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Live single-cell transcriptional dynamics via RNA labelling during the phosphate response in plants

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1 **RNA labelling in live plants reveals single cell transcriptional dynamics: application to**
2 **phosphate signaling**

3

4 #Sahar Hani¹, #Laura Cuyas^{1,2}, #Pascale David¹, David Secco³, James Whelan³, Marie-
5 Christine Thibaud¹, Rémy Merret⁴, Florian Mueller⁵, Nathalie Pochon¹, Hélène Javot¹, Orestis
6 Faklaris⁶, Eric Maréchal⁷, Edouard Bertrand^{8,9,10*} and Laurent Nussaume^{1*}

7 #These authors equally contributed to the publication

8 *For correspondance: lnussaume@cea.fr and edouard.bertrand@igh.cnrs.fr

9

10 1 Aix Marseille Univ, CEA, CNRS, BIAM, UMR7265, SAVE (Signalisation pour
11 l'Adaptation des Végétaux à leur Environnement), Saint-Paul lez Durance, France.

12 2 Centre Mondial de l'Innovation, Groupe Roullier, 18 avenue Franklin Roosevelt, Saint-
13 Malo, France.

14 3 Department of Animal, Plant and Soil Sciences, Australian Research Council Centre of
15 Excellence in Plant Energy Biology, School of Life Sciences, La Trobe University, Bundoora,
16 Victoria, Australia.

17 4 UMR5096 CNRS/Université de Perpignan, Laboratoire Génome et Développement des
18 Plantes, 58 Avenue Paul Alduy, Perpignan, France

19 5 Unité Imagerie et Modélisation, Institut Pasteur and CNRS UMR 3691, 28 rue du Docteur
20 Roux, 75015 Paris; France

21 6 MRI, BioCampus Montpellier, CRBM, Univ. Montpellier, CNRS, Montpellier, France.

22 7 UMR 5168 CNRS-CEA-INRA-Université Grenoble Alpes, Laboratoire de Physiologie
23 Cellulaire et Végétale, iRIG, CEA-Grenoble, 17 rue des Martyrs, Grenoble, France.

24 8 Institut de Génétique Moléculaire de Montpellier, Univ. Montpellier, CNRS, Montpellier,
25 France.

26 9 Institut de Génétique Humaine, Univ. Montpellier, CNRS, Montpellier, France.

27 10 Equipe labélisée Ligue Nationale Contre le Cancer, Montpellier, France.

28 **Abstract**

29 Plants are sessile organisms constantly adapting to ambient fluctuations through spatial and
30 temporal transcriptional responses. Here, we implemented the latest generation RNA imaging
31 system and combined it with microfluidics to visualize transcriptional regulation in living
32 Arabidopsis plants. This enabled quantitative measurements of the transcriptional activity of
33 single loci in single cells, real time and changing environmental conditions. Using phosphate
34 responsive genes as model, we found that active genes displayed high transcription initiation
35 rates ($\sim 3s$) and frequently clustered together in endoreplicated cells. We observed gene
36 bursting and large allelic differences in single cells, revealing that at steady-state, intrinsic
37 noise dominated extrinsic variations. Moreover, we established that transcriptional repression
38 triggered in roots by phosphate, a crucial macronutrient limiting plant development, occurred
39 with unexpected fast kinetics (\sim minutes) and striking heterogeneity between neighboring
40 cells. Access to single cell RNA polymerase II dynamics within live plants will benefit future
41 studies of signaling processes.

42 **Introduction**

43 Plants are sessile organisms permanently coping with environmental variations.
44 Transcriptional reprogramming¹ plays a key role in these responses as illustrated by the
45 number of plant transcription factors (5.4% in Arabidopsis, <https://agris-knowledgebase.org/>).
46 Most physiological studies quantify mRNA abundance in organs. Accessing specific cell
47 types is possible by expressing a fluorescent marker in cells of interest, to FACS sort them
48 and perform sequencing analysis². Nevertheless, this requires long enzymatic digestion to
49 generate protoplasts preventing fast kinetic studies. Transcriptional fusions between
50 promoters and reporter genes such as GFP provide cellular resolution, but the time required to
51 accumulate detectable level of mature fluorophore (often in the range of tens of minutes³)
52 prevents the rapid transcriptional monitoring. This is moreover highly variable and depends
53 on the strength of the promoter studied and the microscopy setup, leading to confounding
54 effects. These considerations are even more crucial when studying transcriptional inhibition.
55 The above-mentioned experiments provide access to total RNA (or protein), resulting from
56 the balance between synthesis and degradation. Many fluorescent proteins decay in the range
57 of hours and RNA degradation is highly variable from one gene to another^{4,5}. For instance,
58 the median half-life value of *Arabidopsis* mRNAs is 107 min, but exceeds one day for many
59 messengers⁴. This is far too long for transcriptional responses occurring within seconds such
60 as for instance during light stress⁶.
61 All these issues can be resolved with a method originally developed for yeast and animal
62 granting direct real time access to transcriptional activity at the level of single cells⁷⁻¹⁰. It is
63 based on a fusion between GFP and the bacteriophage MS2 coat protein (MCP-GFP)^{7,8} or on
64 related systems¹¹. The MCP-GFP recognizes a specific RNA stem-loop inserted in multiple
65 copies into a reporter RNA, promoting MCP-GFP multimerization to provide signal bright
66 enough for single RNA visualization⁹. Importantly, binding occurs during RNA synthesis and

67 monitors transcription in real time. In plants, this technology would offer major advantages
68 for physiological studies such as adaptations to biotic and abiotic stresses. Indeed, it grants
69 access to the variability of all cell types providing ways to understand how the activity of
70 single cells is integrated within tissues or organs and thus to better understand gene
71 regulation. Real time quantitative analysis can be performed for transcription initiation,
72 elongation or gene bursting. This last phenomenon can further be used to identify promoter
73 states that are rate-limiting for transcription initiation, and thus likely points of regulation¹².
74 The MS2 technology also allows the identification of extrinsic and intrinsic sources of
75 transcriptional noise
76 Therefore, we implemented it into plants using state-of the art MS2x128 repeats to tag a
77 single RNA with ~250 to 500 GFPs for optimal detection sensitivity¹³. We used this system to
78 analyze the transcriptional response to a major macronutrient: inorganic phosphate (Pi). Pi
79 deficiency triggers major transcriptional modifications affecting plant development and
80 metabolism^{14,15}. These are mainly controlled by master regulator genes of the PHR1 family¹⁵⁻
81 ¹⁷. Being constitutive, their regulatory activity relies on inhibitors of the SPX family tuned by
82 Pi uptake^{18,19} as they inhibit PHR1 activity only in the presence of Pi metabolites^{20,21}.

83 Here we used the promoter of early Pi responding genes to drive transcription of an
84 MS2x128 reporter. We combined fast quantitative imaging and microfluidics²², to precisely
85 control Pi delivery while providing stable environmental conditions. It revealed a high
86 heterogeneity of responses between adjacent cells and identified the rapid perception of Pi by
87 the root (within 3/5 min) validating the power of the MS2 technology to dissect plant
88 transcriptional regulation.

89
90
91

92

93 **Results**

94 **Phosphate resupply promotes rapid transcriptional modifications**

95 To identify early markers sensitive to phosphate, we starved plants and performed Pi
96 refeeding experiments. After addition of Pi in the liquid culture medium, we harvested roots
97 and leaves after 30, 60 and 180 min for RNA-seq analysis. In roots, only 22 genes exhibited a
98 significant two-fold reduction of their transcript level over the three time points (Fig. S1A-B).
99 Analysis of the shoot samples revealed a delayed reduction of all these markers in aerial parts
100 (only one third were repressed after 30 min) indicating that Pi was first perceived by roots.
101 Independent experiments analyzed by RTqPCR using well known markers for Pi
102 starvation^{15,23}, involved in Pi uptake (*PHT1;4*) or Pi-induced metabolic remodelling (*SQD2*),
103 confirmed these results (Fig. 1A and S1B). The rapid down-regulation observed also
104 highlighted the fast turn over of these transcripts (half-life estimated to 15-30 minutes; Fig.
105 S1A-B), in regard to the Arabidopsis median value of 3.8 hours⁵. For the rest of this study we
106 selected two genes (*SPX1* and At5G20790, named hereafter *UN1* for Unicorn1), which
107 combined the highest levels of expression in Pi depleted medium (-Pi) with broad dynamics
108 as measured by +Pi/-Pi fold change.

109 Regulation was assumed to occur mainly transcriptionally, as the transcriptional
110 inhibitor cordycepin mimicked effect of Pi resupply (Fig. 1A). This was confirmed by
111 transcriptional fusions with luciferase, which conferred to the reporter gene a similar temporal
112 response to Pi resupply (Fig. S1C), whereas fusing a CaMV 35S constitutive promoter to the
113 coding region of the markers did not reveal any significant difference of RNA levels between
114 +Pi (after 180 min refeeding) or -Pi conditions (Fig. S1D), except for short transient induction
115 promoted by the stress of Pi addition observed during first hour following resupply. A
116 sequence analysis of the 22 genes identified above revealed the presence of P1BS regulatory

117 boxes¹⁶ in 95% of the cases. This box is the binding site of master regulators belonging to
118 PHR1/PHL1 family. Addition of Pi promotes synthesis of inositol pyrophosphates, which act
119 as a molecular tether to fix members of the SPX family to PHR1 and inhibit its activity^{20,21}
120 (Fig. 1B). Consistently, the induction of *SPX1* and *UNII* during Pi starvation was nearly fully
121 abolished in *phr1/phl1* double mutants, which suppress a majority of PHR1/PHL proteins
122 (Fig. 1C). Moreover, this was observed for 91% of the 22 genes identified (Fig. S1A¹⁷). SPX
123 family members inhibit PHR1 and the analysis of *spx1/spx2* double mutants further revealed
124 that reducing the SPX protein pool delayed the repression triggered by Pi addition (Fig. 1D).
125 Altogether, these results demonstrated that the fast decrease of the 22 transcripts identified
126 here resulted from transcriptional control involving several key players such as PHR or SPX
127 family of proteins. Of note, classical transcriptional fusion with Luciferase did not report a
128 fast transcriptional repression (Fig. 1E). More than one hour was required to observe a
129 significant decrease of the signal, whereas RTqPCR failed to detect a significant reduction of
130 mRNA levels for at least 15-20 minutes after resupply (data not shown). To better
131 characterize the transcriptional response to Pi resupply, we implemented the MS2 system for
132 RNA labeling, a technology so far restricted mostly to animal and yeast but offering unique
133 spatio-temporal resolution to study transcriptional regulation.

134

135 **Generation and validation of MS2-tagged Arabidopsis lines**

136

137 We used the last generation MS2 tag containing 128xMS2 repeats, originally developed to
138 image single-molecule of HIV-1 RNA¹³. To improve RNA folding and prevent plasmid
139 instability, this construct is made of 32 distinct MS2 stem loops replicated four times, with
140 each stem-loop binding dimers of the MCP protein with sub-nanomolar affinity¹³ (Fig. 2A).
141 In animal cells, this extended tag provides about a five-fold improved sensitivity over the
142 original MS2x24 tag and allows single molecule visualization for extended periods of times

143 even at high frame rates¹³. Moreover, this construct bears the lower affinity variant of the
144 MS2 stem-loops (U instead of C in third position of the loop), providing excellent RNA
145 visualization while preserving normal RNA degradation²⁴.

146 We developed Moclo vectors adapted to the Golden Gate system²⁵ to facilitate the
147 cloning and use of the MS2x128 recombinogenic sequence (Fig S2A), and we generated
148 transcriptional fusions with the *SPX1* and *UNII* promoters (Fig. 2A). We then introduced into
149 the binary vector used for plant transformation a gene expressing a nuclear targeted MCP-
150 eGFP fusion protein¹³ under the control of the weak constitutive Ubiquitin-10 promoter²⁶ (Fig
151 S2A). For the two constructs, genetic analysis selected homozygous transformants exhibiting
152 single locus insertions. We first focused on the homozygous pSPX1::MS2x128 line named 'S'
153 for strong and exhibiting the best signals. Molecular analysis revealed the insertion of three
154 copies of the transgene at a single locus in the S line (Fig S3). The presence of the cap and the
155 polyA tail, crucial elements for RNA stability, was also verified for MS2x128 transcripts (Fig
156 S4). Single-molecule fluorescence in situ hybridization (smFISH) was performed on root
157 squashing to analyze the phosphate response of the transgenes. Root squashes allows good
158 probe penetration in tissues and causes some cells to fall off the root and adhere to coverslips
159 as a monolayer²⁷, allowing high quality imaging using wide-field microscopy with high
160 numerical aperture objectives (Fig. 2B and S2B). The fluorescent oligonucleotide probes
161 hybridizing against the MS2 stem-loops labelled the reporter RNA in most tissues of the root
162 grown on Pi depleted medium, including the root cap and mature tissues (Fig. 2B and see
163 below Fig. 3), and they did not detect signals in Col0 negative controls lacking the transgene
164 (Fig. S5). Interestingly, the smFISH signals were rarely detected in the cytosol and mostly
165 stained nuclei where transcription sites were visible as bright spots. This nuclear localization
166 could be due to either a nuclear retention of the MS2-labelled RNA, or a reduced stability in
167 the cytosol. In any case, the smFISH signal disappeared when plants were grown in the

168 presence of phosphate (Fig. 2B, right panels). Quantification of the signals indicated that 74%
169 of the cells expressed the pSPX1::MS2x128 transgene in absence of phosphate (>20
170 RNA/cell), and none in its presence (Fig. 2B and 2D). To further validate these results, we
171 visualized the endogenous *SPX1* transcripts with a mix of 24 fluorescent oligonucleotide
172 probes specific for *SPX1*. In Pi depleted conditions, the probes identified the transcription
173 sites and single mRNA molecules present in the cytoplasm, as expected for a translated
174 transcript (Fig 2C and S2B). Similar to the pSPX1::MS2x128 reporter, the endogenous *SPX1*
175 mRNA was expressed throughout the root, including the cap and mature tissues²⁸, with a
176 number of positive cells similar to that of the reporter (Fig. 2D). Importantly, *SPX1* smFISH
177 signals were very low in plants growing on Pi rich medium (Fig 2D and 2C, right panels) and
178 completely absent in a *spx1spx2* double mutant used as negative control (Fig S6). Overall,
179 these data indicated that the MS2 transgene driven by the *SPX1* promoter faithfully reported
180 on the expression of *SPX1*, with a similar regulation and spatial expression pattern.

