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► To cite this version:

Christian Godon, Caroline Mercier, Xiaoyue Wang, Pascale David, Pierre Richaud, et al.. Under phosphate starvation conditions, Fe and Al trigger accumulation of the transcription factor STOP1 in the nucleus of Arabidopsis root cells. *The Plant Journal*, 2019, 99 (5), pp.937-949. 10.1111/tpj.14374 . cea-02149165

HAL Id: cea-02149165

<https://cea.hal.science/cea-02149165>

Submitted on 24 Jun 2019

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the plant journal

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Journal:	<i>The Plant Journal</i>
Manuscript ID	TPJ-01389-2018.R1
Manuscript Type:	Original Article
Key Words:	phosphate, iron, aluminum, root, STOP1, ALMT1, Arabidopsis, ALS3

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Running head

Fe and Al trigger nuclear accumulation of STOP1

Under phosphate starvation condition, Fe and Al trigger the transcription factor STOP1 to accumulate in the nucleus of Arabidopsis root cells

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Abstract

Low-phosphate (Pi) condition is known to repress the primary root growth of Arabidopsis, in a low-pH and Fe-dependent manner. This growth arrest requires the accumulation of STOP1 transcription factor in the nucleus where it activates the transcription of the malate transporter gene *ALMT1*; exuded malate is suspected to interact with extracellular iron to inhibit root growth. In addition, ALS3 –an ABC-like transporter first identified for its role for tolerance to toxic aluminum– represses nuclear accumulation of STOP1 and the expression of *ALMT1*.

Until now, it was unclear whether phosphate deficiency itself or iron activates STOP1 to accumulate in the nucleus.

Here, by using different growth media to dissociate the effects of Fe from Pi deficiency itself, we demonstrate that Fe is sufficient to trigger the accumulation of STOP1 in the nucleus, which in turn, activates the expression of *ALMT1*. We also show that a low pH is necessary to stimulate the Fe-dependent accumulation of nuclear STOP1. Furthermore, pharmacological experiments indicate that Fe inhibits proteasomal degradation of STOP1. We also show that Al acts like Fe for nuclear STOP1 accumulation and *ALMT1* expression, and that the overaccumulation of STOP1 in the nucleus of the *als3* mutant grown in low-Pi could be abolished by Fe deficiency. Altogether, our results indicate that, under low-Pi condition, Fe^{2/3+} and Al³⁺ act similarly to increase the stability of STOP1 and its accumulation in the nucleus where it activates the expression of *ALMT1*.

Significance Statement

Low-phosphate, low-pH and excess aluminum are three major stresses inhibiting root growth. The Arabidopsis transcription factor STOP1 has a major role in root growth

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3 51 under these different stresses, but how STOP1 is regulated is not known. Here we
4 52 show that Fe –instead of low-phosphate– and Al promote the accumulation of
5 53 STOP1 in root nuclei, in a low-pH-dependent manner. This work unveils an important
6 54 regulatory step in the response to Al and Fe stresses.
7 55

8 56 **Keywords**

9 57 STOP1, ALMT1, ALS3, phosphate, iron, aluminum, pH, root, nucleus, Arabidopsis.
10 58

11 59 **Introduction**

12 60 In many plant species, Pi-deficiency (-Pi) alters root growth and architecture
13 61 promoting top-soil foraging: the growth of the primary root is reduced whereas lateral
14 62 root emergence and growth is stimulated toward a more horizontal direction.
15 63 Combined, these responses result in root systems that explore relatively better the
16 64 upper soil horizons where Pi is more concentrated (Lynch, 2001).

17 65 The Arabidopsis primary root stops growing when its apex encounters the -Pi
18 66 substratum (Svistonoff et al., 2007). Split-root and feeding experiments showed that
19 67 this root growth inhibition does not correlate with Pi accumulated inside the root
20 68 (Thibaud et al., 2010). Instead, it depends of the Pi concentration in the growth
21 69 medium. This phenomenon was described as the so-called –Pi local response, by
22 70 opposition to the -Pi systemic responses that are governed by Pi concentration
23 71 present inside the plant tissues (Puga et al., 2017). While the -Pi systemic response
24 72 is mainly controlled by the two master regulatory genes *PHOSPHATE STARVATION*
25 73 *RESPONSE 1 (PHR1)* and *PHR1-like 1 (PHL1)*, the -Pi local response is not
26 74 (Balzergue et al., 2017). This local growth inhibition response critically depends of
27 75 the Fe-content of the growth medium (Svistonoff et al., 2007, Ward et al., 2008,
28 76 Muller et al., 2015), and iron accumulates in the root tip (Muller et al., 2015,
29 77 Balzergue et al., 2017, Mora-Macias et al., 2017).

30 78 Inherent to its chemical properties, Pi can form complexes with metallic cations such
31 79 as $Fe^{2/3+}$ and Al^{3+} (Hinsinger, 2001). This has important consequences for the
32 80 mobility and bioavailability in soil of Pi, Fe and Al as well as for their homeostasis in
33 81 plants (Hirsch et al., 2006, Misson et al., 2005, Briat et al., 2015, Bouain et al., 2014).
34 82 In the medium, reflecting the antagonistic Fe-Pi interactions, the Pi/Fe ratio is
35 83 important: the lower the ratio the higher the inhibition, and when the nutrient solution
36 84 lacks Fe, the root growth is no more inhibited (Ward et al., 2008, Muller et al., 2015,
37 85 Svistonoff et al., 2007).

38 86 During the last decade, major advances contributed to a better understanding of the
39 87 cellular events underlying the “local” response in Arabidopsis. When exposed to low
40 88 external Pi (i.e. a low Pi/Fe ratio), the short primary root results from a combination of
41 89 rapid as well as long-term cellular responses at the root tip. Few hours, if not
42 90 minutes, after the root tip encounters a Pi-depleted zone, cell elongation decreases.
43 91 This is correlated with the deposition of iron and accumulation of reactive oxygen
44 92 species (ROS) in the cell wall of the stem cell niche (SCN) and the elongation zone
45 93 (EZ), and with a peroxidase-dependent stiffening of the cell wall of the EZ.

46 94 In the long term (hours to days), callose accumulates in cell wall of the root apical
47 95 meristem (RAM) and EZ, this occludes plasmodesmata and restricts cell-to-cell
48 96 trafficking. Progressively, root cell proliferation ceases until the exhaustion of the
49 97 RAM by the on-going cell differentiation (Balzergue et al., 2017, Muller et al., 2015,
50 98 Mora-Macias et al., 2017).
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3 100 Genetics and molecular analysis identified several proteins governing the root growth
4 101 inhibition under -Pi. Identified as a major quantitative trait locus, LOW PHOSPHATE
5 102 ROOT 1 (LPR1) codes a cell wall multicopper oxidase with ferroxidase activity
6 103 (Svistoonoff et al., 2007, Ticconi et al., 2009, Muller et al., 2015, Reymond et al.,
7 104 2006). LPR1 has a critical role in the growth arrest because loss-of-function
8 105 mutations in *lpr1* desensitize the root to -Pi. In particular, they accumulate reduced
9 106 amount of Fe in their root tip. Upon extended Pi-deprivation, the LPR1-dependent Fe
10 107 accumulation promotes RAM exhaustion and differentiation by the down-regulation of
11 108 the root patterning transcription factors SHORT ROOT (SHR) and SCARECROW
12 109 (SCR). This regulation operates via increased expression of CLAVATA 4 (CLV4), a
13 110 secreted peptide detected by the plasma membrane receptors CLV2 and
14 111 CLV2/PEP1 RECEPTOR 2 (PEPR2) (Gutiérrez-Alanís et al., 2017). A higher level of
15 112 regulation coupling LPR1 with brassinosteroid (BR) signalling pathways has been
16 113 recently unveiled. In particular, the root growth of seedlings with the constitutively
17 114 active *bzr1-D* mutation (*BZR1* for *BRASSINAZOLE RESISTANT1*) are unrepressed
18 115 in -Pi and has reduced expression of *LPR1* (Singh et al., 2014, Singh et al., 2018). In
19 116 addition, in the wild type (WT), Fe enhances the accumulation of the BR-signalling
20 117 inhibitor BKI1 (BRASSINOSTEROID KINASE INHIBITOR1), thereby closing a
21 118 feedback regulatory loop between LPR1 activity and BR signalling.
22 119 *PHOSPHATE DEFICIENCY RESPONSE 2* (*PDR2*) was the first gene identified in
23 120 the local response (Ticconi et al., 2004). *PDR2* is the only Arabidopsis P5-type
24 121 ATPase; it is located in the endoplasmic reticulum and its substrate is not yet known
25 122 (Ticconi et al., 2009). Compared to *lpr1* mutations, a mutation inactivating *PDR2*
26 123 confers an opposite effect (i.e. over accumulation of Fe) (Muller et al., 2015). The
27 124 *lpr1* mutations are epistatic over *pdr2* indicating a functional interaction between
28 125 LPR1 and PDR2.
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30 127 A large-scale forward genetics screen for seedlings with a primary root less sensitive
31 128 to -Pi inhibition identified several *stop1* and *almt1* mutants (in addition to *lpr1*)
32 129 (Balzergue et al., 2017). *STOP1* (SENSITIVE TO PROTON RHIZOTOXICITY1) is a
33 130 C₂H₂ zinc finger transcription factor necessary for the expression of *ALUMINUM*
34 131 *ACTIVATED MALATE TRANSPORTER 1* (*ALMT1*), coding a plasma membrane
35 132 transporter exuding malate (Hoekenga et al., 2006, Iuchi et al., 2007). While the
36 133 *stop1* and *almt1* mutants have reduced accumulation of Fe in the EZ, they still
37 134 accumulate large amount of Fe in the stem cell niche (SCN) (Balzergue et al., 2017,
38 135 Wang et al., 2019), although others observed reduced accumulation of Fe in SCN
39 136 (Mora-Macias et al., 2017). This is correlated with some remaining root inhibition
40 137 upon long-term -Pi deprivation, presumably mediated by the Fe-dependent RAM
41 138 differentiation and exhaustion. These observations show that the STOP1-ALMT1
42 139 module is mainly involved in the inhibition of cell elongation whereas the LPR1-PDR2
43 140 module influences all aspects of the local response. Supporting independent genetic
44 141 regulations of these two modules, the *lpr1* mutation does not alter the expression of
45 142 *ALMT1* and, reciprocally, *stop1* mutants display WT expression of *LPR1*. It seems
46 143 that, at least in the elongation zone, the convergence point of these two modules is in
47 144 the apoplast compartment where the ALMT1-exuded malate somehow helps the Fe
48 145 to participate in the generation of ROS.
49 146

50 147 In the root tip, Pi deprivation enhances the transcript level of *ALMT1* but not of
51 148 *STOP1*, suggesting a post-translational regulation of STOP1 protein. Indeed, the -Pi
52 149 condition stimulates the accumulation of STOP1 in the nucleus (Balzergue et al.,

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3 150 2017). This nuclear accumulation of STOP1 represents therefore an important
4 151 control step in this pathway for which a first, and unexpected, regulatory component
5 152 has been identified recently. A genetics screen for mutants hypersensitive to -Pi
6 153 induced root growth inhibition retrieved an *als3* mutant. The ALS3 protein
7 154 (ALUMINUM SENSITIVE 3) interacts with STAR1 (SENSITIVE TO ALUMINUM
8 155 RHIZOTOXICITY 1) to form a putative ATP-binding cassette (ABC) transporter
9 156 complex located in the tonoplast (Dong et al., 2017). Consistently, in -Pi, the *star1*
10 157 mutant behaves like *als3*. Interestingly, the root hypersensitivity of *als3* and *star1*
11 158 correlates with higher accumulation of STOP1 in root nuclei and overexpression of
12 159 *ALMT1* in the root tip. By contrast, in -Pi, the overexpression of the ALS3-STAR1
13 160 fusion protein represses the accumulation of STOP1 in the nucleus and improves
14 161 root growth of WT. Moreover, a suppressor screen of *als3* identified *stop1* and *almt1*
15 162 mutants (as well as *lpr1*) (Wang et al., 2019). Taken together, these results show
16 163 that, in combination, ALS3 and STAR1 attenuate the root growth inhibition in -Pi by
17 164 repressing the accumulation of STOP1 in the nucleus. This led us to hypothesize that
18 165 ALS3-STAR1 depletes an unknown cytosolic compound (toward the vacuole) that
19 166 enhances the accumulation of STOP1 in the nucleus (Wang et al., 2019).
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24 168 Before the discovery of their implication in the response to -Pi, STOP1, ALMT1,
25 169 ALS3 and STAR1 were all known for their major role in Arabidopsis resistance
26 170 against toxic aluminum. Loss-of-function mutations in any of these genes severely
27 171 impair root growth in the presence of Al³⁺ (Larsen et al., 2005, Iuchi et al., 2007,
28 172 Huang et al., 2010, Hoekenga et al., 2006). It is therefore tempting to deduce that in
29 173 Pi deprived conditions, Fe^{2/3+} is the responsible metallic ion triggering nuclear
30 174 accumulation of STOP1, thereby participating to root growth repression in -Pi.
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33 176 In this work, we found growth conditions with limited Pi allowing to distinguish the
34 177 effect of Fe from the -Pi *per se*. This enabled us to compare the effect of Fe with Al
35 178 on STOP1 and ALMT1. Our results demonstrate that Fe, as well as Al, triggers the
36 179 accumulation of STOP1 in the nucleus and the expression of *ALMT1*.
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39 181 Results

40 182 We previously showed that the low-Pi condition stimulates the expression of the
41 183 *ALMT1* gene, and this stimulation relies on the transcription factor STOP1 (Balzergue
42 184 et al., 2017). In addition, under low-Pi, omitting Fe in the nutrient solution prevents
43 185 the root growth arrest, showing that Fe is essential for this growth response
44 186 (Svistoonoff et al., 2007, Ward et al., 2008, Dong et al., 2017, Muller et al., 2015,
45 187 Mora-Macias et al., 2017). However, the -Fe conditions previously tested did not
46 188 completely suppress the expression of *ALMT1*.
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49 189 Fe, but not Pi deficiency *per se*, induces *ALMT1* expression

50 190 We used the *pALMT1::GUS* (GUS, β -glucuronidase) construct to investigate the role
51 191 of Fe because this visual reporter sensitively allows to test the activity of the STOP1
52 192 signalling in the root tip (Balzergue et al. 2017). We first tested the influence of the
53 193 Pi/Fe ratio on the expression of *ALMT1*. Seedlings were first grown 3 days on a -Pi
54 194 medium without Fe added. To prevent the expression of *ALMT1* (and thus the
55 195 accumulation of the GUS protein) during this pre-culture, the growth medium was
56 196 buffered at pH 6.7. Indeed, at pH around neutrality we observed no or very little GUS
57 197 staining in the root tip of *pALMT1::GUS* seedlings (Balzergue et al. 2017 & Figure
58 198 S1). Seedlings were then transferred from this pre-culture condition, to media
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3 200 differing by their Fe to Pi ratio, at pH 5.5. Twenty-four hours after transfer, the
4 201 seedlings were stained for GUS activity. In a medium supplemented with 15 μM of
5 202 Fe, and without Pi added, there is a strong expression of *ALMT1* (Figure 1a).
6 203 Increasing the Pi content reduces the expression of *ALMT1*. However, at the highest
7 204 Pi concentration tested (250 μM), the expression of *ALMT1* is still induced. This
8 205 result confirms that *ALMT1* expression is higher in -Pi compared to +Pi condition.
9 206 However, increasing the Fe content in a medium containing 250 μM Pi, increases the
10 207 expression of *ALMT1* (Figure 1b). This confirms result of Müller et al (2015) and
11 208 suggests that Fe, instead of Pi deficiency *per se*, stimulates the expression of
12 209 *ALMT1*.

