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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

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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 ( $\sim 3$ s) 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 reprogramming<sup>1</sup> 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 analysis<sup>2</sup>. 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 minutes<sup>3</sup>)  
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<sup>4,5</sup>. For instance,  
58 the median half-life value of *Arabidopsis* mRNAs is 107 min, but exceeds one day for many  
59 messengers<sup>4</sup>. This is far too long for transcriptional responses occurring within seconds such  
60 as for instance during light stress<sup>6</sup>.  
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 cells<sup>7-10</sup>. It is  
63 based on a fusion between GFP and the bacteriophage MS2 coat protein (MCP-GFP)<sup>7,8</sup> or on  
64 related systems<sup>11</sup>. 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 visualization<sup>9</sup>. 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 regulation<sup>12</sup>.  
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 sensitivity<sup>13</sup>. 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<sup>14,15</sup>. These are mainly controlled by master regulator genes of the PHR1 family<sup>15-</sup>  
81 <sup>17</sup>. Being constitutive, their regulatory activity relies on inhibitors of the SPX family tuned by  
82 Pi uptake<sup>18,19</sup> as they inhibit PHR1 activity only in the presence of Pi metabolites<sup>20,21</sup>.

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 microfluidics<sup>22</sup>, 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<sup>15,23</sup>, 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 hours<sup>5</sup>. 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 boxes<sup>16</sup> 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<sup>20,21</sup>  
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. S1A<sup>17</sup>). 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 RNA<sup>13</sup>. 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 affinity<sup>13</sup> (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 rates<sup>13</sup>. 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 degradation<sup>24</sup>.

146 We developed Moclo vectors adapted to the Golden Gate system<sup>25</sup> to facilitate the  
147 cloning and use of the MS2x128 recombinogenic sequence (Fig S2A), and we generated  
148 transcriptional fusions with the *SPX1* and *UNI1* 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 protein<sup>13</sup> under the control of the weak constitutive Ubiquitin-10 promoter<sup>26</sup> (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 monolayer<sup>27</sup>, 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 tissues<sup>28</sup>, 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-<br/>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 cells<sup>29</sup>, 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<sup>9,12,30,31</sup>. 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<sup>32,33</sup>. 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 minute<sup>13</sup> (assuming an  
231 elongation rate of 2 kb/min<sup>30</sup>). 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*<sup>13</sup>. 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 on<sup>13</sup>.

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 16C<sup>29</sup>.  
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 epidermis<sup>29</sup> (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 tissue<sup>29</sup> (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 cells<sup>34</sup>.

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 mammals<sup>35</sup>. 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 regulation<sup>12</sup>. This stochasticity causes variations in gene  
280 expression among identical cells (ie. gene expression noise), which has sometimes important  
281 phenotypic consequences<sup>12</sup>. 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<sup>36,37</sup>, 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 version<sup>38</sup> of the RootChip microfluidic system<sup>22</sup> 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 Synaptored<sup>TM</sup> 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 cells<sup>39</sup>. 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<sup>12,40</sup>. 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 lines<sup>41</sup>. 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<sup>10,35</sup>), 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 minute<sup>42</sup>. 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 animals<sup>35</sup>, 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)<sup>29</sup>. 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 territory<sup>43</sup>. 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 cells<sup>34</sup>. 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<sup>32,33</sup>, 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  $\mu\text{M}$  (+P) or 13  $\mu\text{M}$  (-P)  $\text{KH}_2\text{PO}_4$  in a culture chamber under a  
450 16-hr- light/8-hr-dark regime (25°C/22°C), as previously described<sup>44</sup>.

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 cordycepin<sup>45</sup>. For each condition three independent biological replicates were analyzed.  
462 Plant DNA extraction and genetic segregation analysis were performed as previously  
463 described<sup>46</sup>.

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 described<sup>47</sup>. Quantitative RTqPCR experiments were  
469 performed as previously described<sup>48</sup> 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 described<sup>49</sup> 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 technique<sup>25</sup>. 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 bp<sup>28</sup> 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 Dest<sup>26</sup> was assembled with MCP contained in pDONR201<sup>8</sup> 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 published<sup>44</sup>.

497

#### 498 **Production of transgenic lines**

499 Transformants produced by floral dipping<sup>50</sup> 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 repeats<sup>13</sup>. 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/ $\mu$ 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 described<sup>51</sup>. After addition of probes, samples were covered  
526 with a coverslip and remained overnight at 37°C as previously described<sup>51</sup>. 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  $\mu$ m or 0.4  $\mu$ 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  $\mu$ 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 Imjoy<sup>52</sup>. 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 HotSpot<sup>40</sup>. This is a user-friendly MatLab software that allows to quantify smFISH signals in  
550 very noisy images, unlike FISH-quant<sup>53,54</sup>. 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  $\mu m$  and a minor axis length of 6  $\mu m$ ,  
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<sup>36,37</sup>. 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<sup>22,38</sup>, 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. Synaptored<sup>TM</sup>C2 (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 control<sup>45</sup>; 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 log<sub>10</sub> 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  $\mu\text{m}$  (left and right panels), and 4  $\mu\text{m}$  (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  $\mu\text{m}$ .

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  $\mu\text{m}$  (top panels) and 10  $\mu\text{m}$   
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  $\mu\text{m}$  (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  $\mu\text{m}$ . 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  $\mu\text{m}$ .

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  $\mu\text{m}$ .

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  $\mu\text{m}$ .

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  $\mu\text{m}$ .