181

182 **Imaging transcription in fixed tissues of whole plants with MCP-GFP**

183 We then turned to spinning disk confocal microscopy to image plants through the tissue depth
184 (reaching 100µm) and to assess in detail the performance of MCP-GFP to image
185 transcription. We first used the brightest homozygous pSPX1::MS2x128 line (called S) to
186 image expression of the reporter in the root (Fig. 3A; a viewer with a zoomable high
187 resolution image can be accessed at [https://imjoy.io/lite?plugin=muellerflorian/hani-
188 ms2:hani-ms2-sample-3](https://imjoy.io/lite?plugin=muellerflorian/hani-ms2:hani-ms2-sample-3) and [https://imjoy.io/lite?plugin=muellerflorian/hani-ms2:
190 hani-ms2-
191 sample-1](https://imjoy.io/lite?plugin=muellerflorian/hani-ms2:hani-ms2-
189 sample-1), which is a continuously growing organ with a well-defined architecture. At the
apex, the root cap encapsulates the meristematic zone where cells divide rapidly until they
enter the transition zone (TZ). Cells then elongate (EZ) and initiate tissue differentiation

192 according to a well defined radial pattern, generating a number of different cell types (Fig.
193 3A).

194 First, we tested whether MCP-GFP could faithfully report on the MS2-tagged RNA
195 and we performed smFISH together with MCP-GFP imaging. In the root cap, which is mostly
196 composed of diploid cells²⁹, smFISH against the MS2 stemloops revealed both single
197 molecule and transcription sites, visible as bright spots in the nucleus (Fig. 3B, bottom panels;
198 pink and orange arrows, respectively). In both root cap (Fig. 3B) and mature tissue (Fig. 3C),
199 we observed colocalization between the smFISH signals revealed by MS2 probes and the
200 fluorescent signal produced by MCP-GFP. The transcription sites were easily detected (Fig.
201 3B and C, orange arrows), and MCP-GFP could also reveal the brightest single mRNAs (Fig.
202 3B, pink arrows), indicating that the sensitivity of the system approaches that of single
203 molecules (see also below live cell experiments). To assess the reliability of detection, we
204 counted transcription sites in both colors, and found that 90-95% of the sites detected by
205 MCP-GFP were also labelled by MS2 smFISH.

206 Finally, we extended these observations to other *Arabidopsis* lines, using plants having
207 either the pUnicorn1::MS2x128 reporter or another single locus transformant of the
208 pSPX1::MS2x128 transgene (J line). The J line turned out to also present multiple T-DNA
209 copies (Fig S3B), but it exhibited reduced levels of fluorescence when compared to the S line.
210 The transcription sites of the reporters were readily detected with MCP-GFP and co-localized
211 with the MS2 smFISH signals (Fig. S7). Overall, these results validated the MS2/MCP
212 system as a robust and sensitive system to image transcription in whole plants tissues.

213

214 **Single molecule counting by smFISH gives insights into the dynamics of transcription in**
215 ***Arabidopsis thaliana***

216 The ability of smFISH to detect single RNAs enables a quantitative analysis of the RNA
217 polymerase II transcription cycle^{9,12,30,31}. We therefore measured the brightness of
218 transcription sites and used the visible single molecules to compute the absolute number of
219 nascent RNA molecules at active transcription sites (Fig. 4A and B; see Methods). We
220 obtained an average of 37 molecules at the transcription site of the pSPX1::MS2x128 reporter
221 for the S lines, with a main peak at ~20 molecules (Fig. 4A). We also designed a set of 21
222 fluorescent oligonucleotides against the sequence immediately downstream the
223 polyadenylation site of the reporter (see Fig. S12). We however failed to detect any smFISH
224 signal, indicating that 3'-end processing was rapid as compared to transcription. This agrees
225 with previous GRO-seq experiments that revealed that RNA polymerases terminate
226 transcription very rapidly after polyA sites^{32,33}. We repeated these measurements for the
227 endogenous *SPX1* mRNA and found an average of 11.3 molecules at transcription sites, with
228 a main peak at 9 molecules. Given that the *SPX1* gene is 1.4 kb long, this number suggests
229 that active *SPX1* genes have one polymerase every 77 bases if 3'-end processing is immediate,
230 and one polymerase every 120 bases if 3'-end processing takes a minute¹³ (assuming an
231 elongation rate of 2 kb/min³⁰). For the 3kb pSPX1::MS2x128 reporter, similar numbers were
232 found if one considers that the peak of 25 molecules at a transcription site corresponds to a
233 single active copy (Fig. 4A). These numbers indicate that active *SPX1* genes have an initiation
234 event every 2.3 to 3.5 seconds on average (see Methods and schematic in Fig. 4B). This is in
235 the high range of previous estimates obtained in human cells and *Drosophila*¹³. It suggests
236 that transcription in *Arabidopsis* is rapid and occurs in the form of polymerase convoys,
237 produced by initiation events rapidly occurring one after another when the gene turns on¹³.

238

239 **Quantitation of *SPX1* transcription in fixed roots reveals cell polyploidy in mature tissue**
240 **as well as large allelic differences**

241 Endoreplication occurs frequently in root with DNA cell contents ranging from 2C to 16C²⁹.
242 This phenomenon increased as one moves away from the root tip. Being involved in the size
243 of the cells, it also varies and increases from the central to the expanded external cell layers
244 such as cortex or epidermis²⁹ (Fig. 3A). The number of transcription sites present in each
245 nucleus highlighted this endoreplication. In the root apex of the pSPX1::MS2x128 S line,
246 cells of the columella (extremity of the root cap) exhibited in the vast majority of cases no
247 more than two transcription sites for the MS2 reporter or the endogenous *SPX1* gene, as
248 expected for a mainly diploid tissue²⁹ (Fig. 3B and Fig. 4C for quantifications). In contrast,
249 images recorded in the differentiated part of the root revealed the more complex nature of
250 older tissues with the presence of cells with many transcription sites, indicating polyploidy
251 (Fig. 3C and 2C). Quantification of the number of active alleles per cell indicated that in
252 diploid columella cells, only 3% of the cells had more than 2 transcription sites, while in the
253 mature tissue, 35% had more than two sites, with 2% having as many as 7 (Fig. 4C and 4D).
254 Similar numbers were obtained with the endogenous *SPX1* gene (Fig. 4D). Interestingly, we
255 noted that in cells with many active alleles, the *SPX1* transcription sites frequently clustered
256 in the nucleoplasm, most often forming two groups (see Fig. 4F for an example). To explore
257 this further, we measured the distance between all visible transcription sites in cells having 4
258 to 8 active sites, and we compared the resulting distance distribution to a situation where we
259 simulated 8 sites with a random location (Fig. 4E, see Methods). This showed that the
260 transcription sites had a non-random distribution and were frequently close to one another,
261 with 40% of the distances falling within 1.5 microns. This phenomenon was also visible when
262 we compared the brightness of transcription sites in cells having one or two transcription
263 sites, to cells having four or more sites (Fig. S2C). Cells with one or two *SPX1* transcription
264 sites had a single peak of 10 nascent RNAs, while cells with four or more sites had a second
265 peak at 20 nascent RNAs (Fig. S2C). This suggests that in these cells, some transcription sites

266 had coalesced into a single spot, reminiscent of the 'transcription factories' previously
267 described in human cells³⁴.

268 Next, we compared the activities of the different alleles of single cells, focusing first
269 diploid root cap cells. Surprisingly, an important number of cells exhibited only a single
270 active transcription site (30 % of the cells; Fig. 4C), or no transcription at all (39%). This was
271 also observed with the endogenous *SPXI* gene (Fig. 4D), indicating that it is a feature of this
272 Arabidopsis gene and not an artifact of the MS2 reporter. Since the cells with no transcription
273 sites contained smFISH signal in the nucleoplasm or the cytoplasm (Fig. 3), transcription had
274 been active in these cells, therefore indicating that *SPXI* promoter activity was discontinuous.
275 Such discontinuous transcription is the result of gene bursting and has been observed in many
276 organisms including yeast, *Drosophila* and mammals³⁵. To our knowledge, it has not been
277 reported so far in plants. Gene bursting involves the stochastic switching of a promoter
278 between active and inactive states. It depends on mechanistic aspects of transcription
279 initiation as well as transcriptional regulation¹². This stochasticity causes variations in gene
280 expression among identical cells (ie. gene expression noise), which has sometimes important
281 phenotypic consequences¹². Commonly, two sources of noise are distinguished: intrinsic and
282 extrinsic. Intrinsic noise is due to the stochastic nature of biochemical reactions involving a
283 single molecule of DNA and the transcription factors acting on it, and it occurs independently
284 on each allele. In contrast, extrinsic noise modulates similarly both alleles as the result of
285 events affecting the entire cell (such as cell cycle or activation of a signaling pathway).
286 Interestingly, our capability of accessing several alleles within a cell raised the possibility to
287 discriminate between the two causes of transcriptional noise. For each cell that had only one
288 or two active alleles, we plotted the brightness of one *SPXI* allele as a function of the other
289 (Fig. 4G). This revealed both correlated (cells on the diagonal) and uncorrelated (cells off the
290 diagonal) transcriptional activities of these alleles. To quantify this phenomenon further, we

291 measured the total, intrinsic and extrinsic noise^{36,37}, using cells having either exactly two, or
292 3-4 active transcription sites (Fig. 4H, see Methods). As expected from the results described
293 above, we found that the total noise had significant intrinsic and extrinsic contributions, with
294 intrinsic noise being the dominant source. This highlights the quantitative importance of
295 transcriptional noise for Arabidopsis.

296

297 **Real-time visualization of SPX1 transcription reveals gene bursting and Pi mediated** 298 **repression**

299 We used a simplified version³⁸ of the RootChip microfluidic system²² to combine live-cell
300 imaging with the capacity to change the phosphate solution rapidly (Fig. 5A and Movies).
301 Imaging was performed with spinning disk microscopy, which offered the best compromise
302 between image quality, size of the field of view and image acquisition speed. A typical
303 experiment recorded at each time point a z-stack of 200 images with a z-spacing of 500 nm,
304 allowing us to image at high resolution the entire root and thus access all its tissues. One
305 image stack was recorded every 2-3 minutes for ~1 hour, and maximum intensity projection
306 produced clear images where multiple transcription sites could be analyzed. Using the
307 brightest S line expressing pSPX1::MS2x128 and grown without Pi, images from the first
308 time point confirmed the transcriptional heterogeneity between cells, with neighboring cells
309 exhibiting zero, one, two or even more active transcription sites and with the intensity of
310 transcription sites varying by a factor of 4 (Fig. 5-7 and Movies 3-7). Observation of the root
311 constantly supplied with -Pi solution directly demonstrated bursting of the SPX1 promoter,
312 with transcription sites coming on and off over periods of minutes (Fig. 6, Movies 1 and 2).
313 Interestingly, this phenomenon was mainly observed in the root cap, and only very rarely in
314 mature tissues. Remarkably, the arrest of transcription after a burst provided an opportunity to
315 visualize the release of single RNAs from the transcription sites (Fig. 6D, Movie 2),

316 highlighting the dynamics of the process and confirming single molecule sensitivity in live
317 plants.

318 Next, we analyzed the response to Pi resupply. To this end, the S line was first grown
319 during a few days in -Pi liquid medium, before receiving phosphate rich medium combined
320 with the SynaptoredTM fluorescent dye. This allowed to precisely record the arrival of the
321 supplemented media on the imaging field, thereby defining the time 0 of the time course. The
322 movement of nuclei in the cells and the displacement of cells themselves complicated
323 transcription site tracking. To simplify the analysis of the time course, we therefore used only
324 cells with one or two active transcription sites. The repression of transcription upon Pi
325 resupply proved to be rapid (Fig. 5 A-C, S8, S9 and Movies 3-7). Signals were then
326 normalized to the value obtained at time 0 (Fig. 7C) and an average response curve was
327 produced (Fig. 7D). The measurements were repeated with independent samples and they all
328 showed similar curves with a signal starting to decrease between 0 to 6 minutes after Pi
329 provision at the root surface (Fig. 7E). Similar results were obtained with independent
330 transgenic J line (Fig. S10, Movie 8), revealing the extraordinary sensitivity and rapidity of
331 the regulatory cascade triggered by Pi.

332 The decrease of fluorescence starting with Pi resupply reached the root continued until
333 it reached a plateau 18 to 25 minutes later (Fig. 7F and S10). Interestingly, cells presenting
334 multiple transcription sites revealed on average a close coordination of the repression between
335 these sites, indicating that extrinsic factors dominate gene regulation when Pi repression
336 initiates, as expected for a signaling pathway affecting the entire cell (Fig. 5C, 7B and S8).
337 Controls continuously supplied with Pi depleted solution in mature tissue did not show
338 significant changes (Fig. 7 G, H and S11) over 25 min observation period.

