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1 Imaging of labile Fe²⁺ and Fe³⁺ in living *Arabidopsis* 2 *thaliana* roots

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11 Dear Editor,

12 Imaging of iron (Fe) in living organisms is challenging and ways to visualize Fe are limited to
13 sophisticated elemental methods and/or fixed tissues. As a transition metal, Fe cycles between
14 two oxidation states, Fe²⁺ and Fe³⁺, losing or donating an electron in doing so. This property
15 enables Fe to participate in key metabolic pathways (Briat et al., 2015). Imaging of the redox
16 species of Fe is therefore of interest to decipher its biological functions. Cellular Fe is
17 partitioned into two distinct pools (Koppenol and Hider, 2019): static Fe, which is tightly bound
18 to its ligands, and labile Fe, which is weakly bound and can be exchanged between ligands
19 rather effortlessly. To date, there are no reports describing the distribution of Fe²⁺ and Fe³⁺
20 labile pools in living organisms. The Perls-DAB histochemical method stains Fe in fixed
21 tissues, chiefly the Fe³⁺ form (Roschzttardtz et al., 2009), but it mainly detects the static Fe
22 fraction since labile Fe is likely lost during tissue fixation. In order to address the dynamics of
23 Fe²⁺ and Fe³⁺ labile pools in live plants, we have established a method combining two probes,
24 which enables specific detection of the redox state of the labile Fe pools.

25 To that aim we have selected two fluorescent probes, SiRhoNox-1 (Hirayama et al., 2017) and
26 MPNBD (Park et al., 2014), which we used to image labile Fe²⁺ and Fe³⁺, respectively, in
27 *Arabidopsis* (*Arabidopsis thaliana*) roots (Supplemental Methods). The two probes were
28 chosen in such a way that their spectral properties do not overlap, allowing their simultaneous
29 utilization without any crosstalk (Supplemental Figure S1). The specificity of each probe was
30 reconfirmed *in vitro* albeit in an aqueous buffer adapted for plant applications (Supplemental
31 Figure S2). The fluorescence of the two probes, though depending on pH, was found rather
32 stable at physiological pH (6.0-6.5) (Supplemental Figure S2G,H). Fluorescence intensity
33 fluctuations must therefore be interpreted cautiously. The selectivity of the probes toward the
34 redox state of Fe was tested by applying them to various Fe species *in vitro*. The mixed probes