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  $\mu\text{m}$  (left), and 4  $\mu\text{m}$  (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  $\mu\text{m}$  (1<sup>st</sup> and 3<sup>d</sup> panels), and 4  $\mu\text{m}$  (2<sup>d</sup> and 4<sup>th</sup> 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  $\mu\text{m}$  (1<sup>st</sup> and 3<sup>d</sup> panels), and 4  $\mu\text{m}$  (2<sup>d</sup> and 4<sup>th</sup> 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  $\mu\text{m}$  (top panels) and 4  $\mu\text{m}$  (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  $\mu\text{m}$ . 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  $\mu\text{m}$ .

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  $\mu$ 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  $\mu$ 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  
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930

### 931 **Declaration of Interests**

932 The authors declare no competing interests.

933

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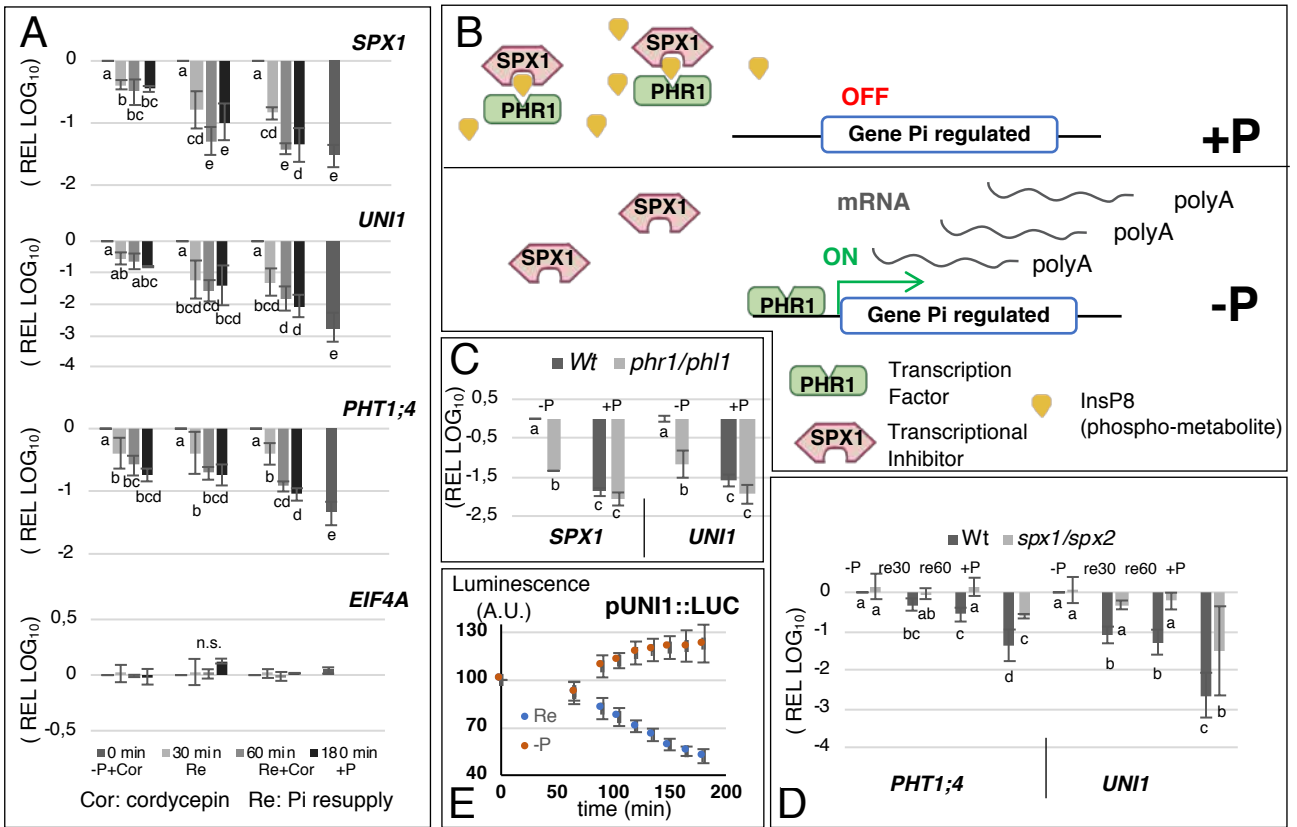
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1085