339 The comparison between the MS2 and RTqPCR results revealed a delay of 20-30
340 minutes for the RT-qPCR to show the maximum repression, which is in agreement with the

341 *SPXI* mRNA half-life previously estimated in the range of 15-20 min (Fig. 1A and S1A,B).
342 Overall, these results demonstrate the unexpectedly fast dynamics of the plant response to Pi
343 resupply, and nicely illustrates the capacity of the MS2 system to access transcriptional
344 regulation in live plants. It should be noticed that in mature tissues, we restricted the analysis
345 to nuclei exhibiting one or two active alleles to avoid misattribution during the analysis
346 (resulting from nuclei movement during analysis). This favors more central cell (less affected
347 by polyploidy) representation, which are known to be less affected by Pi repression compared
348 to cortex or epidermis cells³⁹. This may explain the slight difference (around 10%) of average
349 extent of the decrease in expression between RTqPCR and MS2 live imaging.

350

351 **Discussion**

352

353 **Large MS2 arrays and microfluidics reveal single cell transcriptional dynamics in live** 354 **plants**

355 Fluorescent RNA technologies provide access to single molecule studies offering invaluable
356 insights into gene expression mechanisms^{12,40}. A previous attempt to use these technologies in
357 plants studied a highly abundant viral RNA in transient transformants and low signal-to-noise
358 ratio prevented their use in stable plant lines⁴¹. The MS2x128 construct developed here
359 (which uses six time more MS2 loops) solves this problem and provides a direct access to
360 transcriptional activities of live plants, at the level of single alleles and with a sensitivity
361 nearing single RNAs. Nevertheless, single molecule detection remains difficult and not
362 exhaustive, due in particular to the necessity of limiting illumination power to prevent
363 bleaching and phototoxicity when acquiring 200 z planes for tens of time points. We hope that
364 new constructions currently underway, where the number of MS2 loops is doubled (256) or
365 MCP-GFP expression is optimized, will provide important future improvements. Originally
366 developed for yeast or mammalian cell lines, fluorescent tagging of RNA has been
367 introduced in only few multicellular organisms (*Drosophila* embryos in particular^{10,35}), mostly

368 to study transcriptional regulation during embryonic development and in absence of external
369 perturbations. Here, the implementation of microfluidics allowed a complete control of
370 environmental conditions and the investigation of the root response to phosphate starvation
371 and resupply. Measurements of transcriptional inhibition often suffer from both indirect
372 measurements introducing a considerable lag time in repression detection, and ensemble
373 measurements averaging effects. In contrast, the tools developed here allow visualization of
374 repression in real time and single cells, and demonstrate that it is a very rapid process that
375 takes place in minutes.

376 Plants are sessile organisms that developed exquisitely efficient regulation systems
377 to ensure homeostasis in face of changing environmental conditions. Due to the fast and
378 sensitive tuning of transcriptional mechanisms, microfluidics offers the ideal solution to
379 control environmental conditions. This strategy could be extended to a variety of other
380 stresses, including biotic ones, and to all plant organs as various microfluidic systems exist to
381 study aerial parts. The technological developments made here thus open entirely new
382 possibilities by allowing the direct visualization of transcriptional regulations in living plants,
383 in real time and at the level of single cells and single molecules.

384

385 **Integration of single cell responses to phosphate signaling at the tissular level**

386 The capacity to dissect transcriptional responses of individual cells within a tissue and
387 between different tissues within an organ is a major step forward. The use of real-time
388 phosphate radioisotope imaging revealed that Pi enters and accumulates in the root tip within
389 less than one minute⁴². The present study thus directly links this arrival with transcriptional
390 repression, demonstrating the close concomitance between the two phenomena. Moreover,
391 our analysis, owing to unprecedented cellular resolution in plants, also identified the
392 heterogeneity of transcriptional responses. Indeed, we observe strikingly different responses

393 to phosphate resupply in different tissues of the root: a cell can repress *SPXI* transcription
394 within minutes while its neighbor may continue transcribing unabatedly (Fig. 3B, 5C and 7).
395 The fact that the alleles of a cell show a coordinated response indicates that this heterogeneity
396 in repression arises mainly from extrinsic sources, as expected for a regulation involving a
397 cellular signaling pathway. Interestingly, the cell-to-cell heterogeneity of *SPXI* transcription
398 is already visible at steady state when plants are starved from phosphate: a third of root cap
399 cells contain *SPXI* mRNAs while not being transcriptionally active, indicating discontinuous
400 promoter activity. This peculiar phenomenon of gene bursting is an important driver of cell
401 heterogeneity and has already been described in animals³⁵, but not plants. Our analysis
402 suggested that this phenomenon may differ greatly between cell types. Using specific cell
403 layer markers in the future will help to investigate more precisely this phenomenon and
404 decipher putative driving mechanisms. We show here with *SPXI* that transcriptional noise in
405 plants has both intrinsic and extrinsic components, suggesting that it arises from both
406 stochastic promoter dynamics and cellular regulatory pathways. At steady state, in absence of
407 Pi, intrinsic noise appears to be dominant, while extrinsic factors appear to take over during Pi
408 refeeding, generating heterogeneity in repression kinetics and in the extent of repression. This
409 needs to be investigated in different organs, developmental stages, physiological statuses and
410 environmental contexts. Because of its role in cell heterogeneity, transcriptional noise is
411 clearly a key point to understand how the activity of individual cells is integrated within entire
412 tissues.

413

414 **Transcription imaging in polyploid cells reveals clustering of active alleles**

415 Endoreplication in plants has been associated with high metabolic activity or fast biomass
416 development, economically important for most vegetables and fruits. Recent analysis of
417 *Arabidopsis* illustrated the extent of this process affecting many root cells during their

418 development and differentiation, the highest level of endoploidy (16C) being observed either
419 in most developed cells (epidermis hair cell) or in highly active regions associated with
420 distribution of nutrients (phloem companion cells)²⁹. So far endoploidy detection uses
421 destructive techniques such as flow cytometry or histochemical staining of endocycles
422 markers or DNA. Here, we overcame such limitations providing the opportunities to access
423 kinetics of endoploidy taking into account development, cell fate, biotic or abiotic stresses.
424 Our results highlighted the magnitude of somatic polyploidy in a root, revealing that many
425 alleles of polyploid cells can be simultaneously transcriptionally active. We found that in
426 absence of phosphate, active *SPX1* alleles of polyploid cells tended to cluster together,
427 possibly because endoreplicated chromatids generally occupy the same chromosome
428 territory⁴³. More surprisingly, our data also suggest that active *SPX1* alleles have a propensity
429 to coalesce together, a phenomenon reminiscent of transcriptional factories reported in animal
430 cells³⁴. In the future, it will be important to confirm the coalescence of active alleles by
431 directly observing this phenomenon in live cell. It will be also interesting to determine
432 whether this is related to the very high transcriptional activity of the *SPX1* gene in these
433 conditions (one initiation event every 2-4 seconds), and to understand how this process occurs
434 and whether it contributes to gene regulation. Note that these initiation rates are based on
435 polymerase elongation speed measured in animals or yeast. They do not take into account
436 possible modifications affecting transcription elongation, pausing and termination, which are
437 poorly described in plants despite recent advances^{32,33}, and will require additional experiments
438 to account for them.

439

440 Overall, the technology presented here offers direct access to the RNA polymerase II activity
441 in live cells and could be used to study crucial plant phenomena affecting transcription
442 (silencing, heterozygosity, impact of gametophyte origin ...). This unprecedented spatio-

443 temporal resolution for plant transcription therefore provides new horizons to multiple
444 applications hitherto inaccessible for plant physiology.

445 **Material and Methods**

446 **Plant materials and growth conditions**

447 Wild type *Arabidopsis thaliana* Col-0 seeds were sterilized and grown vertically on
448 Murashige and Skoog medium diluted 10-fold (MS/10) in Petri dishes supplemented with a Pi
449 source containing either 500 μ M (+P) or 13 μ M (-P) KH_2PO_4 in a culture chamber under a
450 16-hr- light/8-hr-dark regime (25°C/22°C), as previously described⁴⁴.

451 For global transcriptomic analysis, 7 days old seedlings were germinated on a sterile
452 nylon mesh deposited at the surface of the culture medium to facilitate the transfer between
453 media. For resupply experiment, plantlets were transferred from -P to +P for 30, 60 or 180
454 minutes. In order to minimize stress related transfer, control plants in -P and +P media were
455 also transferred for similar period of time on same media.

456 In order to get closer to microfluidic conditions and limit the stresses related to the
457 solid media transfer, we modified the protocol for subsequent experiments (RTqPCR
458 analyses), by transferring the 7 days old seedling from agar plate to liquid MS/10 for 3 days.
459 Then, -P plants were resupplied with +P solution for up to 3 hours before collecting the roots
460 for RNA extraction. Transcription inhibition experiments were performed by addition of 0.6
461 mM cordycepin⁴⁵. For each condition three independent biological replicates were analyzed.
462 Plant DNA extraction and genetic segregation analysis were performed as previously
463 described⁴⁶.

464

465 **RNA-seq library preparation and analysis (RNA seq and RTqPCR)**

466 The extraction of total RNA from *Arabidopsis* roots or shoots, the synthesis of RNA-seq
467 libraries and their analysis using Illumina sequencing technology (Illumina, San Diego, CA)
468 were performed as previously described⁴⁷. Quantitative RTqPCR experiments were
469 performed as previously described⁴⁸ with primers listed in the Supplemental Table 1.

470 For 7-Methylguanosine RNA Immunoprecipitation (m7G-RIP) experiment, total RNA
471 was extracted using Monarch Total RNA Miniprep kit including DNase treatment on column
472 (New England Biolabs). m7G-RIP was then performed as previously described⁴⁹ with slight
473 modifications. 5 µg of total RNA was used as starting material. Elution was performed using
474 Guanidium 8M 10 minutes at 65°C followed by RNA precipitation. As negative control,
475 pyrophosphatase treatment was performed on total RNA prior to m7G-RIP. Reverse-
476 transcription was then performed on input and eluate fractions using 500 ng of RNA and oligo
477 dT primer. PCR was then performed using primers against MS2 transgene or endogenous
478 SPX1 using oligos described in Supplemental Table 1.

479

480 **Transgene constructs**

481 The pSPX1::MS2x128 and pUNI1::MS2x128 constructs were built using the Golden gate
482 cloning technique²⁵. MS2x128 stem loops (pMK123-MS2X128-XbaI) and NOS terminator
483 were inserted into level 0 vectors pICH41308, and pICH41421 respectively. The promoters of
484 *SPX1* (At5G20150) and *UNICORN1* (At5g20790) were PCR amplified (1452 bp²⁸ and
485 2161bp upstream of the start codon) using oligos described in Supplemental Table 1. These
486 sequences were introduced in level0 vectors (pICH41295). The levels 0 modules were
487 assembled directly into a level2 expression vector pICH86966.

488 The Ubiquitin10 promoter (At4g05320) driving eGFP expression derived from pUBN-
489 Dest²⁶ was assembled with MCP contained in pDONR201⁸ using the Gateway cloning
490 technique (Invitrogen) creating plasmid pUb::eGFP/MCP-NLS. This construct was excised
491 from the Gateway vector using PmeI and SmaI (Invitrogen) and introduced in level2 T-DNA
492 pSPX1::MS2x128 or pUNI1::MS2x128 vectors using PmeI restriction sites. A clone having
493 the two constructs in opposite orientations creating pMCP/proSPX1::MS2 was used to
494 transform *Agrobacterium tumefaciens* strain C58C1. Figure S2A summarizes the cloning

495 strategy. The reporter line carrying Luciferase in fusion with the UNICORN1 promoter was
496 generated as previously published⁴⁴.

497

498 **Production of transgenic lines**

499 Transformants produced by floral dipping⁵⁰ were selected on Hoagland/2 media
500 complemented with 50 mg/L kanamycin. The progeny exhibiting 3:1 segregation were carried
501 to T3 generation to identify homozygous lines carrying a single insertion locus. At least 5
502 independent lines were obtained for each construct.

503

504 **Single molecule Fluorescence *In Situ* Hybridization**

505 *Arabidopsis* seedlings were grown on +P and -P agar media. The roots of 7 to 10 day old
506 seedlings were collected and fixed for 30 min with 2-(*N*-morpholino) ethane sulfonic acid
507 (MES) buffer solution pH 5.7 containing 4% paraformaldehyde (32% methanol-free solution;
508 Electron microscopy Sciences). pH was adjusted to 5.7 with KOH solution.

509 Roots were rinsed twice with MES buffer (pH 5.8) and put onto a microscopic slide
510 with a coverslip. They were smoothly squashed (to splay them and produce a single cell layer)
511 and submerged briefly in liquid nitrogen. Then, after removing coverslips, they were left out
512 to dry at room temperature for one hour. To permeabilize the samples, the slides were
513 immersed in 70% ethanol overnight on a rotary shaker prior to hybridization. The ethanol was
514 evaporated at room temperature before washing the roots with MES.

