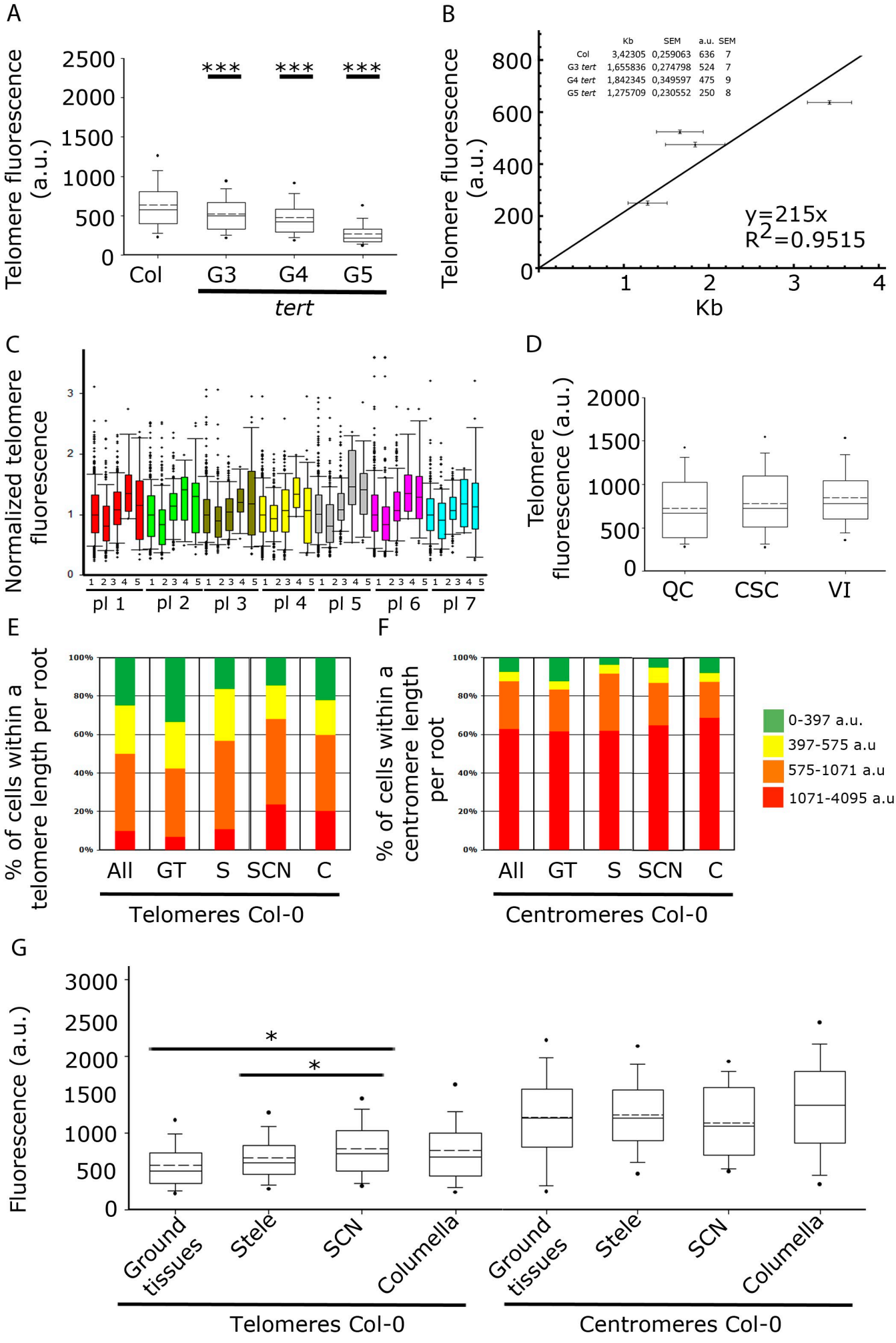

A
Telomere fluorescence (a.u.)
2500
2000
1500
1000
500
0
***
***
***
Col
G3
G4
G5
tert
B
Telomere fluorescence (a.u.)
800
600
400
200
0
Kb SEM a.u. SEM
Col 3,42305 0,259063 636 7
G3 tert 1,655836 0,274798 524 7
G4 tert 1,842345 0,349597 475 9
G5 tert 1,275709 0,230552 250 8
y=215x
R2=0.9515
1
2
3
4
Kb
C
Normalized telomere fluorescence
3
2
1
0
1 2 3 4 5
pl 1
pl 2
pl 3
pl 4
pl 5
pl 6
pl 7
D
Telomere fluorescence (a.u.)
2000
1500
1000
500
0
QC
CSC
VI
E
% of cells within a telomere length per root
100%
80%
60%
40%
20%
0%
All
GT
S
SCN
C
Telomeres Col-0
F
% of cells within a centromere length per root
100%
80%
60%
40%
20%
0%
All
GT
S
SCN
C
Centromeres Col-0
0-397 a.u.
397-575 a.u
575-1071 a.u
1071-4095 a.u
G
Fluorescence (a.u.)
3000
2500
2000
1500
1000
500
0
*
*
Ground tissues
Stele
SCN
Columella
Ground tissues
Stele
SCN
Columella
Telomeres Col-0
Centromeres Col-0

Figure S2. Related to Figures 2-4. Validation of Q-FISH technique in the Arabidopsis root.

(A) Boxplots showing the telomere length distributions in all cells of WT compared to *tert* mutants. Boxes represent the interquartile range (25^{th}-75^{th} percentiles, median indicated by the black horizontal line, average indicated by the dashed line) of the distribution, whiskers extend to the 10^{th} and 90^{th} percentiles and outliers are represented by black dots. ***p<0.001.

(B) Calibration plot to convert arbitrary units of fluorescence (a.u.) into Kb of DNA. SEM values are represented. The inset shows the table with the average telomere length expressed in kilobases (Kb) of DNA in the WT and *tert* mutants, as determined by PETRA assays, vs telomere fluorescence obtained by Q-FISH in the Arabidopsis root tips.

(C) Boxplots showing the cell type specific telomere length pattern inside each individual Col-0 plant analyzed (each color corresponds to one plant). On the Y axis are represented the raw values normalized with the median value of the telomere length distribution corresponding to all tissues, for each plant. On the X axis, 1-5 numbers stand for: 1. All tissues, 2.Ground tissues, 3. Stele, 4. SCN, 5. Columella. It can be easily observed that all plants follow the same pattern. Boxes represent the interquartile range (25^{th}-75^{th} percentiles, median indicated by the black horizontal line, average indicated by the dashed line) of the distribution, whiskers extend to the 10^{th} and 90^{th} percentiles and outliers are represented by black dots. *p<0.05.