# Figure 1



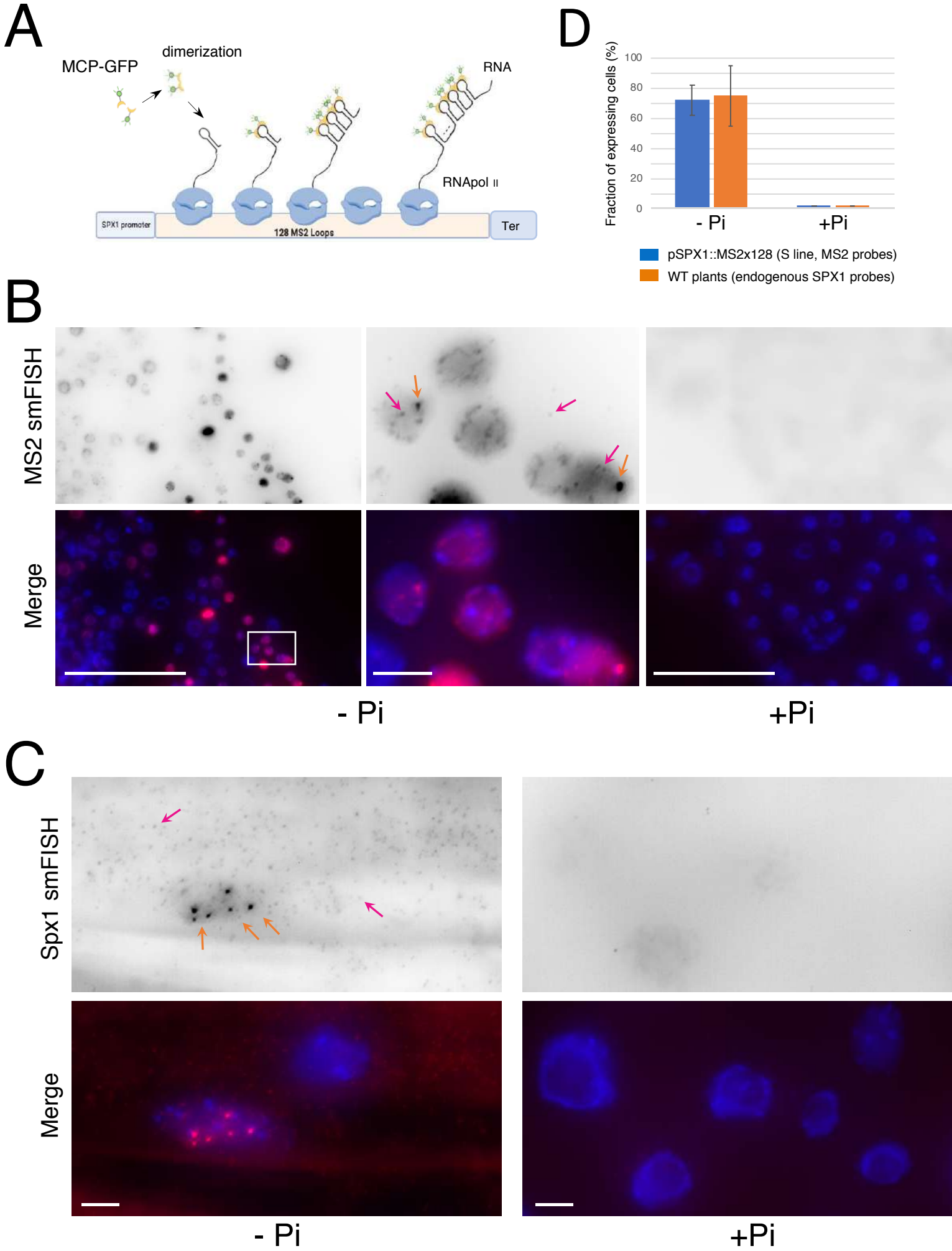


Figure 2