515 SmFISH was used for the detection of MS2 repeats and endogenous *SPX1* mRNAs.
516 The MS2 probe was made of a mix of 10 pre-labelled fluorescent oligos directed against
517 32xMS2 repeats¹³. Each oligo contains 2 to 4 molecules of Cy3 and hybridizes 4 times across
518 128xMS2 repeats, allowing the binding of 40 probes to each single RNA molecule. The set of
519 *SPX1* probes was made of a mix of 24 fluorescent oligonucleotides carrying 2 to 3 Cy3

520 fluorophores and covering the entire SPX1 transcript including 5' and 3' UTR (Table S2).
521 The pre-hybridization was performed in 1xSSC/40% formamide buffer for 15 min at room
522 temperature. Hybridization was performed with 2 ng/ μ l probe and in 40% formamide for
523 MS2, whereas for *SPX1* only 15% formamide was used. The smiFISH probe mix directed
524 against the post-polyA region of the MS2 construct was made of 21 oligonucleotides (Table
525 S2) and hybridized as previously described⁵¹. After addition of probes, samples were covered
526 with a coverslip and remained overnight at 37°C as previously described⁵¹. For rinsing, after
527 coverslip removal, root samples were washed twice during 45 min at 37°C with freshly
528 prepared 1xSSC/40% formamide buffer and rinsed at room temperature with MES pH 5.8
529 buffer and dried. A drop of Prolong Diamond antifade mounting medium (Invitrogen)
530 containing DAPI was added prior to observation. For long-term storage, slides were kept at -
531 20°C.

532

533 **Fluorescence imaging of fixed plants**

534 SmFISH and MCP-GFP images were taken using either a spinning disk confocal or a wide
535 field microscope. For spinning disk microscopy, we used a Dragonfly (Oxford instrument)
536 equipped with four laser lines (405, 488, 561, 637 nm) and an ultrasensitive EMCCD camera
537 (iXon Life 888, Andor) mounted on a Nikon Eclipse Ti2 microscope body, using a 40x, NA
538 1.3 Plan Fluor oil objective or a 60x, NA 1.4 Plan Apochromat oil objective coupled with a
539 supplementary lens of 2x, using z-stacks with a 0.5 μ m or 0.4 μ m step. For wide field
540 imaging, we used a Zeiss Axioimager Z1 wide-field upright microscope equipped with a
541 camera sCMOS ZYLA 4.2 MP (Andor), using a 100x, NA 1.4 Plan Apochromat oil objective.
542 For these z stacks, a step of 0.3 or 0.4 μ m was used. Maximal image projections (MIP) were
543 generated with ImageJ, and figures were realized using Adobe Photoshop and Illustrator. The

544 mosaic of Figure 3A is accessible with a viewer run with Imjoy⁵². Note that plant fixation and
545 smFISH both reduced the GFP signals.

546

547 **Analysis and quantifications of smFISH images**

548 Quantification of the brightness of transcription sites was made with a modified version of
549 HotSpot⁴⁰. This is a user-friendly MatLab software that allows to quantify smFISH signals in
550 very noisy images, unlike FISH-quant^{53,54}. Briefly, the user can navigate in 2D or 3D bicolor
551 images to manually select transcription sites and single RNAs, and the script finds the local
552 maxima and fits 2D or 3D gaussians to the selected transcription spots. The images of single
553 RNAs are averaged and also fitted to 2D or 3D gaussians, and the integrated intensity of the
554 transcription sites are divided by the intensity of average of single RNAs, yielding the exact
555 number of molecules present at transcription sites. In this work, quantifications were done in
556 2D on MIPs using 100-500 single RNA per image to generate the average. HotSpot is
557 available on GitHub (<https://github.com/muellerflorian/hotspot>).

558 To calculate initiation rates, we assumed that initiation occurs at random with a
559 constant rate. This produces a uniform distribution of RNA polymerases along the *SPXI* gene
560 following arithmetic series. Because the MS2 and *SPXI* probes are distributed
561 homogeneously along the corresponding pre-mRNAs, incomplete nascent RNAs are labelled
562 with only a fraction of the probes. If one assumes that the pre-mRNAs immediately leave the
563 transcription site once polymerases reach the 3'-end of the gene (in agreement with
564 experimental data), the barycenter of the polymerase distribution is the middle of the gene,
565 and the average brightness of a nascent pre-mRNA is thus half that of a full-length pre-
566 mRNA. In this case, the number of polymerase on the gene, N_{pol} , is thus twice the brightness
567 of transcription sites, expressed in number of full-length pre-mRNA (noted TS_{RNA}). Assuming
568 an elongation rate of 2kb/min, the time to transcribe the gene, t_{el} , is 42 s, and the average

569 initiation rate is N_{pol} divided by t_{el} . If the pre-mRNA takes t_{proc} seconds to be 3'-end
570 processed and released from the transcription site, then N_{pol} is $TS_{RNA} * [2 * t_{el} / (t_{el} + t_{proc}) +$
571 $t_{proc} / (t_{el} + t_{proc})]$, and the initiation rate is N_{pol} divided by $(t_{el} + t_{proc})$.

572 To analyze the distribution of distances separating active transcription sites within
573 single nuclei, we computed for each nuclei all the distances between all the visible
574 transcription sites. To simulate a random distribution of transcription sites, *Arapidopsis* nuclei
575 were simulated as ellipses with a major axis length of 12 μm and a minor axis length of 6 μm ,
576 and we randomly selected points using a uniform distribution in the ellipse surface.

577 To measure total (n_{tot}), extrinsic (n_{ext}) and intrinsic (n_{int}) noise for cells with any
578 number of alleles, we used an extension of the approaches previously developed^{36,37}. For a
579 population of cells with n alleles, we considered the brightness of the transcription sites of the
580 n alleles, expressed in number of full-length pre-mRNA molecules, as n random variables.
581 We computed the corresponding variance-covariance matrix of these n variables and defined
582 n_{tot} as the root square of the mean of the variances given by the matrix diagonal, n_{ext} as the
583 root square of the mean of the covariances obtained by the non-diagonal values of the matrix,
584 and n_{int} as the root square of $n_{tot}^2 - n_{ext}^2$. The error in n_{tot} , n_{int} and n_{ext} was estimated by
585 randomly attributing each of the n transcription sites to the n alleles, calculating n_{tot} , n_{int} and
586 n_{ext} and repeating this 100 times to calculate the standard deviation of the values obtained.

587 In Figure 2D, the number of expressing cells (i.e. cells having at least 20 RNA
588 molecules in the nucleus or the cytoplasm), as well as the number of transcription site per cell
589 (see Fig. 4C-D), was counted manually from 2D MIP smFISH images. The plot of Figure 4
590 were generated in R and incorporated in the figure using Adobe Illustrator.

591

592 **Rootchips and *in vivo* live plants imaging experiments**

593 For these experiments, we used brightest line obtained with *SPXI* promoter (the S and J
594 lines). Transgenic seeds were germinated in conical cylinders produced from micropipette tips
595 filled with -P agar and inserted into sterile -P agar plates. 5 to 7 day old Pi deficient seedlings
596 were then transferred into a RootChips^{22,38}, grown vertically and fed with a Pi deficient
597 nutritive solution at a low flow pressure until the root grew in the channel. For refeeding
598 experiment, the nutritive solution was changed from -Pi to +Pi. SynaptoredTMC2 (5µg/mL,
599 Ozyme) was added into the +P solution to detect the arrival time of Pi (defining t=0). In vivo
600 movies were recorded with an Andor Dragonfly spinning disk mounted on a Nikon Eclipse
601 Ti using 40x Plan-Apo water objective (1.15 NA; 0.6 mm DT). Z-stacks were made every 3
602 minutes with a scan size of 100-150µm (0.5µm step size; ~200 steps per Z-stack). The data
603 was analyzed with the ImageJ software using the TrackMate plugin to track transcription sites
604 on 2D MIPs. The intensity of *SPXI* transcription sites was assessed for several nuclei per
605 sample. In each nucleus, the mean intensity of the nuclear background was subtracted to the
606 maximum intensity of *SPXI* transcription site, and the resulting intensities were plotted
607 against time.

608

609

610 **Figure Legend**

611

612 **Figure 1: identification of fast responsive transcripts regulated at transcriptional level**
613 **by phosphate resupply.**

614 (A) RTqPCRs of roots of seedlings grown for 7 days either in the presence of Phosphate
615 (+Pi), in the absence of Phosphate (-Pi), or in the absence of Phosphate for 7 days followed by
616 Phosphate resupply for 30 minutes (Re30) or 60 minutes (Re60). *EIF4A*, a translation
617 initiation factor known not to react to cordycepin addition was used as control⁴⁵; n =2-3.

618 (B) Model depicting the transcriptional regulation by phosphate.

619 (C) RTqPCRs of roots of WT and *phr1/phl1* seedlings grown during 7 days under +Pi and –
620 Pi; n=2-3.

621 (D) RTqPCRs of roots of WT and *spx1/spx2* seedlings grown during 7 days under +Pi or -Pi,
622 and supplemented with Pi for 30 or 60 minutes; n=3-5.

623 (E) Kinetic of luminescence measurements in pUNI1::LUC transgenic seedlings between –Pi
624 and resupplied sample, value are relative to –Pi; n=5.

625 For all RTqPCR experiments *TUBULIN* was used as a housekeeping reference gene for
626 normalization. Values are log₁₀ relative expression levels (REL) normalized to 1 for -Pi levels
627 at time zero represented. Different letters indicate significantly different means (SNK one-
628 way ANOVA, p<0.05, Rstudio). Error bar represent standard deviation and n is the number of
629 biological replicates used for RNA extraction.

630

631 **Figure 2: Validation of pSPX1::MS2x128 transgenic plants.**

632 (A) Principle of the MS2-MCP system. The transgene is under the control of the *SPX1*
633 promoter and synthesizes a reporter RNA bearing 128 MS2 stem-loops, which are recognized
634 by the MCP protein fused to a fluorescent marker (eGFP).

635 (B) Microscopy images of squashed root caps of the transgenic S line expressing
636 pSPX1::MS2x128 and processed for smFISH with probes hybridizing to the MS2x128
637 sequence. Left: Pi depleted sample (-Pi) and zoomed over the boxed area in the middle
638 panels. Right: sample grown on Pi rich medium (+Pi). Top: smFISH signals; bottom: smFISH
639 signals colored in red and merged with Dapi (blue). Images are maximal projections of z-
640 stacks (widefield microscopy). Scale bars: 40 μm (left and right panels), and 4 μm (middle
641 panels). Transcription sites and single RNA molecules are indicated by orange and pink
642 arrows, respectively.

643 (C) Legend as in B except that smFISH was performed with probes hybridizing against the
644 endogenous *SPX1* mRNAs in WT squashed roots in the cap (right panel) and mature tissue
645 (left panel). Scale bars: 4 μm .

646 (D) Bar plot depicting the number of cells expressing the endogenous *SPX1* mRNA (orange,
647 WT plants), or the pSPX1::MS2x128 reporter RNA (blue, S plants) in root (data for root cap
648 and mature root area are combined), with (+Pi) or without (-Pi) phosphate. Mean and standard
649 deviation estimated from 12 fields of view for *SPX1* probes (n=300) and 23 for MS2 probes
650 (n=3087). Expressing cells are defined here as cells having at least 20 RNA molecules in the
651 nucleus or the cytoplasm.

652

653 **Figure 3: imaging *SPX1* transcription in fixed plant tissues reveals allelic differences in**
654 **the root cap and polyploid expression in mature tissue.**

655 (A) Organization of Arabidopsis root, with the name of the various root parts and tissues
656 overlaid on a microscopy mosaic image of a homozygous S plant with MCP-GFP (green)
657 and DNA (blue). Inset: ploidy of the root tissue. Note that a viewer with a zoomable high
658 resolution version of the mosaic can be accessed at
659 <https://imjoy.io/lite?plugin=muellerflorian/hani-ms2:hani-ms2-sample-3>.

660 (B) Images are maximal image projections of z-stack from a root cap of a homozygous S
661 plant grown without Pi and imaged by spinning disk microscopy in three colors. Left (and red
662 in the Merge panel): smFISH signals obtained with probes against the MS2 repeat; middle
663 (and green in the Merge panel): MCP-GFP signals. Blue: nuclei stained with Dapi. The
664 bottom panels are zooms of the boxed area in Merge panel. Pink arrows: single RNA
665 molecule; orange arrows: transcription sites. Scale bar: 100 μm (top panels) and 10 μm
666 (bottom panels).

667 (C) Legend as in B, except that a mature part of the root is imaged. Note that images in A, B
668 and C come from different plantlets.

669

670 **Figure 4: quantitation of *SPX1* transcription in fixed cells gives insights into**
671 **transcription dynamics, ploidy and intrinsic vs. extrinsic noise.**

672 (A) Graph depicting the distribution of the brightness of active transcription sites for the
673 endogenous *SPX1* gene (left), or the SPX1-MS2x128 reporter (right) both detected by
674 smFISH. Brightness values are expressed in number of full-length RNA molecules (x-axis),
675 and the y-axis represent the number of cells with these values.

676 (B) Model of *SPX1* transcription, based smFISH data labelling the endogenous *SPX1*
677 mRNAs.

678 (C) Graph depicting the number of active transcription sites per cell for the SPX1-MS2x128
679 reporter detected by smFish on 4 plants (488 nuclei) for mature tissue and 3 plants (641
680 nuclei) for root cap. x-axis: number of active transcription sites per cell; y-axis: fraction of
681 cells with these values. Orange bars: diploid root cap cells; blue bars: differentiated, polyploid
682 cells. Error bars represent standard deviation.

683 (D) Graph depicting the number of active transcription sites per cell for the endogenous *SPX1*
684 mRNA detected by smFish on 9 plants (300 nuclei) for mature and 3 plants (137 nuclei) for
685 root cap. Legend as in C.