13 210 We observed that even without supplementing the -Pi growth medium with Fe, at pH
14 211 5.5, there was still a strong expression of *ALMT1* (Figure 2a, first picture). It
15 212 prompted us to assay putative presence of iron in the agar using a quantification by
16 213 ICP-AES (Inductively Coupled Plasma – Absorption Emission Spectrometry). This
17 214 analysis revealed the presence of 38 $\mu\text{g Fe}\cdot\text{g}^{-1}$ agar (5.5 μM Fe in the final growth
18 215 medium) (Table S1).

19 216 To further test the hypothesis that, in -Pi condition, the Fe that was contained in the
20 217 agar powder is the trigger of *ALMT1* expression, we reduced the availability of Fe in
21 218 the growth media using a siderophore. Supplementing the medium with 100 μM
22 219 deferoxamine (DFO), a potent Fe-chelator, strongly diminished *ALMT1* expression,
23 220 (Figure 2a). DFO was effective in chelating Fe since addition of 15 μM Fe did not
24 221 stimulate expression of *ALMT1* (Figure 2a). To avoid the potential toxicity of a high
25 222 concentration of DFO that could interfere with gene expression, an alternative
26 223 approach was used to reduce bioavailable iron from the agar. The agar powder was
27 224 mixed with DFO and then washed (see Experimental procedures). We then tested
28 225 whether the level of expression of *ALMT1* remained low. In -Pi plates made with this
29 226 washed, DFO-treated agar, the expression of *ALMT1* is almost fully abolished, and
30 227 addition of 15 μM Fe restored a high level of expression (Figure 2b). We conclude
31 228 that a strong chelator of Fe suppresses the expression of *ALMT1*. Altogether, these
32 229 results show that the stimulation of *ALMT1* expression in -Pi condition is triggered by
33 230 Fe but not by the Pi-deficiency *per se*.

34 231
35 232 This result prompted us to compare the expression of *ALMT1* with that of *SPX1*
36 233 (*SYG1/Pho81/XPR1*), a classical marker of the -Pi stress. *SPX1* encodes a protein
37 234 regulating the activity of the transcription factor *PHR1* –a master regulator of the -Pi
38 235 stress, and is frequently used as a marker of the -Pi transcriptional response. To
39 236 monitor its expression, we used WT seedlings containing the *pSPX1::GUS* marker
40 237 (Duan et al., 2008). The *pALMT1::GUS* and *pSPX1::GUS* seedlings were grown in +
41 238 or -Pi, at pH 5.5 or 6.7, and with two different agars or an agarose; in all these media
42 239 the nutrient solution was not supplemented with Fe.

43 240 As already shown (Balzergue et al., 2017), on an agar containing Fe, the expression
44 241 of *ALMT1* is induced at pH 5.5 but not at pH 6.7, in both -Pi and +Pi (Figure 3a). This
45 242 expression is much less stimulated in media that contain very low amount of Fe
46 243 (DFO-treated agar or on the agarose Seakem, Figure 3a and Supplementary Table
47 244 1). By contrast, the expression of *SPX1* is induced only in -Pi conditions, whatever
48 245 the pH or the gelifying agent used to prepare the growth medium (Figure 3a). All
49 246 these observations were confirmed by qRT-PCR (Quantitative Reverse
50 247 Transcriptase-Polymerase Chain Reaction) (Figure 3b and Figure S2), and with the
51 248 *PPsPase1* (pyrophosphatase [PPi]-specific phosphatase1) gene as an additional
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3 249 marker whose expression is highly stimulated by Pi-deficiency (Hanchi et al., 2018)
4 250 (Figure 3b).

5 251 We also monitored the expression of the *IRT1* (IRON-REGULATED
6 252 TRANSPORTER 1) gene, a well-known indicator of Fe deficiency (Vert et al., 2002).
7 253 This marker indicates that, as expected, when grown on the Sigma-DFO agar plates,
8 254 seedlings are more Fe starved than on the other plates (Figure 3b). On plates made
9 255 with the Seakem agar, that contains very low amount of Fe, *IRT1* is also more
10 256 expressed than with the Sigma agar (Figure 3b). These analyses of genes
11 257 expression further show that *ALMT1* is not stimulated by the -Pi *per se*, and strongly
12 258 suggest that the signal stimulating the expression of *ALMT1* is distinct from that of
13 259 the SPX1 pathway. By contrast to *ALMT1* mRNA, Al, Fe and pH do not modulate the
14 260 level of *STOP1* mRNA accumulation (Figure S3).
15 261

18 262 **Fe stimulates STOP1 accumulation in the nucleus**

19 263 With the *pSTOP1::GFP-STOP1* reporter, we previously showed that STOP1
20 264 accumulates in the nuclei at the primary root tip seedlings grown in -Pi (Balzergue et
21 265 al., 2017, Wang et al., 2019). Our gene expression analysis of *ALMT1* incited us to
22 266 test whether Fe is involved in this nuclear accumulation of STOP1.

23 267 Seedlings carrying the *pSTOP1::GFP-STOP1* marker were grown 3 days in non-
24 268 inducible conditions (i.e. agarose Seakem without addition of Pi and Fe that do not
25 269 stimulate the expression of *ALMT1*, as shown in Figure 3a), transferred in the same
26 270 medium supplemented or not with Fe before examining the GFP-fluorescence. We
27 271 first assessed the range of Fe concentrations that stimulate nuclear accumulation of
28 272 STOP1 in acidic condition (pH 5.5). Figure 4a (and Figure S4a for an independent
29 273 experiment) shows that from 0 to 60 μ M Fe GFP-fluorescence in the nuclei increases
30 274 with Fe concentration; a plateau is reached around 60 μ M Fe. Note that without Fe,
31 275 we observe small fluorescent dots in cells (first picture in Figure 4a). These dots are
32 276 autofluorescence of root cell components (i.e. not GFP fluorescence) since a WT
33 277 seedling that does not carry the GFP marker displays similar dots (Figure S5).

34 278 Since in non-inducible conditions we do not detect GFP fluorescence in the
35 279 cytoplasm, we hypothesized that the accumulation of GFP-STOP1 protein in the
36 280 nucleus results from reduced proteasomal degradation (instead of i.e. translocation
37 281 form cytosol). We then tested the effect of MG132, an inhibitor of the 26S
38 282 proteasome, on the accumulation of GFP-STOP1 in seedlings grown in a moderate
39 283 amount of Fe (Agar Sigma without addition of Fe, see Table S1). When treated with
40 284 MG132, the GFP fluorescence substantially accumulated in the nucleus (Figure 4b,
41 285 see also Figure S6 for additional pictures of two independent experiments). This
42 286 result therefore supports the idea that Fe somehow inhibits the degradation of
43 287 STOP1 by the 26S proteasome.
44 288

45 289 We then performed a kinetic analysis, at pH 5.5, of the nuclear accumulation of
46 290 STOP1. Figure 4c (and Figure S4b) shows that in seedlings transferred in a medium
47 291 without Fe added (-Fe), the level of nuclear GFP-fluorescence remains low through
48 292 the 3 h of the kinetic. By contrast, after transfer of seedlings onto plates containing 60
49 293 μ M Fe, the GFP fluorescence in nuclei increases after 30 min and becomes higher
50 294 than in the -Fe control after 1h; and the fluorescence further increases with time. This
51 295 confocal analysis, performed in -Pi condition for all conditions, indicates that Fe, and
52 296 not the -Pi condition *per se*, rapidly promotes the accumulation of STOP1 in the
53 297 nucleus.
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3 298 We have shown previously that the acidity of the growth medium is a crucial
4 299 parameter to stimulate *ALMT1* expression (Balzergue et al., 2017). We therefore
5 300 tested whether low pH on its own also promotes the nuclear accumulation of STOP1.
6 301 Figure 4d (and Figure S4c) shows that, indeed, in presence of 60 μM Fe, the level of
7 302 nuclear GFP-fluorescence of GFP-STOP1 is higher in acidic conditions, whereas at
8 303 pH 6.7 it is low and does not significantly differ from the -Fe control. This confirms the
9 304 result shown in Figure 4a: an acidic pH (below 6.1) without Fe does not stimulate
10 305 accumulation of GFP-STOP1 in the nucleus. These measures show that a low pH is
11 306 required, but not sufficient, to stimulate nuclear accumulation of STOP1; the
12 307 accumulation occurs only in acidic conditions supplemented with Fe. This comforts
13 308 results shown in Figure 3 about the expression of *ALMT1*.
14 309

17 310 **Aluminum triggers STOP1 accumulation in the nucleus**

18 311 STOP1 and *ALMT1* participate to resistance against Al^{3+} toxicity, and Al^{3+} stimulates
19 312 the expression of *ALMT1*. As, for Fe (Figure 2b), in seedlings grown on plates made
20 313 with the washed, DFO-treated agar, the expression of *ALMT1* is restored by addition
21 314 of 15 μM Al^{3+} (Figure S7). We therefore asked whether Al^{3+} also stimulates STOP1 to
22 315 accumulate in the nucleus. Seedlings were grown 3 days in agarose Seakem plates,
23 316 and transferred for 2 h in plates supplemented or not with Al^{3+} before observing GFP-
24 317 fluorescence of GFP-STOP1. Preliminary observations showed GFP-fluorescence in
25 318 the nucleus after the transfer in Al-plates. A dose-response curve indicated that 15
26 319 μM Al^{3+} is sufficient to detect an accumulation of GFP in the nucleus, and a plateau is
27 320 reached at about 30 μM Al^{3+} (Figure 5a and Figure S8a for an independent
28 321 experiment). A kinetic indicates that 1 h after transfer the GFP signal is already
29 322 significantly higher than the control not supplemented with Al (Figure 5b, Figure S8b).
30 323 The signal further increases through the 3 h kinetic.

31 324 As for Fe, a low-pH (from 5.5 to 6.7) is required to Al to promote the nuclear
32 325 accumulation of the GFP-STOP1 (Figure 5c and Figure S8c). These experiments
33 326 show that Al, as Fe, rapidly triggers nuclear accumulation of STOP1 when the growth
34 327 conditions are acidic.
35 328

39 329 **The overaccumulation of STOP1 in root nuclei of *als3* is Fe-dependent**

40 330 Using the *pSTOP1::GFP-STOP1* marker we previously shown that in the *als3* and
41 331 *star1* mutants, the accumulation of GFP-STOP1 in root nuclei is much higher than in
42 332 the WT (Wang et al., 2019). We thus asked whether this overaccumulation depends
43 333 of Fe. We confirmed that, when grown on agarose Seakem plates supplemented with
44 334 60 μM Fe, the *als3* mutant accumulates much more GFP-STOP1 in nuclei than the
45 335 WT control (Figure 6). In -Fe, this accumulation is suppressed in both the WT and the
46 336 *als3* mutant (Figure 6). This result was confirmed with the DFO-agar (Figure S9).
47 337 Therefore, the enhanced accumulation of nuclear GFP-STOP1 in *als3* root nuclei
48 338 depends of Fe. Our results show that Fe is critical for STOP1 to accumulate in
49 339 nucleus, and ALS3 repress this accumulation in a Fe-dependent manner.
50 340

53 341 **Discussion**

54 342 We, and others, have previously shown that several aspects of the root growth arrest
55 343 under -Pi conditions are Fe-dependent (Svistoonoff et al., 2007, Ward et al., 2008,
56 344 Dong et al., 2017, Muller et al., 2015, Gutiérrez-Alanís et al., 2017, Singh et al., 2018,
57 345 Wang et al., 2019), but the relationships between -Pi and Fe were unclear. Here, we
58 346 focused our work on the STOP1 signalling and succeed to dissociate the effect of -Pi
59 347 itself from the role of Fe in the STOP1 signalling.