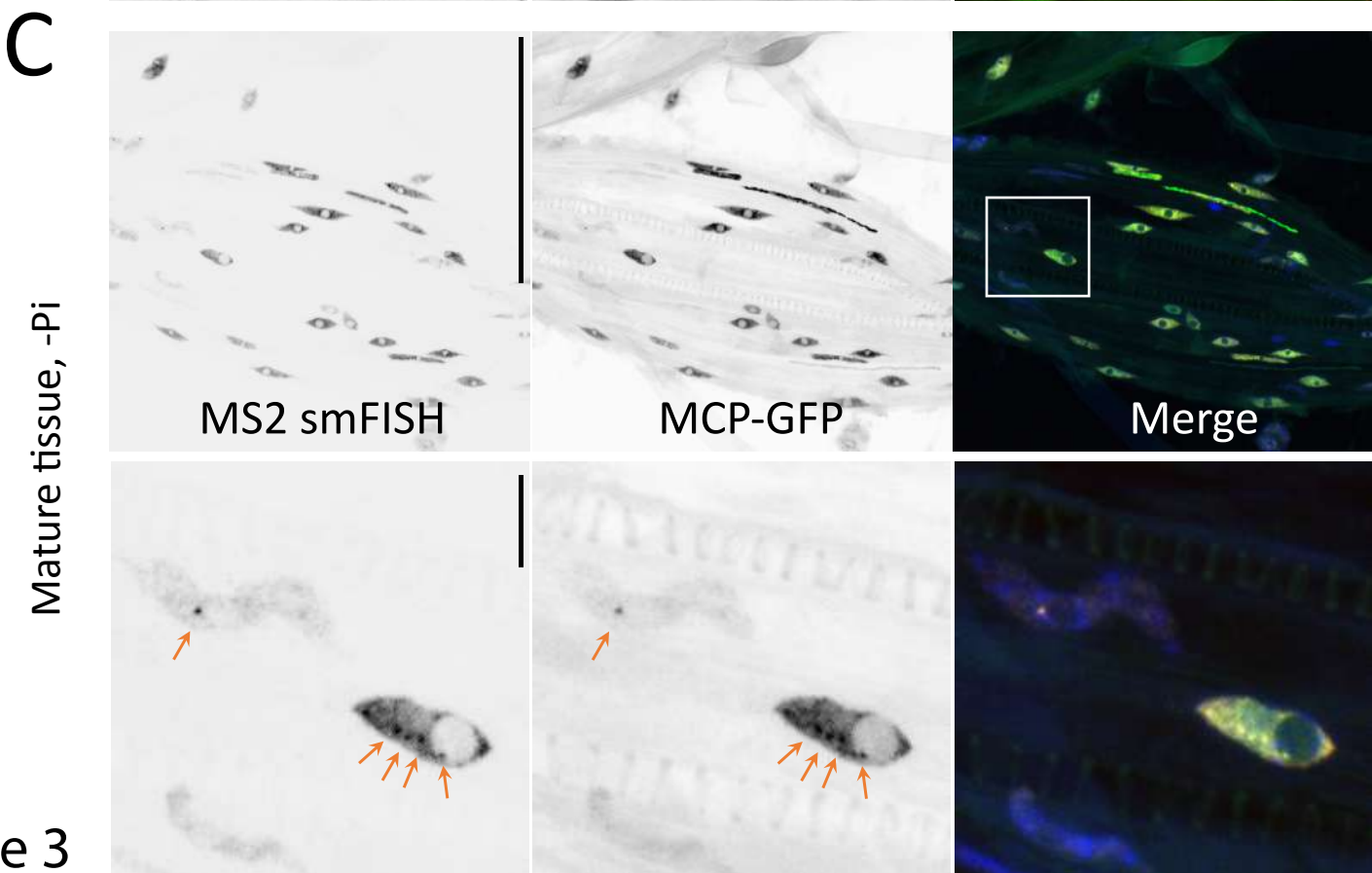
