



HAL
open science

Low pH-induced cell wall disturbances in *Arabidopsis thaliana* roots lead to a pattern-specific programmed cell death in the different root zones and arrested elongation in late elongation zone

Jonathas Pereira Graças, Mariana Belloti, Joni Esrom Lima, Lázaro Eustáquio Pereira Peres, Vincent Burlat, Elisabeth Jamet, Victor Alexandre Vitorello

► To cite this version:

Jonathas Pereira Graças, Mariana Belloti, Joni Esrom Lima, Lázaro Eustáquio Pereira Peres, Vincent Burlat, et al.. Low pH-induced cell wall disturbances in *Arabidopsis thaliana* roots lead to a pattern-specific programmed cell death in the different root zones and arrested elongation in late elongation zone. *Environmental and Experimental Botany*, 2021, 190, pp.104596. 10.1016/j.envexpbot.2021.104596 . hal-03437134

HAL Id: hal-03437134

<https://ut3-toulouseinp.hal.science/hal-03437134>

Submitted on 2 Aug 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Low pH-induced cell wall disturbances in *Arabidopsis thaliana* roots lead to a pattern-specific programmed cell death in the different root zones and arrested elongation in late elongation zone

1 **Jonathas Pereira Graças^{1,3,4*}, Mariana Belloti², Joni Esrom Lima¹, Lázaro Eustáquio Pereira**
2 **Peres³, Vincent Burlat⁴, Elisabeth Jamet⁴, Victor Alexandre Vitorello^{2*}**

3 ¹Present address: Botany Department, Federal University of Minas Gerais, Avenida Antônio Carlos,
4 6627 Bloco I- 2 Sala 316, 31270-901, Belo Horizonte, Brazil

5 ²Laboratory of Cell and Molecular Biology, Centro de Energia Nuclear na Agricultura, University of
6 São Paulo, CP 96, Av. Centenário 303, 13416-000 Piracicaba, SP, Brazil

7 ³Laboratory of Hormonal Control of Plant Development, Escola Superior de Agricultura “Luiz de
8 Queiroz”, University of São Paulo, Piracicaba, Brazil

9 ⁴Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Auzeville
10 Tolosane, France

11

12 * Corresponding Authors:

13 jonathasgracas@gmail.com (J.P. Graças), victor@cena.usp.br (V.A. Vitorello).

14 **Abstract**

15 Low pH stress (pH < 5.5) triggers sensitivity responses in roots such as cell death (CD) or arrest in
16 elongation which are detrimental to the development of many plant species. The involvement of
17 the cell wall (CW) with these sensitive responses and their spatiotemporal dynamics in the distinct
18 root zones remains poorly understood. The spatio-temporal analysis of primary roots of *Arabidopsis*
19 *thaliana* upon low pH (pH 4.6) revealed that the CD repeatedly started in cells of the transition zone
20 (TZ). Then, CD dynamically moved downward to the meristematic zone (MZ) and upward to the
21 early elongation zone (EZ). The dead cells exhibited coordinated *in situ* DNA fragmentation,
22 highlighted by the deformity in the nuclei of dead cells and positive reaction for Terminal
23 Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL). This DNA fragmentation
24 strongly suggests that the low pH-driven CD occurred through a programmed cell death (PCD)
25 pathway. In addition, a decreased CW stiffness in TZ epidermal cells before the onset of CD
26 suggests that low pH first triggers cell wall disturbances (CWDs) in these root cells. Disruption in CW
27 integrity caused by incorrect cellulose deposition in *cobra* (*cob-1*) mutant enhanced CD in TZ and
28 early EZ. Supporting this notion, an increase in calcium concentration upon low pH alleviated the CD
29 in roots, probably due to its role in stabilization of the pectin crosslinking in CWs and likely
30 counteracting the low pH-induced CWDs. Moreover, the CD was significantly decreased when roots
31 were exposed to low pH under reduced CW tension, however, a sudden increase in turgor pressure
32 and CW tension (hypoosmotic treatment) combined with low pH accelerated CD in TZ and early EZ.
33 The CD was not observed in the wild type late EZ trichoblasts upon low pH but rather an
34 orchestrated arrest in elongation. An increase in calcium concentration inhibited this elongation
35 arrest upon low pH, suggesting that the onset of this response also required CWDs. Altogether,
36 these results indicate that low pH-induced CWDs triggered sensitive responses within defined root
37 zones. The low pH-activated PCD and an orchestrated elongation arrest occurring in different root
38 zones suggest the occurrence of yet to be identified signaling cascades.

39

40 Keywords: acidic stress, cell mortality, cell wall integrity, cell wall stiffness, root zone.

41 Abbreviations

42 CD Cell Death

43 CW Cell Wall

44 CWD Cell Wall Disturbance

45 CWI Cell Wall Integrity

46 DAPI 4',6-Diamidino-2-phenylindole dihydrochloride

47 HO Hypoosmotic

48 HYO Hyperosmotic

49 LEH Length of the first Epidermal cell with visible root Hair bulge

50 OG Oligogalacturonide

51 TUNEL Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling

52

53 1. Introduction

54 More than 40 % of the world's arable soils are acidic (pH < 5.5) (Von Uexküll and Mutert, 1995).
 55 Acid stress, *i.e.*, exogenous low pH, causes sensitive responses in plants such as arrest in root
 56 growth and cell death (CD) in root tip cells (Koyama et al., 2001) and consequently has a
 57 detrimental impact on agricultural ecosystems. These sensitive responses remain poorly
 58 understood at the tissular and molecular levels (Kobayashi et al., 2013; Graças et al., 2016; Graças
 59 et al., 2020). Thus, understanding the sensitivity of different root cells to low pH is both a
 60 fundamental issue in plant biology and a way to improve agriculture.

61 The pH dynamics across different cellular compartments is crucial for several processes
 62 (reviewed by Shavrukov and Hirai, 2016). The elongation of plant cells has been proposed to
 63 depend on a mechanism of apoplast acidification known as the "acid growth theory" (Rayle and
 64 Cleland, 1992). Acidification caused by proton extrusion activates enzymes/proteins secreted in the
 65 cell wall (CW) which in turn increase the extensibility of CWs through modifications of
 66 polysaccharides and of their interactions, thus allowing further cell elongation (Spartz et al., 2014;
 67 Cosgrove, 2015; Barbez et al., 2017; Hocq et al., 2017). This apoplast acidification during elongation
 68 is transitory and tightly controlled (Bibikova et al., 1998).

69 Root growth is reduced in treatment solutions with low pH and low ionic strength, simulating
 70 natural stressful soil conditions (Koyama et al., 2001, Kobayashi et al., 2014; Graças et al., 2016).
 71 This apparent discrepancy with the cell elongation mechanism proposed by the "acid growth
 72 theory" might arise when exogenous low pH occurs at the wrong place or time and reaches a stress
 73 threshold. The mechanism of root growth arrest under exogenous low pH stress remains elusive,
 74 however, it was associated with a coordinated response to circumvent CD in root tip cells (Graças et
 75 al., 2016).

76 In root, four zones can be distinguished above the root cap, from meristematic to fully-
 77 differentiated cells: meristematic zone (MZ), transition zone (TZ), fast elongation zone (EZ) and
 78 growth-arrested zone (AZ) (Verbelen et al., 2006). Three coordinated cellular processes determine
 79 root growth rates: cell division, duration of cell cycle and cell elongation (Verbelen et al., 2006;
 80 Tsukagoshi et al., 2010). Cell division and the duration of the cell cycle regulate the number of cells
 81 that exit MZ to TZ. In TZ, root cells undergo an increase in cell volume (Verbelen et al., 2006). TZ is

82 an active site for the crosstalk between auxin and cytokinin that antagonistically regulates the
83 balance between cell division and cell differentiation (Kong et al., 2018). In EZ, CW modifications
84 prime root cells to increase their size by an order of magnitude under a constant turgor pressure,
85 triggering the cellular elongation (Verbelen et al., 2006; Cosgrove, 2015; Somssich et al., 2016). The
86 coordination of cell elongation contributes to the rapid regulation of root growth (Le et al., 2001;
87 Verbelen et al., 2006).

88 The occurrence of CD in root tip cells upon low pH stress was previously reported (Koyama et al.,
89 2001; Kobayashi et al., 2013; Graças et al., 2016). However, whether a mechanism coordinates this
90 CD upon low pH remains largely elusive. Necrotic CD is the result of multiple uncontrolled events
91 caused by severe cellular stresses and displays features such as cell swelling (Reape et al., 2008).
92 Conversely, programmed cell death (PCD) is an orchestrated event characterized by hallmarks such
93 as nuclear condensation, DNA cleavage and cell shrinkage (Reape et al., 2008; Watanabe and Lam,
94 2008). PCD is part of root development involved in xylogenesis, control of the number of root cap
95 cells and death of senescent tissues (Bagniewska-Zadworna and Arasimowicz-Jelonek, 2016).
96 Furthermore, PCD is a coordinated response to biotic or abiotic stresses (Olvera-Carrillo et al.,
97 2015). Thus, it is relevant to determine if the low pH-induced CD is caused by necrotic death or
98 through a PCD mechanism. Moreover, to date, it is not clear whether CD due to low pH occurs
99 randomly or according to a defined pattern in the four root zones described above.

100 Indirect evidence suggesting the involvement of the CW in root tissues to mediate low pH
101 responses was revealed examining the role of ions in the alleviation of low pH stress. Increasing
102 calcium and boron concentration in culture media restore root growth and prevents CD in root tip
103 cells upon low pH stress (Koyama et al., 2001; Kobayashi et al., 2013). Calcium and boron play
104 critical roles in crosslinking of pectic homogalacturonan and rhamnogalacturonan II, respectively
105 (Willats et al., 2001), and therefore, likely acting upon CW structure by limiting cell wall
106 disturbances (CWDs) caused by excessive proton concentration. A possible CW remodeling in roots
107 upon low pH was suggested by analysis of the expression of CW-related genes. Genes encoding two
108 polygalacturonase-inhibiting proteins (PGIPs) were found to be regulated by SENSITIVE TO PROTON
109 RHIZOTOXICITY 2 (STOP2), a transcription factor involved in tolerance to protons and aluminum
110 (Kobayashi et al., 2014). Accordingly, the binding of PGIPs to homogalacturonan was proposed to
111 contribute to the stabilization of CWs upon low pH exposure, thus decreasing cell sensitivity to
112 stress. Furthermore, CW loosening or strengthening likely involves the enzymatic activity of
113 apoplastic proteins such as class III peroxidases (CIII Prxs) (Francoz et al., 2015). Recently, the
114 *Arabidopsis thaliana* CIII Prxs *AtPrx62* was shown to be positively and spatiotemporally involved
115 with the CD induced upon exogenous low pH stress in roots (Graças et al., 2020).

116 The perception of CWDs by sensor proteins is crucial for plant development as well as in
117 response to stresses to maintain cell wall integrity (CWI) (Liu et al., 2015; Vaahtera et al., 2019). The
118 large number of these proteins and their overlapping and complementary roles make it difficult to
119 find candidates to be investigated in low pH response. However, *bona fide* CW architecture, besides
120 being relevant for CWI (Liu et al., 2015), may also contribute to the sensitivity of roots to low pH
121 toxicity. Mutants impaired in COBRA, a GPI-anchored protein, display unusual cell expansion in
122 roots caused by poorly organized cellulose microfibrils, thus impacting root development (Benfey et
123 al., 1993; Roudier et al., 2002). We hypothesize that CWD can be crucial for the occurrence of CD
124 upon low pH. Therefore, proteins related to CW *bona fide* architecture (e.g., COBRA) might be
125 relevant players for the occurrence of CD in response to low pH.