686 (E) Histogram depicting the distance distribution between active transcription sites in single
687 cells, for the endogenous *SPX1* gene and for polyploid cells having 4 or more sites. Distances
688 are expressed in μm (x-axis), and the y-axis represent the number of cells with these values.
689 Pink curve: distance distribution obtained by simulating a random location of 8 spots within
690 Arabidopsis nuclei.

691 (F) Image is a maximal image projection of a z-stack from a differentiated root cell labelled
692 by smFISH with probes against the endogenous *SPX1* mRNA (widefield microscopy). Red:
693 smFISH signals; blue: nucleus stained with Dapi. Scale bar: 5 microns.

694 (G) Graph depicting the correlation of activities of *SPX1* alleles, in cells having one or two
695 active transcription sites. x-axis: number of molecules at the first transcription site; y-axis:
696 number of molecules at the second transcription site.

697 (H) Graph depicting the levels of total, intrinsic and extrinsic noise for cells with either 2
698 active transcription sites (left), or 3-4 (right). Error bars represent the error estimated by
699 bootstrapping (see Methods).

700

701 **Figure 5: combining microfluidic and MS2 technology reveals the fast transcriptional**
702 **repression triggered by Pi supply in pSPX1::MS2x128 transgenic plants.**

703 (A) Principle of experiment combining RootChip microfluidic system with spinning disk
704 microscopy to analyze the transcriptional response to Pi refeeding in real time.

705 (B) Image is a maximal image projection from a time-lapse movie recorded in 3D (200 z-
706 planes), which displays MCP-GFP fluorescence in S plants at the start of Pi refeeding ($t=0$).

707 Scale bar: 100 μm . Inset: identical image with a higher contrast to display the tissue structure.

708 (C) Images are maximal image projection from the time-lapse movie shown in B, taken at the
709 indicated time points after Pi refeeding and displaying MCP-GFP fluorescence. Scale bar: 10
710 μm .

711

712 **Figure 6: the SPX1 promoter generates bursts of activity in root cap cells grown at**
713 **steady-state in absence of phosphate.**

714 (A) Image is a maximal image projection from a time-lapse movie recorded in 3D (44 z-
715 planes 600 nm apart), which displays MCP-GFP fluorescence in S plants. Scale bar: 20 μm .

716 (B and C) Images are maximal image projection (MIP) on XY (A) or XZ (B) axes from the
717 time-lapse movie shown in A, taken at the indicated time points and displaying MCP-GFP
718 fluorescence. Arrows pinpoint transcription sites where the signal remains constant (green),
719 increases (blue) or turns off and then on (orange) during time-laps. Scale bar: 10 μm .

720 (D) Images are maximal image projection (MIP) on XY (A) or XZ (B) axes of a nucleus
721 where transcription decreases at transcription site (orange arrow) and individual mRNA
722 released can be seen (pink arrows). Scale bar: 5 μm .

723

724 **Figure 7: analysis of transcription site activity following Pi supply in pSPX1::MS2x128 S**
725 **line transgenic plants.**

726 (A) Intensity of fluorescence signals at transcription sites in arbitrary units (a.u.), and
727 recorded every 3 minutes after Pi refeeding, for cells exhibiting one or two active
728 transcription sites (n=22) recorded in 14 mature root cells located 1mm above the root tip.

729 (B) Intensity of fluorescence of transcription sites over time, for 8 cells exhibiting two active
730 transcription sites (plotted in same color).

731 (C) Normalized intensities of transcription sites over time, for the same cells as in A.
732 Normalization is done according the value measured at $t=0$.

733 (D) Average normalized intensities of transcription site, for the 22 transcription sites shown in
734 panels A and B (mean and standard deviation).

735 (E) Same average curve of normalized transcription site intensities as (D) obtained with
736 independent plant samples.

737 (F) Average curve obtained by analysis of 67 transcription sites analyzed on 5 plants.

738 (G) Intensity of fluorescence signals at transcription sites in arbitrary units (a.u.), and
739 recorded every 3 minutes for cells exhibiting one or two active transcription sites of plant
740 supplied with Pi depleted nutrient solution, (n=14)

741 (H) Normalized intensities of transcription sites over time, for the same cells as in G.
742 Normalization is done according the value measured at t=0. Standard deviation is provided
743 (n=14).

744

745 **Supplemental Figure 1: identification of genes rapidly responding to Pi resupply.**

746 (A) List of the fastest genes (presenting a $|\text{Fold Change (Log}_2)| > 1$) common in all the
747 replenish RNAseq points in roots (30, 60 and 180 min), accompanied with the values obtained
748 in shoots. Their possible regulation by PHR1/PHL1 has also been tested: presence of P1BS
749 box 3kb upstream or 3 Kb downstream, and misregulation in *phr1/phl1* double mutant (data
750 from Bustos et al., 2010, or analyzed by RTqPCRs and labelled* (data not shown).

751 (B-C-D) RTqPCR analysis of the effect of Pi replenishing for different markers WT (B-C-D)
752 or transgenic pSQD2::LUC (C) or p35S::SPX1-GFP (D) lines. In the p35S::SPX1-GFP line,
753 repression triggers by Pi resupply does not affect SPX1-GFP transcript whereas SPX3 mRNA
754 (close homologue of SPX1, identified as early responding genes in the S1A list) remains
755 strongly regulated as observed in the WT background.

756 The X-axis legend refers for (B) to markers analyzed in the WT background, whereas for (C
757 and D) it refers to genetic background used (WT or transgenic), markers analyzed (LUC,
758 GFP, SPX3) are mentioned above the graphs.

759 *TUBULIN* was used as housekeeping gene. Values are \log_{10} relative expression to -Pi levels
760 (REL), which are normalized to 1; n=3-5. Different letters indicate significantly different
761 means (SNK one-way ANOVA, $p < 0.05$, Rstudio). Error bar represent standard deviation and
762 n the number of biological replicates used for RNA extraction.

763

764 **Supplemental Figure 2: implementation and validation of the MS2 system in plants.**

765 (A) Moclo vectors adapted to the Golden Gate system and cloning strategy used to introduce
766 the MS2 system into plants.

767 (B) Images are maximal image projections of microcopy images from Arabidopsis roots
768 grown in Pi depleted medium and processed for smFISH with probes hybridizing against the
769 endogenous *SPX1* mRNAs. Top: SmFISH signal; bottom: merge with Dapi (blue); right
770 panels: zoom over the boxed area. Scale bars: 40 μm (left), and 4 μm (right). Pink arrows:
771 single RNA molecules; orange arrows: transcription sites.

772 (C) Graph depicting the distribution of the brightness of active transcription sites for the
773 endogenous *SPX1* gene, for cells having 1 or 2 transcription sites (yellow bars), or more than
774 3 (blue bars). Brightness values are expressed in number of RNA molecules (x-axis), and the
775 y-axis represents the frequencies of these values. a.u.: arbitrary units.

776

777 **Supplemental Figure 3: genetic and molecular analysis of selected lines (J, S) expressing**
778 **the pSPX1:MS2x128 transgene.**

779 (A) Segregation analysis of Kanamycine resistance in the T2 progeny. Seeds were germinated
780 on Hoagland/2 medium supplemented with kanamycin (50 mg/l). (+) = growth, (-) = death. c^2

781 values are provided for theoretical segregation ratio (3:1). The critical value for a p level of
782 0.05 is 3.84).

783 (B) Molecular analysis by qPCR of T-DNA presence in the Col, S and J lines. Set of primers
784 used are described in the Supplemental Table1 to amplify GFP, MCP and the SPX1 promoter
785 (the only gene present in all lines). Values are presented as relative amounts (REL)
786 normalized to S line (100%). The endogenous SPX1 gene was used as reference gene to
787 normalize the DNA amount between samples.

788

789 **Supplemental Figure 4: the RNA produced by the SPX::MS2x128 transgene is capped**
790 **and polyadenylated.**

791 RT-PCR amplification of the MS2x128 and endogenous SPX1 RNA after total RNA
792 immunoprecipitation using m7G antibody (m7G-RIP). After RIP, RNAs were reverse-
793 transcribed using oligo(dT) prior to PCR amplification. Sets of primers targeting transgene or
794 endogenous gene were used. Wild-type (Col0) and two different transgenic lines (S and J)
795 were used. As negative control, total RNAs were treated with pyrophosphatase to remove the
796 cap prior to RIP experiment and RT-PCR. I : Input fraction. E : Eluate fraction. L : Ladder.

797

798 **Supplemental Figure 5: MS2 smFISH in control Col0 plants and homozygous**
799 **pSPX1:MS2x128 S line grown without Pi.**

800 (A) Microscopy images of squashed root caps of Col0 (right two panels) and transgenic S line
801 (left two panels), grown without Pi and processed for smFISH with probes hybridizing to the
802 MS2x128 sequence. Top: smFISH signals; bottom: smFISH signals colored in red and
803 merged with Dapi (blue). Images are maximal projections of z-stacks (widefield microscopy).
804 Scale bars: 40 μm (1st and 3^d panels), and 4 μm (2^d and 4th panels, zooms corresponding to the

805 boxed area of the other panels). Transcription sites and single RNA molecules are indicated
806 by orange and pink arrows, respectively.

807 (B) Legend as in A, but imaging was done on root mature tissue.

808

809 **Supplemental Figure 6: SPX1 smFISH in Col0 and control spx1/spx2 mutant plants**
810 **grown without Pi.**

811 (A) Microscopy images of squashed root caps of Col0 (left two panels) and spx1/spx2 control
812 plants (right two panels), grown without Pi and processed for smFISH with probes
813 hybridizing to the SPX1 mRNA. Top: smFISH signals; bottom: smFISH signals colored in
814 red and merged with Dapi (blue). Images are maximal projections of z-stacks (widefield
815 microscopy). Scale bars: 40 μm (1st and 3^d panels), and 4 μm (2^d and 4th panels, zooms
816 corresponding to the boxed area of the other panels). Transcription sites and single RNA
817 molecules are indicated by orange and pink arrows, respectively.

818 (B) Legend as in A, but imaging was done on root mature tissue.

819

820 **Supplemental Figure 7: imaging pUnicorn1::MS2x128 and pSPX1:MS2x128 J line in**
821 **fixed plant tissues.**

822 (A) Microscopy images of squashed mature tissue of the homozygous J line expressing
823 pSPX1::MS2x128 grown without Pi and processed for smFISH with probes hybridizing to the
824 MS2 sequence. Images are maximal image projections of z-stacks taken in three colors. Left
825 (and red in the Merge panel): smFISH signals obtained with probes against the MS2 repeat;
826 middle (and green in the Merge panel): MCP-GFP signals. Blue: nuclei stained with Dapi.
827 The bottom panels are zooms of the boxed area in Merge panel. Orange arrows: transcription
828 sites. Scale bars: 40 μm (top panels) and 4 μm (bottom panels).

829 (B) Legend as in B, except that a pUnicorn1::MS2x128 transgenic plant was imaged.

830

831 **Supplemental Figure 8: combining microfluidics and MS2 technology reveals the fast**
832 **transcriptional repression triggered by Pi supply in the S line.**

833 (A) Image is a maximal image projection from a time-lapse movie recorded in 3D (200 z-
834 planes) in the pSPX1::MS2x128 S line, and displays MCP-GFP fluorescence in plants at the
835 start of Pi refeeding ($t=0$; set when the Synaptored dye is detected). Scale bar: 50 μm . Inset:
836 identical image with a higher contrast to display the tissue structure.

837 (B and C) Images are maximal image projection from the time-lapse movie shown in A, taken
838 at the indicated time points after Pi refeeding and displaying MCP-GFP fluorescence. Scale
839 bar: 10 μm .

840

841 **Supplemental Figure 9: number of active transcription sites before and following Pi**
842 **resupply in the pSPX1::MS2x128 S line.**

843 (A) During time lapse, the root is growing and moving in the microfluidic channel of the chip
844 and some nuclei get out of focus. The blue curve depicts the number of nuclei still tracked at
845 each time point (normalized to the initial number of nuclei; $n_n=113$). The orange curve
846 indicated the number of active transcription sites detected in those nuclei, normalized to
847 number of transcription sites present when the recording started ($n_t=107$).

848 (B) Biological replicates of (A) with $n_n= 62, 66, 94, 81$ and $n_t= 49, 72, 45, 47$ for plants 2 to 5
849 respectively.

850

851 **Supplemental Figure 10: transcription site activity following Pi resupply in the**
852 **pSPX1::MS2x128 J line transgenic plants.**

853 (A) Normalized intensities of fluorescence signals at transcription sites over time, recorded
854 every 3 minutes after Pi refeeding, for cells exhibiting one or two active transcription sites
855 (n=16). Normalization is done according to the value measured at t=0.

856 (B) Average normalized intensities of transcription sites, for the 16 transcription sites shown
857 in panel A (mean and standard deviation).

858 (C) Normalized intensities of fluorescence signals at transcription sites over time, and
859 recorded every 3 minutes after Pi refeeding, for cells exhibiting one or two active
860 transcription sites (n=13) in an independent plant than in (A). Normalization is done
861 according to the value measured at t=0

862 (D) Average normalized intensities of transcription sites, for the 13 transcription sites shown
863 in panel B (mean and standard deviation).

864

865 **Supplemental Figure 11: transcription site activity upon continuous exposure to Pi**
866 **depleted medium in pSPX1::MS2x128 S line transgenic plants.**

867 (A) Maximal image projection from a time-lapse movie recorded in 3D (200 z-planes) and
868 displaying MCP-GFP fluorescence in S plants in steady-state conditions (-Pi condition) from
869 a mature root area. Scale bar: 100 μ m. Inset: identical image with a higher contrast to display
870 the tissue structure.