(D) Boxplots showing the telomere length distributions in the quiescent centre (QC), columella stem cells (CSC) and vascular initials (VI) of Col-0 roots. No statistical differences were observed.

(E-F) Col-0 percentage of cells within a telomere and centromere length interval for each cell type. All=all tissues, GT=ground tissues, S=stele, SCN=stem cell niche, C=columella

(G) Boxplots showing the heterogeneity between cell types in telomere length distributions compared to centromere length distribution. Boxes represent the interquartile range (25^{th}-75^{th} percentiles, median indicated by the black horizontal line, average indicated by the dashed line) of the distribution, whiskers extend to the 10^{th} and 90^{th} percentiles and outliers are represented by black dots. *p<0.05.

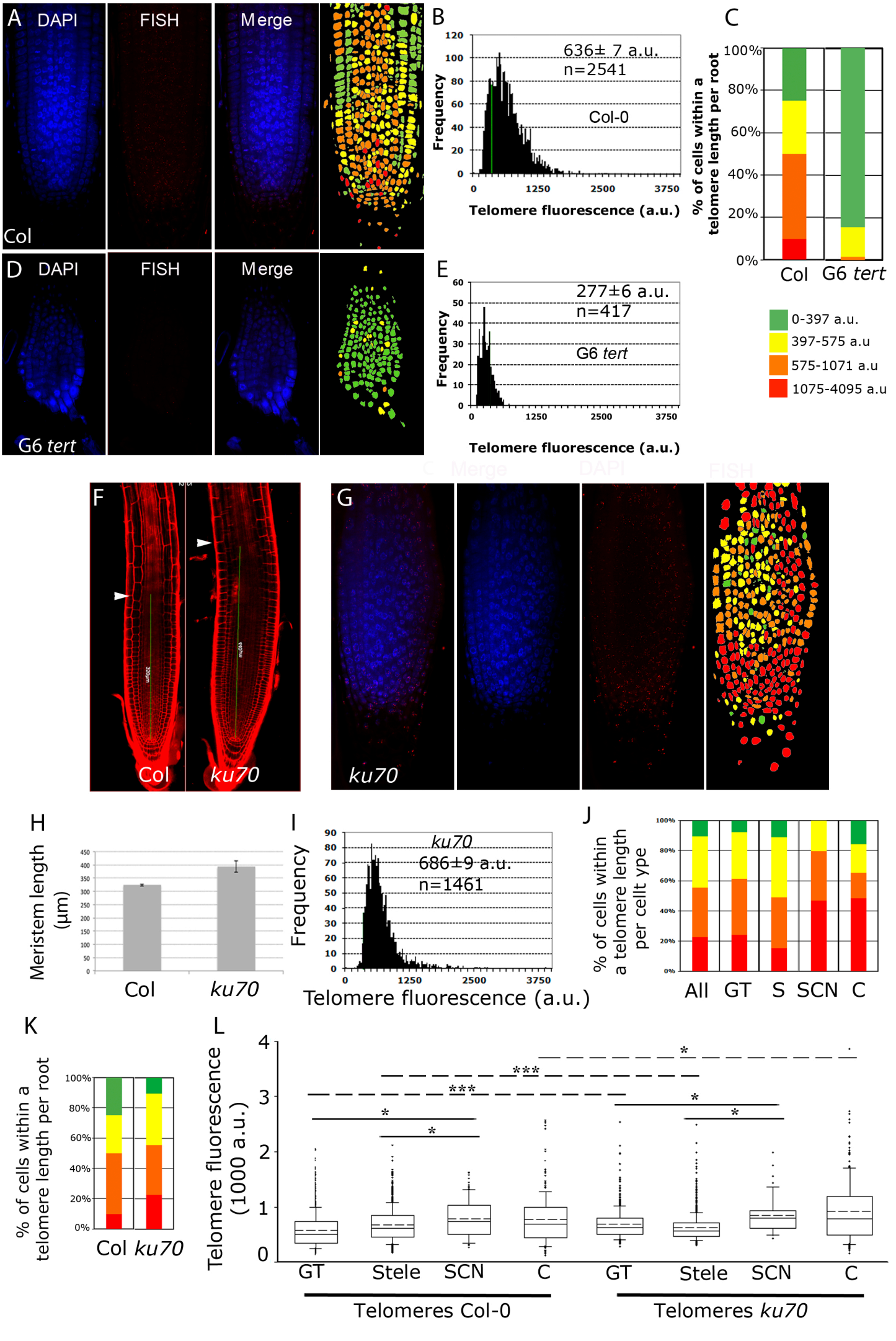

A DAPI
FISH
Merge
Col
B
636± 7 a.u.
n=2541
Col-0
Frequency
Telomere fluorescence (a.u.)
C
% of cells within a telomere length per root
Col
G6 tert
0-397 a.u.
397-575 a.u
575-1071 a.u
1075-4095 a.u
D DAPI
FISH
Merge
G6 tert
E
277±6 a.u.
n=417
G6 tert
Frequency
Telomere fluorescence (a.u.)
F
Col
ku70
G
ku70
H
Meristem length (µm)
Col
ku70
I
ku70
686±9 a.u.
n=1461
Frequency
Telomere fluorescence (a.u.)
J
% of cells within a telomere length per cellt ype
All
GT
S
SCN
C
K
% of cells within a telomere length per root
Col
ku70
L
Telomere fluorescence (1000 a.u.)
GT
Stele
SCN
C
Telomeres Col-0
Telomeres ku70

Figure S3. Related to Figures 2-4. Telomere-length analysis by whole-mounted root Q-FISH of telomere defective mutants.

(A-E). **G6 *tert* mutant analysis.** (A,D) Q-FISH in WT six-day-old roots (A), and G6 *tert* plants (D) telomeric PNA probe stained with Cy3, cell nuclei stained with DAPI and a merged image for both channels. Representative telomere length pseudo-color images of WT and G6 *tert* plants. Nuclei are colored according to their average telomere fluorescence in arbitrary units. (B,E) Telomere Q-FISH histograms showing telomere fluorescence frequencies in WT (B) and G6 *tert* (E) roots. Average telomere fluorescence, SEM and the number of nuclei n are indicated. (C) Percentage of cells showing a given fluorescence in WT and G6 *tert*. Note that *tert* mutants are enriched in cells with the shortest telomeres (green color).

(F-L). Phenotypic analysis and telomere length gradients of *ku70* mutants.