126 Evidence that the CW is a target for exogenous low pH stress also comes from studies of cell
127 expansion in trichoblast cellular structures. Tip-growing root hairs burst upon low pH treatment (pH
128 4.5), but this response does not occur in root hairs that have ceased growth (Bibikova et al., 1998)

129 or are treated at high pH (pH 8.0) (Monshausen et al., 2007). Under constant turgor pressure and
 130 CW tension, a sudden rupture between polysaccharide bonds could occur in CW, thus causing root
 131 hair bursting. This apparent loosening and yielding of CW could be interpreted as CWDs in root
 132 hairs. Whether the CW undergo CWDs due to exogenous low pH stress in complex root tip tissues
 133 such as MZ, TZ, EZ or AZ and if this is linked to CD or arrest in root elongation remains elusive.

134 We present evidence that low pH-induced CWDs are crucial for the occurrence of CD and arrest
 135 in elongation in different tissues of *A. thaliana* primary roots. Root CD triggered within a defined
 136 spatiotemporal pattern in MZ/TZ/early EZ involves the CWI-related gene *COBRA* and is likely
 137 coordinated through a PCD mechanism. Furthermore, the low pH-induced elongation arrest in late
 138 EZ is not a direct effect of low pH, but rather a plant coordinated response.

139 2. Material and methods

140 2.1. Plant material and growth conditions

141 We used 5-day-old *A. thaliana* seedlings of ecotype Columbia-0 (Col-0) as wild type (WT). We
 142 also examined the *cobra-1* mutant phenotype, a previously confirmed homozygous mutant
 143 obtained by EMS mutagenesis (<https://abrc.osu.edu/>; Germplasm stock: CS8541, Locus:
 144 *AT5G60920*) (Hauser et al., 1995).

145 Seeds were disinfected in a commercial sodium hypochlorite solution (5%) for 10 min under
 146 stirring and then washed 5 times with distilled water. The seeds were then transferred to Petri
 147 dishes containing a modified Hoagland's solution at pH 5.8 and 0.8% agar. This solution was
 148 composed as described by Graças *et al.* (2020), except for iron that was 30 μ M Fe-EDDHA as Libfer®
 149 SP (Royal Brinkman, 's-Gravenzande, the Netherlands).

150 The growth temperature was 22°C and the light intensity was approximately $\pm 110 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.
 151 For each treatment, at least ten 5-day-old seedlings were incubated in a 250 mL Erlenmeyer
 152 containing 20 mL of treatment solution composed of 0.5 mM CaCl_2 and 0.6 mM Homo-PIPES
 153 (homopiperazine-1,4-bis(2-ethanesulfonic acid) adjusted to required pH, as previously performed
 154 (Graças et al., 2016). For the calcium treatment, only the CaCl_2 concentration was changed. The
 155 solution was continuously gently aerated.

156 All the analyses described below were performed in primary roots, which showed a length of
 157 approximately 2 cm after five days in our growth conditions.

159 2.2. Evaluation of cell death in root tips

160 We examined CD using Evans blue (Baker and Mock, 1994), a large molecule that only
 161 penetrates dead cells which have lost membrane selectivity. After low pH treatments, seedlings
 162 were soaked in a 0.25 % (w/v) solution of Evans blue for 15 min. Then, they were washed thrice for
 163 5 min with distilled water. Immediately, bright field images were taken, or the roots were frozen in
 164 liquid nitrogen and stored at -20°C for later quantification of Evans blue uptake.

165 For quantitative evaluation of CD as indicated by Evans blue uptake, we performed minor
 166 modifications in the method described by Baker and Mock (1994). We added 300 μ L DMSO
 167 (dimethyl sulfoxide) to ten frozen excised root tips (0.2 cm) from each treatment for 1 h. Then, the
 168 plastic tubes containing this mix were centrifuged at 8.000 $\times g$ for 3 min, and the liquid was used to
 169 read the fluorescence at 690 nm after excitation at 645 nm. Thus, fluorescence reads could be
 170 compared between control (pH 5.8) and low pH treatments. The occurrence of interfering
 171 background or contamination was examined each time by measuring the fluorescence of DMSO
 172 alone, as a negative control.

173

174 **2.3. Identification of root zones related to low pH-induced sensitivity responses**

175 First, we determined the meristem size in our treatment conditions, as detailed in Casamitjana-
176 Martinez *et al.* (2003). Following, TZ, early or late EZ and AZ were defined according to Verbelen *et*
177 *al.* (2006).

178

179 **2.4. Manipulation of root cell wall tension with osmotic solutions**

180 To decrease cellular turgor pressure, the seedlings were transferred from growth medium (Ψ_s of
181 -0.032 MPa) to hyperosmotic (HYO) solutions composed of 0.5 mM CaCl_2 and mannitol (150 mM) to
182 reach water potential of -0.370 MPa Ψ_s , at pH 5.8 or pH 4.6 . To increase the turgor pressure upon
183 the CW, the seedlings were transferred from growth medium to solutions composed of 0.5 mM
184 CaCl_2 and different mannitol concentrations with water potential ranging from Ψ_s of -0.220 MPa to
185 -0.470 MPa for 2 h. This transient HYO treatment was followed by a hypoosmotic treatment (HO) by
186 transferring plants to new solutions at Ψ_s of -0.005 MPa pH 5.8 or pH 4.9 (mild low pH stress).

187 As a control, plants were treated during the duration of the whole experiment in solution at $-$
188 0.005 MPa at pH 5.8 or pH 4.9 .

189

190 **2.5. Evaluation of incipient plasmolysis**

191 Roots treated at pH 5.8 -0.005 MPa Ψ_s were placed for 5 min in mannitol solutions with a Ψ_s
192 ranging from -0.740 MPa to -1.480 MPa. Roots treated at pH 5.8 and -0.420 MPa were exposed for
193 5 min to mannitol solutions (300 to 600 mM) ranging from Ψ_s of -0.990 MPa to -1.480 MPa. Then,
194 after exactly 5 min, the epidermal cells from TZ and early EZ of primary roots were imaged (Leica
195 LM 6000B, Wetzlar, Germany). The plasmolysis point was determined when approximately half of
196 the observed cells were plasmolyzed. For each treatment, at least 20 TZ epidermal cells were
197 observed.

198

199 **2.6. Evaluation of cell wall stiffness in TZ epidermal root cells**

200 We evaluated CW stiffness by examining the decrease in cell length in response to a HYO
201 treatment after low pH stress. We used the method described by Kierzkowski *et al.* (2012) with
202 modifications. After treatment at pH 5.8 or pH 4.6 in solutions at a Ψ_s of -0.005 MPa, root CWs
203 were stained with calcofluor white (0.1% w/v Sigma[®], Saint Louis, Missouri, USA) for 5 min, rinsed in
204 water, and then finally mounted in water on a glass slide with a coverslip. Then, TZ epidermal cells
205 were imaged with an epifluorescence microscope (Leica LM 6000B, Mannheim, Germany,
206 excitation 340 nm/ 40 nm, emission 470 nm/ 40 nm). We gently removed the water from the roots
207 with a filter paper in contact just with the glass keeping the cells of interest in focus. Then, a HYO
208 solution at Ψ_s of -0.480 MPa (200 mM mannitol) was added with a pipette. After exactly 5 min of
209 this treatment, the same cells were imaged again. All procedures were done monitoring the roots
210 under the microscope to avoid damages and to prevent the roots from drying out.

211 The variation in the maximum length of the cells after this mannitol treatment was calculated (Δ
212 length %). The result was interpreted as indicative of CW stiffness in TZ epidermal root cells in
213 response to the HYO treatment after previous incubation at pH 5.8 or 4.6 .

214

215 **2.7. Evaluation of cell elongation in roots**

216 Cell elongation was examined in the late EZ through the length of the first epidermal cell with
217 visible root hair bulge (LEH). LEH was measured in these trichoblasts as described by Le *et al.* (2001)
218 with minor modifications. After the treatment, roots were fixed in a modified Karnovsky solution
219 (2.5 % glutaraldehyde, 2.0 % paraformaldehyde, 0.05 M cacodylate buffer, 0.001 M CaCl₂) (Román
220 *et al.*, 2004) overnight at 4°C. Then, CWs were stained with calcofluor white (0.1% w/v) for 5 min,
221 and cells were imaged by epifluorescence microscopy as described above for evaluation of CW
222 stiffness.

223

224 **2.8. Evaluation of PCD in roots treated at low pH**

225 TUNEL assays were performed to examine the occurrence of PCD (Watanabe and Lam, 2008).
226 The DeadEnd™ Fluorometric TUNEL System (G3250) from Promega® (Madison, Wisconsin, USA)
227 was used. Roots were fixed overnight in 4 % paraformaldehyde diluted in phosphate buffer saline
228 (PBS 1X). Then, they were washed with PBS for 5 min and treated with Triton X-100 for 20 min.
229 Other procedures were in accordance with the manufacturer's instructions. After the TUNEL
230 procedure, some roots were also stained with 1 μM DAPI (4',6-Diamidino-2-phenylindole
231 dihydrochloride) for 5 min and immediately imaged.

232 For the evaluation of nuclei morphology with solely DAPI, roots were fixed in a modified
233 Karnovsky solution (see above) overnight at 4°C. Roots were then stained with DAPI (1 μM) for 5
234 min in the dark and immediately imaged.

235

236 **2.9. Statistical analysis**

237 The experimental design was randomized in all experiments. Each analysis was composed of at
238 least three independent biological replicates. Each replicate was composed of at least ten plants.
239 For the comparison of means, normalized data were subjected to analysis of variance (ANOVA),
240 followed by Duncan's test ($p < 0.05$). When necessary, the difference between two means was
241 evaluated by Student's t-test at the 5% significance level.

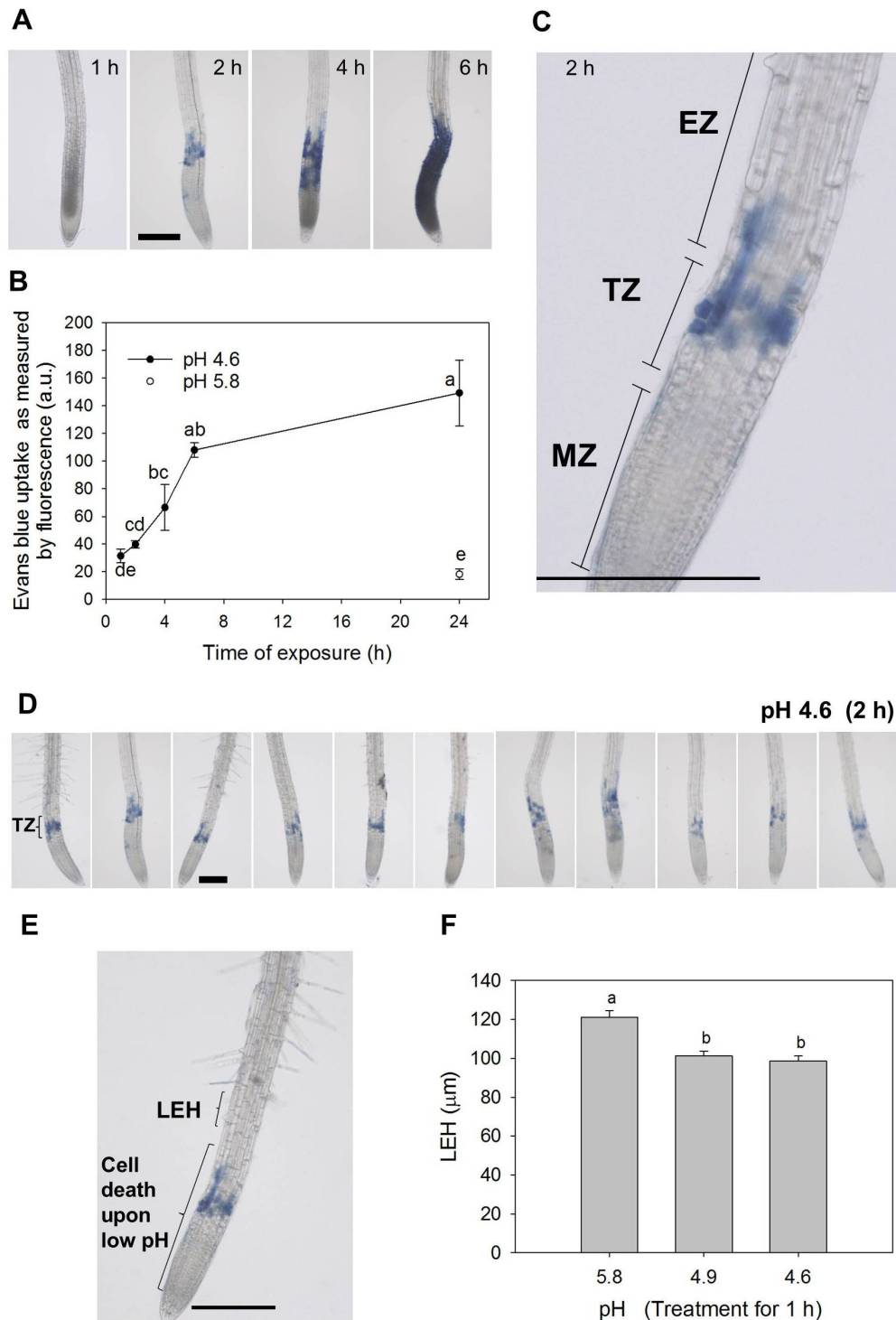
242 **3. Results**

243 **3.1. Analysis of cell death and cell elongation in roots treated at low pH**