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3 348 We compared the expression of *SPX1* and *PPsPase* genes as marker of the
4 349 systemic -Pi stress with that of *ALMT1* that belongs to the local -Pi-stress. We show
5 350 that, in the root, Fe stimulates the expression of *ALMT1* regardless the concentration
6 351 of Pi, and the -Pi-Fe as well as the +Pi-Fe conditions decrease the expression of
7 352 *ALMT1*. In addition, we demonstrate that the accumulation of STOP1 in root tip
8 353 nuclei is Fe-dependent. By contrast, the expression of *SPX1* and *PPsPase* happens
9 354 only under the -Pi condition and it is not correlated with the availability of Fe in the
10 355 medium (Figure 3 and Figure S2). Supporting this conclusion, our previous results
11 356 showed that the expression of *ALMT1* in -Pi does not depend of *PHR1* and *PHL1*
12 357 genes (Balzergue et al., 2017), two targets of *SPX1* regulation (Puga et al., 2014).
13 358 We thus demonstrate that, under -Pi conditions, it is the Fe and not the -Pi condition
14 359 itself that stimulates the accumulation of STOP1 in root nuclei and the transcription of
15 360 *ALMT1*. Under +Pi, Fe is probably associated with Pi molecules, thus hampering the
16 361 Fe-dependent regulation of nuclear STOP1 and the catalysis of ROS production,
17 362 whereas under -Pi condition, Fe is more available to stimulate these two processes.
18 363 Therefore, the STOP1 signalling pathway is clearly distinct from the systemic -Pi
19 364 signalling, at least the *PHR1*- and *PHL1*-dependent pathway.
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24 366 **Fe and Al stimulate the accumulation of STOP1 in the nucleus**

25 367 Our work demonstrates that Fe and Al^{3+} stimulate the accumulation of STOP1 in the
26 368 nucleus. In previous works by others, the cellular localisation of Arabidopsis STOP1
27 369 and STOP1 homologues was assessed by transient expression in onion cells,
28 370 Arabidopsis protoplasts, protoplasts derived from rice callus, tobacco leaves or
29 371 *Nicotiana benthamiana* leaves. In all these assays, the STOP1 proteins, fused to the
30 372 GFP, were localized in the nucleus (Sawaki et al., 2009, Sawaki et al., 2014, Fan et
31 373 al., 2015, Daspute et al., 2018, Huang et al., 2018, Wang et al., 2017, Wu et al.,
32 374 2018, Yamaji et al., 2009, Che et al., 2018). In these assays, the growth media used
33 375 were not phosphate deficient or particularly enriched in Fe or Al^{3+} . This suggests that,
34 376 in these experimental conditions, the pathway stimulating or repressing the
35 377 accumulation of STOP1 in the nucleus is constitutively active or inactive,
36 378 respectively. Supporting this last idea, an immunostaining experiment showed a
37 379 constitutive nuclear localization of OsART1 in WT root cells (i.e. not affected by Al
38 380 treatment) (Yamaji et al., 2009). Another possibility is that overexpression of the
39 381 STOP1 protein saturates the putative regulatory mechanism governing its nuclear
40 382 accumulation. Alternatively, these STOP1 proteins are differently regulated in these
41 383 cells compared to the root cells of Arabidopsis.
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45 384
46 385 In the past, we have shown that under neutral pH condition the root growth is not
47 386 arrested in low-Pi (Svistoonoff et al., 2007) and *ALMT1* is not expressed (Balzergue
48 387 et al., 2017). These two phenomena are now explained (at least partially) as we
49 388 show that under low-pH without Fe or Al^{3+} , STOP1 does not accumulate in the
50 389 nucleus and, reciprocally, in the presence of Fe or Al^{3+} , but at pH 6.7 STOP1 does
51 390 not accumulate in the nucleus either (Figures 4 and 5). The low pH is thus a
52 391 necessary but not sufficient condition to stimulate nuclear STOP1.

53 392 Our qRT-PCR result shows that *IRT1* — a gene whose expression is induced when
54 393 Fe is poorly available, like on the DFO-agar (Figure 3b) — is not or poorly induced at
55 394 pH 6.7 (Figure 3b, agar Sigma and agarose Seakem), indicating that seedlings are
56 395 not Fe-deficient. Nevertheless, this pH prevents the accumulation of STOP1 in the
57 396 nucleus. This suggests that different Fe signalling or different pools or forms of Fe
58 397 regulate *IRT1* expression and STOP1 nuclear accumulation. Knowing that $Fe^{2/3+}$ and
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3 398 Al^{3+} are soluble only under low pH, STOP1 regulation seems sensitive to the cationic
4 399 form of Fe and Al.

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6 401 The first *stop1* mutant was originally identified for its defective root growth on agar
7 402 plates made with a MS medium at pH 4.3 (Iuchi et al., 2007). The authors confirmed
8 403 the hypersensitivity to low-pH by using hydroponic culture; this growth medium
9 404 contained only submicromolar concentration of Fe (and no Pi). According to the
10 405 results presented here, under acidic conditions without Fe (or Al^{3+}), STOP1 poorly
11 406 accumulates in the nucleus. Then, if STOP1 does not accumulate in nucleus under
12 407 these growth conditions, how does it participate to resistance against low-pH? One
13 408 hypothesis is that a very low amount of Fe, at pH 4.3, is sufficient to stimulate the
14 409 accumulation of STOP1 for triggering the expression of genes for H^+ tolerance.
15 410 Alternatively, STOP1 might have already started promoting the expression of genes
16 411 involved in H^+ tolerance before (i.e. during embryogenesis) seedlings encounter
17 412 acidic conditions. A transcriptomic study on seedlings grown in acidic environment
18 413 shown that *stop1* mutant is defective for the expression of several genes, including
19 414 genes involved in cell wall composition or modelling (Sawaki et al., 2009).
20 415 Furthermore, physiological studies suggested that *stop1* seedlings are defective in a
21 416 mechanism alleviating H^+ toxicity, via perhaps Ca^{2+} stabilization of the cell wall
22 417 (Kobayashi et al., 2013b). It is therefore tempting to speculate that already during WT
23 418 embryogenesis, STOP1 participates in the making of root cells with a cell wall able to
24 419 tolerate H^+ during few days after germination, and in the *stop1* seeds the root cell
25 420 wall are somehow altered, leading to reduced root growth under acidic conditions.
26 421

27 422 **ALS3 and STAR1 repress the accumulation of STOP1 in the nucleus**

28 423 In a previous work, Wang et al demonstrated that, together, ALS3 and STAR1
29 424 repress the accumulation of STOP1 in the nucleus (Wang et al., 2019). ALS3 and
30 425 STAR1 associate to form an ABC-type transporter located in the tonoplast. We thus
31 426 inferred a model whereby a cytosolic metabolite stimulates the accumulation of
32 427 STOP1 in the nucleus, and that ALS3-STAR1 transporter pumps this metabolite from
33 428 the cytosol to the vacuole. According to this model, the cytosolic concentration of this
34 429 metabolite is higher in *als3* and *star1* mutants than in WT, thereby increasing nuclear
35 430 STOP1.

36 431 We showed here that the overaccumulation of STOP1 in the nucleus of *als3* mutant
37 432 is abrogated when the seedling grows in a Fe-depleted medium (Figure 6). Since our
38 433 results on the nuclear STOP1 with Al^{3+} are similar to those with Fe, this metabolite
39 434 could be the trivalent metal (Fe^{3+} or Al^{3+}) or a Fe (or Al^{3+})-containing molecule. This
40 435 hypothesis would fit with the role of ALS3-STAR1 in Al^{3+} tolerance. However, we
41 436 cannot exclude that this metabolite does not contain Fe (or Al^{3+}).
42 437

43 438 The *STOP1*, *ALS3* and *STAR1* genes are all expressed in the root tip (Larsen et al.,
44 439 2005, Dong et al., 2017, Balzergue et al., 2017, Mora-Macias et al., 2017); this is
45 440 coherent with their role as a shared functional unit.
46 441

47 442 **The two effects of Fe on root growth arrest**

48 443 A two-branched regulatory pathway modulates the root growth arrest: the
49 444 STOP1/ALMT1/ALS3/STAR1 branch and the LPR1/PDR2 branch (Balzergue et al.,
50 445 2017, Abel, 2017, Wang et al., 2019). These two branches are converging on the Fe-
51 446 dependent production of ROS in the cell wall. In the *lpr1* mutant the expression of
52 447 *ALMT1* is not altered compared to WT (Balzergue et al., 2017). This means that in

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3 448 the *lpr1* mutant, STOP1 is as active as in the WT. Since the *lpr1* root tip accumulates
4 449 far less extracellular Fe and ROS than in the WT, ROS and extracellular Fe seem not
5 450 crucial for activating STOP1 for *ALMT1* expression. Instead, our present work with
6 451 ALS3 indicates that an intracellular metabolite (containing Fe or Al³⁺?) activates the
7 452 STOP1 branch.

8 453 In Figure 7 a model summarizes our current knowledge of this two-branched
9 454 pathway. The phosphate ions inactivate Fe by forming a complex with it. Under -Pi
10 455 condition, Fe is released from this complex. Fe –or an unknown compound–
11 456 accumulates in the cell where it stimulates the accumulation of STOP1 in the nucleus
12 457 by inhibiting its proteasomal degradation. Inside the nucleus STOP1 activates the
13 458 transcription of *ALMT1*. The *ALMT1* protein exudes malate in the apoplast where,
14 459 together with Fe and the ferroxidase LPR1, they generate ROS that inhibit cell wall
15 460 expansion via the cross-linking activity of cell wall peroxidases. In the tonoplast
16 461 membrane, ALS3 and STAR1 pump Fe –or the unknown compound– from the
17 462 cytosol to the vacuole compartment. It follows that the concentration of Fe, or the
18 463 unknown compound, in the cytosol decreases and this reduces the accumulation of
19 464 STOP1 in the nucleus, and therefore reduces also the expression of *ALMT1*.

20 465 Note that Fe has two effects: it directly or indirectly activates the accumulation of
21 466 STOP1 in the nucleus and it also participates to the generation of ROS in the
22 467 apoplast. These two effects can be uncoupled; for example, the *lpr1* mutant still
23 468 expresses *ALMT1* but its root growth is not inhibited under -Pi.

24 469 For the aluminium, the model only partially applies because the exuded malate
25 470 prevents the inhibitory effect of toxic Al³⁺ on root growth. In addition, we do not know
26 471 whether ALS3 and STAR1 pump Al³⁺. In any case, Fe and Al³⁺ share several
27 472 characteristics in the nuclear accumulation of STOP1: dependency to low-pH, similar
28 473 kinetics, negative role of ALS3-STAR1. This could mean that Fe and Al³⁺ have
29 474 some common signalling steps activating STOP1. Many points remain obscure or not
30 475 yet demonstrated in this model. In particular, how cellular Fe (and Al³⁺) regulates
31 476 STOP1.

32 477

33 478 **How the Fe and Al stimulate STOP1 accumulation in nucleus?**

34 479 In plants, few Fe-sensing proteins have been identified (Kobayashi & Nishizawa,
35 480 2012). The HRZ (Haemerythrin motif-containing Really Interesting New Gene
36 481 (RING)-and Zinc-finger) proteins BRUTUS (BTS), BTS-like, OsHRZ1 and OsHRZ2
37 482 are E3 ligases involved in the low-Fe response (Kobayashi et al., 2013a, Long et al.,
38 483 2010, Hindt et al., 2017). They target to proteasomal degradation several basic helix-
39 484 loop-helix transcription factors like the POPEYE (PYE) and PYE-like such as
40 485 bHLH104 and bHLH105, which are positive regulators of the low-Fe response. Under
41 486 Fe-sufficient condition, the binding of Fe to the hemerythrin domains destabilizes the
42 487 HRZ proteins, thereby relieving the transcriptional response to low-Fe (Selote et al.,
43 488 2015, Kobayashi et al., 2013a). Also involved in transcriptional regulation of the Fe-
44 489 homeostasis are the IDEF1 and IDEF2 proteins that bind Fe, as well as zinc
45 490 (Kobayashi et al., 2012).

46 491 Here we have shown that MG132, an inhibitor of the 26S proteasome, substantially
47 492 increases the level of GFP-STOP1 accumulated in the nucleus in seedlings grown in
48 493 poorly inductive conditions (i.e. low-Fe; Figure 4b and Figure S6). This indicates that
49 494 under poorly or non-inductive conditions, STOP1 is degraded by the 26S-proteasome
50 495 pathway. Recently, Zhang et al. (Zhang et al., 2019) found an F-box protein, RAE1,
51 496 promoting the degradation of STOP1 via the 26S-proteasome pathway. The RAE1
52 497 protein interacts with and triggers the 26S-proteasome-dependent degradation of

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3 498 STOP1. Compared to the WT, seedlings homozygous for the loss-of-function *rae1*
4 499 mutation accumulate more STOP1 proteins and *ALMT1* mRNAs. When grown under
5 500 -Pi condition, the *rae1* seedlings have a primary root shorter than that of the WT, and
6 501 when grown with toxic amount of Al it is longer. RAE1 is therefore a strong candidate
7 502 mediating STOP1 degradation in our Fe- and Al-depleted growth conditions.
8 503

9 504 The RAE1 and STOP1 proteins do not contain an obvious Fe- or Al-binding domain.
10 505 How Al³⁺ is sensed in plant is not known (Kochian et al., 2015) and thus the
11 506 activation of STOP1 by Al³⁺ remains an open question. But, since both Fe and Al³⁺
12 507 stimulate the accumulation of STOP1 in the nucleus with similar kinetics, in the same
13 508 root cells and only under acidic conditions, it is tempting to speculate that these two
14 509 cations share a sensing mechanism. Further work will be needed to understand this
15 510 mechanism.
16 511

17 512 **Conclusion**

18 513 In acidic soils, toxic Al³⁺ and Fe, in conjunction with Pi-deficiency limit crops growth
19 514 (Kochian, 2004). There are increasing evidences that, at the cellular level, toxic Al³⁺
20 515 share some common targets and processes with the effects of Fe cations in acidic
21 516 and low-Pi conditions (Abel, 2017). We now demonstrate that Al³⁺ and Fe also share
22 517 at least one signalling step: STOP1 accumulation in the nucleus. Our study therefore
23 518 contributes to further dissection of the sensing and signalling pathways of Al and Fe
24 519 in plants.
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27 522 **Experimental procedures**

28 523 **Plant material.**

29 524 The Arabidopsis wild-type (*Col^{er105}*), *pALMT1::GUS_{#2}*, *pSTOP1::GFP-STOP1_{#B10}* and
30 525 *als3*; *pSTOP1::GFP-STOP1_{#B10}* lines were described previously (Balzergue et al.,
31 526 2017, Wang et al., 2019); *pSPX1::GUS* is in the Col-0 background (Duan et al.,
32 527 2008).
33 528

34 529 **Seedling growth.**

35 530 Seeds were surface-sterilised 5 min in a solution containing 70% ethanol and 0.05%
36 531 sodium dodecyl sulfate, and washed twice with ethanol 96%. The nutrient solution
37 532 was as previously (Balzergue et al., 2017). The agar (8 g l⁻¹) and agarose (7 g l⁻¹) for
38 533 plates was from Sigma-Aldrich (A1296 #BCBL6182V) and Lonza (Seakem LE
39 534 agarose) respectively. The media were buffered at pH ranging from 5.5-6.8 with
40 535 3.4 mM 2-(*N*-morpholino) ethane sulfonic acid. The different plates were prepared by
41 536 mixing the melted autoclaved 1X agar or agarose medium (composition as above)
42 537 with the buffer. Solutions of AlCl₃ and FeCl₂ were sterilized by filtration before adding
43 538 to the plates.
44 539

45 540 For GFP fluorescence experiments, five seeds were sown on a piece of sterile nylon
46 541 square (1 cm X 1 cm, mesh size 10 μm) itself lying on the agar plate. After 3 days of
47 542 growth on the agar plate, the nylon meshes carrying the seedlings were transferred
48 543 on the new plates containing or not different concentrations of aluminium or iron.

49 544 For the experiments with MG132, nylon meshes carrying the 3 days old seedlings
50 545 were transferred on 10 μl droplet (liquid extracted from Sigma-Aldrich agar (A1296
51 546 #BCBL6182V) from which the seedlings were pre-grown) containing or not different
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3 547 concentration of MG132. In all experiments, the pH of the growth media for
4 548 germination and for the subsequent transfer was identical.