(F) Confocal images of six-day-old WT and *ku70* mutants roots stained with PI. Arrows mark the boundary between the proximal meristem and the elongation zone of the root. Scale bars: 100 μm. (G) Representative telomere length pseudo-color images of in *ku70* six-day-old roots. (H) The meristem length of 6-day-old roots of *ku70* mutants compared to WT. Asterisks indicate significant differences relative to WT for each day. (I) Telomere Q-FISH histograms showing telomere fluorescence frequencies in *ku70* roots. Average telomere fluorescence, SEM and the number of nuclei n, pooled form 3 plants, are indicated. (Note that *ku70* mutants are enriched in cells with longest telomeres (red and orange color). (J) *ku70* percentage of cells within a telomere length interval for each cell type. All=all tissues, GT=ground tissues, S=stele, SCN=stem cell niche, C=columella. (K) Percentage of cells showing a given fluorescence in WT and *ku70*. (L) Boxplots showing telomere length distributions of Col-0 cell types compared to *ku70*. Boxes represent the interquartile range (25^{th}-75^{th} percentiles, median indicated by the black horizontal line, average indicated by the dashed line) of the distribution, whiskers extend to the 10^{th} and 90^{th} percentiles and outliers are represented by black dots. Black lines represent internal comparisons between Col-0 cell types and *ku70* cell types, with significant differences between SCN and ground tissues and between SCN and stele in both cases. Dashed lines represent comparisons between corresponding cell types in Col-0 and *ku70*. Telomere lengths in columella cells and ground tissues in ku70 were larger than their correspondent cell types in Col-0, meanwhile the telomeres in stele were shorter, with an overall telomere length larger in *ku70* compared to Col-0. ***$p<0.001$ *$p<0.05$.

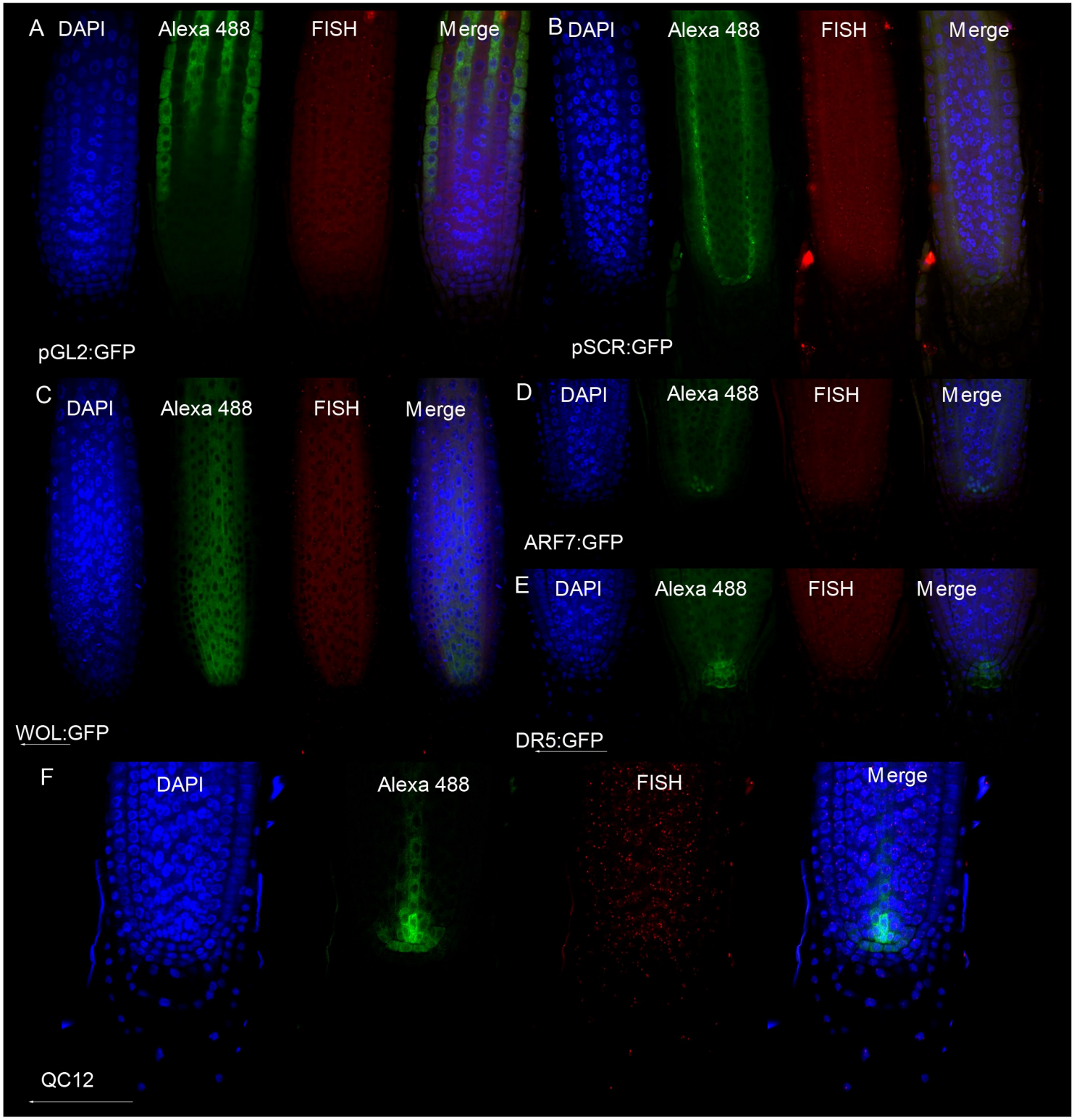
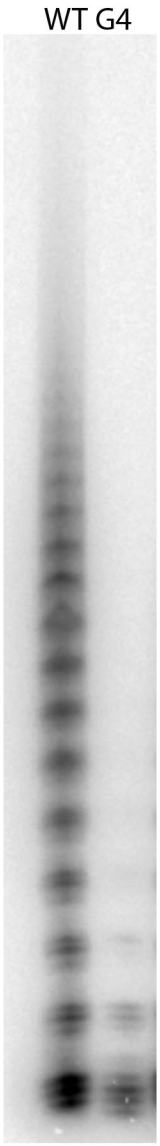
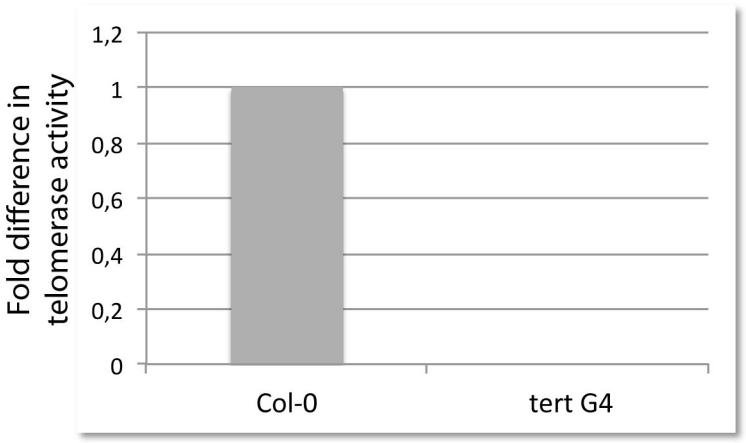