244 We first meticulously examined the CD and cell elongation in primary roots upon low pH. After 2
245 h of exposure to pH 4.6, root cells located in TZ started dying (Fig. 1A, C-E). These cells were located
246 on average 387 μm (± 49 μm) from the meristem quiescent center (Fig. S1C). Loss of cell viability
247 then progressed towards the apex after 4 h of low pH treatment. By 6 h, CD extended downward
248 over almost the entire MZ and upward to the early EZ. Almost no further CD was observed between
249 6 and 24 h of exposure to pH 4.6 (Fig. 1B).

250 Root CD was not found in fully-expanded cells in late EZ, *e.g.*, those located in the same spatial
251 position of root hair initiation (Fig. 1E; Fig. S2). In addition, we examined the effects of low pH stress
252 upon cell elongation in late EZ by measuring LEH (Fig. 1E), which was indicative of cell elongation in
253 epidermal cells and strongly correlated with overall root growth (Le *et al.*, 2001). After 1 h of low pH
254 treatment, the LEH size decreased by nearly 18 % upon mild (pH 4.9) or strong (pH 4.6) low pH
255 stress compared to the LEH size of roots treated at pH 5.8 (Fig. 1F). This indicated an elongation
256 arrest in late EZ epidermal root cells upon low pH stress. The arrest of cell elongation could explain,
257 in part, the decrease of the overall root growth upon low pH, which dropped almost linearly in pH
258 treatments below pH 4.9 and was found negligible after 24 h at pH 4.0 (Fig. S1A). The roots upon

259 pH 4.9 (24 h) or pH 4.6 (6 h) did not show a lignification stress response, as revealed by
 260 phloroglucinol staining (data not shown).
 261



262

263 **Fig. 1. Spatio-temporal analysis of cell death and cell elongation in the primary roots of *A. thaliana* (Col-0) exposed to severe low pH**
 264 **stress (pH 4.6).** The blue staining or increase in fluorescence is indicative of cell death. (A) Bright field images of root tips stained with
 265 Evans blue after treatment at pH 4.6 for different times. The images are representative of at least 15 roots; (B) Quantification of
 266 Evans blue uptake in root tips after treatment at pH 4.6 for different times. As controls, roots were treated at pH 5.8 for 24 h. The
 267 bars represent standard errors of three independent experiments. Statistical analysis was done with Duncan's test; (C) First cells from
 268 root tip exhibiting cell death (blue) after 2 h of treatment at pH 4.6 in TZ and early EZ; (D) The first cells sensitive to low pH were
 269 invariably located in the TZ and early EZ zones in the observed roots (n=11); (E) The area of the regions where low pH-induced cell

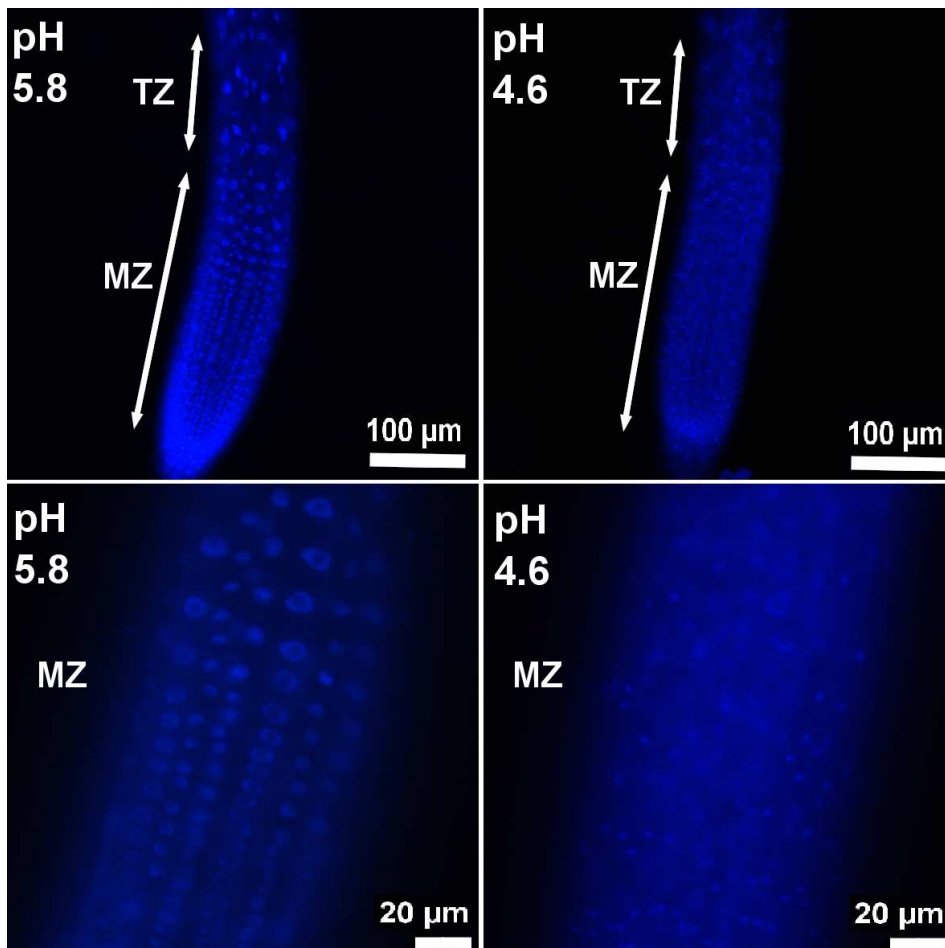
270 death occurred and the length of the first epidermal cell with visible root hair bulge (LEH) was measured in roots, based on all
 271 replicates from (A) and (B); (F) Analysis of cell elongation in roots by measurement of LEH after 1 h of treatment at different pH. The
 272 bars represent the standard errors of three independent experiments. The statistical analysis was done with Duncan's test. Scale
 273 bars: 200 μ m.

274

275 **3.2. Analysis of PCD in roots treated at low pH**

276 We observed a consistent pattern of CD in primary roots upon low pH treatments, always
 277 starting in TZ and then moving towards MZ. This pattern was well-defined and repeatedly observed
 278 (Fig. 1A, D; Fig. S2). To better understand whether this response is merely necrotic CD, we further
 279 examined the occurrence of a PCD mechanism in primary roots under low pH stress. The DAPI-
 280 stained cells from roots treated at pH 4.6 for 3 h showed fragmented or half-moon-shaped nuclei in
 281 TZ and MZ compared to cell nuclei of roots treated at pH 5.8 (Fig. 2; Fig. 3). This disruption in nuclei
 282 morphology seems ubiquitous in cells dying through a PCD mechanism (Watanabe and Lam, 2008;
 283 Guan et al., 2019).

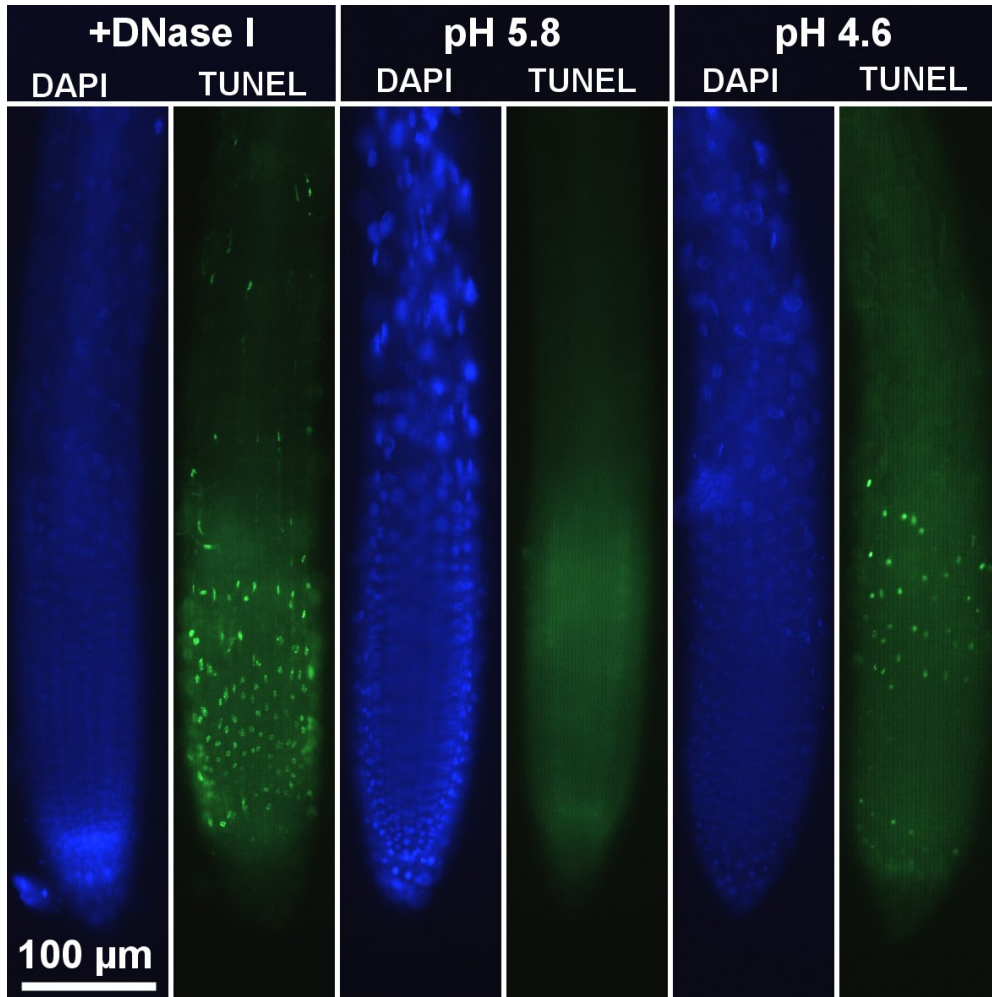
284 To characterize the CD mechanism, we performed a TUNEL assay which allows the labeling of
 285 DNA ends at their 3'-OH end by incorporation of fluorescein-12-dUTP, *i.e.*, the coordinated DNA
 286 fragmentation which is indicative of PCD (Reape et al., 2008). Finding positive nuclei would favor
 287 the hypothesis of PCD (Watanabe and Lam, 2008; Guan et al., 2019). As a positive control, roots of
 288 seedlings were taken from the growth medium and exposed to DNase I and showed positive TUNEL
 289 labeling throughout the primary root tip (Fig. 3). The roots treated at pH 4.6 for 3 h had a positive
 290 reaction for the TUNEL assay in TZ and MZ (Fig. 3). Conversely, no positive nuclei were found in the
 291 roots of seedlings treated at pH 5.8 (Fig. 3). Hence, the TUNEL positive nuclei observed in roots
 292 treated at pH 4.6 indicated that CD triggered by low pH is coordinated by a PCD mechanism.
 293



294

295
296
297
298
299

Fig. 2. Nuclei morphology of cells from primary roots of *A. thaliana* (Col-0) exposed to low pH stress. After treatment at pH 5.8 or pH 4.6 for 4 h, roots were fixed and then stained with DAPI. Control cells (pH 5.8) show defined oval nuclei morphology in TZ and distal part of MZ. Cells treated with severe low pH stress (pH 4.6) show deformed and fragmented nuclei. The upper row shows MZ and TZ. The bottom row focuses on the distal part of MZ.