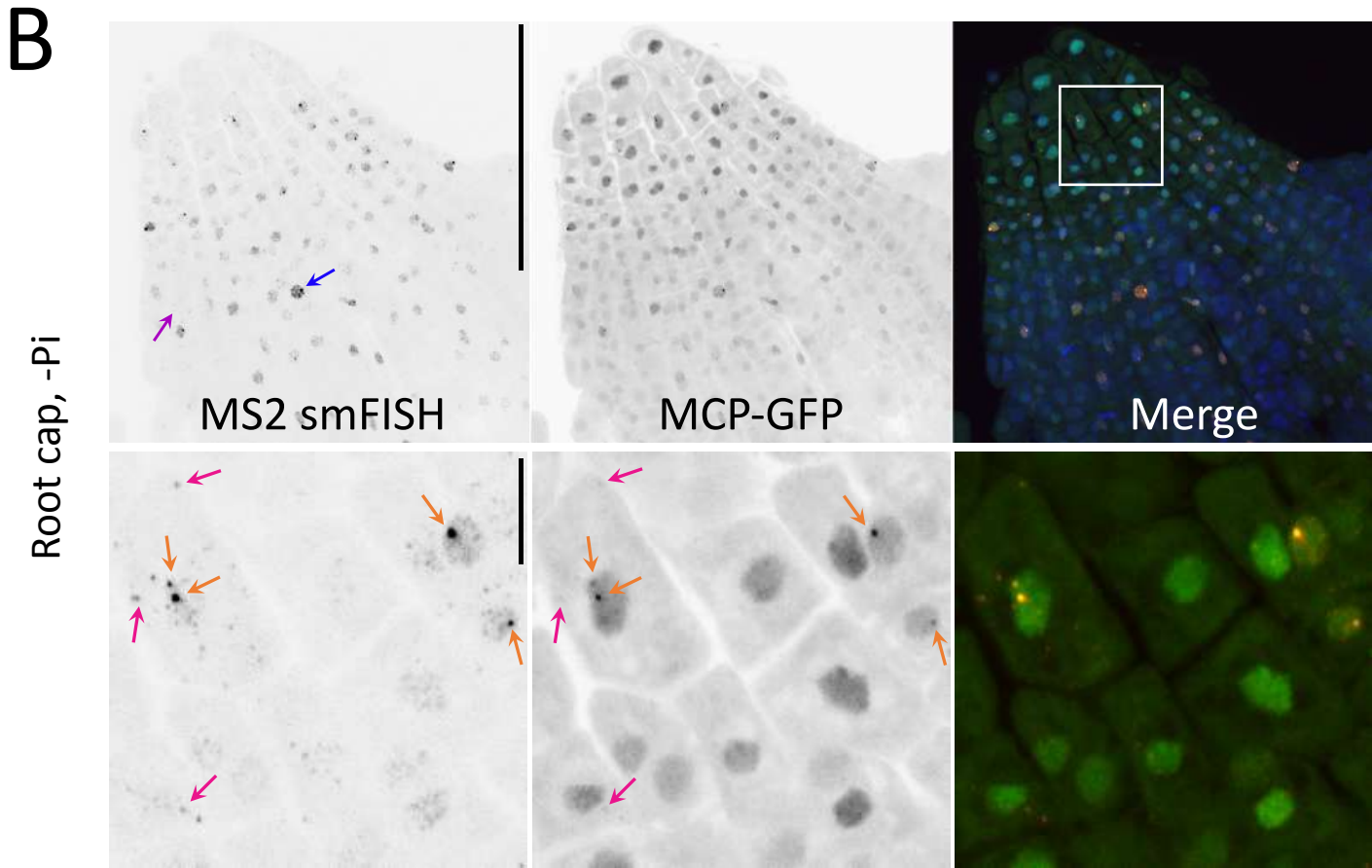
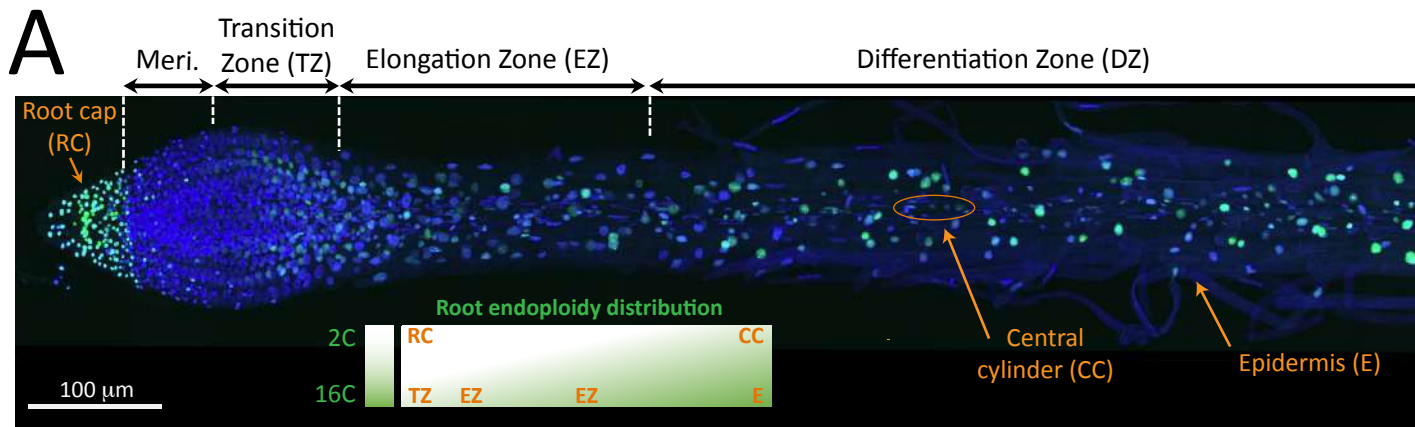