35 detected labile Fe species (Fe(II)-acetate, Fe(II)-SO₄, Fe(III)-NO₃, Fe(III)-Cl₃), but not the Fe
36 species involved in strong chelates such as EDTA or citrate (Fig. 1A). *In vitro* ascorbate-
37 mediated reduction of Fe³⁺ species into Fe²⁺ was successfully monitored by the probes (Fig.
38 1B), suggesting that the method is suitable to assess reductase activity *in vivo*.
39 SiRhoNox-1 and MPNBD were applied in combination to 7 day-old plants and compared with
40 Perls-DAB staining (Fig. 1C-K). The fluorescent signals observed with the two probes were
41 heterogeneously distributed along the entire primary root and were distinct from each other
42 (Supplemental Figure S3). Three root zones representative of the distribution of Fe²⁺ and Fe³⁺
43 were observed at higher magnification (Fig. 1D,G and J). Fe³⁺ was markedly predominant in
44 the primary root apex (Fig. 1J) but absent in the young lateral root (Supplemental Figure S4A-
45 C). Likewise, Perls-DAB did not stain the emerging root, suggesting that if Fe is present at this
46 stage, its level is under the detection threshold of the two methods (Supplemental Figure S4D).
47 The primary root apex exhibited no Fe²⁺ signal (Fig. 1J). In contrast, in the differentiation zone,
48 a strong Fe²⁺ fluorescent signal was observed at the cell periphery (Fig. 1G), suggesting an
49 apoplastic localization. This observation is in agreement with previous studies reporting
50 elemental analyses of cellular fractions (Bienfait et al., 1985; Ye et al., 2015; Liu et al., 2023).
51 Plasmolysis of root cells confirmed the apoplastic localization of Fe²⁺ (Supplemental Figure
52 S5). Moreover, colocalization of FM4-64 and SiRhoNox-1 revealed the presence of Fe²⁺ at the
53 plasma membrane (Supplemental Figure S5F). 3D images of each root zone emphasized the
54 variation of distribution of the two Fe redox species between cell layers and according to root
55 age (Supplemental Figure S6).
56 The Fe redox imaging method was applied to the Fe homeostasis ferric reduction oxidase 2
57 mutant (*fro2*), the Fe³⁺/Fe²⁺ ratio of which is imbalanced owing to a loss of its ability to reduce
58 Fe³⁺ at the root surface (Robinson et al., 1999; Connolly et al., 2003). Compared to wild-type,
59 the differentiation and mature zones of the *fro2* root expectedly exhibited a dramatic decrease
60 of fluorescence with SiRhoNox-1, confirming the specificity of the SiRhoNox-1 probe for Fe²⁺
61 *in vivo* (Fig. 1E,H, Supplemental Figure S7). The penetration ability of the probes was
62 examined using confocal microscopy. Fluorescence of SiRhoNox-1 and MPNBD was visible
63 in most layers of the root including the vascular cylinder (Fig. 2A-E), indicating that the two
64 probes are able to penetrate all the tissues of the root. In addition, fluorescence signals of the
65 Fe probes were detected inside the cells, showing the permeability of the plasma membrane
66 toward these probes. In epidermal cells, MPNBD fluorescence filled the symplast (Fig. 2F,H),
67 whereas SiRhoNox-1 fluorescence surrounded the cells (Fig. 2G). Upon Fe limitation,
68 SiRhoNox-1 produced intracellular punctuate signals (Fig. 2I,J)(Hirayama et al., 2013; 2017).

69 Interestingly the method highlighted a polarized pattern of Fe^{2+} at the external side of epidermal
70 cells in the differentiation zone (Fig. 2K-M), a feature that had not been reported previously.
71 Such Fe^{2+} polarization is reminiscent of the polar localization of FRO2 in the same cell type
72 (Martín-Barranco et al., 2020). Remarkably, this polar distribution shifted to the inner side of
73 the epidermal cells in the mature zone (Fig. 2, compare K-O and P-T) where Fe^{2+} was observed
74 in the apoplast (Fig. 2T, red arrows). Quantification of the fluorescence signals allowed
75 detecting subtle changes in the balance between Fe states, as shown in Fe-sufficient and Fe-
76 deficient conditions in wild-type and the *fro2* mutant (Supplemental Figure S7).
77 In summary, combining fluorescent probes for Fe^{2+} and Fe^{3+} represents an original method to
78 distinguish the redox species of Fe within live tissues, reveals their distribution in root , and
79 uncovers a remarkable polarization of Fe^{2+} . Because this method can detect subtle differences
80 of Fe charges in the tissues, it will become useful to characterize actors of the redox status of
81 Fe, such as oxido-reductases, hence equipping the community with a powerful tool to explore
82 Fe homeostasis in plants.

83

84 **SUPPLEMENTARY DATA**

85 Supplementary Figure S1. Excitation and emission spectra of SiRhoNox-1 and MPNBD
86 probes at pH 6.0.

87 Supplementary Figure S2. *In vitro* characterization of the Fe redox SiRhoNox-1 and MPNBD
88 fluorescent probes.

89 Supplementary Figure S3. Distribution of Fe along the primary root of *A. thaliana* grown on
90 0.5xMS containing 50 μM Fe-EDTA.

91 Supplementary Figure S4. The lateral root apex is not stained by the two Fe probes.

92 Supplementary Figure S5. Fe is localized in the apoplastic space of root epidermal cells.

93 Supplementary Figure S6. Differential spatial distribution of Fe^{2+} and Fe^{3+} according to the
94 developmental stage of the root in 7 day-old plants grown in Fe replete conditions.

95 Supplementary Figure S7. Changes in Fe redox state are dependent on growth conditions and
96 Fe homeostasis.

97 Supplementary Methods.

98

99

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108

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 115 (<https://academic.oup.com/plphys/pages/General-Instructions>) is Tou Cheu Xiong (tou-
 116 cheu.xiong@inrae.fr).