871 (B) The panels are zooms of the boxed area from (A) recorded in the time-lapse movie taken
872 at the indicated time points and displaying MCP-GFP fluorescence. Scale bar: 20 μ m.

873

874 **Supplemental Figure 12: location of smFIH oligonucleotide probes on their respective**
875 **targets RNA**

876 (A) SPX1 mRNA.

877 (B) MS2x128 RNA.

878 (C) Post polyA region of the pSPX1::MS2x128 reporter gene.

879

880 **Supplementary Table 1: primer sequences used for RTqPCR and PCR reaction.**

881

882 **Supplementary Table 2: primer sequences for smFISH experiment.**

883 (A) Primers used to detect *SPX1* mRNA. Cy3 fluorescent dye is grafted on amino-modified
884 uridine analogue C6dT (labelled X on the sequence).

885 (B) Primers used in smiFISH to detect the post polyA region of the pSPX1::MS2x128
886 transcripts.

887 (C) Primers used to detect MS2x128 transcripts.

888

889 **Movie 1 and 2: bursting activity of the pSPX1::MS2x128 reporter**

890 Movie of root cap cells of Arabidopsis S line expressing pSPX1::MS2x128 and MCP-GFP,
891 and continuously grown without Pi. Maximal image projection (XY and XZ) are from a time-
892 lapse movie recorded in 3D (44 z-planes). Time (in min) is indicated. Movie 1 illustrates gene
893 bursting while Movie 2 illustrates the release of single RNAs in the nucleoplasm when
894 promoter activity stochastically turns off when a burst ends.

895

896 **Movies 3 to 8: Transcriptional repression of the pSPX1::MS2x128 reporter triggered by**
897 **Pi resupply.**

898 Movie of root cells of Arabidopsis S (2 to 7) and J (8) line transformed with
899 pSPX1::MS2x128 and MCP-GFP, after receiving a Pi rich solution at time t=0 min. Maximal
900 image projection from a time-lapse movie recorded in 3D (200 z-planes). Acquisitions lasted
901 39 min for Movies 3 and Movie 4 (Movie 4 zooms on few cells cropped from Movie 3), 54

902 min for Movie 5 to Movie 7 (Movies 6 and 7 are magnifications deriving from Movie 5), and
903 48 min for Movie 7.

904

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922

923 **Author contributions**

924 E.B. provided MS2 and MCP original constructs and LN conceived the experiments. L.C.,
925 S.H., P.D. performed all the experiments under LN supervision for physiological part and EB
926 supervision for cell biology. RNA seq data were produced by D.S. and J.W. and analyzed by

927 L.N., L.C. M-C.T. and E.M. Luminescence experiments were performed by N.P. under H.J.
928 supervision. H.J. also implemented microfluidic technique in the SAVE team. Manuscript
929 was written by LN and EB with help from S.H., P.D. and L.C.

930

931 **Declaration of Interests**

932 The authors declare no competing interests.

933

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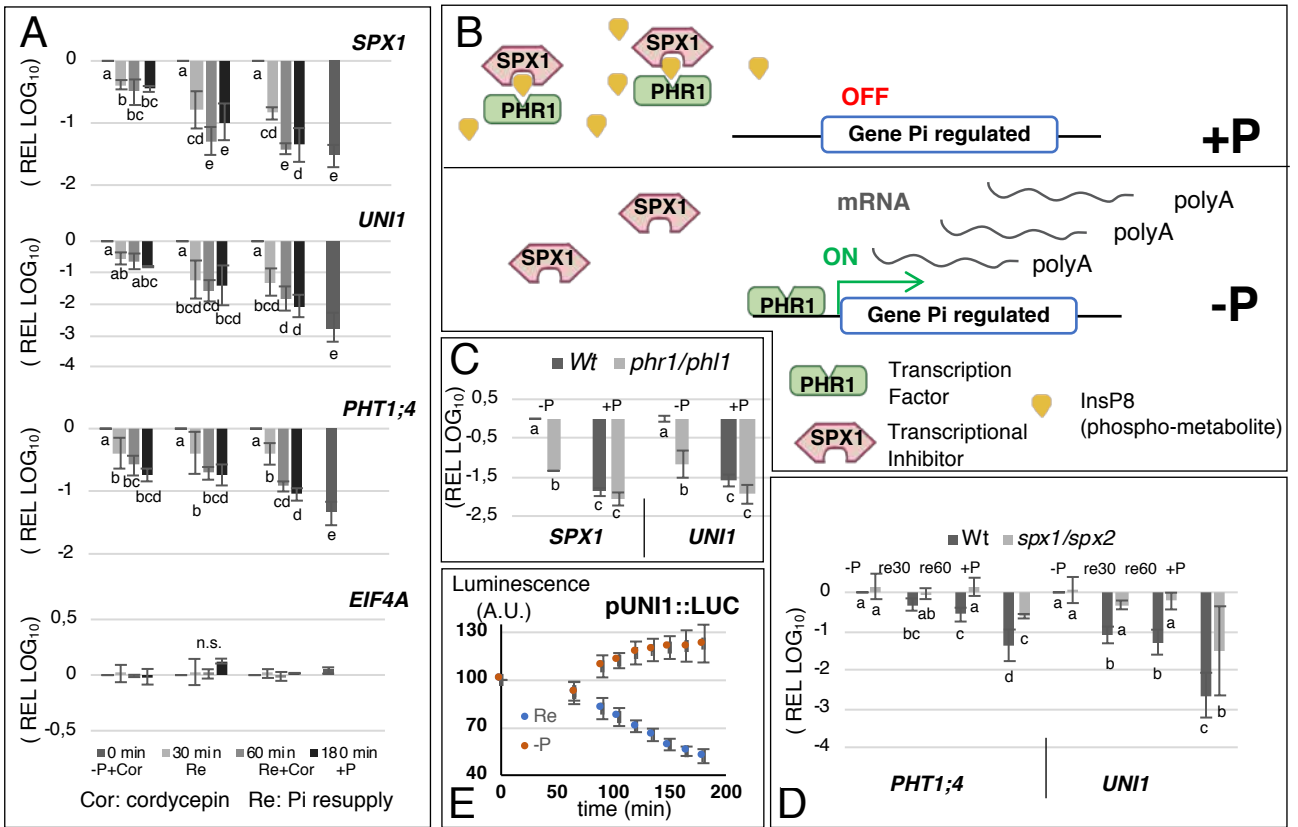
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Figure 1