300

301

302

303

304

305

306

307

308

309

310

311

312

313

314

315

316

317

Fig. 3. TUNEL assay for the detection of nuclei positive for PCD in primary roots of *A. thaliana* (Col-0) treated at pH 5.8 or pH 4.6 for 3 h. Nuclei stained with DAPI showing blue fluorescence. The markedly fluorescent green dots are indicative of nuclei positive for the TUNEL assay. As positive control, roots were directly taken from the growth medium and treated with DNase I.

3.3. Effect of low pH upon cell wall stiffness in epidermal cells of the root transition zone

We provided evidence above that TZ cells were highly sensitive to CD driven by low pH. This zone possesses a particular high CW dynamic (Verbelen et al., 2006; Somssich et al., 2016). Thus, to further investigate whether the low pH-induced CD could be related to CWDs in TZ, we measured CW mechanical properties at the cellular level. Briefly, we adapted a previously described method (Kierzkowski et al. 2012) that consists of measuring cell length before and after a decrease in turgor pressure caused by a hyperosmotic solution (HYO) (Ψ_s of -0.480 MPa). Therefore, a greater decrease in cell length would be indicative of CW loosening, meaning that stiffness decreased.

In primary roots pretreated at pH 4.6 and subsequently placed in the HYO solution, the length of TZ epidermal cells decreased by 9% whereas in roots treated at pH 5.8 followed by HYO solution the length of TZ epidermal cells was little affected (Table 1). These observations indicated that low pH likely decreased CW stiffness of TZ epidermal cells. Importantly, we performed these measurements after 90 min of exposure to pH 4.6, before the prominent occurrence of CD in these

318 cells (Fig. 1). Since TZ cells died in the low pH conditions used, the CW loosening mediated by the
 319 HYO treatment is likely to be a sign of formerly CWDs in roots treated at pH 4.6.

320

321

322

323

324

325

326

327

328

Table 1. Changes in the cell length as indicative of cell wall stiffness of epidermal cells from TZ of *A. thaliana* WT primary roots in response to low pH stress followed by HYO treatment. The roots were first treated at pH 5.8 or 4.6 for 90 min in a solution at a Ψ s of -0.005 MPa. Cell walls were stained with calcofluor white for 5 min and TZ epidermal cells were imaged. Then, roots were exposed to a solution at a Ψ s of -0.480 MPa (HYO) for exactly 5 min and the same cells were immediately imaged again. The change (Δ) in maximum cell length was recorded as a measurement of cell wall changes during a previous low pH treatment. At least ten plants were examined for each treatment. In each plant, 2 to 3 cells were imaged.

Formerly pH treatment	Change in cell length upon HYO treatment	Standard error
5.8	-0.39 % ^b	1.10 %
4.6	-9.08 % ^a	2.53 %

329

¹Different letters indicate significant differences between treatments by Student *t*-test.

330

331

3.4. Sensitivity of *cob-1* roots to low pH

332

333

334

335

336

337

338

339

340

341

342

343

344

345

346

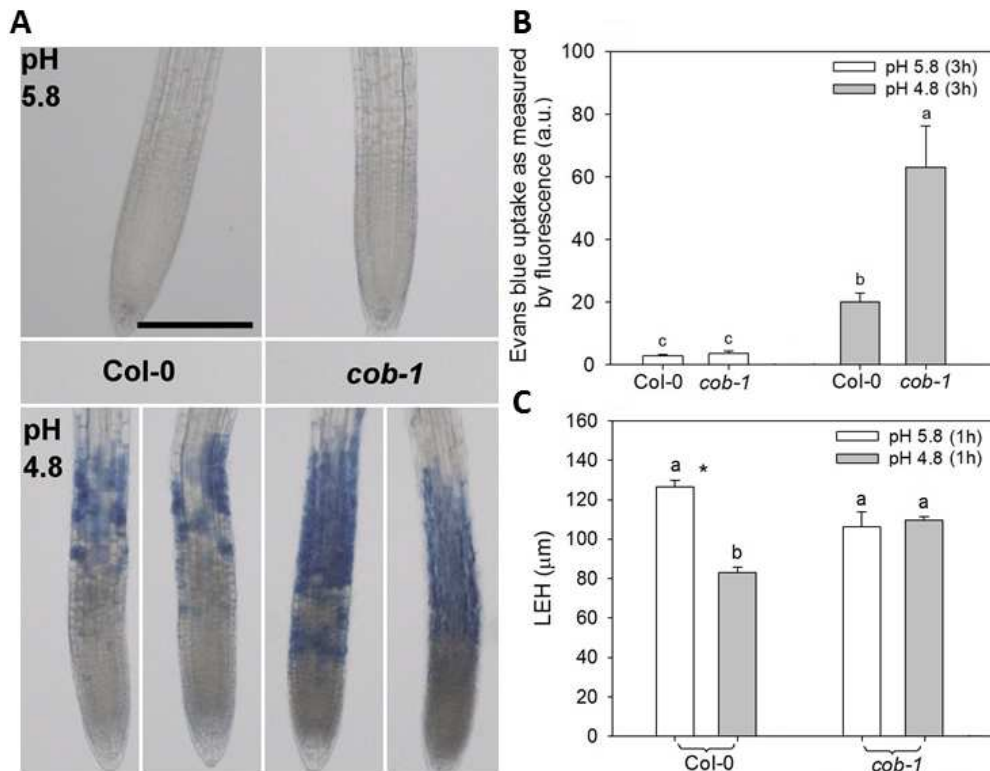
347

348

We next investigated the influence of the CW architecture upon CD or cell elongation in roots in response to exogenous low pH. Roots of the *cobra* mutant (*cob-1*) impaired in cellulose deposition in root CWs and the control Col-0 were treated for 3 h at pH 4.8. This pH value was chosen as intermediate stress between mild (pH 4.9) and severe (pH 4.6) low pH stress (Fig. S1). Severe low pH stress (pH 4.6) would only reveal genotypes highly tolerant to the stress.

Primary roots of *cob-1* showed high sensitivity to pH 4.8 as indicated by strong Evans blue staining in TZ and MZ (Fig. 4A) and three-fold higher Evans blue uptake than WT (Fig. 4B). Cell elongation was also examined after treatment at pH 4.8 by measuring LEH. Roots from Col-0 plants showed a decrease of 34 % in LEH after 1 h of treatment, whereas, in the *cob-1* roots, there was no significant decrease in LEH (Fig. 4C).

The disruption in CW architecture caused by a disturbance in cellulose deposition, *i.e.*, a loss of CWI in *cob-1* could trigger higher mortality due to low pH while there was no significant arrest in cell elongation, as shown by LEH measurement. Together, these results indicate that correct CW architecture differentially affected the low pH responses in MZ/TZ (CD) or in early EZ (elongation arrest) in roots.

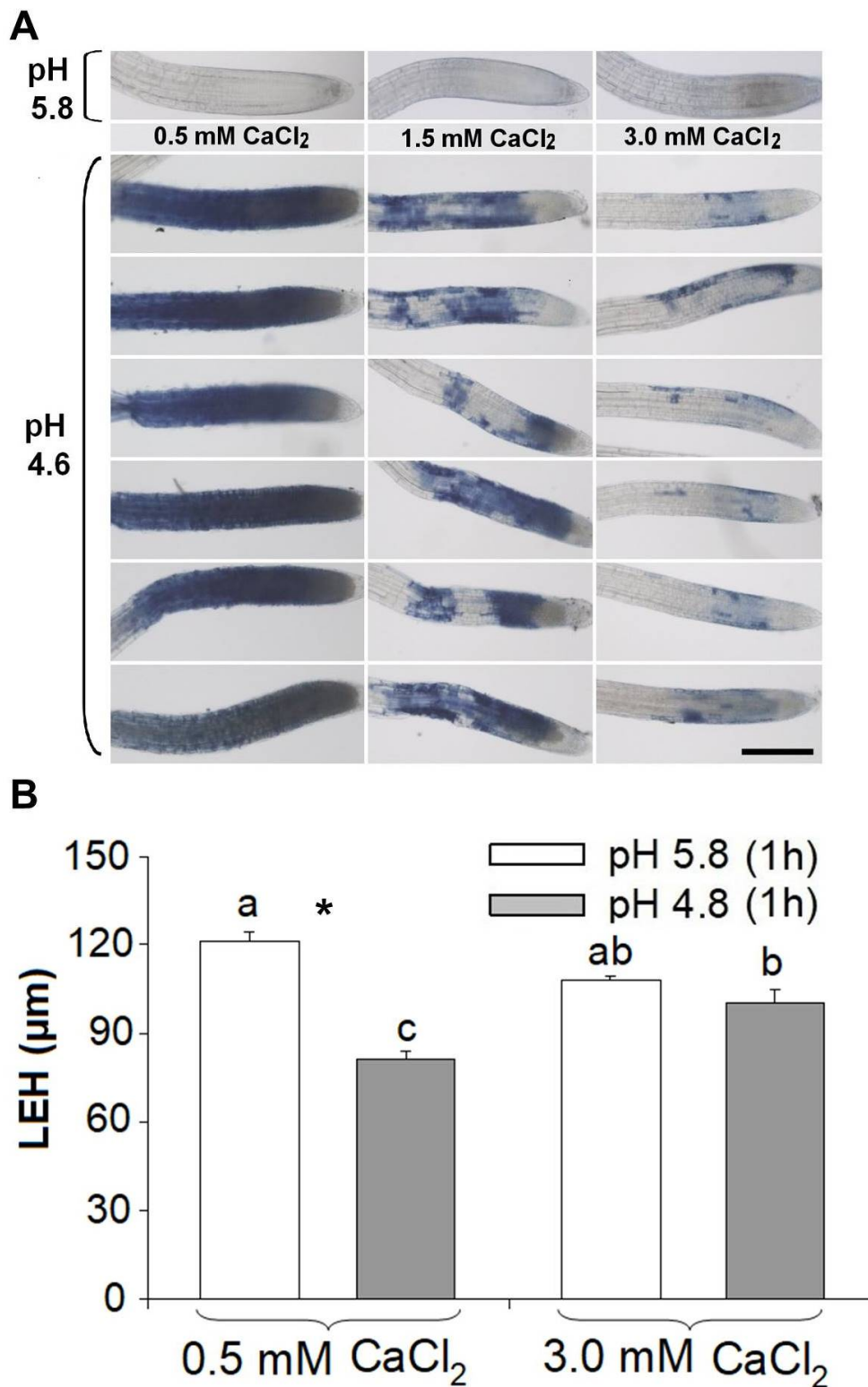


349
 350 **Fig. 4. Cell mortality and cell elongation in primary roots of *A. thaliana* (Col-0) or *cob-1* seedlings treated at intermediate low pH stress**
 351 **(pH 4.8).** The blue staining or increase in fluorescence is indicative of cell death. (A) Roots were stained with Evans blue. In upper
 352 panels, roots were treated at pH 5.8 (3 h) whereas in the bottom panels roots were treated at pH 4.8 (3 h). Scale bar: 200 μm; (B)
 353 Quantification of Evans blue uptake in root tips by a fluorometric assay; (C) Analysis of cell elongation in roots by measurement of
 354 LEH after 1 h of treatment at pH 4.8. Bars represent the standard errors of three independent experiments. Letters indicate
 355 significant differences between the treatments by Duncan's test. The asterisk indicates a significant difference for a given genotype
 356 by Student t-test.
 357

358 3.5. Effect of increase in calcium on mortality and elongation of root cells exposed to low pH

359 Our data indicate that either CWDs or correct CW architecture were crucial for either CD or
 360 arrest in elongation upon low pH stress. Calcium has a role in pectin crosslinking (Willats et al.,
 361 2001), and low pH sensitive responses in roots are easily observed in solutions with low ionic
 362 strength and low calcium concentration (≤ 0.5 mM Ca^{2+}) (Koyama et al., 2001; Graças et al., 2016),
 363 simulating acidic soil stress. Therefore, we examined whether the increase of calcium concentration
 364 in the treatment solution (from 0.5 mM to 3.0 mM CaCl_2) could modify the pattern of CD or cell
 365 elongation upon low pH treatment.