Figure 3



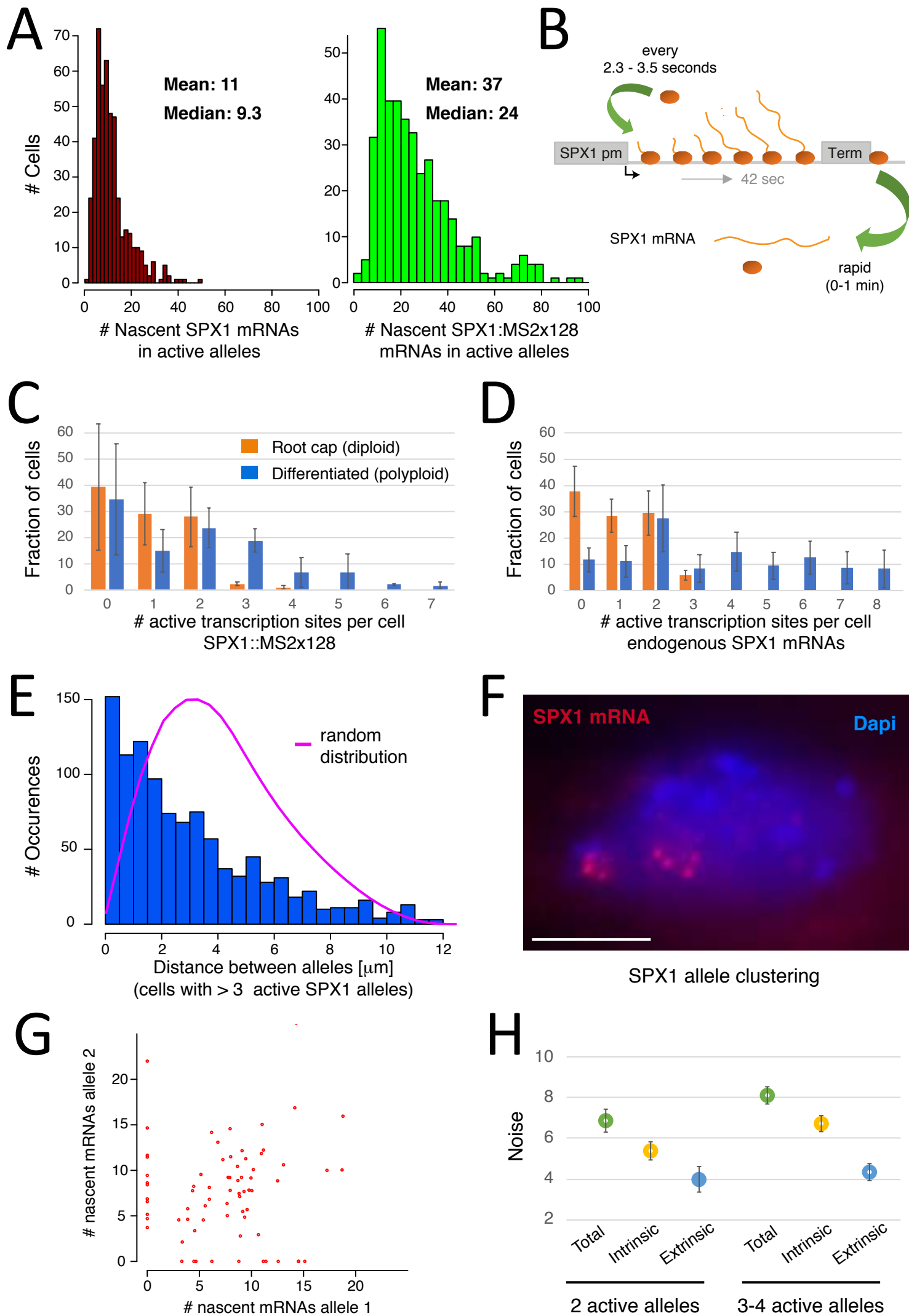


Figure 4

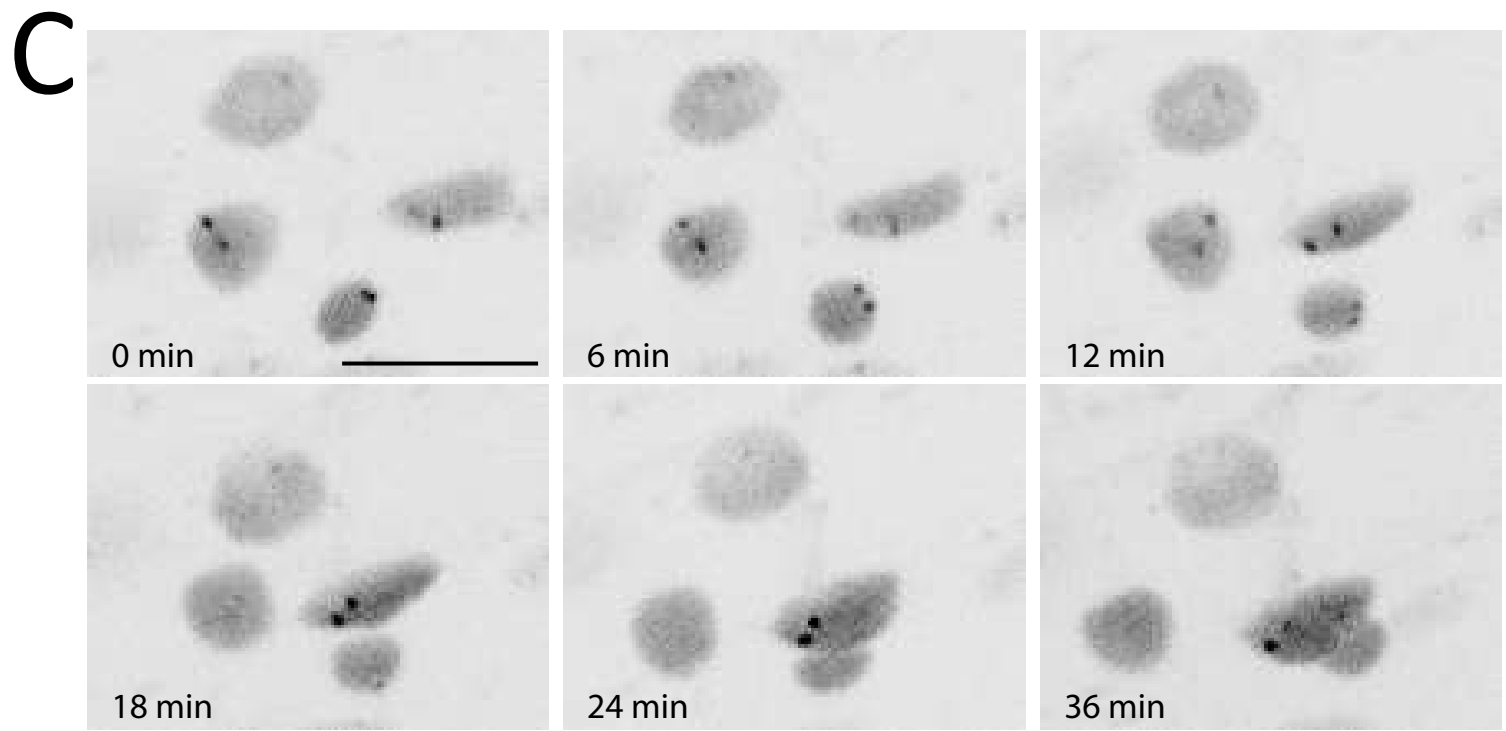