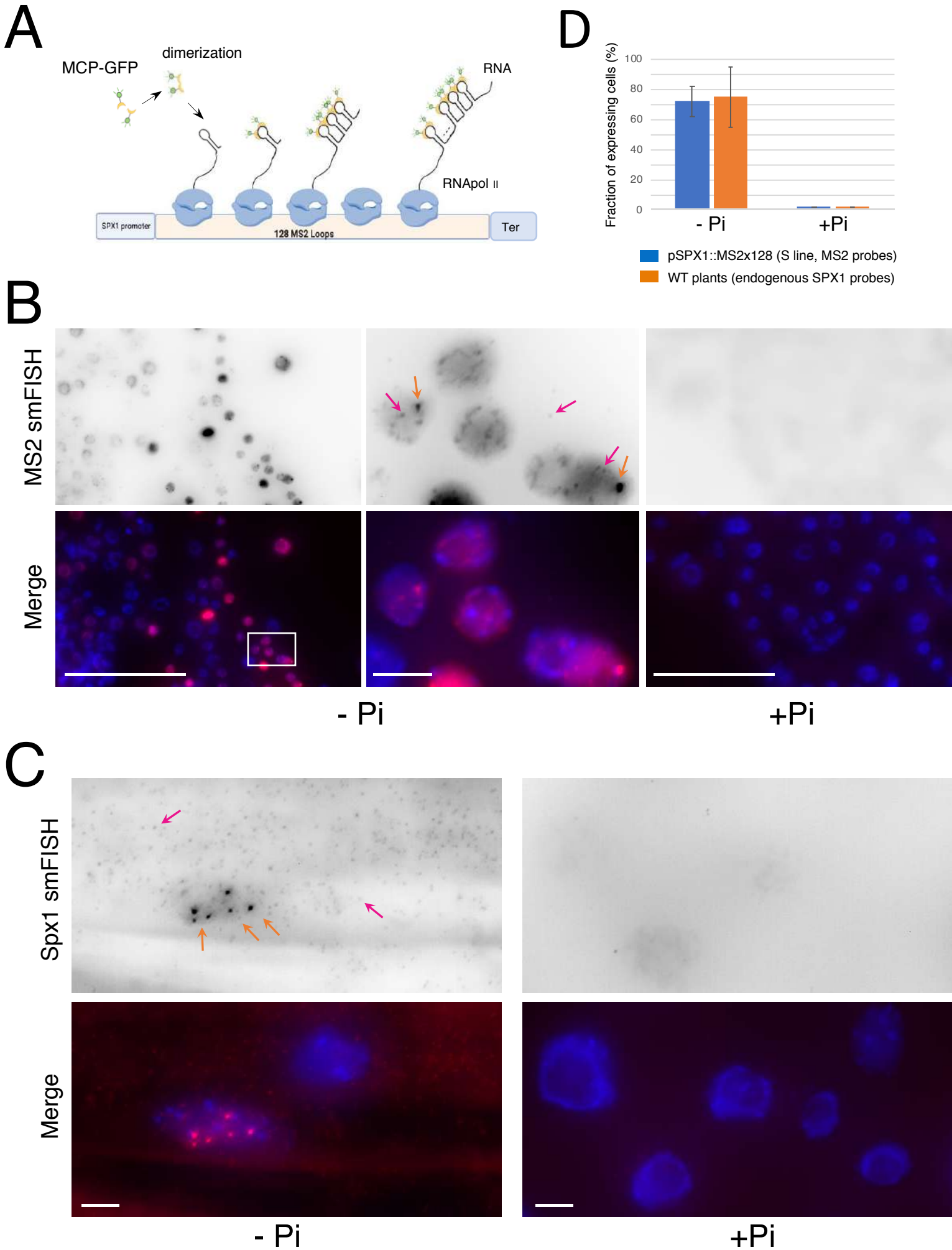


Figure 2

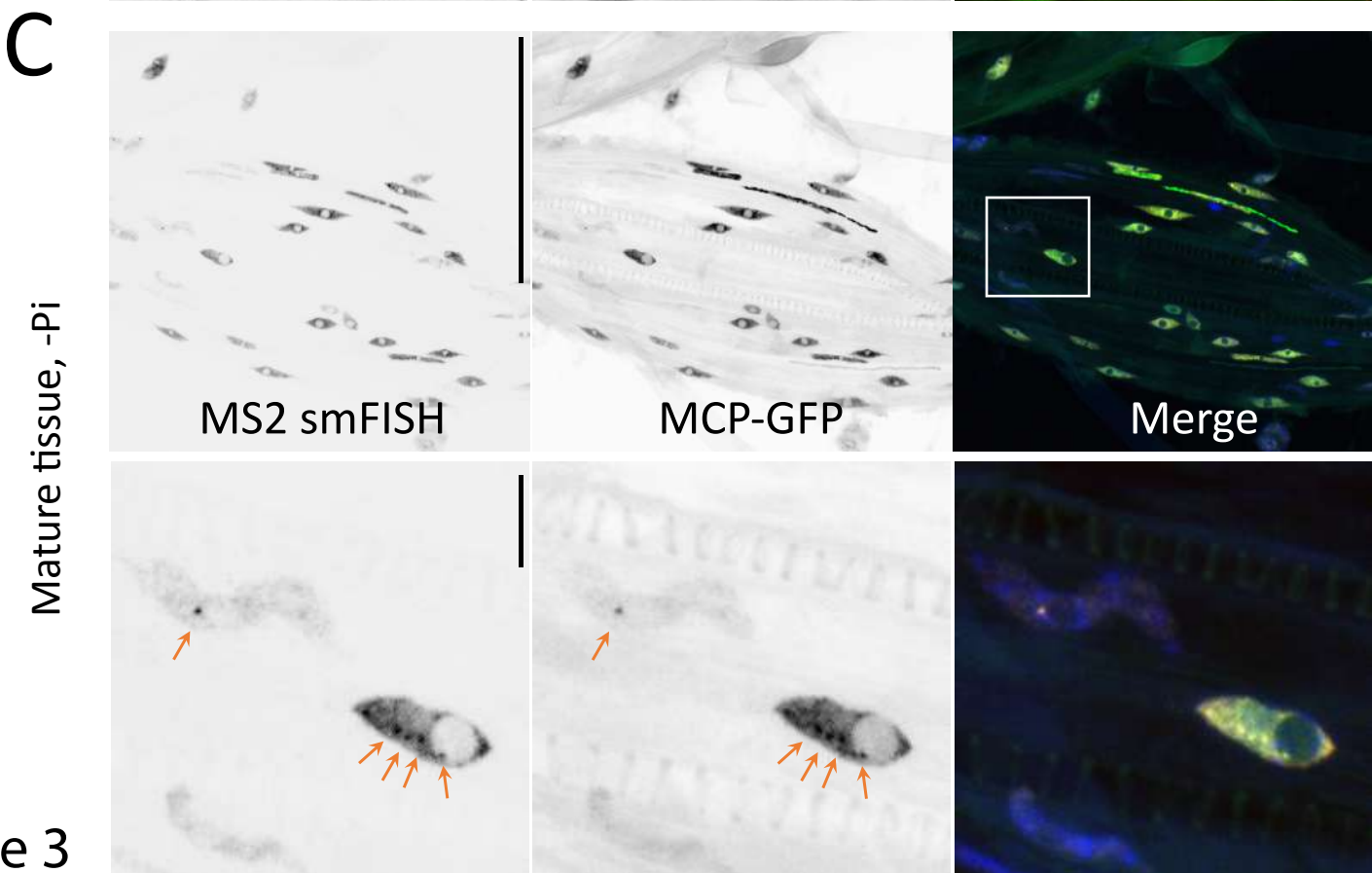
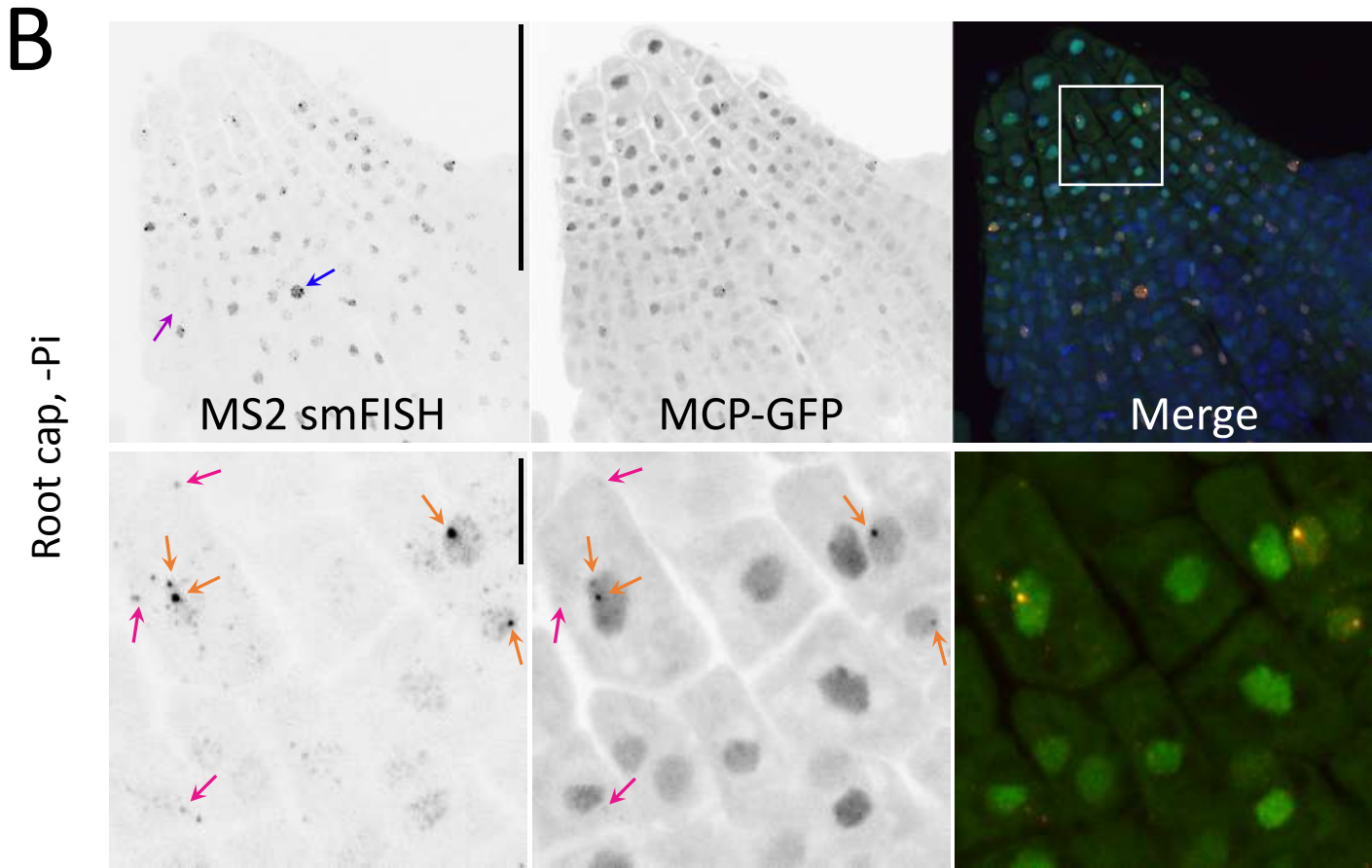
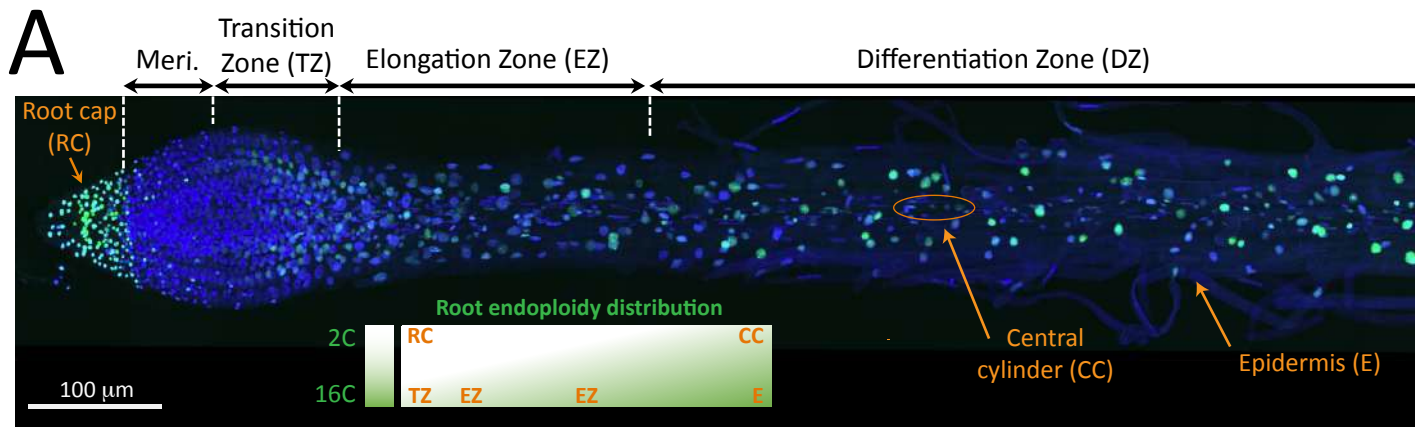