117

118 **AUTHOR CONTRIBUTIONS**

119 CA and TCX performed fluorescence imaging and Perls-DAB staining, designed and carried
 120 out the experiments; AC performed fluorescent probe synthesis; CA, TCX, CC wrote the paper.

121

122

123 **Figure 1: *In vitro* and *in vivo* detection of labile Fe²⁺ and Fe³⁺ using fluorescent probes.**124 **(A,B)** Determination of the specificity of the SiRhoNox-1 and MPNBD probes *in vitro*. **(A)**125 Both probes were applied in combination to 1 mM solutions of a variety of Fe species. **(B)**126 Reduction of Fe³⁺ by addition of 1mM ascorbate (ASC) to the different Fe species allowed127 detecting a change of Fe redox state. **(C-K)** *In vivo* imaging of Fe in the roots of 7 day-old

128 Arabidopsis plants grown on 0.5xMS containing 50μM Fe-EDTA. Labile Fe, detected using

129 combined SiRhoNox-1 and MPNBD **(D, E, G, H, J, K)**, was compared with Fe histochemical130 staining with the Perls-DAB method **(C, F, I)**. In the primary root, the mature zone **(C-E)**,131 differentiation zone **(F-H)** and apex zone **(I-K)** are shown. The projection of maximum132 intensity of the Z-stack of fluorescent pictures is shown for SiRhoNox-1 (Fe²⁺, magenta) and133 MPNBD (Fe³⁺, green) in wild-type (WT) plants **(D, G, J)** and in the *fro2* mutant **(E, H, K)**.

134 Data shown are mean ± SD. Data were collected from 3-4 independent experiments. EDTA=

135 Ethylenediaminetetraacetic acid; a.u.= arbitrary unit; DAB= 3,3'-Diaminobenzidine. All scale

136 bars = 200 μm.

137

138 **Figure 2: Distribution of labile Fe in the different cell layers of the primary root of**
139 ***Arabidopsis thaliana*.** Images were taken from 7 day-old seedlings. (A-E) Representative
140 orthogonal view of the mature zone of the primary root stained with MPNBD (Fe³⁺, A),
141 SiRhoNox-1 (Fe²⁺, B), propidium iodide (Cell wall, C), or a merged image of the 3 probes (D,
142 E). Labile Fe²⁺ and Fe³⁺ are present in most cell types with a signal in the endodermis. An
143 enlarged view of the endodermal layer indicated by a dashed square shows interruption of the
144 apoplastic fluorescent signal at the Casparian strip (arrowheads, E). (F-J): MPNBD and
145 SiRhoNox-1 fluorescent signals in root epidermis showing the presence of Fe³⁺ inside the cell
146 (F) and Fe²⁺ in the apoplast (G) of Fe-replete plants as well as in intracellular dot-like structures
147 of Fe-deficient plants (H-J). (J) Close-up view of the Fe²⁺ dots presented in (I). (K-T): Polar
148 distribution of Fe²⁺ in the epidermal cell wall. Differentiation (K-O) and mature (P-T) zones of
149 the primary root were observed in longitudinal sections. Higher magnification of the epidermal
150 cells (L, Q) shows polar localization of Fe²⁺, albeit in opposite pattern in the two zones, which
151 is confirmed by the line profile of the fluorescence intensity of the probes (M, R). (N, S, O, T):
152 Orthogonal view of the differentiation (N, O) and mature (S, T) root zones, including the
153 corresponding enlarged views (O, T) indicated by dashed areas within panels N and S. (T)
154 SiRhoNox-1 labels the intercellular space in the mature zone (red arrows). (A-G and K-T):
155 Seedlings were grown on 0.5xMS containing 50 μM Fe-EDTA (+Fe). (H-J): Seedlings were
156 grown on 0.5xMS without Fe (-Fe). Magenta LUT: SiRhoNox-1; Green LUT: MPNBD; BIOP-
157 Azure LUT: Propidium iodide. MPNBD = 7-(4-methylpiperazin-1-yl)-4-nitrobenzo-2-oxa-1,3-
158 diazole; a.u.= arbitrary unit; EDTA = Ethylenediaminetetraacetic acid; All scale bars = 20 μm.