A DAPI
Alexa 488
FISH
Merge
pGL2:GFP
B DAPI
Alexa 488
FISH
Merge
pSCR:GFP
C DAPI
Alexa 488
FISH
Merge
WOL:GFP
D DAPI
Alexa 488
FISH
Merge
ARF7:GFP
E DAPI
Alexa 488
FISH
Merge
DR5:GFP
F
DAPI
Alexa 488
FISH
Merge
QC12

Figure S4. Related to Figure 3. Markers for root compartments. (A-F) Immunostaining using anti-GFP antibodies preceded telomere Q-FISH and enabled the distinction of several root cellular domains. Hair epidermis (*pGL2:GFP*) (A), endodermis-quiescent center (*pSCR:GFP*) (B), stele (*pWOL:GFP*) (C), columella and QC (DR5:GFP) (D), vascular initials (*pARF7:ARF7:GFP*) (E) and QC with vascular initials (QC12) (F).

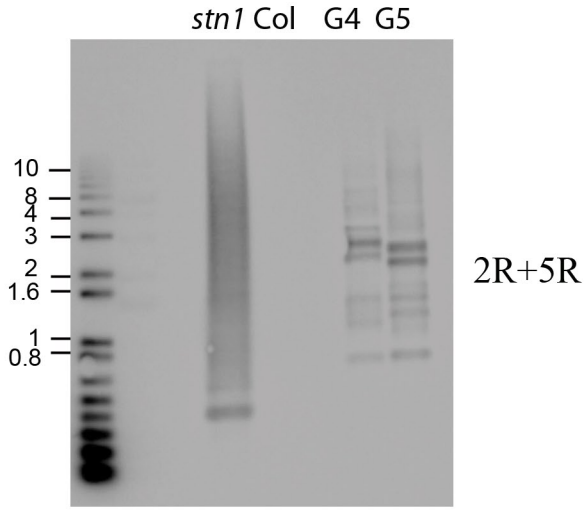
A


WT G4

B


Fold difference in telomerase activity
1,2
1
0,8
0,6
0,4
0,2
0
Col-0
tert G4

C


stn1 Col G4 G5
10
8
4
3
2
1.6
1
0.8
2R+5R

D

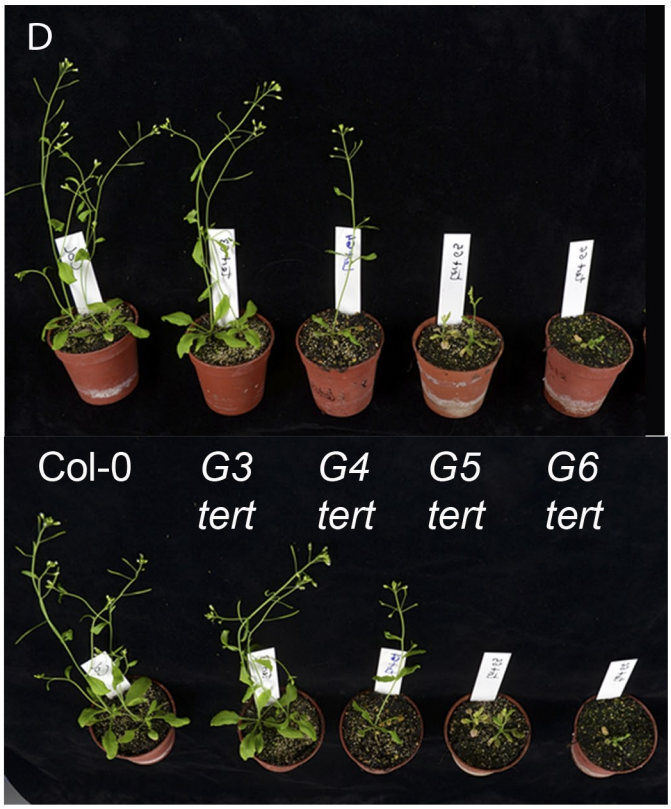

Col-0
G3 tert
G4 tert
G5 tert
G6 tert

Figure S5. **Related to Figures 2,4. TRAP (A) and Q-TRAP (B) analysis in the root tips in WT and G4 *tert* mutants**.

(C) Telomere fusion PCR analysis of *tert* and *stn1* (positive control) are shown. Primers specific for 2R and 5R were used using sectioned Arabidopsis root tips.

(D) Arabidopsis WT and *tert* mutants grown in long day conditions on soil for 3 weeks. Mutants display developmental defects and the progressive depletion of proliferative capacity.