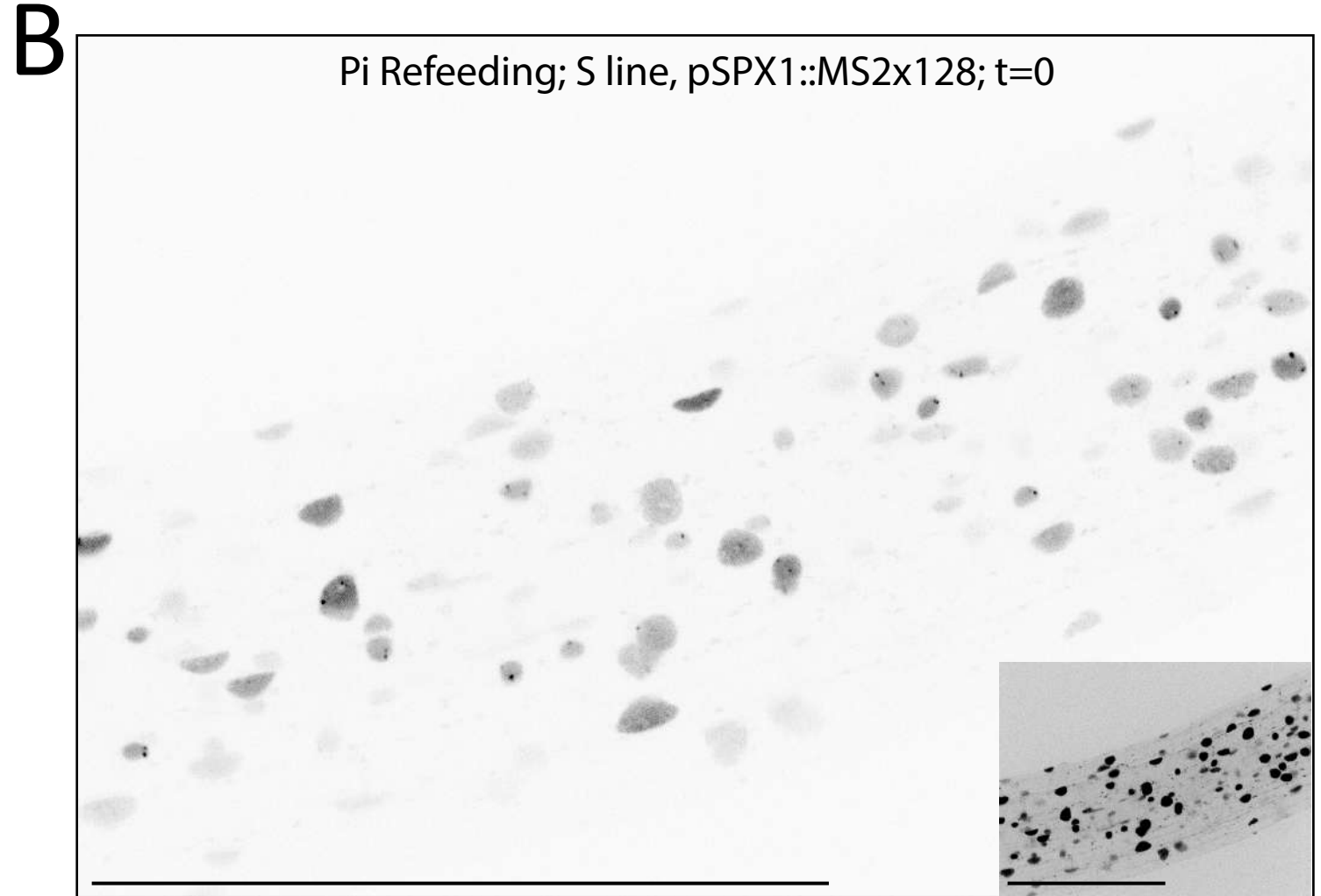
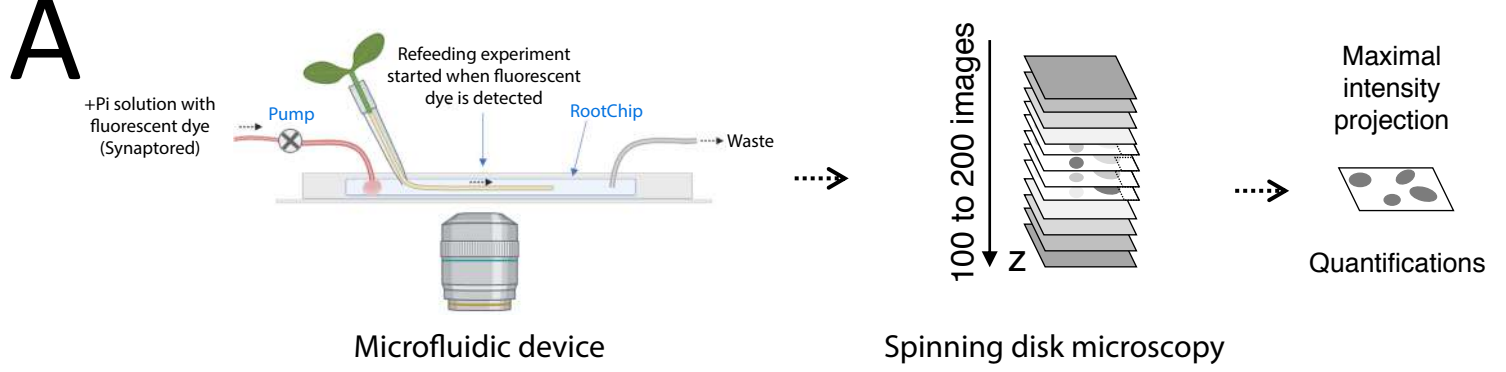