5 549
6 550 For the qRT-PCR experiment, seeds were sown side by side in a line, on a 5 cm
7 551 strip of nylon meshes. Three to 3.5 days after sowing the whole roots of seedlings
8 552 were harvested and mRNA extracted.

9 553

10 554 **Agar treatment with deferoxamine B (DFO)**

11 555 To reduce bioavailable iron in the growth media, we used two methods. For the DFO-
12 556 agar, 100 μ M of deferoxamine B (DFO) was added to the 1x melted autoclaved
13 557 growth media, just before pouring the plates. For the washed DFO-treated agar, we
14 558 prepared the agar as follow: 4 g of Sigma-Aldrich agar (A1296 #BCBL6182V) was
15 559 added to 100 ml of milliQ water containing 600 μ M of DFO and stirred during 15 h.
16 560 The agar was then washed by centrifugation (520 rcf) through a nylon mesh (mesh
17 561 size 10 μ m); this washing step with milliQ water was carried out twice. This washed
18 562 DFO-agar was then used to prepare the growth plates.

19 563

20 564 **Quantification of Al, Fe and P in the agars and agarose**

21 565 Dried samples of agars and agarose were mineralized by addition of 300 μ L HNO₃
22 566 70% (ICP grade, JT Baker) by incubation overnight in an oven at 80 °C. The
23 567 solutions were diluted to a final volume of 5 mL by addition of distilled water. The Al,
24 568 Fe and P were quantified with Inductively Coupled Plasma – Absorption Emission
25 569 Spectrometer (ICP-AES 5110 SVDV, Agilent Technologies). The concentrations of
26 570 metals were determined using standard curves obtained from solutions made with
27 571 ICP-grade elements.

28 572

29 573 **Quantitative RT-PCR.**

30 574 Total RNA were extracted from whole roots using the Direct-Zol™ RNA MiniPrep
31 575 (ZYMO research, USA) and treated with the RNase-free DNase Set (ZYMO
32 576 research, USA) according to the manufacturer's instructions. Reverse transcription
33 577 was performed on 500 ng of total RNA using the qScript™ cDNA SuperMix (Quanta
34 578 Bioscience™). Quantitative PCR (qRT-PCR) was performed on a 480 LightCycler
35 579 thermocycler (Roche) using the manufacturer's instructions with Light cycler 480sybr
36 580 green I master (Roche) and with primers listed in Table S2. We used Tubulin gene
37 581 (At5g62690) as a reference gene for normalization and the quantification of gene
38 582 expression was as according the published method (Pfaffi, 2001).

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40 584 **GUS histochemical staining.**

41 585 The GUS staining of Arabidopsis seedlings was conducted as previously described
42 586 (Balzergue et al., 2017), except that the seedlings were incubated 40 min in the GUS
43 587 staining solution.

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45 589 **Quantification of GFP fluorescence.**

46 590 Images were collected on a Zeiss LSM780 confocal microscope (Carl Zeiss, France)
47 591 using a \times 20 dry objective (Plan Apo NA 0.80). GFP were excited with the argon ion
48 592 laser (488 nm). Emitted light was collected from 493 to 538 nm for GFP, using the
49 593 MBS 488 filter. All nuclei were imaged using the same conditions of gain, offset,
50 594 resolution and with a pinhole setting of 1 a.u. The quantification of GFP fluorescence
51 595 was carried out as follows: a Z-stack of 9 images (separated by 1.8 μ m distance)
52 596 imaged representative nuclei on the surface of the root. Images were acquired in 12

bits using Zen black software (Zen black 2012 SP2 Version 11.0), then converted to a maximal projection image. The average nuclei fluorescence intensities were quantified using the Zen blue software (Zen 2 blue edition, version 2.0.0.0), by drawing identical ROI (region of interest). A 120 μm X 60 μm rectangle was defined at 200 μm from a root tip (roughly in the transition zone), in which a region of interest (6 μm X 6 μm) inside each nucleus was defined for the measurement of fluorescence.

Acknowledgements

B.Alonso, S.Chiarenza, H.Javot and N.Léonhardt (CEA, cadarache) for experimental helps and discussions.

This work was funded by CEA, Investissements d'avenir (DEMETERRES) and ROULLIER/AGRO INNOVATION INTERNATIONAL (C.M.); X.W. and D.L. were funded by The National Natural Science Foundation of China (grant no. 31670256). Support for the microscopy equipment was provided by the Région Provence Alpes Côte d'Azur, the Conseil General of Bouches du Rhône, the French Ministry of Research, the CNRS and the Commissariat à l'Energie Atomique et aux Energies Alternatives. The qRT-PCR machine was funded by Hélobiotech.

The authors declare no competing interests.

Short legends for Supporting Information

Figure S1

Under neutral condition, *ALMT1* is not expressed.

Picture of the root tip after the GUS staining, of WT seedlings carrying the *pALMT1::GUS* marker. Seedlings were grown in low-Pi at pH 5.8 or 7.1, with or without 15 μM Fe. Note that at pH 7.1 no GUS staining is detected. Bar, 1 mm.

Figure S2

Expression (qRT-PCR) of *ALMT1*, *SPX1* and *PPsPase* in seedlings roots. Seedlings were grown 3 days on the indicated media. Mean \pm SD (n = two independent experiments).

Figure S3

Analysis of *STOP1* mRNA expression.

WT seedlings were pre-grown 3 days under low-Pi condition at pH 5.8 or 7 without Fe or Al added, transferred for 3 hours in the same original pH condition with or without Al^{3+} or Fe^{2+} before extraction of root RNAs and qRT-PCR reactions. The conditions for qRT-PCR reactions and the calculations of relative expression were as in Figure 3 and Figure S2. Mean \pm SD (n = two independent experiments). *ALMT1* was used as a control.

Figure S4

Fe promotes the accumulation of GFP-STOP1 in root nuclei.

The GFP fluorescence was measured (a.u.) in nuclei at the root tip of *pSTOP1::GFP-STOP1* seedlings.

a) Three-day-old seedlings were transferred 2 h in -Pi plates with the indicated concentration of Fe.

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3 646 b) Three-day-old seedlings were transferred for the indicated time in -Pi plates
4 647 containing 0 or 60 μM Fe.

5 648 c) Three-day-old seedlings were transferred 2h in -Pi plates buffered at the indicated
6 649 pH, containing 0 or 60 μM Fe.

7 650 d) Picture of GFP fluorescence at the root tip, in seedlings transferred 2h in -Pi plates
8 651 without (left) or with (right) 60 μM Fe.

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10 653 Box plots indicate the median, the 25th to 75th percentiles (box edges) and the min to
11 654 max range (whiskers); Mann-Whitney test; **** $P < 0.0001$; NS, not significant
12 655 ($P > 0.05$); number of nuclei per condition: a) 329-346; b) 291-343; c) 322-392).

13 656 14 657 **Figure S5**

15 658 Non-transgenic WT seedlings were grown on a -Pi-Fe plate made with the DFO-agar,
16 659 transferred to a -Pi plate containing 0 or 60 μM Fe for 2 h, and the fluorescence
17 660 pictured by confocal microscopy (same setting as in Figure S9). Note the small
18 661 autofluorescent dots. Bar, 100 μm .

19 662 20 663 **Figure S6**

21 664 The 26S proteasome inhibitor MG132 promotes GFP-STOP1 accumulation in root
22 665 nuclei.

23 666 Three days old seedlings were treated 6 h (a) or 4 h (b), without or with 60, 125 or
24 667 250 μM (a) or 250 μM (b) MG132, and photographed as in Figure 4b. **a** and **b**
25 668 represent two independent experiments. Bars, 100 μm .

26 669 27 670 **Figure S7**

28 671 Aluminum stimulates the expression of *pALMT1::GUS*.

29 672
30 673 Seedlings were grown four days on a -Pi medium pH 5.5, made with a washed DFO-
31 674 treated agar, supplemented or not with 15 μM Al^{3+} , before GUS staining. Note that
32 675 the 0 Al control is the same as in Figure 2b, since it is part of the same experiment.
33 676 Bar, 1 mm.

34 677 35 678 **Figure S8**

36 679 Al^{3+} promotes the accumulation of GFP-STOP1 in root nuclei.

37 680
38 681 The GFP fluorescence was measured (a.u.) in nuclei at the root tip of pSTOP1::GFP-
39 682 STOP1 seedlings.

40 683 a) Three-day-old seedlings were transferred 2 h in -Pi plates with the indicated
41 684 concentration of Al^{3+} .

42 685 b) Three-day-old seedlings were transferred for the indicated time in -Pi plates
43 686 containing 0 or 30 μM Al^{3+} .

44 687 c) Three-day-old seedlings were transferred 2h in -Pi plates buffered at the indicated
45 688 pH, containing 0 or 30 μM Al^{3+} .

46 689
47 690 Box plots indicate the median, the 25th to 75th percentiles (box edges) and the min to
48 691 max range (whiskers); Mann-Whitney test; **** $P < 0.0001$; number of nuclei per
49 692 condition: a) 315-348; b) 330-399; c) 315-448).

50 693 51 694 **Figure S9**

52 695 ALS3 represses STOP1 accumulation in root nuclei.

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3 696 a) WT and the *als3* mutant seedlings carrying the *pSTOP1::GFP-STOP1* construct
4 697 were grown 3 days on a -Pi-Fe plate, transferred to -Pi or -Pi-Fe plates for 2 h, and
5 698 GFP-fluorescence was pictured by confocal microscopy. Bars, 100 μ m.
6 699

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Table S1: Al, Fe and P content ($\mu\text{g}/100\text{ mg}$ agar or agarose)

	Al	Fe	P
Agar Sigma A1296	5,75	3,82	5,86
Agar Sigma A1296 treated with DFO and washed	5,87	3,50	0,63
Agarose Ionza Seakem	0,42	0,24	0,81

Table S2: primers sequence

gene	primer	sequence (5' to 3')
<i>PPsPase1</i>	AT1G73010_R	GACGACACGTGGATGAATTG
	AT1G73010_F	TCATGATCAAGGCAAACCA
<i>SPX1</i>	AT5G20150_R	GCGGCAATGAAAACACACTA
	AT5G20150_F	CGGGTTTTGAAGGAGATCAG
<i>IRT1</i>	AT4G19690_R	GACGATAGAACTATACTGCCTTGA
	AT4G19690_F	TGCGGAATTGAAATCATGTG
<i>ALMT1</i>	AT1G08430_R	CGATTCCGAGCTCATTCTTC
	AT1G08430_F	GGCAGTGTGCCTACAGGATT
<i>STOP1</i>	AT1G34370_F	AAGTGGCTTTGTTCTGTGG
	AT1G34370_R	GGCTGTGTGGTTTCTTGTT
<i>Tubulin</i>	AT5G62690_R	ACACCAGACATAGTAGCAGAAATCAAG
	AT5G62690_F	GAGCCTTACAACGCTACTCTGTCTGTC

Figure legends

Figure 1

Antagonistic interactions of Pi and Fe on the expression of *pALMT1::GUS*.

a) Effect of the Pi concentration on GUS staining, when Fe is at 15 μ M.

b) Effect of the Fe concentration on GUS staining, when Pi is at 250 μ M.

Seedling were grown 3 days on a pH 6.7 medium not supplemented with Pi and Fe, and transferred 24 h on a pH 5.5 medium supplemented with the indicated concentrations of Pi and Fe, before GUS staining. Bar, 1 mm.

Figure 2

Fe is necessary for the expression of *ALMT1*.

a) DFO inhibits the expression of *pALMT1::GUS*.

b) In a growth medium made with a washed DFO-treated agar, supplementation of Fe restores the expression of *pALMT1::GUS*.

Seedling were grown 4 days on a pH 5.5 medium not supplemented with Pi, and supplemented or not with the indicated concentrations of DFO and Fe, before GUS staining. Bar, 1 mm.

Figure 3

Fe, but not the -Pi condition *per se*, stimulates the expression of *pALMT1::GUS*.

a) GUS staining in the primary root of *pALMT1::GUS* and *pSPX1::GUS* seedlings.

b) Expression (qRT-PCR) of *ALMT1*, *SPX1*, *IRT1* and *PPsPase* in seedlings roots (mean of three technical replicates).

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3 871 Seedlings were grown 5 days (for the GUS staining experiment) or 3.5 days (for the
4 872 qRT-PCR experiment) on a medium not supplemented with Pi and Fe, at pH 6.7 or
5 873 5.5. Bars, 1 mm.

8 875 **Figure 4**

9 876 Fe promotes the accumulation of GFP-STOP1 in root nuclei

10 877
11 878 The GFP fluorescence was measured (a.u.) in nuclei at the root tip of *pSTOP1::GFP-*
12 879 *STOP1* seedlings.

13 880 a) Top: three-day-old seedlings were transferred 2 h in -Pi plates with the indicated
14 881 concentration of Fe. Bottom: representative pictures used for measurements.

15 882
16 883 b) MG132 promotes the accumulation of GFP-STOP1 in nuclei of the root tip.
17 884 Seedlings carrying the *pSTOP1::GFP-STOP1* reporter were grown 3 days in agar
18 885 (Agar Sigma A1296, Table S1). under low-Pi without Fe or Al added. Then, they were
19 886 treated 4 h with or without 250 μ M MG132 before photographed with the confocal
20 887 microscope. One representative picture is shown for each condition (see Figure S6
21 888 for additional pictures). Note that under in the untreated control, STOP1 slightly
22 889 accumulates in the nucleus.

23 890 c) Left: three-day-old seedlings were transferred for the indicated time in -Pi plates
24 891 containing 0 or 60 μ M Fe. Right: representative pictures used for measurements.

25 892 d) Top: three-day-old seedlings were transferred 2 h in -Pi plates buffered at the
26 893 indicated pH, containing 0 or 60 μ M Fe. Bottom: representative pictures used for
27 894 measurements.

28 895
29 896 Box plots indicate the median, the 25th to 75th percentiles (box edges) and the min to
30 897 max range (whiskers); Mann-Whitney test; **** $P < 0.0001$; *** $P < 0.001$; * $P < 0.05$; NS,
31 898 not significant ($P > 0.05$); number of nuclei per condition: a) 94-154; b) 99-119; c) 87-
32 899 121). Bars, 100 μ m.

33 901 **Figure 5**

34 902 Al^{3+} promotes the accumulation of GFP-STOP1 in root nuclei.

35 903
36 904 The GFP fluorescence was measured (a.u.) in nuclei at the root tip of *pSTOP1::GFP-*
37 905 *STOP1* seedlings.