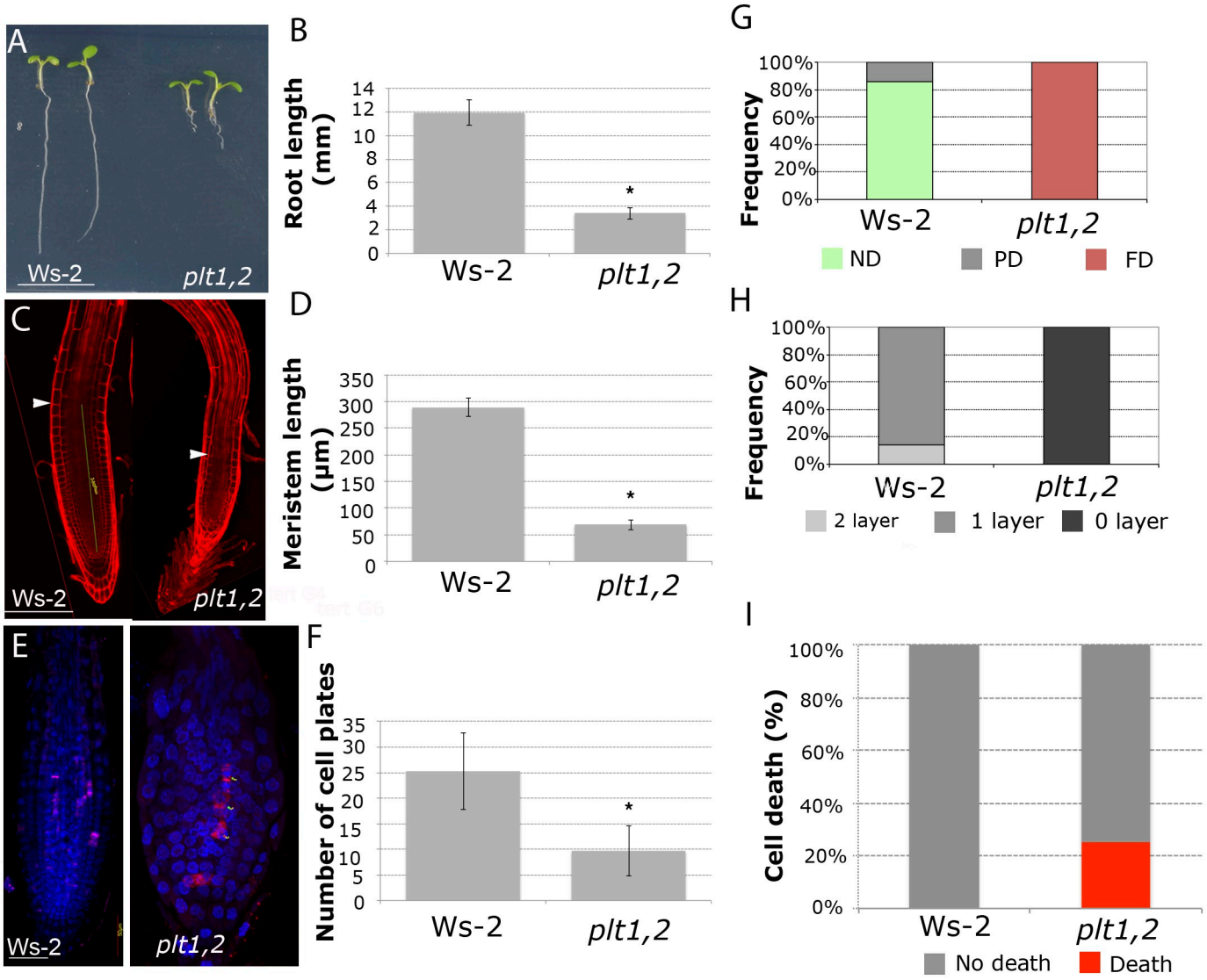

A
Ws-2
plt1,2
B
Root length (mm)
14
12
10
8
6
4
2
0
*
Ws-2
plt1,2
G
Frequency
100%
80%
60%
40%
20%
0%
Ws-2
plt1,2
ND
PD
FD
C
Ws-2
plt1,2
D
Meristem length (µm)
350
300
250
200
150
100
50
0
*
Ws-2
plt1,2
H
Frequency
100%
80%
60%
40%
20%
0%
Ws-2
plt1,2
2 layer
1 layer
0 layer
E
Ws-2
plt1,2
F
Number of cell plates
35
30
25
20
15
10
5
0
*
Ws-2
plt1,2
I
Cell death (%)
100%
80%
60%
40%
20%
0%
Ws-2
plt1,2
No death
Death

Figure S6. Related to Figure 6. Six-day-old phenotypes of *plt1 plt2* roots.

(A) Morphology of six-day-old seedlings of Ws-2 and *plt1 plt2* plants. Scale bar: 10 mm.

(B) Root-length measurements of Ws-2 and *plt1 plt2* seedlings compared to WT. Asterisks denote statistically significant differences relative to WT. (P<0.001)

(C) Confocal images of six-day-old Ws-2 and *plt1 plt2* roots stained with PI. Arrows mark the boundary between the proximal meristem and the elongation zone of the root. Scale bars: 100 μm.

(D) The meristem length of 6-day-old roots Ws-2 and *plt1 plt2* compared to WT. Asterisks indicate significant differences to the WT for each day. (P<0.001).

(E) Whole-mount immunofluorescence with anti-KNOLLE antibodies (Volker et al., 2001) in 6-day-old roots Ws-2 and *plt1 plt2*. Scale bar: 50μm.

(F) Number of cell plates calculated in whole-mount immunofluorescence with anti-KNOLLE antibodies present in Ws-2 and *plt1 plt2*. Asterisks denote a statistically significant difference with WT (P<0.001).

(G) Frequency distribution of QC division in Ws-2 and *plt1 plt2* seedlings compared to Col-0 (WT) at 6 days after germination. PD=partially divided, ND=non-divided, FD=fully divided.

(H) Frequency distribution of the number of cell layers is given between the QC and the first differentiated columella cells that contain starch granules in Ws-2 and *plt1 plt2* seedlings. Note in *plt1 plt2* mutants all (100%) of CSC differentiated.

(I) Cell death in the meristem was measured for intense propidium-iodide (PI) staining to count dead stem cells per root in Ws-2 and *plt1 plt2*.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Related to Figure 1. Confocal optical sections of a 6-day-old Arabidopsis root. Z-stack confocal optical sections at 0.8μm spacing were collected from 6-day-old roots. Immunofluorescence of GFP expressing cells in WOX5:GFP, telomere fluorescence obtained by Q-FISH along the root using a PNA probe stained with Cy3 and cell nuclei stained with DAPI.

Movie S2. Related to Figure 2. 3D reconstruction of single root cells revealing the topology of the fluorescence spots. Desktop recording of the 3D cell model creation process. The user is first prompted to select the cell to be rendered in 3D. Together with the segmentation process that allows detecting the boundaries of the cell nucleus in each stack layer, the layer-wise quantization process is conducted on the cell of choice applying an adaptive fluorescence sensitivity threshold. As a result, the 3D model of the cell is computed and presented to the user, who can freely rotate the cell to study its internal topology.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Telomere-length quantification and statistical analysis of the data

After acquisition in the confocal microscope, the 3 channels images were passed to our custom semi-automatic Matlab program in order to generate the binary mask containing nuclear areas, based on the blue channel (DAPI) (Supporting Information). For final adjustments (final separation of nuclei that were still united) the binary masks were manually processed in Adobe Photoshop CS5 (version 12.0.4, Adobe Systems Incorporated). During this process, the original 8-bit image containing both DAPI and Cy3 channels was used as background and the binary mask obtained in Matlab was used as a layer on top of the background. By changing the transparency of the layer it was possible to visualize all the telomeres and to improve the mask ensuring that all telomeres fall inside their nuclear area.

Once the final binary mask was generated, it was introduced together with the Cy3 image (red channel) in the Metamorph software package (version 6.3r6, Molecular Devices, Union City, CA, USA) to identify and measure telomeres/centromeres intensity inside nuclear area.

The Granularity Metamorph module was used to identify telomeres/centromeres spots and remove background. This measured the intensity of each telomere/centromere inside each nucleus avoiding changes in ploidy or area (Supporting Information). Based on the average telomere/centromere intensity per nucleus, a coloured map was generated and the intensity data for each analysed plant was saved as Excel files for histogram generation and further processing.