Figure 5

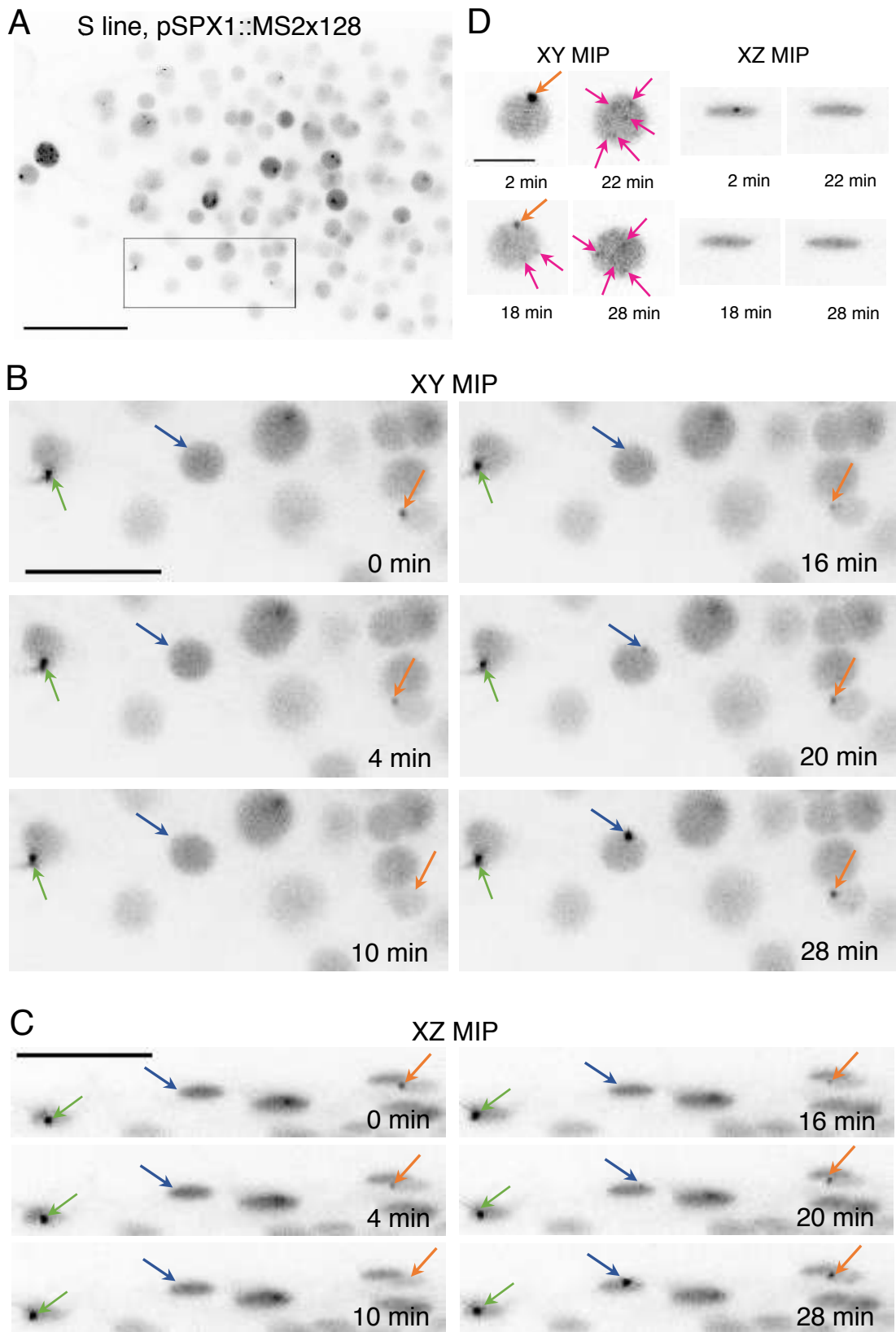
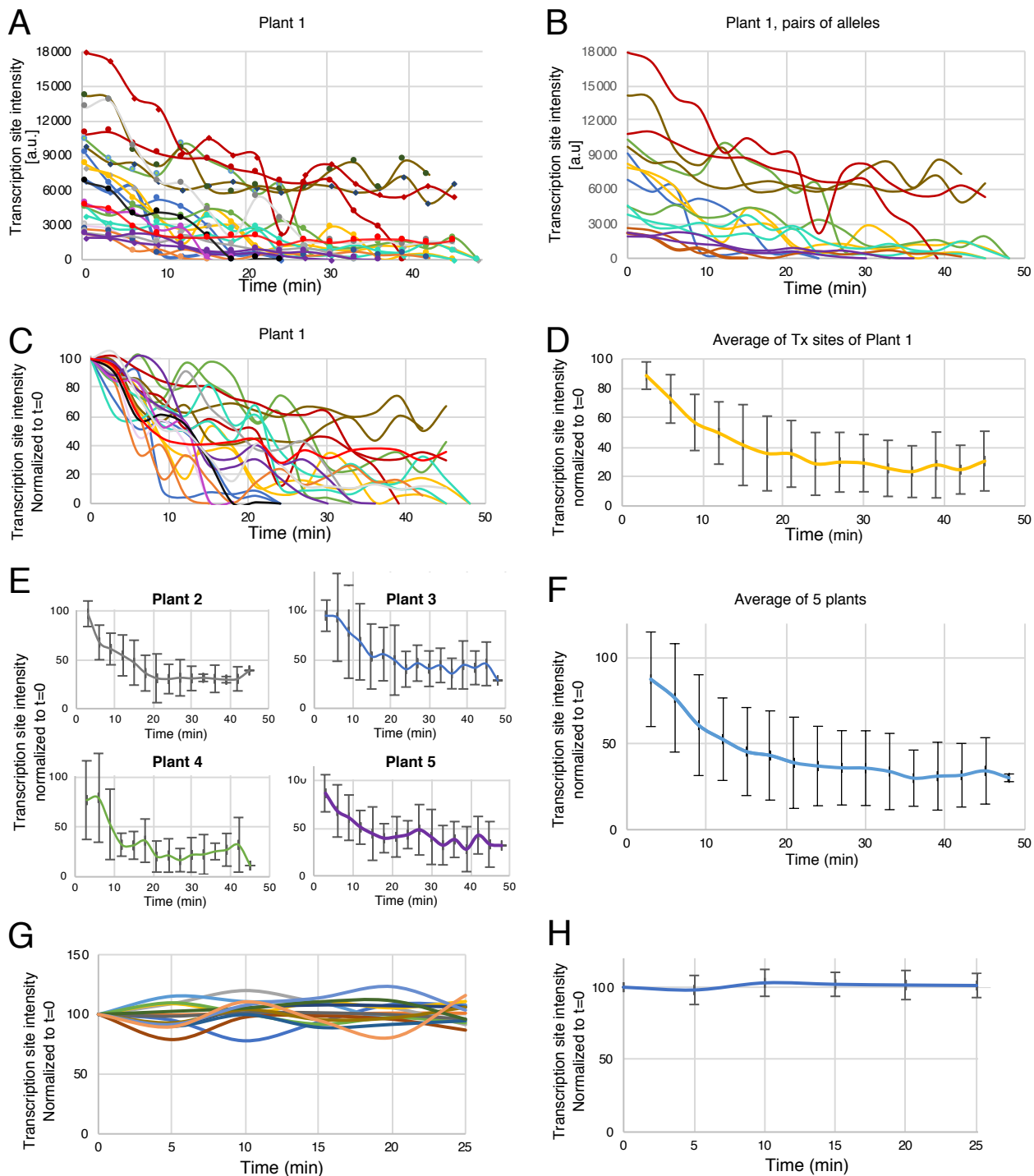


Figure 6



S line, pSPX1::MS2x128

Figure 7