38 906 a) Top: three-day-old seedlings were transferred 2 h in -Pi plates with the indicated
39 907 concentration of Al^{3+} . Bottom: representative pictures used for measurements.

40 908 b) Left: three-day-old seedlings were transferred for the indicated time in -Pi plates
41 909 containing 0 or 30 μ M Al^{3+} . Right: representative pictures used for measurements.

42 910 c) Top: three-day-old seedlings were transferred 2 h in -Pi plates buffered at the
43 911 indicated pH, containing 0 or 30 μ M Al^{3+} . Bottom: representative pictures used for
44 912 measurements.

45 913 Box plots indicate the median, the 25th to 75th percentiles (box edges) and the min to
46 914 max range (whiskers); Mann-Whitney test; **** $P < 0.0001$; ** $P < 0.01$; NS, not
47 915 significant ($P > 0.05$); number of nuclei per condition: a) 80-143; b) 102-125; c) 86-
48 916 121). Bars, 100 μ m.

49 917 **Figure 6**

50 918 ALS3 represses STOP1 accumulation in root nuclei.

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3 921 WT and the *als3* mutant seedlings carrying the *pSTOP1::GFP-STOP1* construct were
4 922 grown 3 days on a -Pi-Fe plate (made with agar Seakem), transferred to -Pi-Fe or -
5 923 Pi + 60 μ M Fe plates for 2 h, and GFP-fluorescence was visualized by confocal
6 924 microscopy. To avoid saturated GFP fluorescence in the images, the microscope
7 925 was set on the highest fluorescence (as detected in *als3*). Bars = 100 μ m.
8 926

9 927 **Figure 7**

10 928 Model depicting the roles of Fe and Al on the STOP1 signalling and root growth in
11 929 relation with Pi availability.
12 930

13 931 Phosphate reversibly inactivates Fe and Al by forming a complex with them. Under -
14 932 Pi condition and low-pH, Fe, Al or another unknown molecule accumulates in the cell
15 933 where it decreases proteasomal degradation of STOP1 thereby stimulating the
16 934 accumulation of STOP1 in the nucleus; nuclear STOP1 activates the transcription of
17 935 *ALMT1*. The tonoplast-anchored ALS3 and STAR1 proteins pump the Fe, Al or the
18 936 unknown molecule from the cytosol to the vacuole compartment, thereby decreasing
19 937 its concentration in the cytosol. This reduces the accumulation of STOP1 in the
20 938 nucleus and therefore the transcription of *ALMT1*. The *ALMT1* transporter exudes
21 939 malate in the apoplast where, together with Fe and the ferroxidase LPR1, they
22 940 generate ROS that inhibit cell wall expansion. Exuded malate also chelates Al³⁺,
23 941 therefore preventing its toxicity (not shown in the scheme).
24 942

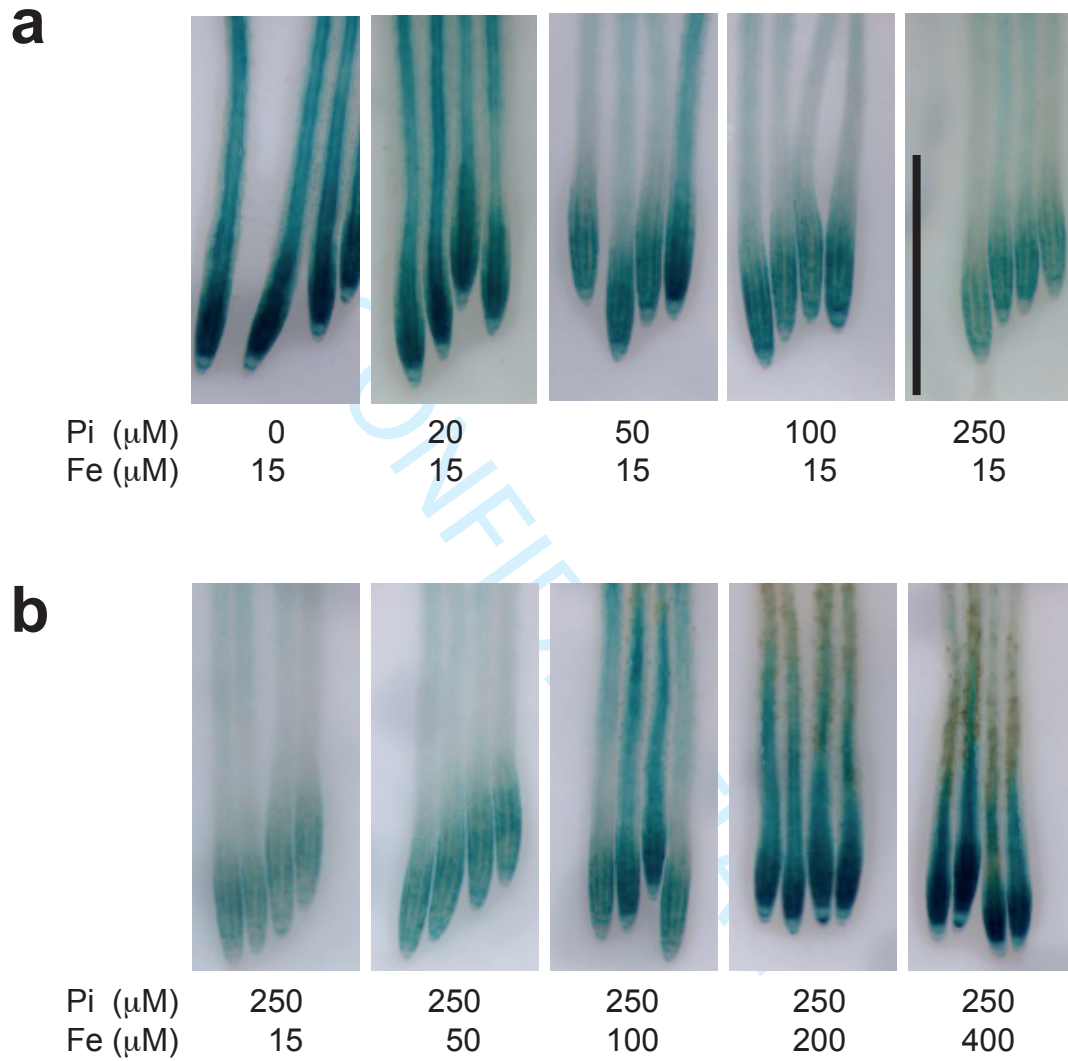
25 943 Red arrows: activation

26 944 Black arrow: proteasomal degradation

27 945 Blue blunt arrows: repression

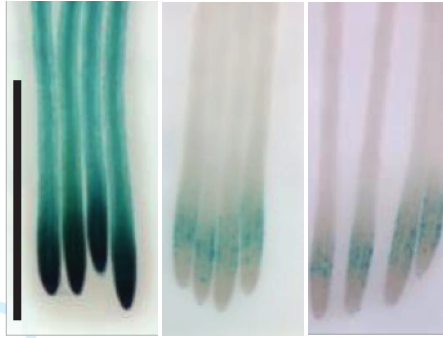
28 946 Dashed arrows: transfer between compartments
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Figure 1



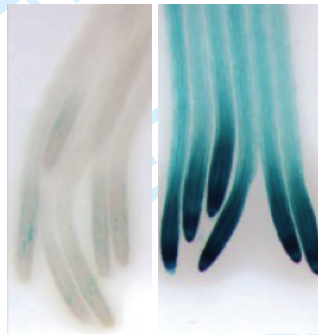
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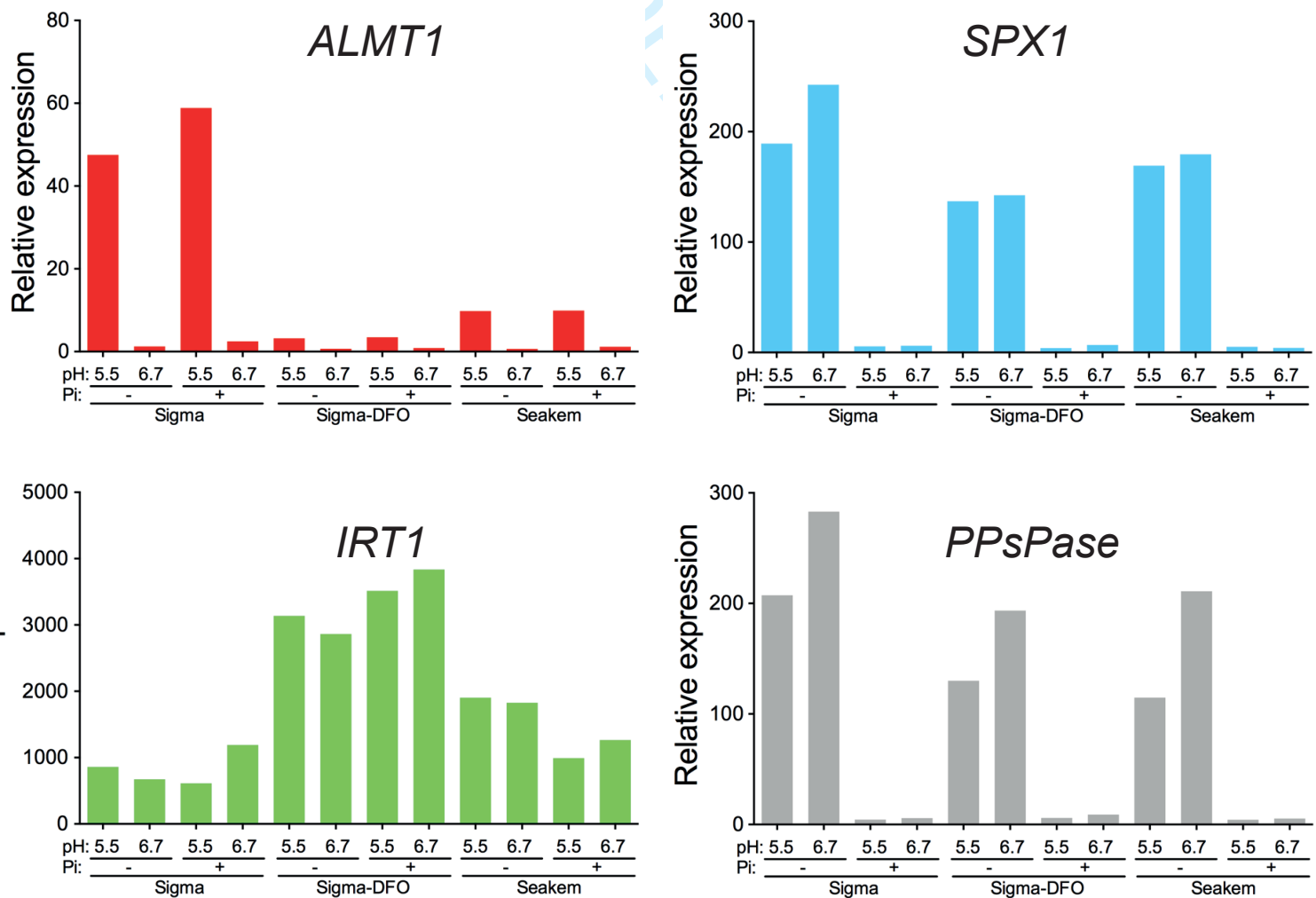
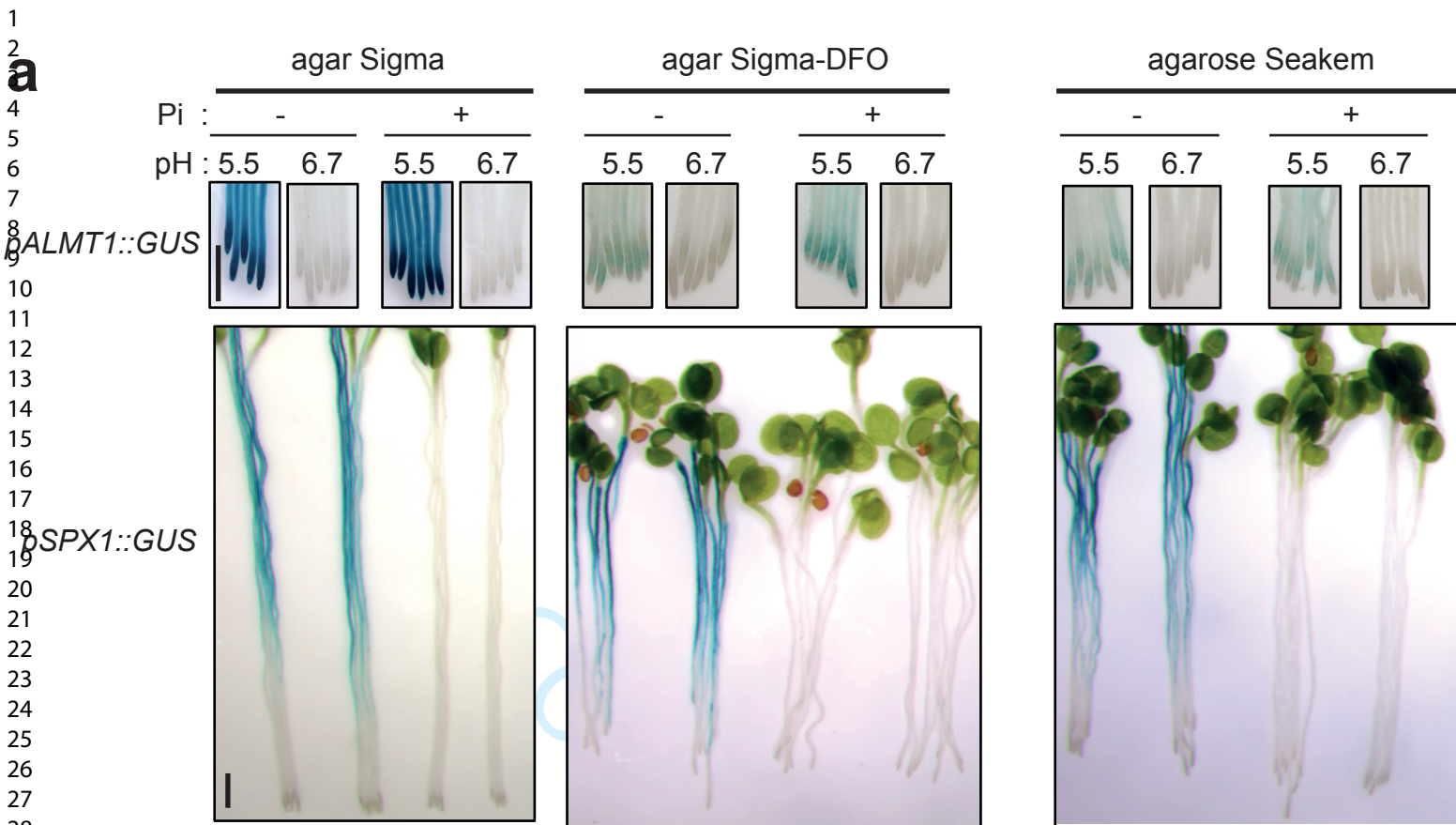
DFO : 0 100 μ M 100 μ M
Fe : 0 0 15 μ M