366 First, strong low pH stress was imposed on roots (pH 4.6 along 6 h) to reveal a possible
 367 protective effect of calcium upon cell viability of primary roots. Under 1.5 mM CaCl_2 , we found a
 368 slightly alleviated CD due to strong low pH stress. However, 3.0 mM CaCl_2 treatment reduced CD
 369 and had a protective effect to low pH stress (Fig. 5A). This concentration was chosen for
 370 comparison with 0.5 mM CaCl_2 regarding cell elongation in roots upon intermediate low pH stress
 371 (pH 4.8), which was sufficient to cause the arrest of cell elongation in Col-0 as described above (Fig.
 372 4C). Interestingly, roots treated at pH 4.8 in the presence of 0.5 mM CaCl_2 exhibited a decrease in
 373 LEH upon low pH as compared to roots treated at pH 5.8, corresponding to an arrest of cell
 374 elongation of about 33 %. However, this prominent arrest did not occur in roots upon low pH in the
 375 presence of 3.0 mM CaCl_2 (Fig. 5B). These results evidence the protective effect of calcium
 376 preventing the CD or arrest of elongation caused by low pH stress.
 377

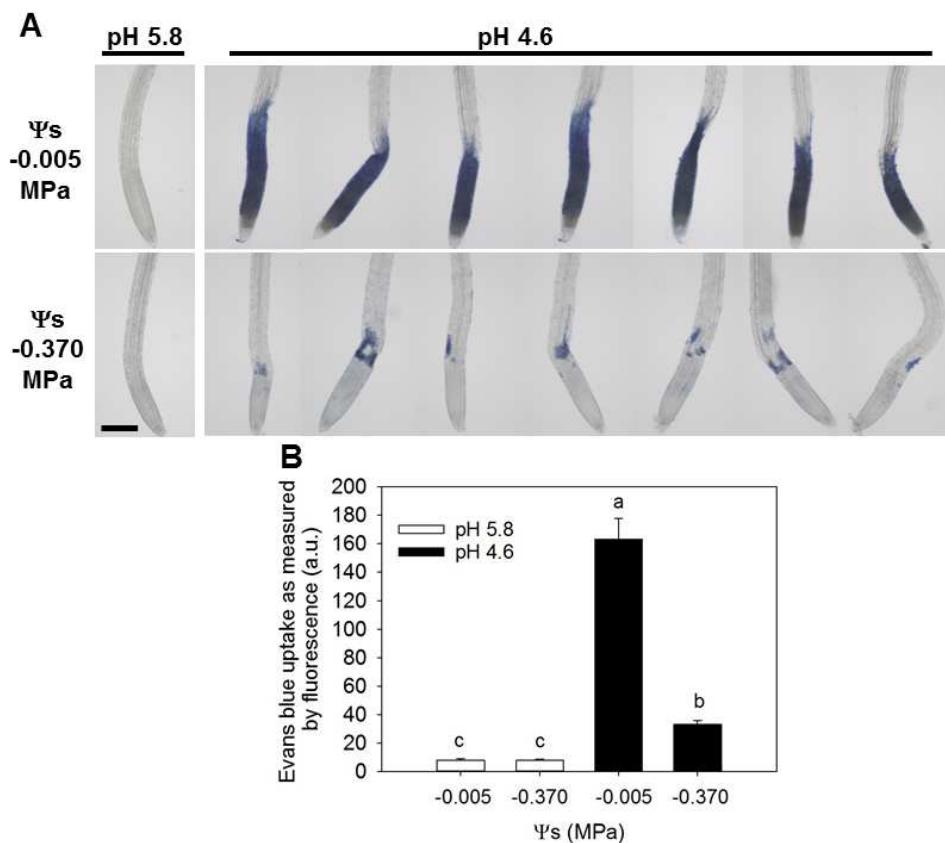


378
379
380 Fig. 5. Cell mortality and cell elongation in primary roots of *A. thaliana* (Col-0) exposed to low pH stress at different concentrations of
381 CaCl₂. Blue color (Evans blue staining) is indicative of cell death. (A) Roots treated at pH 5.8 or pH 4.6 (strong low pH stress) for 6 h.
382 Scale bar: 200 μm. In each case, the images are representative of at least 15 roots. For simplification, a single image is shown for
383 each treatment at pH 5.8, but there was no cell death in the roots of control replicates. Note that only 3.0 mM CaCl₂ caused a clear
384 decrease in cell mortality upon low pH; (B) LHE upon treatment at pH 5.8 or 4.8 for 1 h. Bars represent the standard errors of three
385 independent experiments. Letters indicate significant differences between the treatments by Duncan's test. The asterisk indicates a
386 significant difference between treatments at pH 5.8 as compared to pH 4.8 for a given CaCl₂ concentration by Student *t*-test.

387 **3.6. Effect of reduced cell wall tension on death of root cells exposed to low pH**

388 We demonstrated above that CWDs, CW architecture and CW stabilizing ions such as calcium are
 389 crucial to the sensitivity responses to low pH in roots. To further examine whether reduction of CW
 390 tension could influence CD induced by low pH, roots were exposed to pH 4.6 or pH 5.8 for 6 h in
 391 solutions with different osmotic potentials at Ψ_s of -0.005 MPa (same conditions as those used in
 392 Figs. 1, 2, 3 and 4) or -0.370 MPa (supplemented with 150 mM mannitol).

393 Primary roots exposed to pH 4.6 for 6 h in a -0.005 MPa Ψ_s solution exhibited CD as shown by a
 394 high Evans blue uptake (Fig. 6A, B). In sharp contrast, there was a significant decrease in CD in roots
 395 treated at pH 4.6 for 6 h in a solution at a Ψ_s of -0.370 MPa (Fig. 6A, B). These results indicated that
 396 a reduced pressure/tension upon CWs prevented the death of root cells exposed to severe low pH
 397 stress.



398
 399 **Fig. 6.** Cell mortality in primary roots of *A. thaliana* (Col-0) exposed to severe low pH stress (pH 4.6) under reduced water potential (-
 400 0.370 MPa) or at higher turgor pressure (-0.005 MPa) for 6 h. The blue staining or increase in fluorescence is indicative of cell death.
 401 (A) Bright field images of root tips stained with Evans blue after treatment at pH 5.8 or 4.6 at different Ψ_s . For simplification, one
 402 representative image is shown for each condition of Ψ_s upon pH 5.8 (control). Scale bar: 200 μ m; (B) Quantification of Evans blue
 403 uptake in root tips by a fluorometric assay. Bars represent the standard errors of three independent experiments. Letters indicate
 404 significant differences between the treatments by Duncan's test.

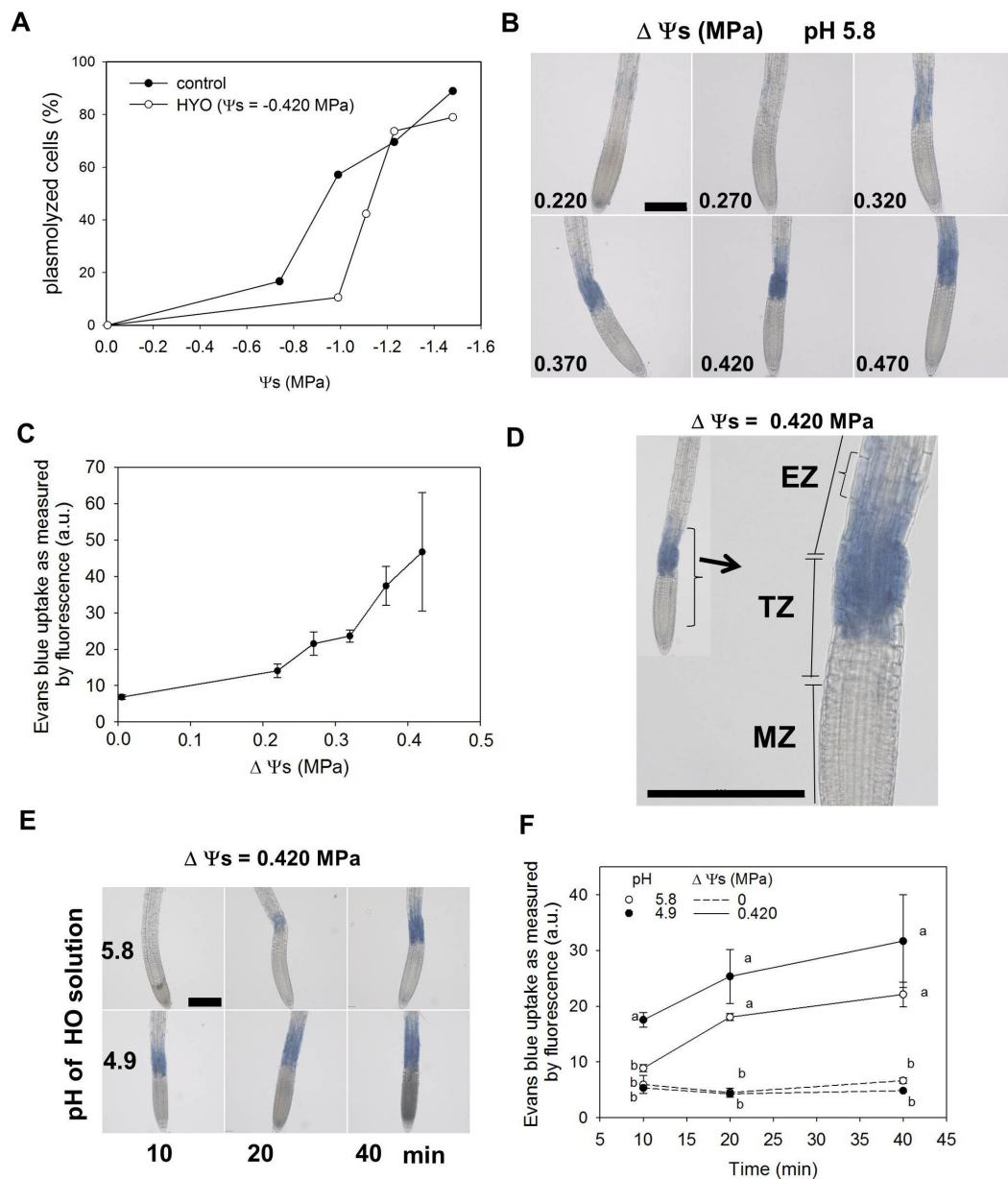
406 3.7. Effect of increased wall tension on death of root cells with or without simultaneous exposure to 407 low pH

408 To examine the effect on CD of increased turgor pressure alone or in combination with low pH,
 409 roots were pre-treated in HYO solutions ranging from a Ψ_s of -0.220 to -0.470 MPa at pH 5.8 for 2
 410 h, to promote osmotic adjustment. Following, we subjected these same roots to a hypoosmotic
 411 (HO) treatment by returning them to a solution at a Ψ_s of -0.005 MPa for 1 h, at pH 5.8 or pH 4.9
 412 (mild low pH stress). Thus, HO treatments of different magnitudes were applied in primary roots by
 413 varying the Ψ_s of the pre-treatment solution. The magnitude of these HO treatments was
 414 expressed as $\Delta\Psi$, *i.e.*, the difference in Ψ_s between HYO and HO solutions. For simplification, the
 415 Ψ_s of the HO solution was considered to be zero.