Telomere data from WT plants (7 independent roots) were compared with telomere data from G3 *tert* (3 independent roots), G4 *tert* (3 independent roots) and G5 *tert* (3 independent roots) using Kruskal-Wallis One Way Analysis on Ranks test. In all cases significant differences with $P<0.001$ were obtained.

The same statistical test was used for centromeres and telomeres-centromeres data comparisons and also for comparisons between cell types.

Root length and meristem length were measured in at least three independent experiments. Roots were scanned and measured with ImageJ software (http://rsb.info.nih.gov/ij/). For comparisons, Student's *t*-tests were performed in all cases.

The folowing steps were folowed to set up the quantification of fluorescence probes:

1. Generation of a Binary mask from the confocal microscopy images

1.1. Select the file of interest and open it with Olympus FV 1000.

1.2. Save all 8 and 16 bit images.

1.3. For 8 bit images: In Output Format, select Merge Channel (using assigned LUT), Merge Method: Amount, Channel Selection: 1 and 2 corresponding to DAPI (nuclei) and Cy3 (telomeres/centromeres).

1.4. For 16 bit images: In Output Format, select Raw data Extracted (without LUT), Extract Method: Raw (16 bit TIFF), Channel Selection: 1 and 2 (corresponding to DAPI and Cy3).

1.5. Select the Z-plane of interest (containing the middle plane of the root corresponding to the QC) with FV1000.

1.6. The 8 and 16 bit folders contain all the images saved at step 1.3 and 1.4 .

1.7. The folder “mask” will be described in the following steps, in section 2.

2. Nuclei segmentation process (Matlab)

As a result, the typical appearance of the Arabidopsis roots images captured by the confocal microscope is the one presented in Figure 1.

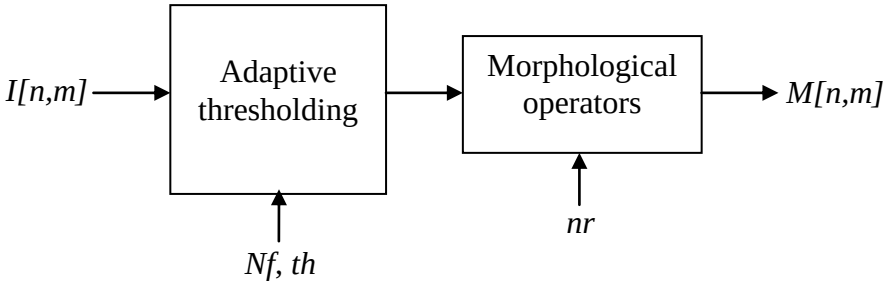
Due to the confocal microscopy capture technique employed, the cell nuclei appear in the blue channel and the telomeric information in the red channel in an 8 bit image. Therefore, a simple channel separation allows obtaining the nuclear area of the root.

As the construction of telomapping requires analyzing cells individually, it becomes necessary to **create a binary mask** that allows isolating each cell from the remaining ones. The automatic construction of such a binary mask is a challenging task, as the objects to segment (i.e. cells nuclei) present fairly distinct gray levels across the image. There is little that can be done in this regard, as these differences are due to the cylindrical geometry of the root, which causes some cells to be more illuminated than others.

Despite this fact, we have opted for the use of histogram based segmentation techniques (i.e. based on setting a gray level threshold for dividing the image into foreground -cells- and background –non-cells) for obtaining the binary mask in this stage of the imaging analysis module development. The motivation for this decision is that these approaches are at the same time simple and adaptable.

The described method has been implemented using Matlab Release 2008a, as a command-line application that allows users tuning its parameters. Subsequently, the resulting binary mask is displayed and the user has the option of saving it into an image file. Running on a standard PC (Intel Core Duo@1.83GHz, 2 GB RAM), the processing of a good resolution image (e.g. 1024x768 pixels or similar) takes less than five seconds.

After testing several segmentation methods, the most accurate one has proved to be a two-step segmentation strategy that combines average filtering based adaptive thresholding segmentation and morphological operators:



The general idea is to create a first version of the binary mask by means of average filtering based adaptive thresholding, and subsequently improving the quality of the mask through the use of morphological operators, aiming at reducing its noise level.

The rationale behind average filtering adaptive thresholding is based on segmenting the image using a locally computed gray level threshold (Wellner, 1993). By proceeding this way, we set a segmentation threshold, which is highly local, thus being fairly immune to the large illumination variations found across the image. To that end, the image is filtered using an averaging filter of size *Nf* x *Nf* pixels (see equation 1). As a result, we obtain an averaged version of the image, where each pixel equals the mean gray level of its *Nf*-sized square neighborhood.

$$I_{avg}[n,m] = I[n,m] * h_{Nf}[n,m], \quad where\ h_{Nf}[n,m] = \frac{1}{Nf^2}\begin{pmatrix} 1 & \cdots & 1 \\ \vdots & \ddots & \vdots \\ 1 & \cdots & 1 \end{pmatrix} \qquad (1)$$

Next, a segmentation decision step based on a sensitivity threshold *th* is conducted (see equation 2), which consists of setting each pixel to white in case it is a *th* percent darker than the average gray level of its neighbors (Wellner, 1993)

.

$$I_{bin}[n,m] = \begin{cases} 0, if\ \ I[n,m] < th \cdot I_{avg}[n,m] \\ 1, if\ \ I[n,m] \geq th \cdot I_{avg}[n,m] \end{cases} \qquad (2)$$

In equations 1 and 2, $I[n,m]$, $I_{avg}[n,m]$, $I_{bin}[n,m]$ refer to the original image, the image resulting from the averaging filtering and the thresholded image, respectively, $h_{Nf}[n,m]$ represents the impulse response of the *Nf* x *Nf* averaging filter, and * stands for the linear convolution operation. It is worth mentioning that the *Nf* and *th* parameters are tunable by the user.

After this process is completed, the binary image $I_{bin}[n,m]$ is an approximate mask which is somewhat noisy, and sometimes tends to present overlapping cells nuclei.

At its current stage of development, cell overlapping can only be solved by means of manual post processing. However, we can alleviate much of the burden of this process by applying morphological operators, aiming to improve the overall quality of the final binary mask ($M[n,m]$). To that end, two morphological operators are sequentially applied: majority and horizontal breaking.