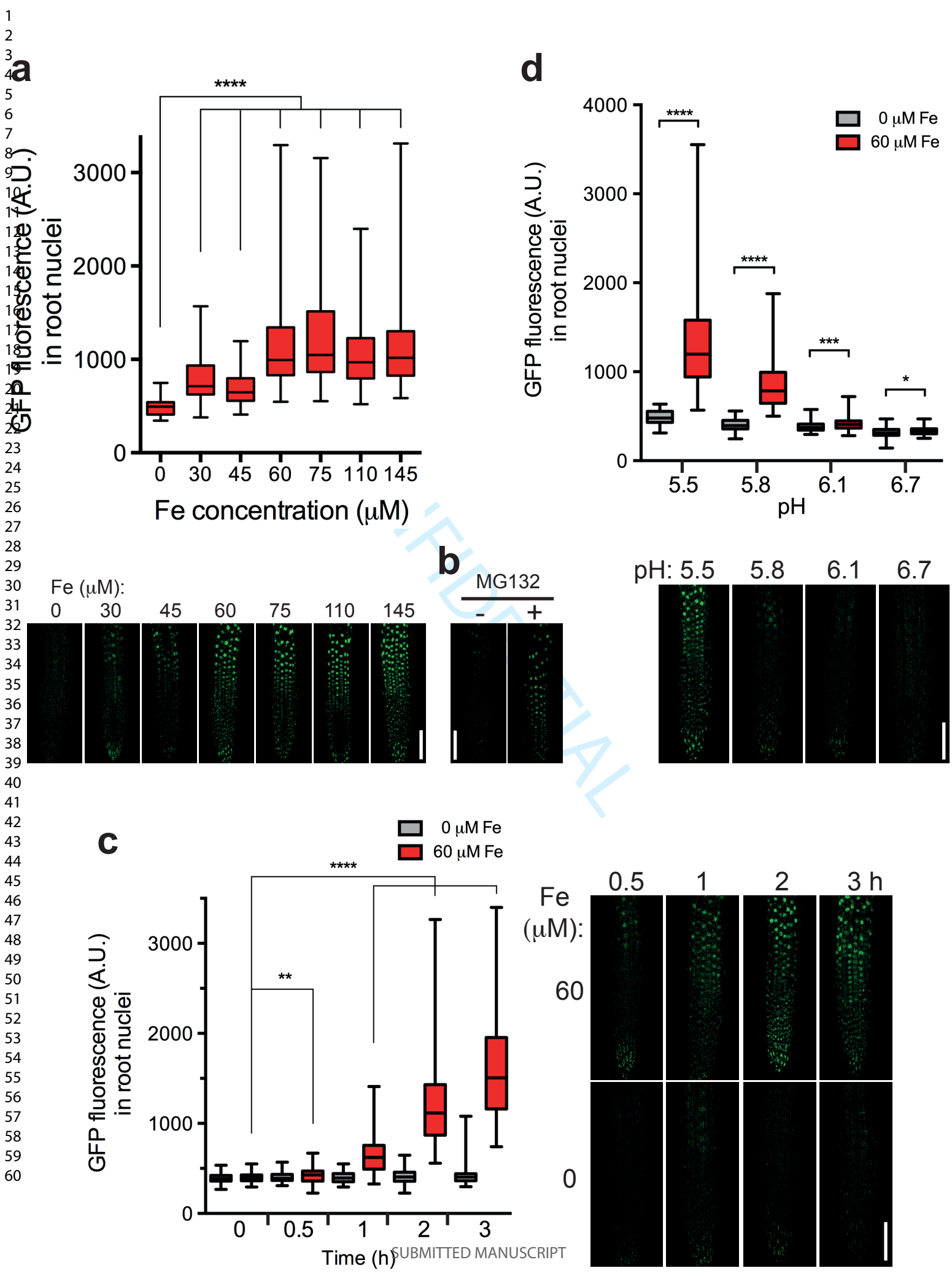
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Fe : 0 15 μ M

Figure 3

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Figure 5

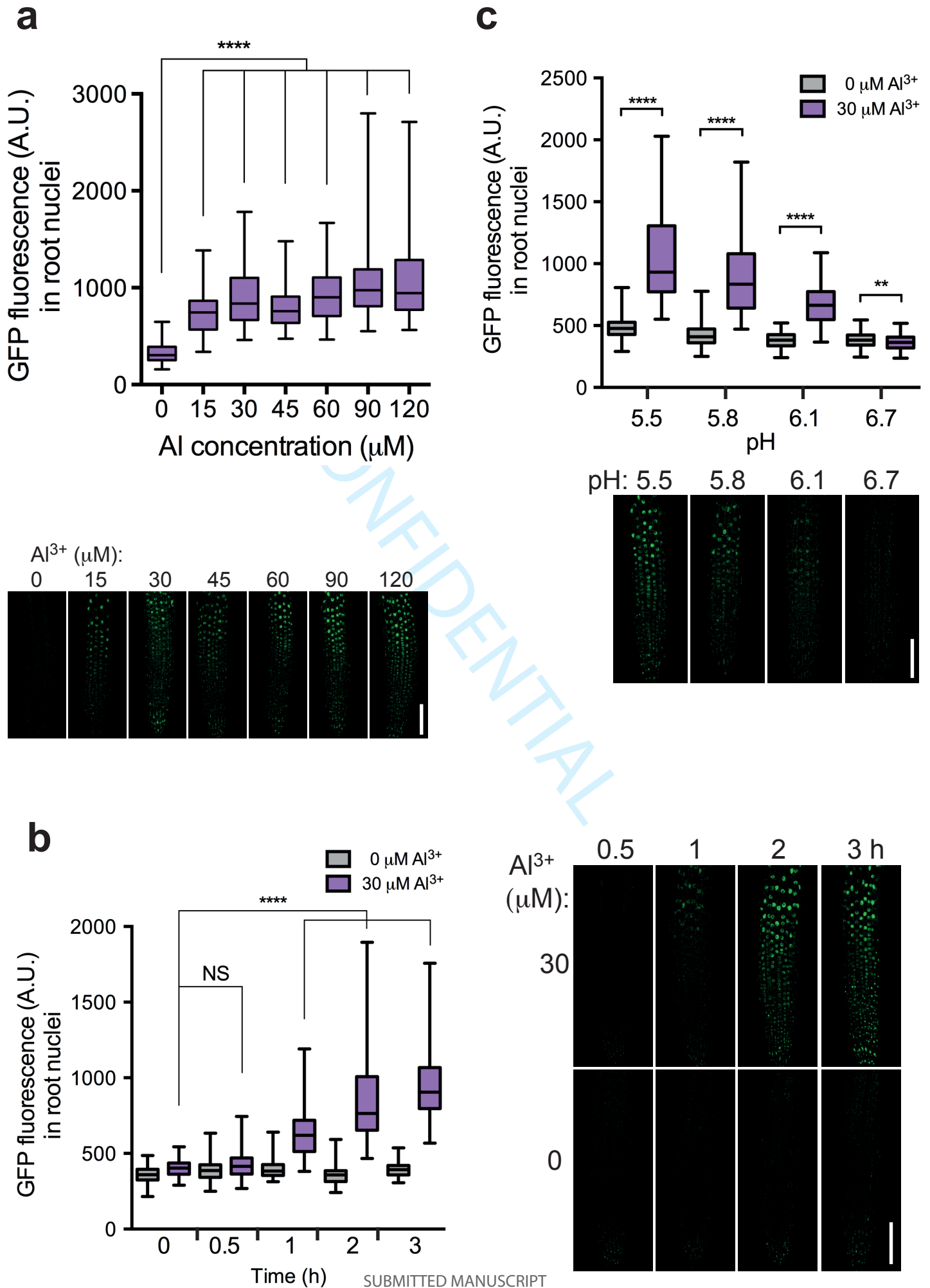


Figure 6

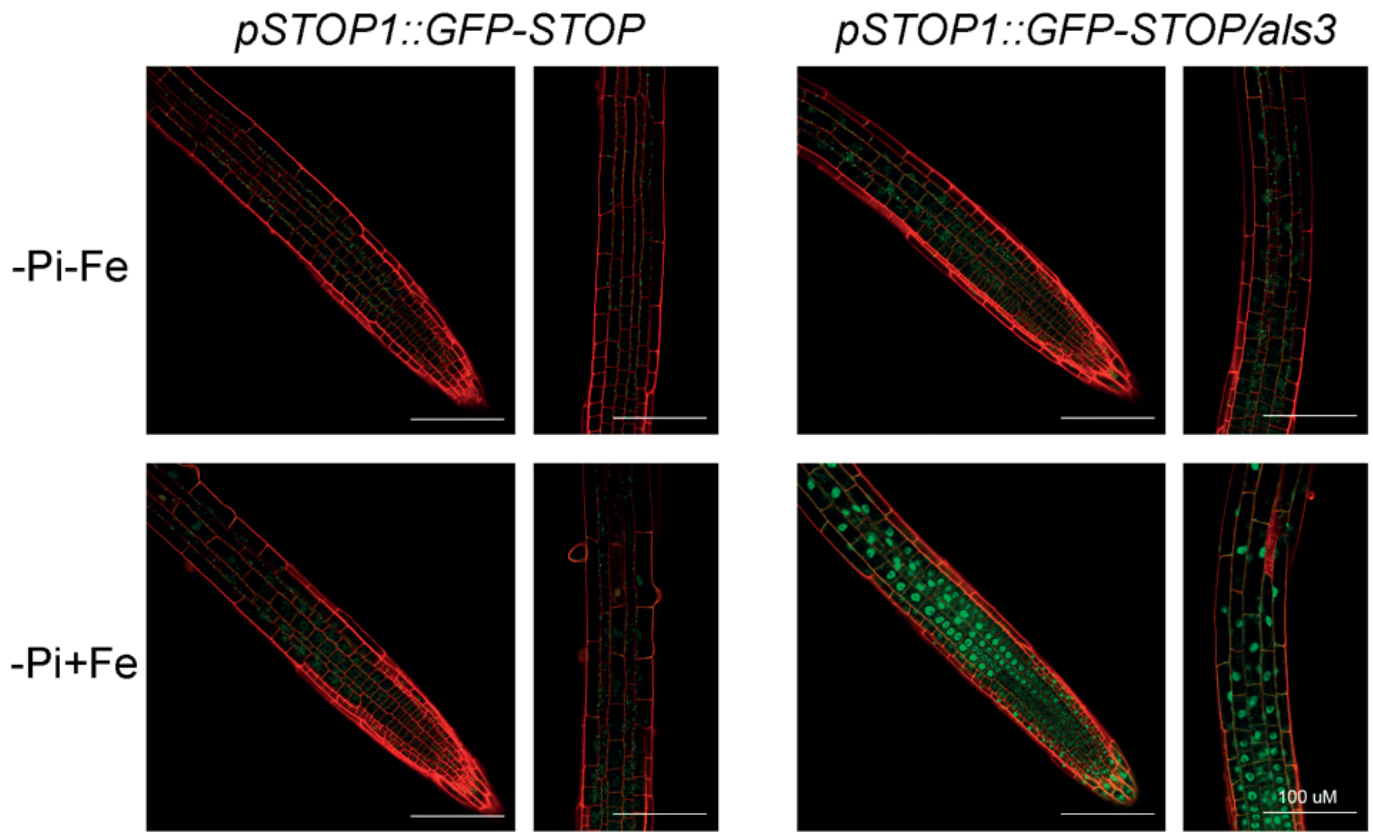
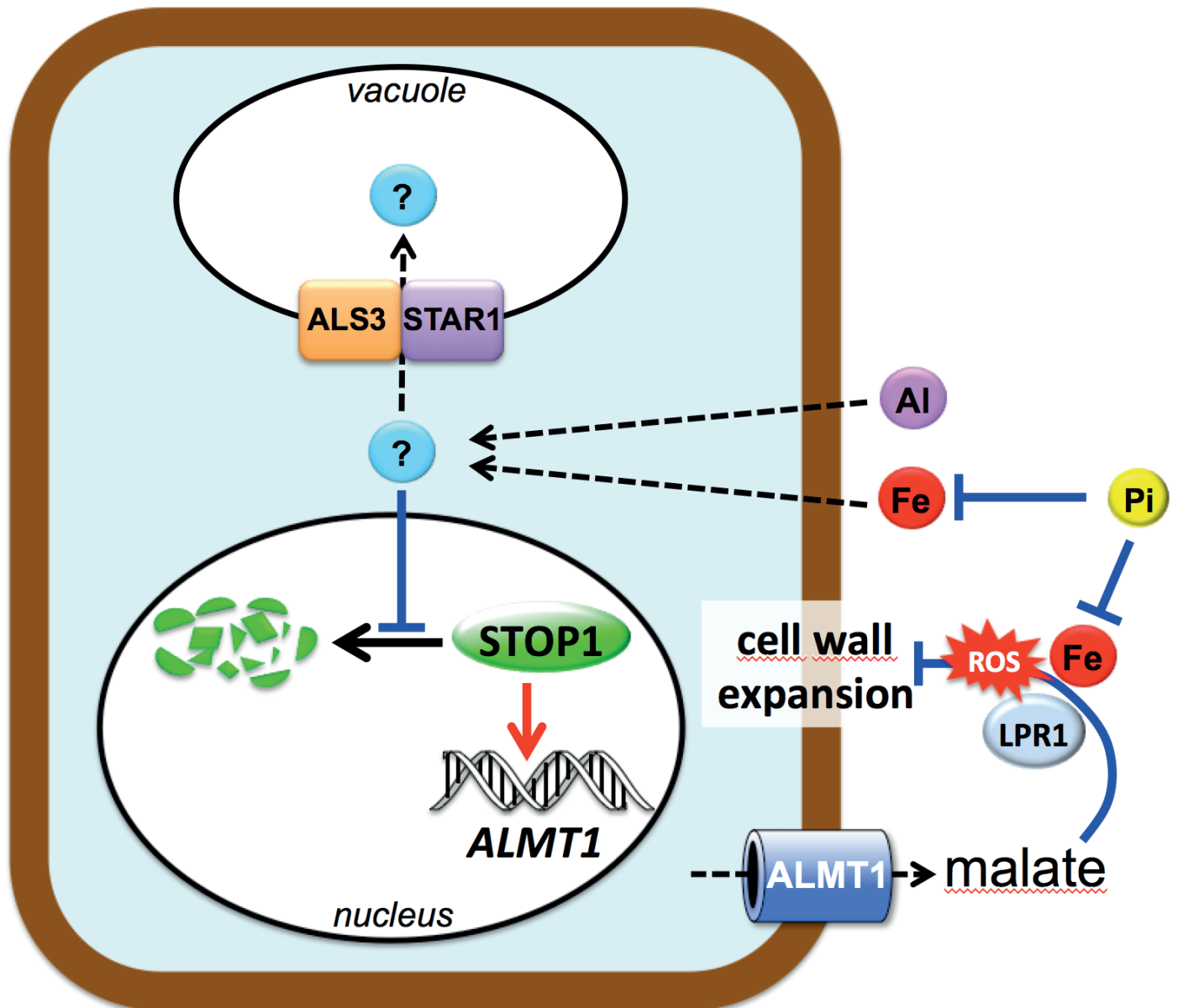