416 To verify whether the HYO pre-treatment promoted osmotic adjustment, an analysis of incipient
417 plasmolysis was performed in cells from early EZ. In roots pretreated with a -0.420 MPa Ψ_s
418 mannitol solution, approximately half of the cells plasmolyzed after transfer to a -1.100 MPa Ψ_s
419 solution (Fig. 7A). Conversely, in control roots pre-treated in a -0.005 MPa Ψ_s solution,
420 approximately half of the cells plasmolyzed in solutions of a Ψ_s of -0.900 MPa. Thus, the -0.420
421 MPa Ψ_s HYO pre-treatment decreased the Ψ_s of cells by roughly -0.200 MPa.

422 Notably, a HO treatment at pH 5.8, following a HYO pre-treatment, caused CD without any low
423 pH treatment (Fig. 7B, C). A HO treatment with $\Delta\Psi_s$ of 0.220 MPa only slightly affected cell viability.
424 However, the CD was clearly observed after Evans blue staining when the $\Delta\Psi_s$ of the HO treatment
425 was at least 0.320 MPa and was prominent at $\Delta\Psi_s$ of 0.420 and 0.470 MPa (Fig. 7B, C). CD due to
426 HO treatment was confined to TZ/early EZ and did not progress toward MZ (Fig. 7D), differently
427 from low pH-induced CD. Another important difference from CD solely due to low pH stress was
428 that CD caused by the HO treatment occurred much faster. It was observable after 20 min of a HO
429 treatment with a $\Delta\Psi_s$ of 0.420 MPa and at pH 5.8 (Fig. 7E, F).

430 To examine whether low pH could modify the pattern of CD due to HO treatment, we performed
431 a HO treatment ($\Delta\Psi_s = 0.420$ MPa) at pH 4.9, a mild low pH stress. Compared to pH 5.8, CD due to
432 HO treatment at pH 4.9 was faster and observable after 10 min but it was confined at the same root
433 zones, in TZ and early EZ (Fig. 7E, F). Altogether, the increase in CW tension driven by a HO
434 treatment was able to cause CD in TZ and early EZ of primary roots. Prominently, the CD was greatly
435 accelerated when roots were subjected to HO treatment combined with low pH stress.



436

437

438

439

440

441

442

443

444

445

446

447

448

449

450

Fig. 7. Cell plasmolysis and cell mortality in primary roots of *A. thaliana* (Col-0) exposed to an increase in magnitude of turgor pressure (HO treatment) combined or not with mild low pH stress (pH 4.9). The blue staining or increase in fluorescence is indicative of cell death. (A) Measurement of incipient plasmolysis in cells from early EZ. The roots remained for 2 h in solution at a Ψ_s of -0.005 (control) or -0.420 MPa (HYO) and were then transferred to solutions ranging from a Ψ_s of -0.740 to -1.480 MPa for 5 min (HYO treatment). Cell plasmolysis was then quickly imaged using bright field microscopy. In (B) and (C) bright field images of Evans blue staining or quantification of its uptake, respectively, in root tips after HO treatment. In (B) and (C) the roots remained for 2 h in HYO solutions at pH 5.8 ranging from a Ψ_s of -0.220 until -0.470 MPa and following were transferred to HO solution at a Ψ_s of -0.005 MPa at pH 5.8 for 1 h; (D) Detail of TZ and early EZ region which showed mortality due to HO treatment at pH 5.8. The key shows an early EZ cell. In (E) and (F) Bright field images of Evans blue absorption or quantification of its uptake, respectively, in root tips after HO treatment combined or not with mild acidic treatment (pH 4.9). In (E) and (F) the roots remained for 2 h in a HYO solution at a Ψ_s of -0.420 MPa at pH 5.8 (HYO) and were then transferred to a HO solution at a Ψ_s of -0.005 MPa at pH 5.8 or pH 4.9 for 10, 20 or 40 min. As a control, roots remained for 40 min in HO solution at pH 5.8 or pH 4.9 at a Ψ_s of -0.005 MPa. All the bars are standard errors of three independent experiments. Statistical analysis was done with Duncan's test. Scale bars: 200 μ m.

451

4. Discussion

452 In this study, we provide accurate spatio-temporal information regarding low pH-induced CD in
 453 the primary roots of *A. thaliana*. The distinct root zones (MZ, TZ, early EZ, and late EZ) responded
 454 differently and sequentially to low pH stress. First, low pH induced a decrease in CW stiffness of TZ
 455 epidermal cells. Following this, CD first occurred in TZ and then expanded toward MZ and early EZ.
 456 We also show that CD due to low pH occurred through a PCD pathway and that it was related to
 457 CWDs and CW architecture. In contrast, low pH stress did not induce CD in late EZ but instead
 458 resulted in a coordinated arrest of epidermal cell elongation. Taken together, our data support a
 459 mechanistic model for the action of low pH in *A. thaliana* roots (Fig. 8).

460

461 **4.1. Low pH induced-cell death within defined root zones is coordinated through PCD and is related to** 462 **acid-induced CWDs**

463 We suspected first that CD due to exogenous low pH stress in the root tip could be similar to
 464 that of the bursting observed in root hairs upon low pH (Bibikova et al., 1998), which is likely due to
 465 instantaneous loss of CWI. Alternatively, the CD could be due to a sum of several cellular damages
 466 leading to necrotic cell death. Our present work indicates that low pH-induced CD in MZ/TZ/early EZ
 467 occurs via a PCD pathway rather than as a direct consequence of loss of CWI or as a necrotic CD.
 468 The changes in the morphology of DAPI-stained nuclei and the positive reaction for the TUNEL assay
 469 in low pH-stressed roots are indeed strongly indicative of PCD. These latter cited methods provided
 470 unambiguous evidence of PCD in roots as a response to drug-induced endoplasmic reticulum stress
 471 (Watanabe and Lam, 2008) or waterlogging stress (Guan et al., 2019).

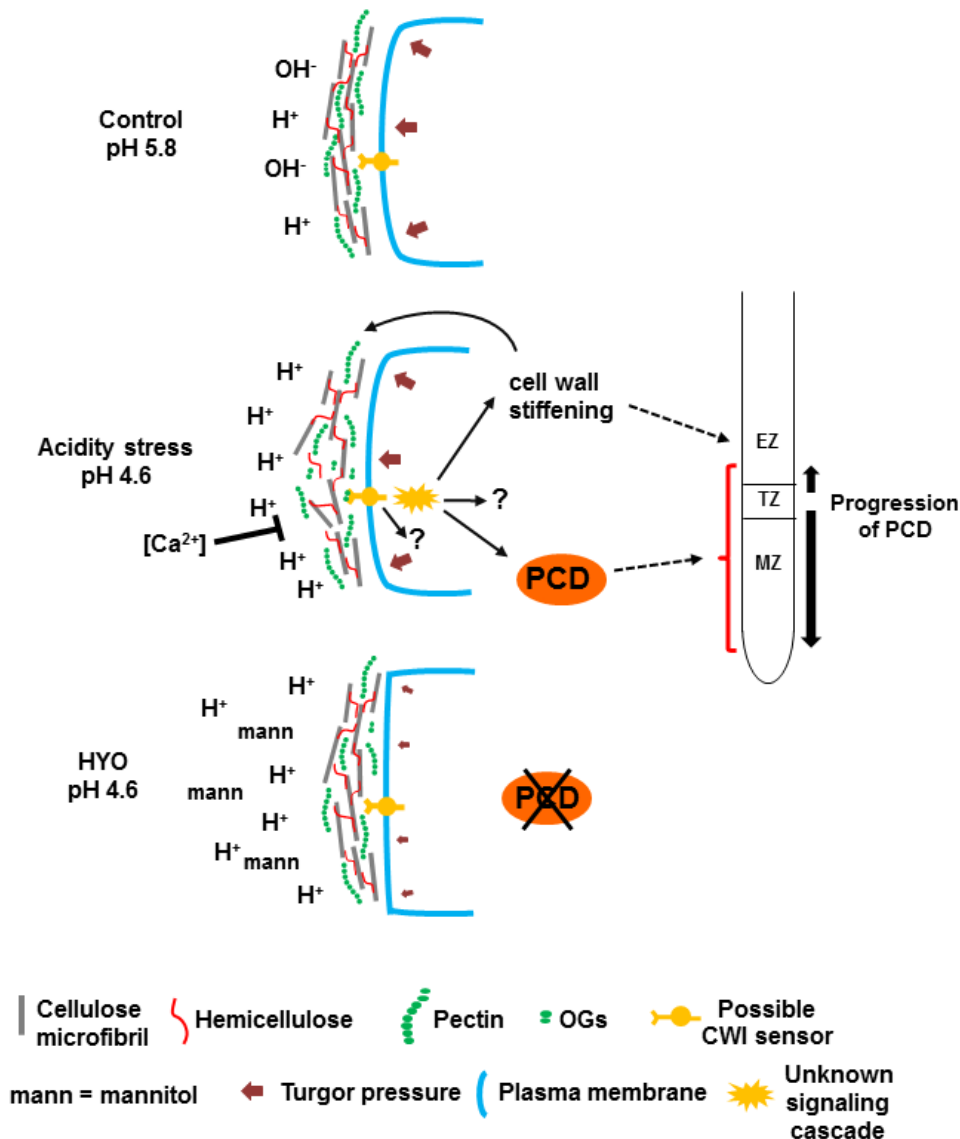
472 The increased CD in the roots of the *cob-1* mutant to low pH suggests that low pH-induced PCD
 473 is caused by CWDs. COB is crucial for the correct organization and deposition of cellulose
 474 microfibrils in the primary CW and controls the direction of cell expansion (Schindelman et al.,
 475 2001; Roudier et al., 2005) that is crucial for CWI (Liu et al., 2015). Thus, CWDs caused by low pH
 476 may be exacerbated in *cob-1* mutants with defective cellulose deposition. Alternatively, *cob-1* may
 477 hamper the ability of the cell to counteract low pH-induced CWDs. Transcriptomics studies also
 478 provide evidence that low pH may cause CWDs. In *A. thaliana*, low pH was shown to cause deep
 479 transcriptional regulation of genes encoding CW remodeling proteins such as those coding for
 480 xyloglucan endotransglycosylases, CIII Prxs, expansins, and pectin methylesterases (Lager et al.,
 481 2010). For instance, the expression level of the genes encoding proteins such as EXPANSIN A17 and
 482 the xyloglucan endotransglycosylase hydrolase 22 (XTH22/TOUCH4) changed 20 min after the
 483 beginning of low pH treatment, and likely resulting in changes in CW structure in roots in response
 484 to the stress.