159

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196 **COMPETING FINANCIAL INTERESTS**

197 The authors declare no competing financial interests.

Figure 1

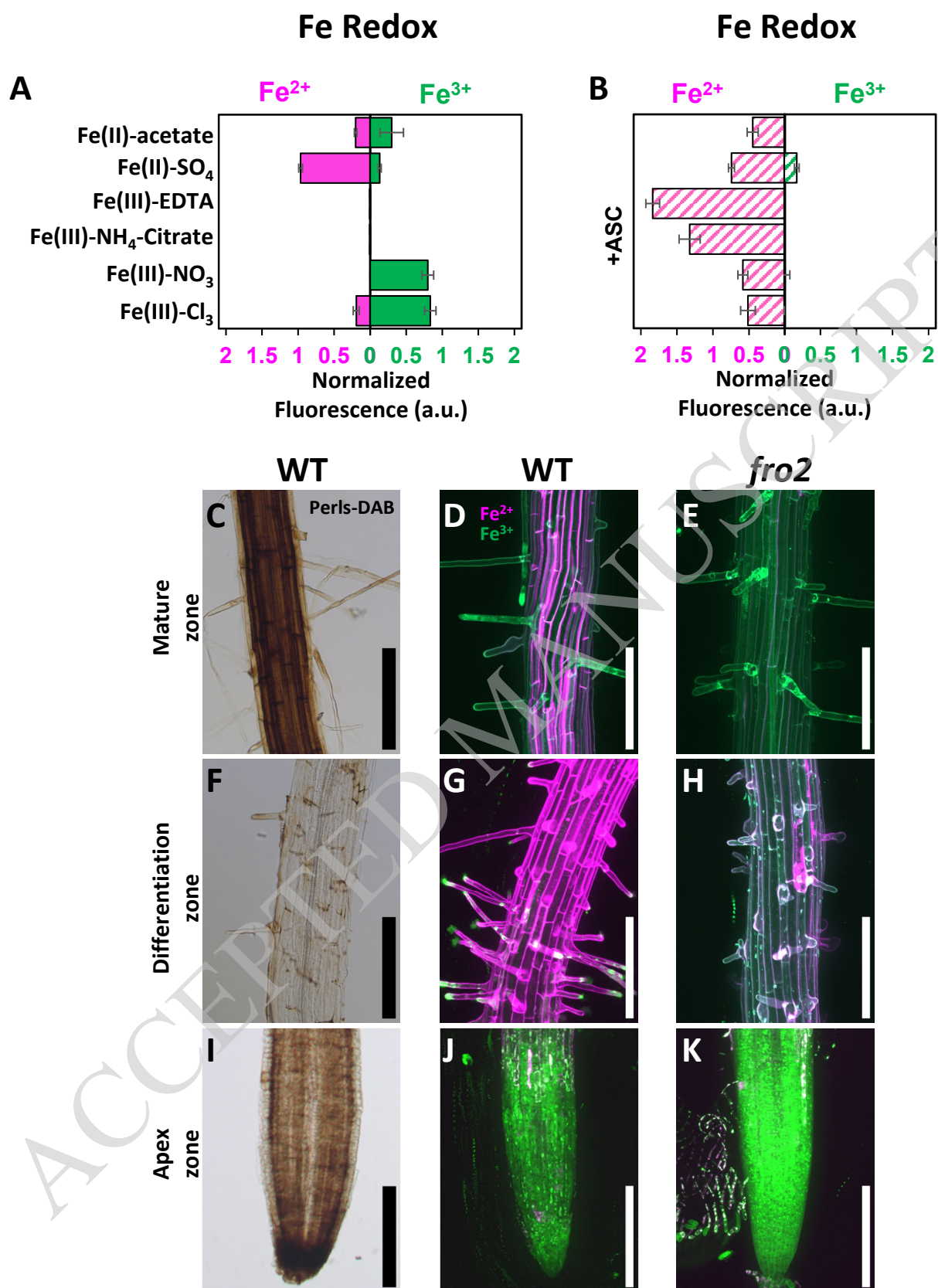
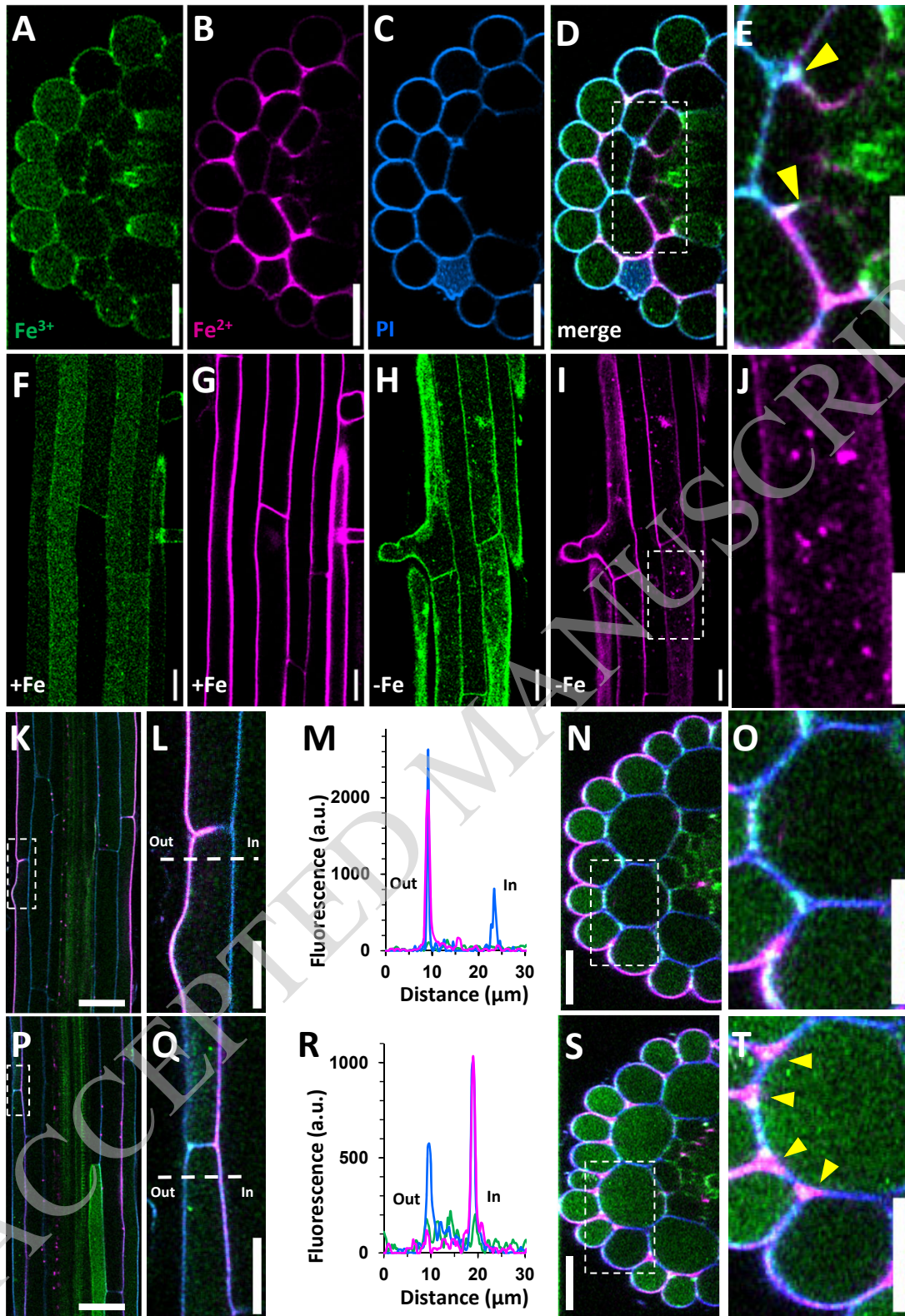


Figure 2



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