Figure 7



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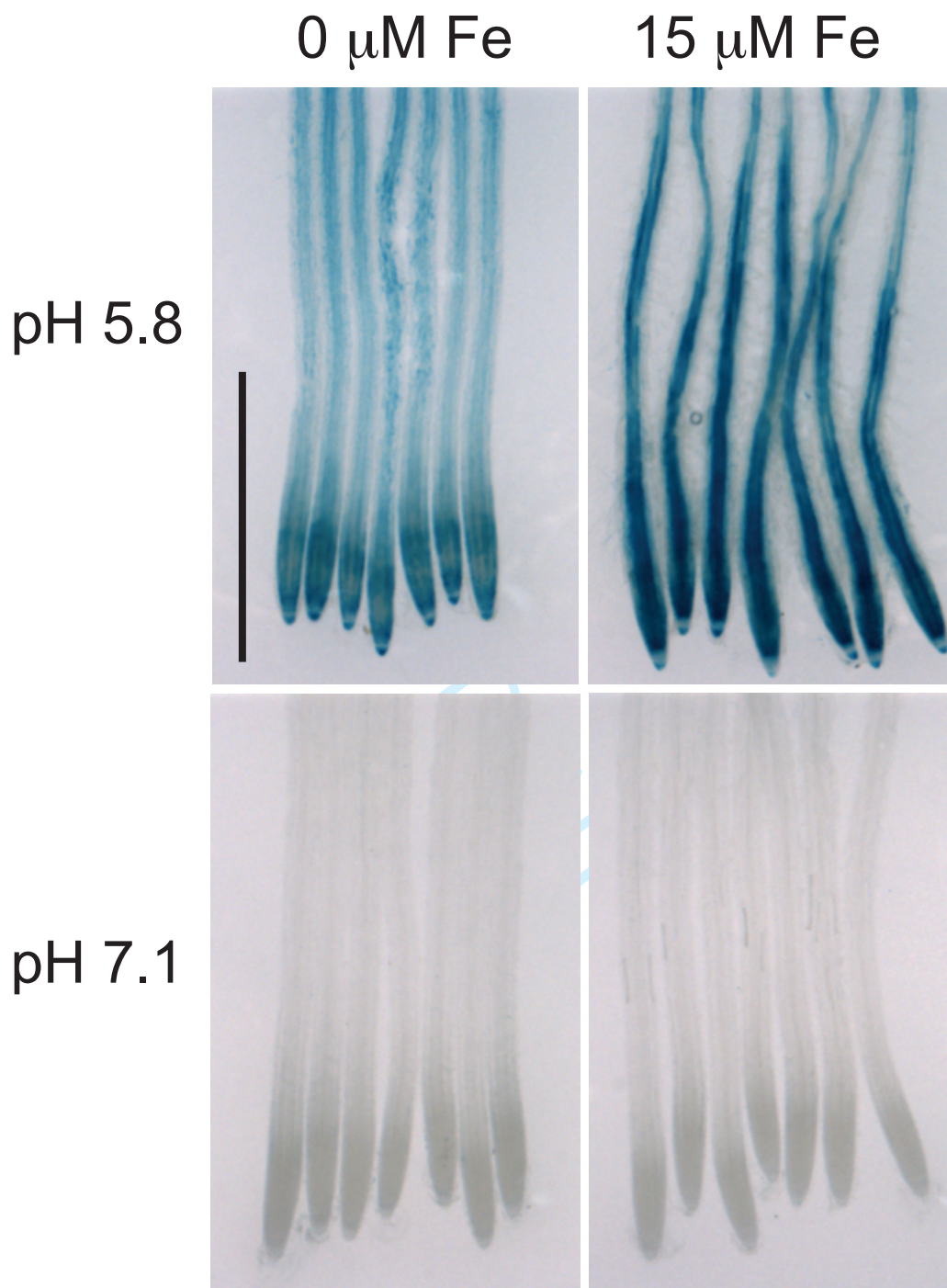
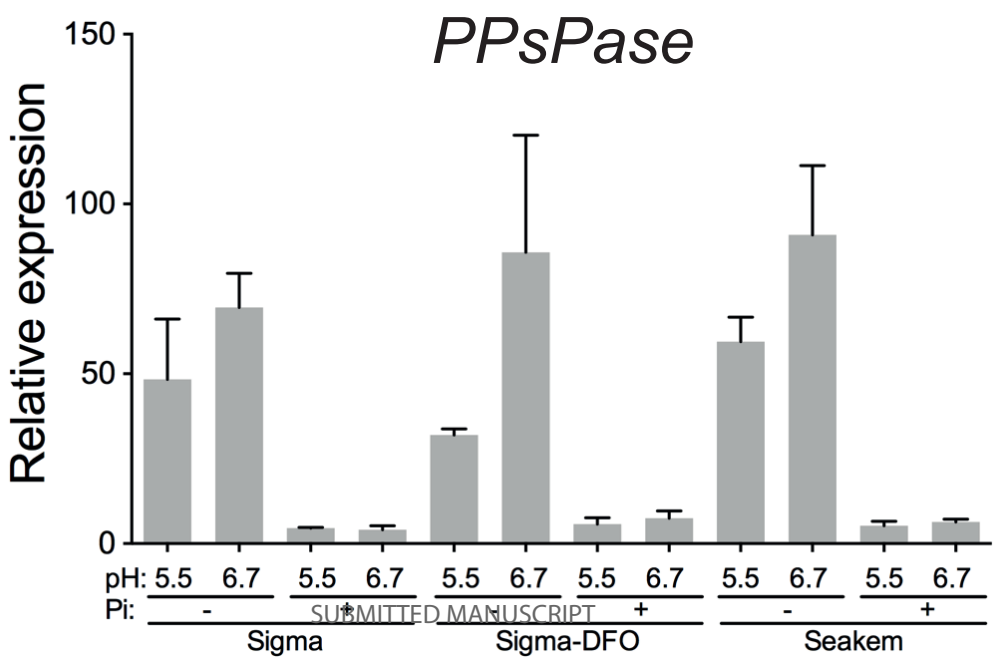
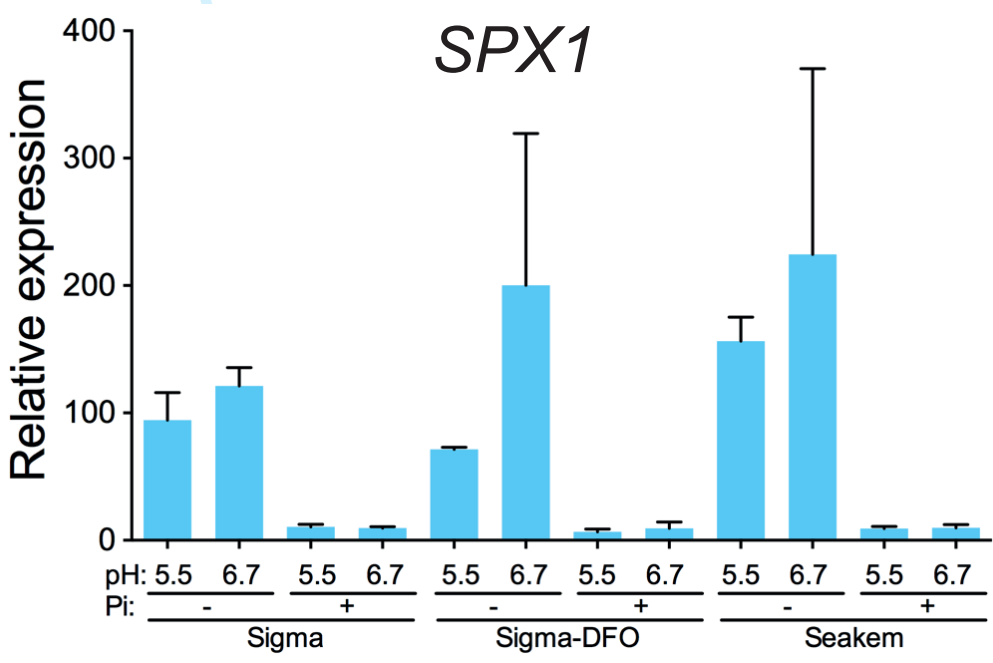
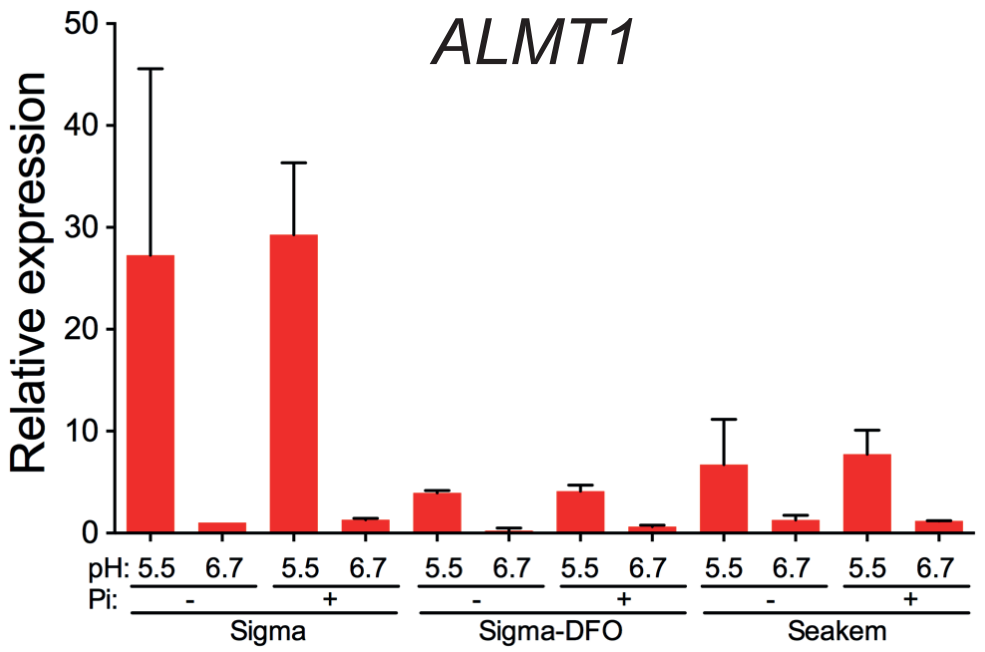


Figure S2

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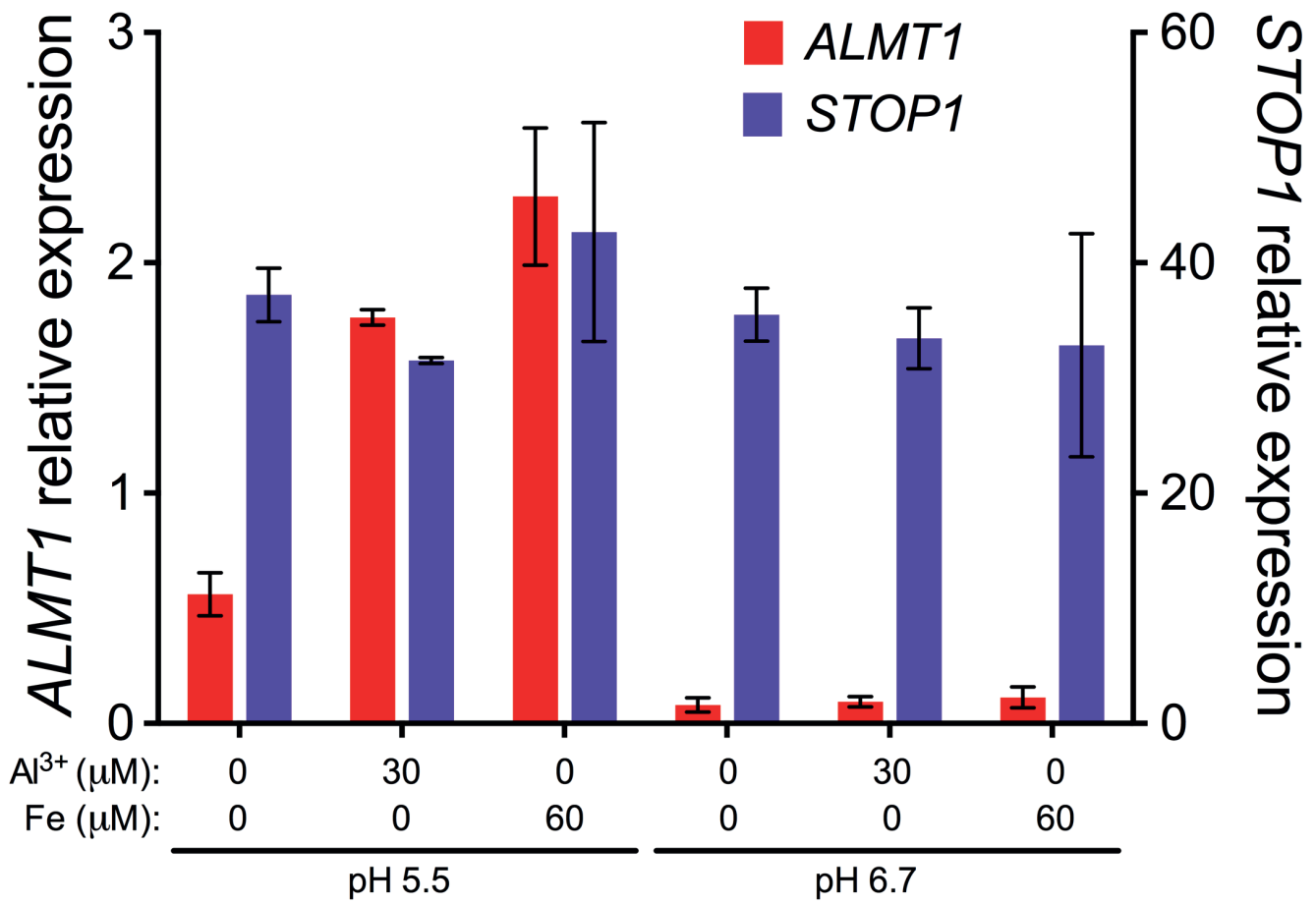
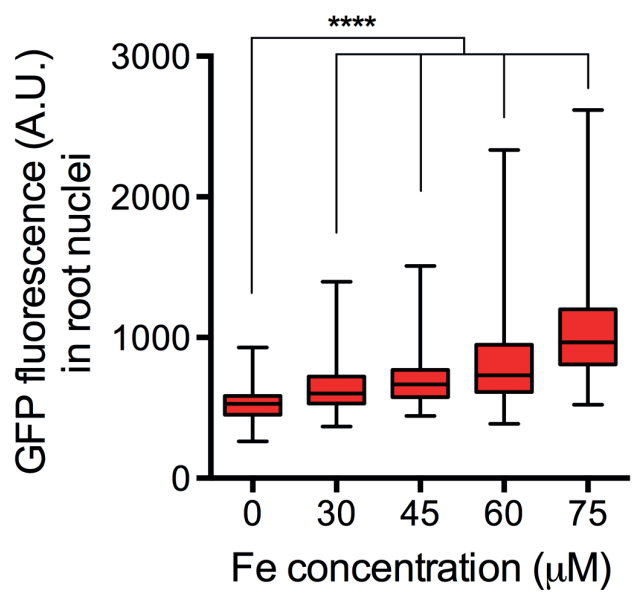