485 In the graphical model (Fig. 8), we summarized the results obtained here and postulated that
 486 exogenous low pH disturbs the normal CW structure, thus threatening CWI. The direct evidence
 487 was that before the onset of CD, low pH decreased CW stiffness of TZ epidermal cells, *i.e.*, the most
 488 sensitive cells to low pH-induced CD. Prominently, the CD decreased in roots treated
 489 simultaneously with low pH and mannitol (Fig. 6). The simplest interpretation of this result is that a
 490 certain level of turgor pressure, and therefore, CW tension is required for low pH to cause CWDs
 491 and then a signaling cascade would be activated for PCD. An additional explanation is that an
 492 increased concentration of osmoticum in the solution could also decrease the level of hydration of
 493 the CW, which in turn could increase its stiffness (Evered et al., 2007; Hansen et al., 2011; Wolf et
 494 al., 2012). However, small molecules such as mannitol, which can penetrate the pores of the CW,
 495 are not expected to affect the CW hydration state (Passioura, 1994; Evered et al., 2007), but this
 496 assumption cannot be ruled out. Either way, the effect of mannitol supports the idea that low pH-
 497 induced CD is caused by CWDs. Supporting this notion, calcium is a key cation that stabilizes
 498 homogalacturonan crosslinking (Willats et al., 2001; Mravec et al., 2017) within 20 min of CaCl₂

499 treatment (Xi et al; 2015) and is therefore critical for the mechanical properties of the CW and the
500 maintenance of CWI and stabilization (Voxeur and Höfte, 2016). Low pH can cause the weakening
501 of cellulose-pectin interactions and pectin-pectin interactions (Phyo et al., 2019). Upon increased
502 calcium concentration in the treatment solution, inhibition or alleviation of low pH-induced CWDs
503 likely prevented the signaling for PCD in MZ/TZ/early EZ (Fig. 5). This latter result agrees with the
504 protective effects of an increase in extracellular calcium concentration that inhibited cellular
505 damages in a *stop1* (*sensitive to proton rhizotoxicity1*) knockout mutant, presumably having less
506 capacity for maintaining CW stability in roots upon low pH stress (Kobayashi et al., 2013).

507 It is unlikely that low pH-induced CD in complex primary root tissues was directly due to yielding
508 of the CW, as in the case of the bursting of single root hairs treated with low pH (Bibikova et al.,
509 1998; Monshausen et al., 2007) or in EZ cells during salt stress (Feng et al., 2018). In addition to the
510 evidence that CD occurred via PCD, the time required for the onset of CD (2 h) was long as
511 compared to that observed for bursting of root hairs (10 min) (Monshausen et al., 2007) or CD
512 caused by HO treatment at pH 5.8 reported here (20 min) (Fig. 7E, F). This HO treatment was
513 performed by formerly placing the roots in a HYO solution, which decreased cellular Ψ_s by
514 approximately 0.2 MPa (Fig. 7A). Most likely, the turgor pressure was increased around this
515 magnitude when roots were placed back in solution with a Ψ_s close to zero at mild low pH. *Triticum*
516 *aestivum* roots subjected to a mannitol treatment transiently reduced the turgor pressure and
517 growth rate that were restored within the exposure time (6 h) (Pritchard et al., 1990). Accordingly,
518 restoration of root growth rate was assumed to be due to cellular acclimation to maintain the
519 turgor pressure. A sudden increase of 0.2 MPa in turgor pressure such as reported here does not
520 appear strong enough to cause CD. However, the elongation capacity upon low water potential
521 such as in HYO solution could be preserved by increasing CW extensibility, especially in EZ (Wu et
522 al., 1994; Wu and Cosgrove, 2000), which coincides with the HO-induced CD reported here (Fig.
523 7D). Hence, besides the sudden increase in turgor pressure, the HO-induced CD reported here
524 could be due to increased loosening of the CW driven by prior incubation in the HYO solution.
525 Furthermore, we reported that mild low pH stress (pH 4.9) only caused significant root CD (10 min)
526 in TZ and early EZ when combined with a sudden increase in turgor pressure due to the HO
527 treatment (Fig. 7E, F). Thus, the CW loosening mediated by the HYO treatment could therefore
528 explain why mild low pH accelerated CD in roots (10 min) when it was applied simultaneously with
529 HO treatment. Either way, the fact that CD is accelerated in the latter case also suggests that low
530 pH stress affects the CW structure in root tip tissues.

531



532
 533 **Fig. 8. Proposed model for the action of low pH in *A. thaliana* root cells.** Low pH causes cell wall disturbances (CWDs) such as a
 534 decrease in cell wall (CW) stiffness that could be due to weakening of pectin-cellulose interactions, as previously proposed (Phyo et
 535 al., 2019). These CWDs are likely perceived by yet to be identified cell wall integrity (CWI) sensor-(s) type-(s). Activation of CWI
 536 receptor (s) triggers a signaling pathway and a cellular response dependent on cell type. In transition zone (TZ) cells, perception and
 537 signaling of CWDs result in programmed cell death (PCD), perhaps by mimicking pathogen attack. Signals are relayed to meristematic
 538 zone (MZ) and early elongation zone (EZ) cells, sequentially triggering PCD. In contrast, in expanding trichoblasts of late EZ,
 539 perception of CWDs does not lead to CD but rather activates a CW remodeling system that is likely already organized and ready to
 540 promote the arrest of elongation (not illustrated here). The coordinated elongation arrest requires correct CW architecture and is
 541 most likely triggered due to rapid CW stiffening, as indicated by decreased LEH upon low pH. Spatial separation between PCD and
 542 elongation arrest may indicate that they are not activated by the same signaling cascade. Given the previously reported involvement
 543 of pectin in low pH-responses, oligogalacturonides (OGs) may be one of the primary CWD signals for these sensitive responses or
 544 even the decrease in CW stiffening that we measured here in TZ cells. Low pH-induced CWDs that trigger PCD require a certain level
 545 of CW tension (conditioned by turgor pressure). Low pH-induced CWDs can be alleviated by increasing concentrations of Ca²⁺ which
 546 stabilize homogalacturonan crosslinks and therefore prevents a signaling cascade (s) triggering PCD in MZ/TZ/early EZ and arrest of
 547 elongation in late EZ (cell wall stiffening).

548
 549 Low pH-induced CD being coordinated through a PCD mechanism rather than a necrotic CD
 550 naturally implicates the occurrence of a signaling process. We propose that CWDs may be the initial
 551 part of this process. Low pH has been suggested to affect the pectin network in root CWs (Koyama
 552 et al., 2001). Root hair tips are very sensitive to low pH (Bibikova et al., 1998) and their CWs are rich
 553 in pectins (Wolf and Greiner, 2012). Pectins also play important roles in CW loosening and cell
 554 expansion (Wolf and Greiner, 2012; Chebli and Geitmann, 2017). The relationship between “acid

555 growth” and pectin has been examined (Hocq et al., 2017). Damages upon pectin can produce
 556 oligogalacturonides (OGs) as pectin fragments (Brutus et al., 2010). It was observed here that low
 557 pH decreased CW stiffness in TZ cells (Table 1). Stiffness reflects the resistance to deformation
 558 (Hocq et al., 2017). Thus, the decrease in stiffness due to low pH can be interpreted as CWDs,
 559 rather than a coordinated increase in CW extensibility that occurs in EZ allowing cell elongation
 560 (Verbelen et al., 2006; Spartz et al., 2014). This is mainly supported by the decrease in CW stiffness
 561 observed before the onset of CD in TZ as reported here. Upon low pH, homogalacturonans charge
 562 neutralization disrupts calcium crosslinking, resulting in their partial aggregation and weakening of
 563 cellulose-pectin interactions (Phyo et al., 2019). Moreover, *COB* is expressed at a high level above
 564 MZ and *COB* drives the correct cellulose deposition (Schindelman et al., 2001; Roudier et al., 2005).
 565 The disruption in correct CW architecture in *cob-1* mutant roots accelerated the CD in TZ under low
 566 pH. Given these considerations, we propose that low pH causes CWDs that could correspond to
 567 polysaccharide disorganization or even to OGs production which could, in turn, be perceived by yet
 568 to be identified CWI/CWDs sensors (Fig. 8). OGs are known as DAMPs (Damage Associated
 569 Molecular Patterns) and play a role as danger signals upon pathogen attack that can activate PCD
 570 (Benedetti et al., 2015; Choi and Klessig, 2016; Davidsson et al., 2017). Thus, upon low pH, sensing
 571 of CWDs in TZ likely triggers a response similar to that activated by pathogen attack, thus leading to
 572 PCD. This hypothesis is supported by similar co-regulation of genes induced by low pH stress and by
 573 pathogen elicitors such as the genes encoding WRKY transcription factors which are important for
 574 plant immunity (Lager et al., 2010). *WAK2* and *WAK4* are genes coding for wall-associated kinases
 575 and are up-regulated upon low pH stress in *Triticum aestivum* (Hu et al., 2018). In *A. thaliana*, WAKs
 576 are involved in sensing CWI and play a crucial role in plant response to pathogens (Anderson et al.,
 577 2001; Wagner and Kohorn, 2001; Voxeur and Hofte, 2016). In TZ cortical cells, the activity of α -
 578 expansin 1 (*EXPA1*) upon apoplast acidification promotes CW loosening resulting in cell size
 579 changes, driving cells towards differentiation in *A. thaliana* roots (Pacifici et al., 2018). Accordingly,
 580 *EXPA1* is also expressed in TZ epidermal cells. Therefore, the low pH stress such as the one reported
 581 here, may also activate a CW loosening driven by expansins, and exacerbates CWDs. Furthermore,
 582 TZ is highly sensitive to environmental stimuli and is a site for hormone crosstalk between auxin,
 583 cytokinin, and other endogenous signals that mediate root growth (Kong et al., 2018). A regulatory
 584 phytohormone network in the root TZ determines root growth by regulating the balance of cell
 585 differentiation and cell division by an antagonistic interaction between auxin and cytokinin (Di
 586 Mambro et al., 2017; Pacifici et al., 2018). Considering that the TZ is highly sensitive to low pH
 587 stress, it will be interesting to elucidate whether hormonal crosstalk integrates into signaling that
 588 triggers sensitive responses in TZ such as CD, or to the regulation of root growth.

589 590 **4.2. Elongation arrest in late EZ is a coordinated response activated upon low pH-induced CWDs**

591 The coordination of cellular elongation is crucial for the rapid control of root growth (Le et al.,
 592 2001; Verbelen et al., 2006; Barbez et al., 2017; Pavelescu et al., 2018). Despite PCD detected in
 593 MZ/TZ/early EZ, late EZ epidermal cells displayed arrested elongation as indicated by
 594 measurements of LEH (in trichoblasts) in primary roots after 1 h of low pH treatment. This sensitive
 595 response in late EZ of wild-type roots was not a direct effect of low pH on the CW since a reduction
 596 of LEH was not observed in roots of *cob-1* mutant. The absence of arrested elongation in *cob-1* was
 597 not due to impairment in the cellular capacity to perform this sensitive response, such as it could
 598 occur because of cell mortality. CD progressed in *cob-1* towards late EZ faster and more intensively
 599 than in WT roots for 3 h. However, even after 3 h of stress, the cells for which LEH was examined
 600 did not display CD due to low pH in *cob-1* roots (Fig. S3). Hence, the absence of cell elongation
 601 arrest in *cob-1* was not a consequence of CD. The primary roots of *Zea mays* exhibit different