The application of the first morphological operator is intended to reduce noisy pixels (i.e. fairly isolated white pixels that do not belong to cells nuclei). Basically, what the majority morphological operator does is setting a pixel to white if five or more pixels in its 3-by-3 neighborhood are white; otherwise, it sets the pixel to black (see equation 3). Therefore, this allows eliminating isolated (i.e. noisy) white pixels resulting from the adaptive thresholding stage.

$$I_{maj}[n,m] = \begin{cases} 0, \textit{if} \sum_{k=n-1}^{n+1} \sum_{l=m-1}^{m+1} I_{bin}[k,l] < 5 \\ 1, \textit{if} \sum_{k=n-1}^{n+1} \sum_{l=m-1}^{m+1} I_{bin}[k,l] \geq 5 \end{cases} \qquad (3)$$

The amount of noise reduction can be increased by the cascaded application of the majority morphological operator. The number of times it is applied determines the noise reduction (*nr*) input parameter that appears in the two-step segmentation strategy, also tunable by the user.

However, the amount of noise reduction achievable through this method has a natural threshold, as after a certain number of iterations this morphological operation becomes idempotent (Gonzalez, 2008). Therefore, care must be taken when setting the *nr* parameter, as a too large value will uselessly increase the computational complexity of the noise reduction process.

Finally, after the noise reduction step, an H-break morphological operator is applied in order to split cells in case they are connected by one pixel, which renders the final mask, *M[n,m]*.

$$M[n,m] = H_{break}\{I_{maj}[n,m]\} \qquad (4)$$

Results

In order to analyze the influence of setting the neighborhood parameter *Nf* to different values, we used different neighborhood sizes (*Nf*=20, 40 and 80) with a fixed threshold (*th*=1%) value.

Almost all the cells in the image were recovered, and no geometric artifacts appeared. Moreover, small neighborhood sizes tended to yield oversegmented and noisy masks. On the other hand, if the neighborhood is large, cells tend to overlap. Bearing this in mind, and considering that the cells nuclei average size is around 40 pixels, setting *Nf*=40 seems a neat decision, which is backed up by empirical evidence.

Next, we set the threshold at different levels (*th*=1%, 10% and 20%) for a fixed neighborhood size (*Nf*=40). The higher the threshold the more undesired information (i.e. background) is retrieved. Empirical evaluation yielded the best results by setting *th* equal to 1.

Finally, the application of the majority and H-break morphological operators aim at reducing the level of noise in the mask obtained from the previous stage. We varied the noise reduction parameter (*nr*) to different values and we observed that the more number of times the majority morphological operator is applied, the greater noise reduction is achieved. Empirical experimentation suggested that the *nr* parameter should be set to 10 or 15, depending on the sample.

It is important to mention that the resulting mask (Fig. 1) needs some manual processing (basically separating overlapped nuclei, holes filling and contour delimitation) in order to obtain the final result.

Due to the high complexity of the sample and the big number of nuclei, the mask automatically generated by Matlab (Fig. 1) was transferred to Adobe Acrobat 9 Professional (CS5) for further manual processing (better separation of joint nuclei and better delimitation of the nuclei contour). During this process, the original 8 bit image containing both DAPI and Cy3 channels was used as background and the binary mask obtained in Matlab was used as a layer on top of the background. By changing the transparency of the layer it was possible the visualization and the inclusion of all the telomeres inside their nuclear area.

3. Metamorph analysis

The 16 bit sets of confocal images were analyzed in Metamorph (version 7.7.0.0, Molecular Devices, Union City, CA, USA). The final mask generated in Photoshop was auto-thresholded for light objects and transformed into a 1-bit binary image. The

morphology filter Fill holes was applied to 1-bit binary image in order to fill the holes corresponding to nucleolus inside each nucleus, generating the Holes image. Granularity module was applied to the Cy3 16 bit image to identify only telomeres (min width 3.5, max width 10, Intensity Above Local Background 50) or centromeres (min width 5, max width 20, Intensity Above Local Background 50) and to assign a value of 0 to the intensity of all the remaining pixels. In this way we efficiently removed any background present in the image. The generated image was titled Granules. The parameters used for Granularity module were set after several measurements of individual telomeres/centromeres size in pixels and the difference in intensity between a real telomere/centromere and the local background. Furthermore, the accuracy of the identification was visually checked in the original image after applying the module.

The Granules image was binarized, combined with the original Cy3 image through LOGICAL AND arithmetical function and thresholded, to create an image with the original grey values of the granules which was called AND. The contours of all the nuclei (regions) in Holes image were transferred to AND image to identify only the telomeres/centromeres inside nuclear areas. Cy3 fluorescence of individual telomeres was measured as ¨Average grey value¨ units (arbitrary units of fluorescence) using the Integrated Morphometry Analysis module and given as the average intensity/nucleus (the sum of all the telomeres/centromeres inside a nuclear area divided by the number of telomeres/centromeres in that area). Telomeric/centromeric intensity values obtained in this way were exported to Excel for frequency histograms generation and further analysis.

In order to visualize and classify the nuclei according to their telomeric intensity, Configure Object Classifier module was used and a code of four colours (green, yellow, orange and red), representing the nuclei having the intensity in the 25^{th}, 50^{th} and 90^{th} quartiles was generated. These intervals were established based on all WT data (7 plants for telomeres and 3 plants for centromeres) and maintained constant for all the analyzed mutants to facilitate comparisons.

Protocol for Metamorph Analysis:

3.1. Open final mask generated in Photoshop-> Auto-threshold for light objects-> 1-Bit binary image

3.2. Process menu->Morphology filters->Fill Holes (1-Bit binary image). Apply ->Holes image

3.3. **Apps -> Granularity (Cy3 image)-> Min width 3,5 max 10 background 50 (for centromeres Min width 5 max 20 background 50). Apply**

3.4. Create a blank 16 bit image to be used to mark nuclei. The easiest way is to use Arithmetic (process menu) to multiply the Cy3 image by 0 and create a 16 bit result. The result image is called Multiply.

3.5. The Granules image should be made into a binary image by using a threshold of 1 – 4095 and then Binarize (Binary Operations, Process menu)

3.6. Use Logical AND to create an image with the original grey values of the granules. Arithmetic dialog (Process menu): Source Image 1 – Original image (Cy3), Source 2-Image – binary result from step 1, Result Bit Depth – whatever the original image was (16bit), Operation – Logical AND.

3.7. Set threshold for the result of step 4 (AND) grey values > 0.

3.8. You can measure the result of step 5 with Integrated Morphometry Analysis (Measure menu) to get the number of granules, the grey values for each granules and the summary for the measurements. In Integrated Morphometry Analysis window, in Preferences, click Measure All regions.

3.9. You can measure the granules specific to each nucleus using the following method.

3.10. **Open Holes**

3.11. Create Regions Around Objects (Regions menu)

3.12. Transfer regions of interest from Holes to the results of step 5 (AND) (granules having the initial grey value and thresholded). Select “All regions”.