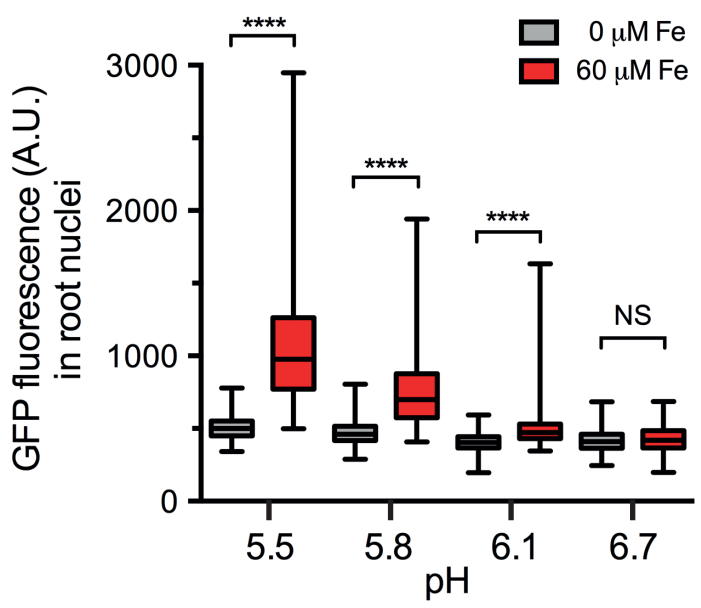
Figure S4

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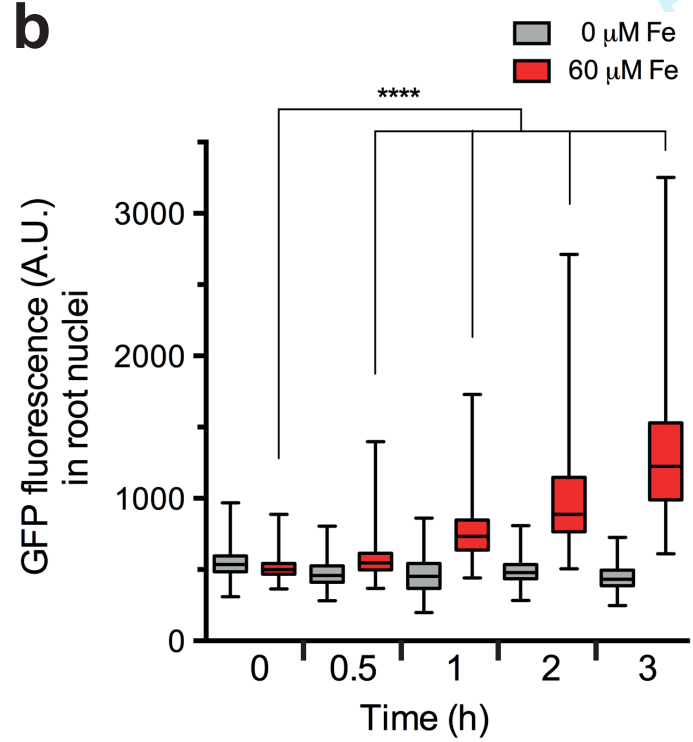


Figure S5

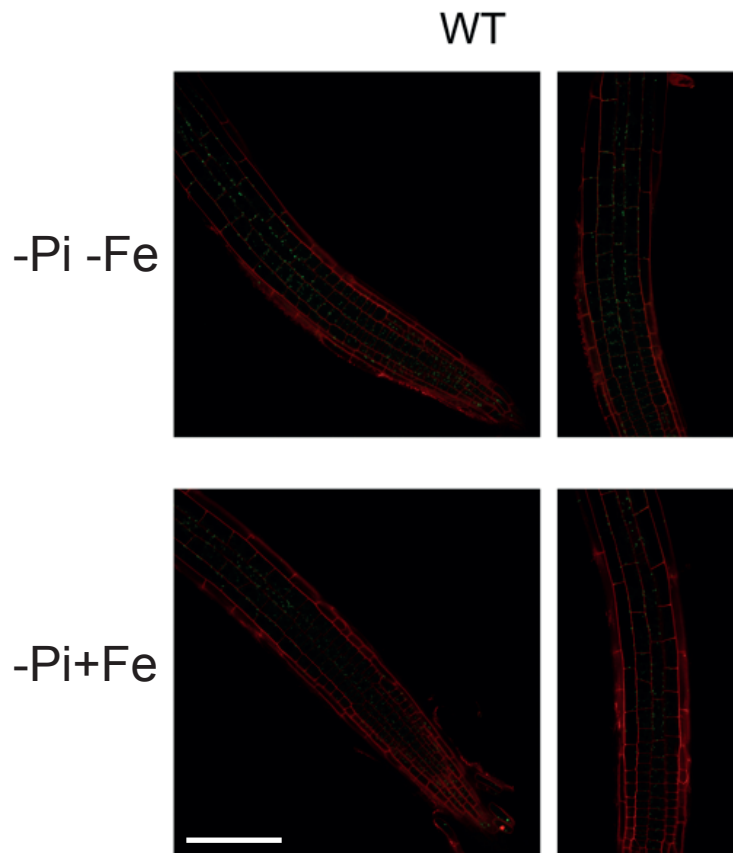
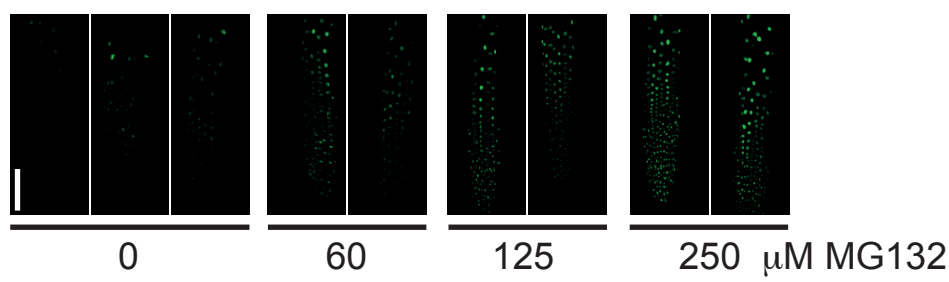


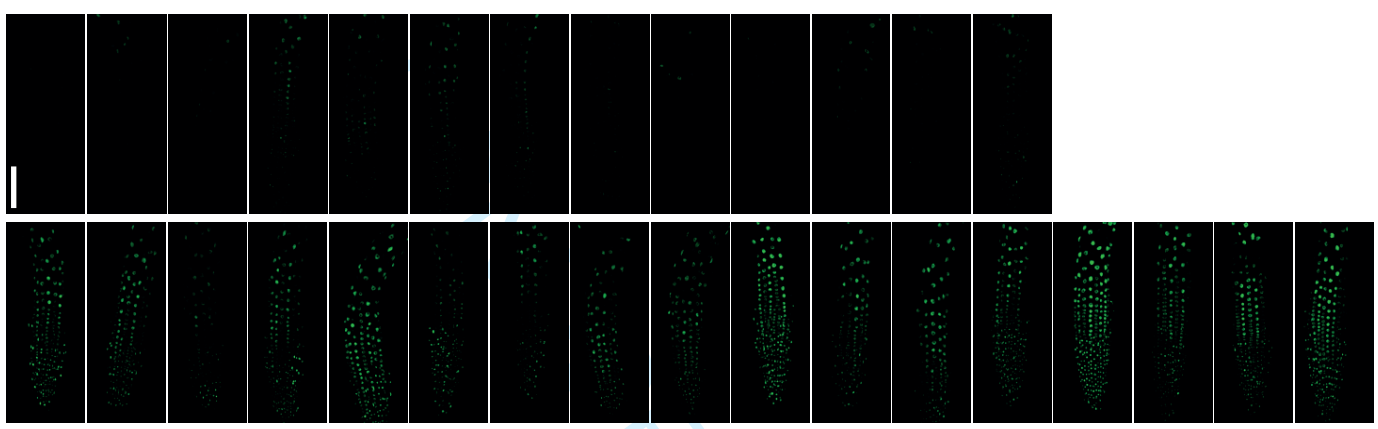
Figure S6

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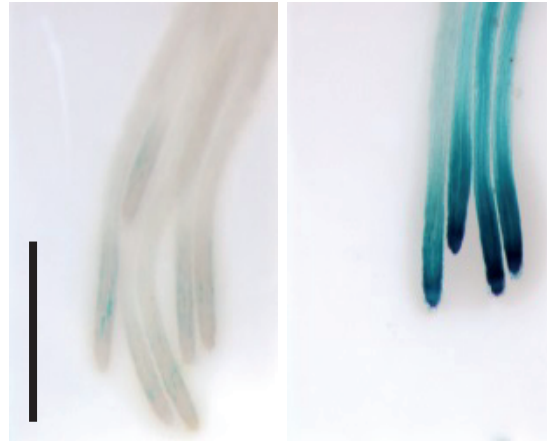


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CONFIDENTIAL

Figure S7



Al³⁺ (μM): 0 15

CONFIDENTIAL

Figure S8

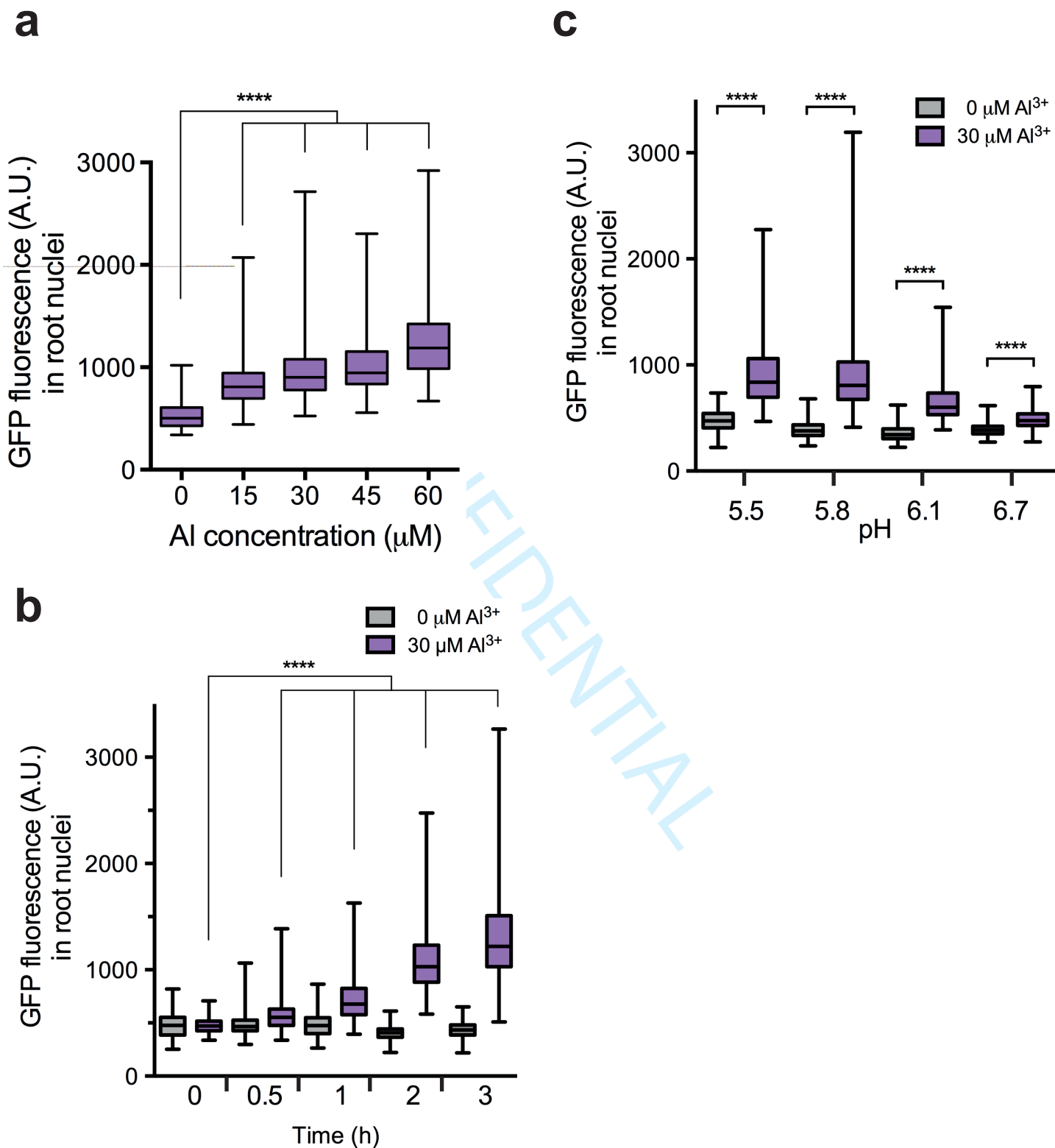


Figure S9