Figure 3

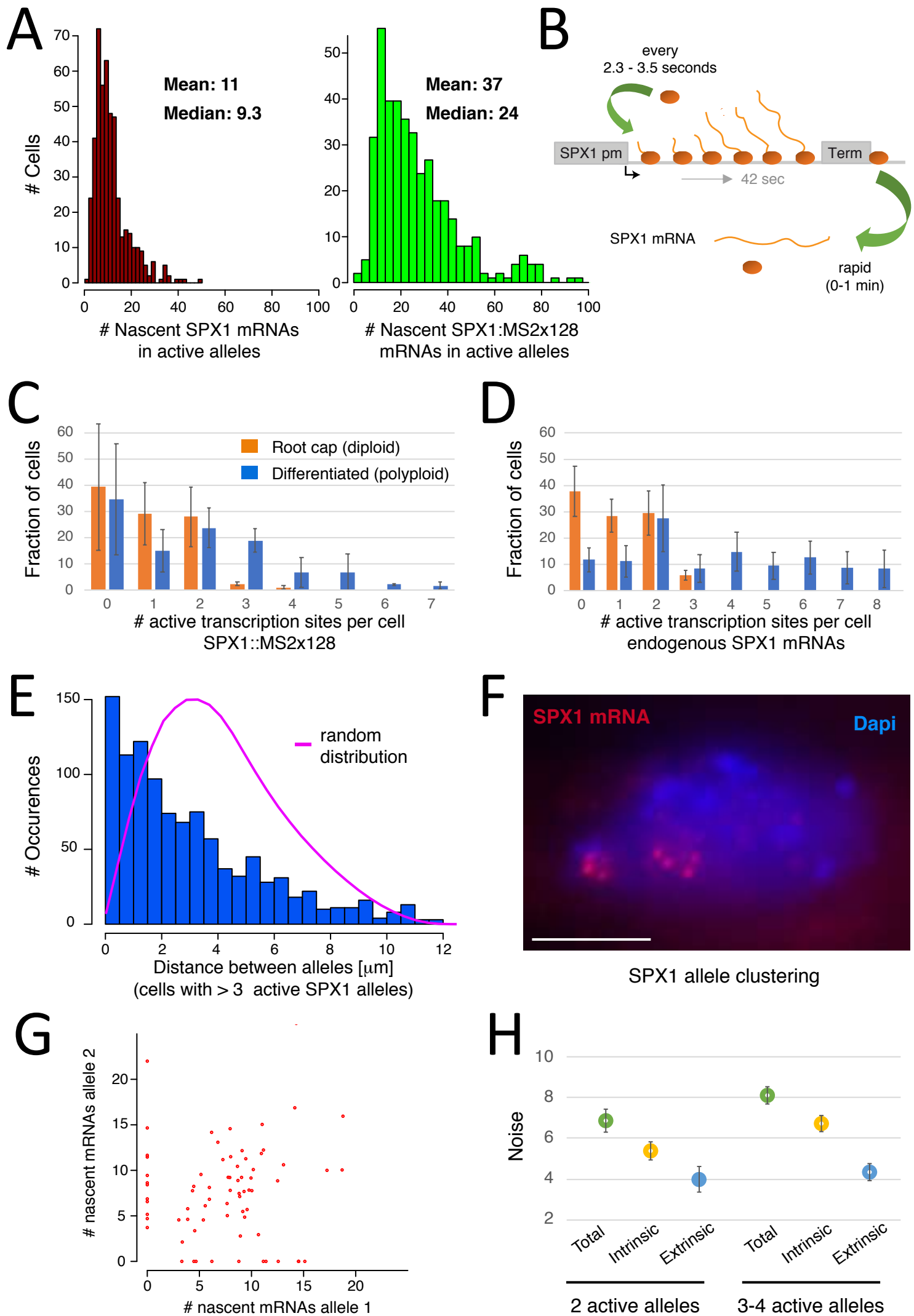


Figure 4

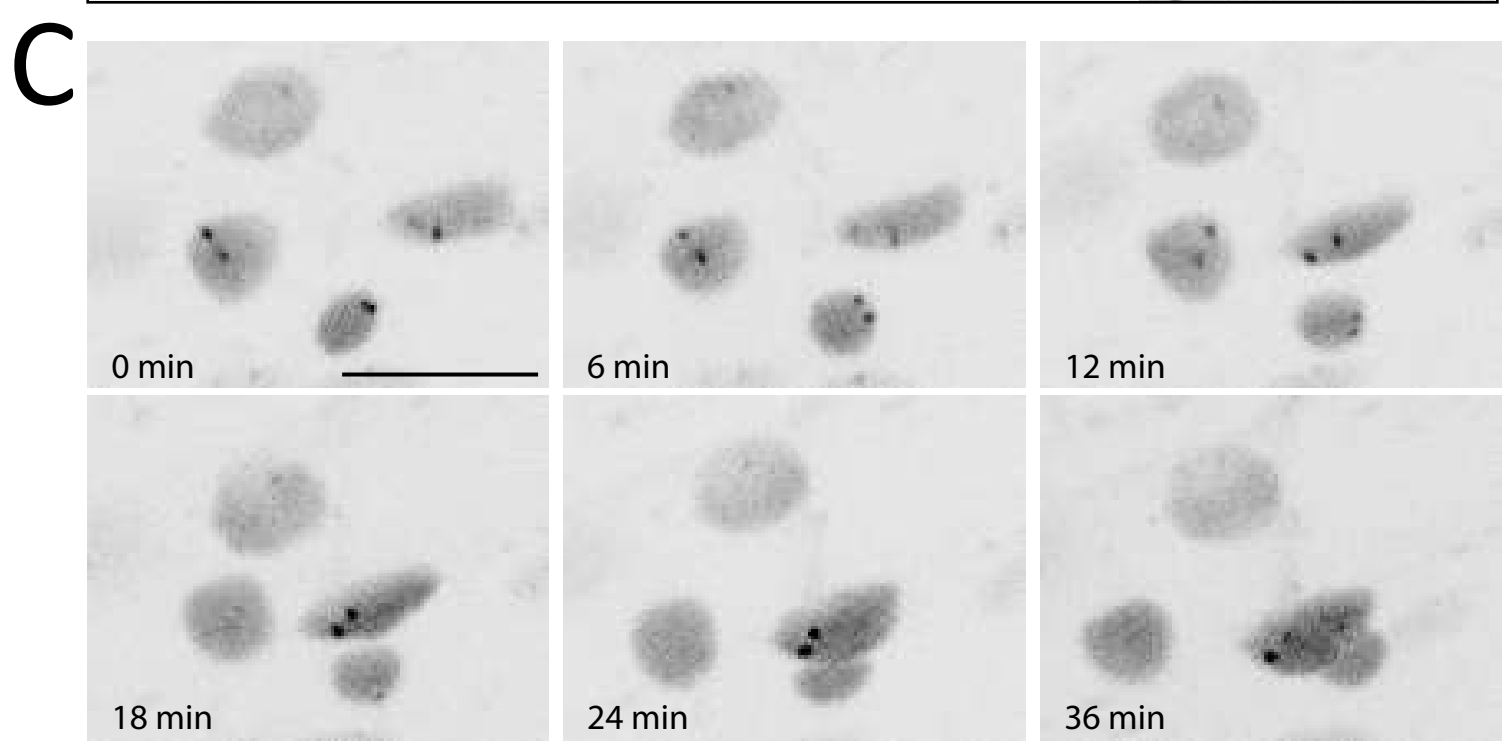
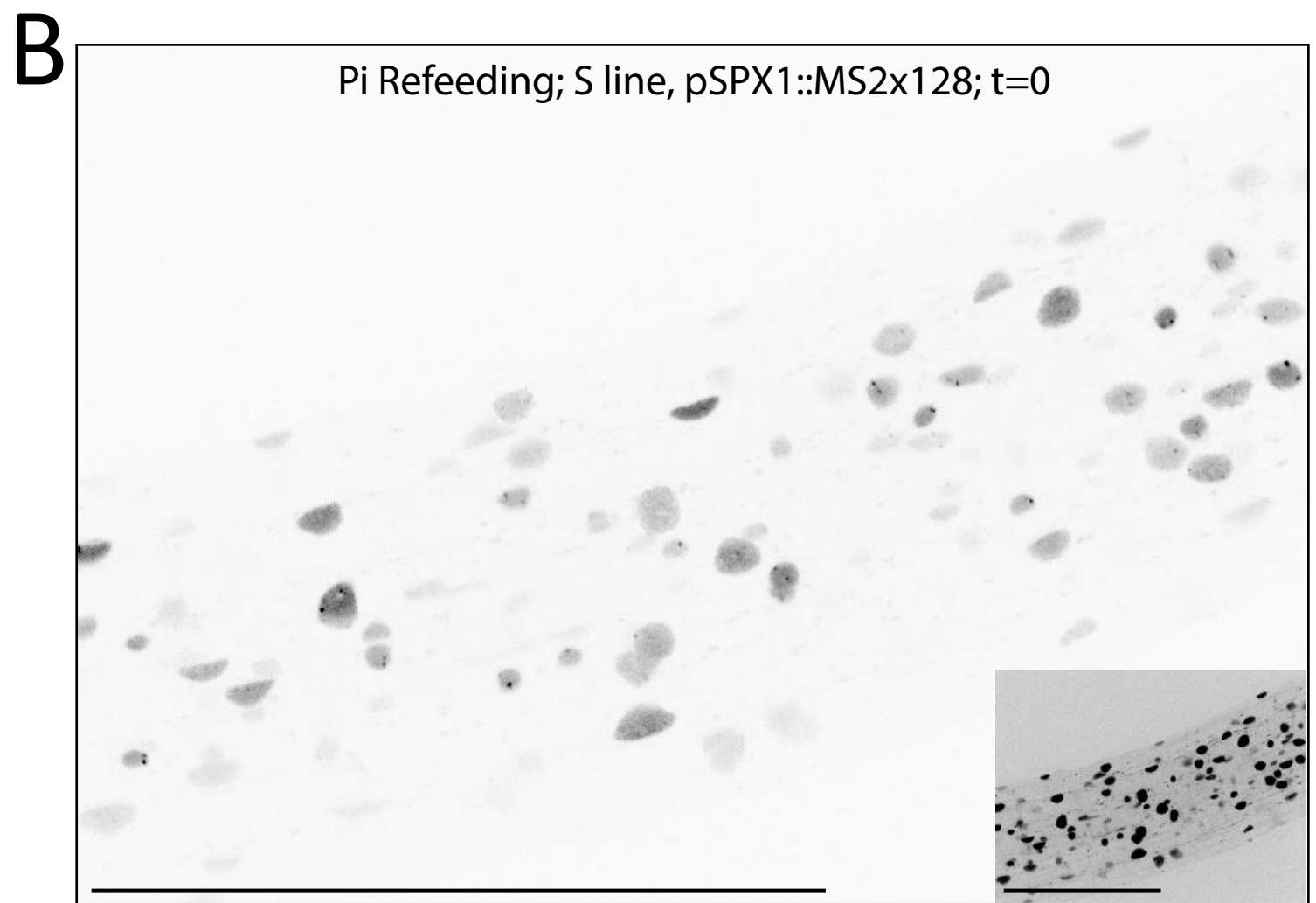
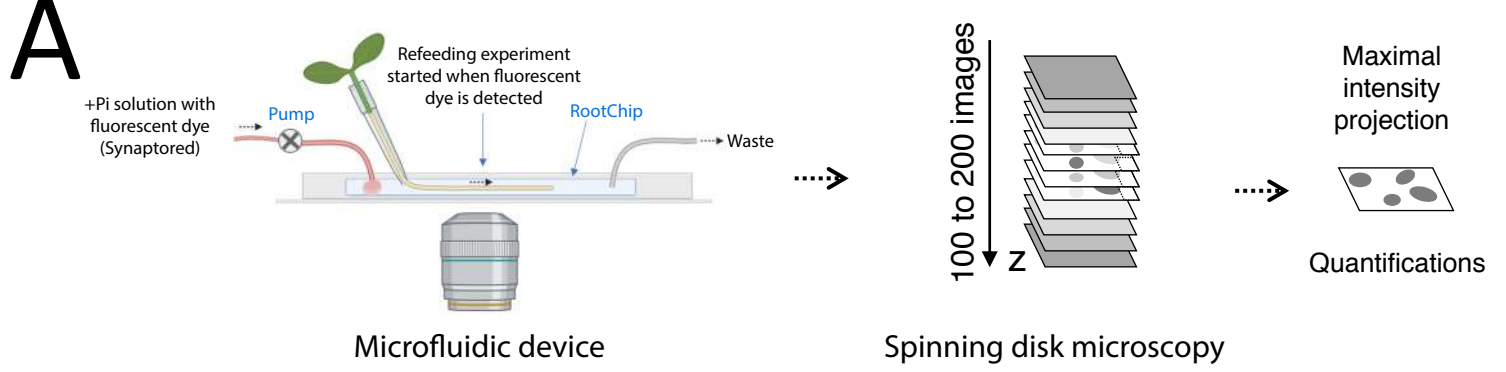


Figure 5

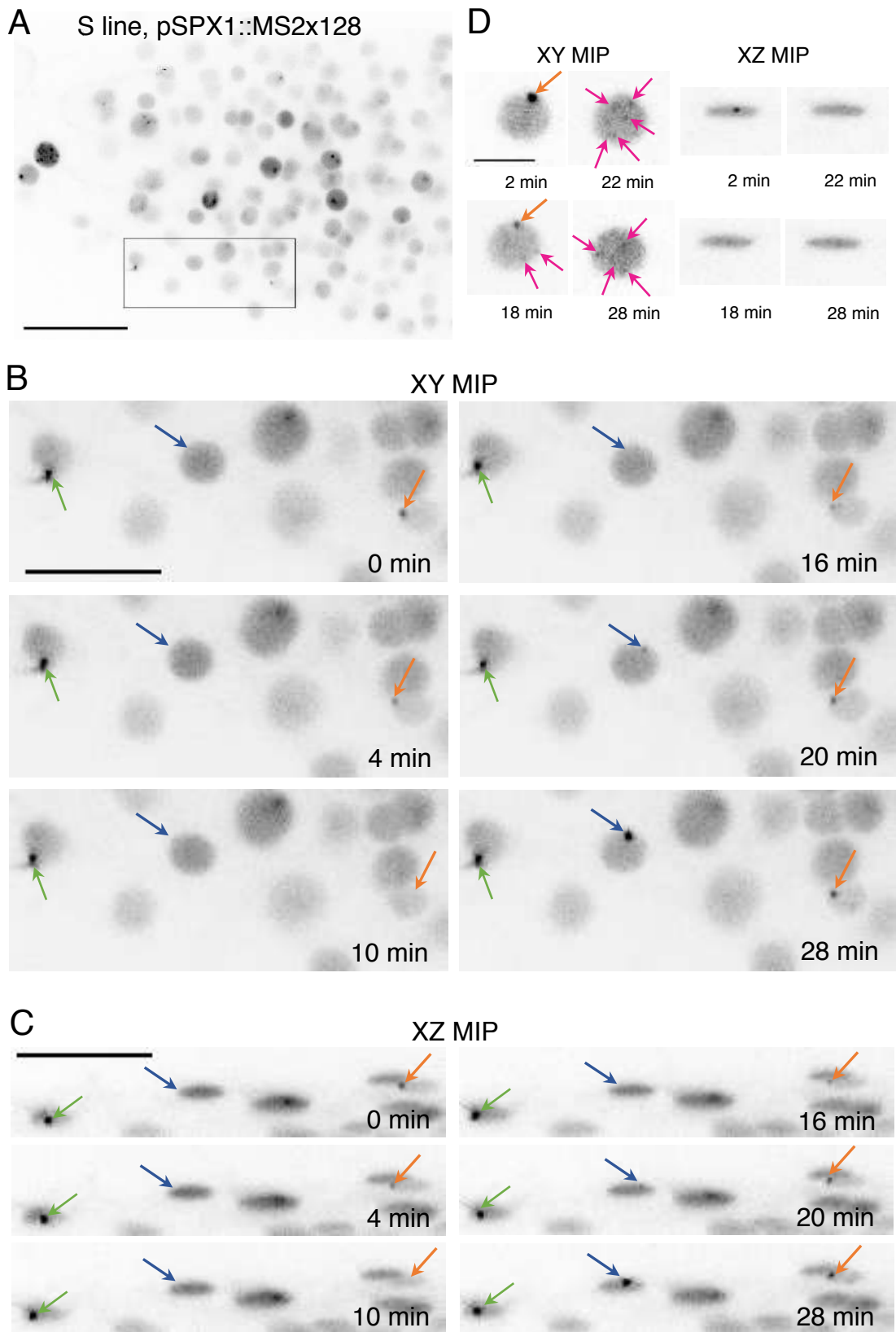
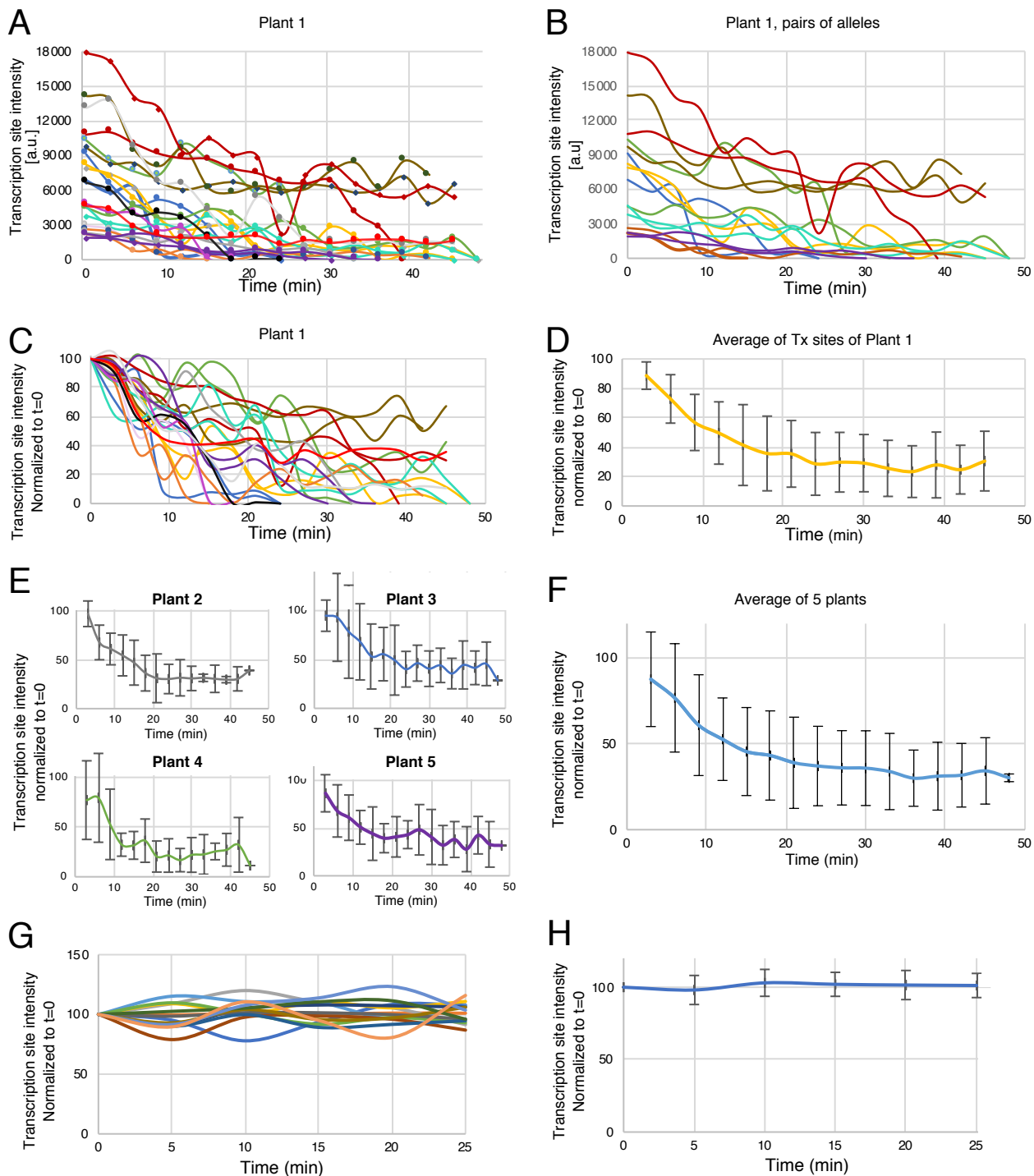


Figure 6



S line, pSPX1::MS2x128

Figure 7