3.13. Measure using Integrated Morphometry Analysis (Measure menu). Only objects within <u>all</u> of the regions of interest (overlapping the holes) will be measured. In Integrated Morphometry Analysis window, in Preferences, click Measure All regions. Also, if you log the Summary Data each region of interest will be logged separately.

3.14. Generating the Excel file which contains the Region Name ,the Average intensity coming from all the points in that region, the area and the count (name of the file_total):

3.14.1. Measurements- Avg int and Total int

3.14.2. Summary-Configure Log- Click only the options Region Name and Average- OK

3.14.3. Open Log- F9-Log Data

3.14.4. Save as name of file_total.xls

3.14.5. Measure with Integrated Morphometry Analysis the image Holes. Measurements – Total area.

3.14.6. Object data. Open Log- F9-Log Data.

3.14.7. Measure with Integrated Morphometry Analysis the image AND Measurements- Avg int and Total int

3.14.8. Summary-Configure Log- Click only Region Name and Count- OK

3.14.9. Open Log- F9-Log Data

3.14.10. Change total intensity by count in the head of the column

3.14.11. Sort all data by Average intensity and remove all the regions with 0 avg int.

3.14.12. Sort the remaining data by Total area of the region and select only the regions which are above 100 area.

Observation: Step 8.12 can be skiped if we are interested only in obtaining the average intensity per nucleus and not the intensity value of each particle.

3.15. Each nucleus can be measured by itself using the following method.

3.16. Save an Integrated Morphometry Analysis state file (Measure menu) that measures the Average Intensity and turns Off the preference to *Measure all regions*. **** It has to be done only once, at the begging

3.17. Save an Integrated Morphometry Analysis state file (Measure menu) that measures the Average Intensity and turns Off the preference to *Measure all regions*. **** It has to be done only once, at the begging

3.18. How to do it: In the IMA window: a) Measurements-> Click only Average intensity; b) Preferences-> Unclick *Measure all regions*; c) Save State->OK-> Save as Average intensity.IMA

3.19. Load the State saved before (Average intensity.IMA)

3.20. In Summary->Open Log. Do not press F9 : Log the data

3.21. Pause logging to the Summary Log (Log menu, Summary sub-menu-> Pause Summary Log)

3.22. Selecting the image result of step 9 (AND with all the Regions) -> Journal menu->Loop sub-menu->Loop for all regions ->Image AND, Select Journal-> Journal_to_loop.jnl

3.22.1. Loop through all regions in an image *Journal to Loop (saved as Journal_to_loop.jnl)*

3.22.2. Select one region in AND to activate it. Measure AND using Integrated Morphometry Analysis (Measure menu). Only objects within the active region of interest will be measured.

3.22.3. Log the Summary Data (Press Record button) for the region of interest (it will not go to the spreadsheet or file because logging was paused on step 15).

3.22.4. Journal menu-> Variables-> Assign variable -> Under Variable and Expression, assign to the variable *Average* the measurement variable *IMASummary.Average*. Press OK.

3.22.5. Transfer the active region of interest from the current image (AND) to Multiply (the blank 16 bit image result of step 2). Select *Clear regions from Destination before transfer* and *Active Region*.

3.22.6. Select the region in *Multiply* (Journal menu, Recording tools->Select Region->Select) to make the transferred region active.

3.22.7. Paint the region from step E. Display menu, Graphics sub-menu-> Paint Region-> Paint Mode: Inside region area; Paint Color: Value from variable, Variable Name: *Average*.

3.23. Clear the regions from the image result of step F (Multiply).

3.24. This image now has objects with grey values equal to the average of the granule grey values.

3.25. Multiply->Threshold-> Inclusive, from 1 to the highest intensity

3.26. Measure Multiply with IMA (Average intensity and Total area) and log the results in an excel file After, unclick Total Area and leave only Avg intensity. If not, in Config Object Classifier it will always activate as a filter Total Area too.

3.27. Select Multiply image->Measure menu-> Morphometry-> Configure Object Classifier: Classifiers (from b to e). Click Active for each of them and set the Filter Range and color:

3.28. In Telomeres case : b) 0-397, Green; c) 397-575, Yellow; d) 575-1071, Orange; e) 1071-4095, Red.

3.29. Click Recalc after setting each of them. Filters: Average gray value

3.30. Observation: Select the image, Measure menu-> Morphometry-> Measure Objects and Load set File-> and click Recalc. It goes faster.

3.31. Select Multiply image->Measure menu-> Morphometry-> Measure Objects-> Resulting image=Measured Multiply --- This image has the colors according to the average intensity of each nucleus.

3.32. Save Multiply and Measured Multiply images in the folder .

4. Excel and statistical analysis

The results generated in Metamorph were first filtered by total area size in order to eliminate all the ROIs that have an area smaller than 100 pixels^2 (the smallest area that a real nucleus can have). In this way, only real nuclei will contribute to the final fluorescence results.

Based on this data, frequency histograms were generated and the 25th, 50th and 90th quartiles were calculated in Excel, generating color coded percentiles graphs which served for further comparison between samples.

Kruskal-Wallis One Way Analysis of Variance on Ranks test was used to evaluate the differences between unpaired samples after all samples failed the Shapiro-Wilk

normality test. Both tests were performed using SigmaPlot`s (Systat Software, Inc.) statistical package.

Telomere data from WT plants (7 plants) was compared with telomere data from *tert* G3 (3 plants), *tert* G4 (3 plants) and *tert* G5 (3 plants) mutants. In all cases significant differences with $p<0.05$ were obtained. The same statistical test was used for centromeres data comparisons and telomeres-centromeres comparisons ($p<0.001$. To make the comparisons between cell types, first we measured telomere fluorescence for each cell type in each sample being analyzed. Next, we gathered the results from all the samples in one phenotype and generated the final data for each cell type for each phenotype. Given that all data failed the Shapiro-Wilk normality test, we compared each cell type with the others (ground tissues vs stele, SCN and columella, stele vs ground tissues, SCN and columella, SCN vs columella) by using the Kruskal-Wallis One Way Analysis of Variance on Ranks in SigmaPlot.

5. Kbs to a.u.f conversion

In order to convert kbs into a.u.f., the values of a.u.f. measured by Q-FISH corresponding to Col-0 and G3-G5 tert mutants were plotted against the values in kbs measured by PETRA again for Col-0 and G3-G5 tert mutants and set to pass through origin (0 a.u.f. correspond to 0 kbs). LinearModelFit function (Mathematica, Wolfram Research) was used to fit a linear polynome to the data, using the standard error of the mean (s.e.m) values to compute the weights as 1/ (s.e.m)^2. The value obtained for the slope gives an approximation for the number of a.u.f corresponding to 1 kb, with $R^2=0.9516$.

SUPPLEMENTAL REFERENCES

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