602 surface pH depending on the root zone that can be either acid, neutral or alkaline compared to the
603 pH of the treatment solution (Peters and Felle, 1999). Moreover, upon a pH 4.2 treatment, the pH
604 at the root surface is more alkaline than the medium except in EZ. Accordingly, an increase in
605 growth upon lowering of the pH produced the highest growth rates only when slowly growing roots
606 were subjected to the treatment, suggesting that modulation of CW by pH and additional factors
607 may contribute to the root growth control. Excised segments of lateral roots of *Pisum sativum* killed
608 by methanol exhibited growth upon pH 3.0 when compared to excised roots treated at pH 6.0, as
609 indicated by creep extension analysis (Tanimoto et al., 2000). These results suggest that low pH
610 decreases the viscosity of the CWs, thus allowing their extension. In sharp contrast, our findings
611 described an elongation arrest in roots upon low pH. However, we used intact roots that showed
612 living cells in late EZ upon low pH stress. Furthermore, we applied low pH stress simulating acidic
613 soil conditions in a low ionic strength solution which imposes a rapid stress (Koyama et al., 2001;
614 Graças et al., 2016), differing from the treatments described in Peters and Felle (1999).
615 Interestingly, these studies are in agreement with a tight coupling between CWs and low pH
616 responses in roots. Here, in support of this assumption, we found that low pH most likely affects
617 CWI, and in response, the cell elongation is inhibited. Multiple hormones and signaling pathways
618 interact to control the cellular elongation in roots. The arrest of elongation has been reported in
619 different situations such as in response to ethylene signaling (Le et al., 2001), production of reactive
620 oxygen species that could also stiffen the CW through an increase in polysaccharides crosslinking
621 (Camacho-Cristóbal et al., 2015), in response to isoxaben driven-CWDs (Tsang et al., 2011), upon
622 phosphate starvation (Balzergue et al., 2017), aluminum toxicity (Ma et al., 2004), and in response
623 to auxin signaling (Růžička et al., 2007). The well-known arrest in cell elongation in EZ driven by
624 ethylene signaling (Le et al., 2001) is partially coordinated by modulation of the expression of genes
625 related to CW polysaccharides crosslinking (Markakis et al., 2012). Moreover, CIII Prxs activity can
626 trigger CW stiffening (Francoz et al., 2015) and is induced in EZ of *A. thaliana* roots upon low pH
627 stress (Graças et al., 2020). Thus, in response to low pH stress, late EZ cells likely stiffen the CWs
628 leading to the observed elongation arrest, contributing to the reduced root growth (Fig. S1A).
629 Therefore, the loss in the correct deposition of cellulose microfibrils and disruption in CW
630 architecture in *cob-1* roots (Schindelman et al., 2001; Roudier et al., 2005; Liu et al., 2015) may
631 hamper the ability of EZ cells to orchestrate this elongation arrest in response to low pH.

632 The low pH-induced elongation arrest in roots was prevented upon increased calcium
633 concentration in the treatment solution (Fig. 5). *Z. mays* roots were able to maintain growth at pH
634 4.5 only when they were formerly soaked in a high calcium concentration solution (10 mM CaCl₂)
635 (Hasenstein and Evans, 1988). Accordingly, upon low pH and low calcium concentration in solution
636 treatment (1 mM CaCl₂), low pH triggered the release of calcium from the tip of *Z. mays* roots
637 resulting in reduced root growth. Indeed, low pH reduces the cation exchange capacity of pectin
638 homogalacturonans since protons can displace cations from their binding sites (White, 2012). The
639 decrease in interaction between galacturonic acid residues and calcium can cause the collapse of
640 the homogalacturonan network upon low pH (Phyo et al., 2019). Increasing calcium concentration
641 restored the overall root growth upon low pH stress, likely due to pectin stabilization (Koyama et
642 al., 2001). Thus, increased calcium concentration in the low pH stress treatment most likely
643 counteracts the low pH-induced CWDs. Given the known role of calcium in the maintenance of CWI
644 (Voxeur and Höfte, 2016), the calcium treatment supports the idea that low pH-induced CWDs may
645 activate a yet to be identified signaling cascade that triggers a coordinated elongation arrest in late
646 EZ. EZ cells possess several mechanisms to tightly control elongation. Root elongation can be rapidly
647 inhibited via transcriptional (Růžička et al., 2007) or non-transcriptional mechanisms that perceive
648 endogenous signals (Fendrych et al., 2018). Ethylene (Le et al., 2001; Markakis et al., 2012), auxin

649 (Růžicka et al., 2007), and brassinosteroid (Pavelescu et al., 2018) crosstalk has a fundamental role
650 in the coordination of cell elongation. However, the signaling mechanism involved in elongation
651 arrest in roots upon low pH stress needs further investigation.

652 5. Conclusions

653 Previous work has suggested the involvement of the CW in roots upon low pH stress (Koyama et
654 al., 2001; Lager et al., 2010; Kobayashi et al., 2013; Graças et al., 2016). Here, we show that events
655 such as CD or arrest of cell elongation, regarded as indicators of toxicity, are not caused by the
656 direct action of low pH on the CW, but rather, the effect of low pH on the CW must be perceived
657 and translated into a cellular response in specific root zones as clearly indicated by the occurrence
658 of PCD in MZ/TZ/early EZ. Thus, further investigation to uncover the actors involved in these tissue-
659 specific processes should contribute to a more detailed elucidation of the cellular and molecular
660 events involved in the sensitivity responses of roots to exogenous low pH stress.

661 Declaration of Competing Interest

662 The authors declare that the research was conducted in the absence of any commercial or financial
663 relationships that could be construed as a potential conflict of interest.

664 Authorship Statement

665 Conceptualization, J.P.G., V.A.V., methodology, J.P.G., V.A.V., J.E.L. and M.B.; software, J.P.G. and
666 M.B.; validation, J.P.G., V.B. and M.B.; formal analysis, J.P.G.; investigation, J.P.G., M.B.; resources,
667 J.P.G., V.A.V. and M.B.; data curation, J.P.G., V.A.V. and V.B.; writing—original draft preparation,
668 J.P.G. and V.A.V.; writing, review and editing, J.P.G., V.A.V., E.J., L.E.P.P., J.E.L. and V.B.; visualization,
669 J.P.G.; supervision, V.A.V., E.J. and L.E.P.P.; project administration, V.A.V.; funding acquisition, V.A.V.
670 and E.J. All authors have read and agreed to the final version of the manuscript.

671 Funding

672 This work was supported through regular CAPES doctorate funding and abroad fellowship
673 granted by CAPES (*Programa de Doutorado-Sanduiche no Exterior*, Grant 88881.135425/2016-01)
674 to J.P.G.

675 Acknowledgments

676 The authors are thankful to CAPES for its financial support. Also, they appreciated the help of
677 Drs. Tsai Sui Mui and Antonio V. O. Figueira (CENA-USP), and Eliot W. Kitajima (NAP/MEPA ESALQ-
678 USP) who unconditionally opened their laboratories to perform our experiments. They would like to
679 thank Pr. Christophe Dunand, Drs. Hervé Canut and Christian Mazars for fruitful suggestions. They
680 also wish to thank the Paul Sabatier-Toulouse 3 University and the CNRS for supporting the
681 research of J.P.G. while visiting student at *Laboratoire de Recherche en Sciences Végétales*. We also
682 thank the anonymous reviewers who contributed with constructive critics toward improving the
683 manuscript organization and discussion.

684
685

686 References

- 687 Anderson, C.M., Wagner, T.A., Perret, M., He, Z.H., He, D.Z., Kohorn, B.D., 2001. WAKs: *cell*
688 *wall-associated kinases* linking the cytoplasm to the extracellular matrix. *Plant Mol. Biol.*
689 47, 197-206. <https://doi.org/10.1023/A:1010691701578>.
- 690 Bagniewska-Zadworna, A., and Arasimowicz-Jelonek, M., 2016. The mystery of underground
691 death: cell death in roots during ontogeny and in response to environmental factors. *Plant*
692 *Biol.* 18, 171-184. <https://doi.org/10.1111/plb.12391>.
- 693 Baker, C.J., and Mock, N.M., 1994. An improved method for monitoring cell-death in cell-
694 suspension and leaf disc assays using Evans blue. *Plant Cell Tissue Organ Cult.* 39, 7-12.
695 <https://doi.org/10.1007/BF00037585>.
- 696 Balzergue, C., Dartevelle, T., Godon, C., Laugier, E., Meisrimler, C., Teulon, J.M., Creff, A.,
697 Bissler, M., Bouchoud, C., Hagège, A., Müller, J., Chiarenza, S., Javot, H., Becuwe-Linka,
698 N., David, P., Péret, B., Delannoy, E., Thibaud, M.C., Armengaud, J., Abel, S., Pellequer,
699 J.L., Nussaume, L., Desnos, T., 2017. Low phosphate activates STOP1-ALMT1 to rapidly
700 inhibit root cell elongation. *Nat Commun.* 8, 15300. <https://doi.org/10.1038/ncomms15300>.
- 701 Barbez, E., Dünser, K., Gaidora, A., Lendl, T., Busch, W., 2017. Auxin steers root cell expansion
702 via apoplastic pH regulation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 114,
703 E4884–E4893. <https://doi.org/10.1073/pnas.1613499114>.
- 704 Benedetti, M., Pontiggia, D., Raggi, S., Cheng, Z., Scaloni, F., Ferrari, S., Ausubel, F.M., Cervone,
705 F., De Lorenzo, G., 2015. Plant immunity triggered by engineered *in vivo* release of
706 oligogalacturonides, damage-associated molecular patterns. *Proc. Natl. Acad. Sci. U.S.A.*
707 112, 5533-5538. <https://doi.org/10.1073/pnas.1504154112>.
- 708 Benfey, P.N., Linstead P.J., Roberts, K., Schiefelbein, J.W., Hauser, M.T., Aeschbacher, R.A.,
709 1993. Root development in *Arabidopsis* - 4 mutants with dramatically altered root
710 morphogenesis. *Development* 119, 57-70. <https://dev.biologists.org/content/119/1/57>.
- 711 Bibikova, T.N., Jacob, T., Dahse, I., Gilroy, S., 1998. Localized changes in apoplastic and
712 cytoplasmic pH are associated with root hair development in *Arabidopsis thaliana*.
713 *Development* 125, 2925-2934. doi: <https://dev.biologists.org/content/125/15/2925.long>.
- 714 Brutus, A., Sicilia, F., Macone, A., Cervone, F., Lorenzo, G.D., 2010. A domain swap approach
715 reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of
716 oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457.
717 <https://doi.org/10.1073/pnas.1000675107>.
- 718 Camacho-Cristobal, J.J., Martin-Rejano, E.M., Herrera-Rodrigues, M.B., Navarro-Gochicoa, M.T.,
719 Rexach, J., Gonzalez-Fontes, A., 2015. Boron deficiency inhibits root cell elongation via an
720 ethylene/auxin/ROS-dependent pathway in *Arabidopsis* seedlings. *J. Exp. Bot.* 66, 3831-
721 3840. <https://doi.org/10.1093/jxb/erv186>.
- 722 Casamitjana-Martinez, E., Hofhuis, H.F., Xu, J., Liu, C.M., Heidstra, R., Scheres, B., 2003. Root-
723 specific *CLE19* overexpression and the *sol1/2* suppressors implicate a CLV-like pathway in
724 the control of *Arabidopsis* root meristem maintenance. *Curr. Biol.* 13, 1435-1441.
725 [https://doi.org/10.1016/s0960-9822\(03\)00533-5](https://doi.org/10.1016/s0960-9822(03)00533-5).
- 726 Choi, H.W., and Klessig, D.F., 2016. DAMPs, MAMPs, and NAMPs in plant innate immunity.
727 *BMC Plant Biol.* 16, 232. <https://doi.org/10.1186/s12870-016-0921-2>.
- 728 Chebli, Y., and Geitmann, A., 2017. Cellular growth in plants requires regulation of cell wall
729 biochemistry. *Curr. Opin. Cell Biol.* 44, 28–35. <https://doi.org/10.1186/s12870-016-0921-2>.
- 730 Cosgrove, D.J., 2015. Plant cell wall extensibility: connecting plant cell growth with cell wall
731 structure, mechanics, and the action of wall-modifying enzymes. *J. Exp. Bot.* 67, 463-476.
732 <https://doi.org/10.1093/jxb/erv511>.

- 733 Davidsson, P., Broberg, M., Kariola, T., Sipari, N., Pirhonen, M., Palva, E.T., 2017. Short
734 oligogalacturonides induce pathogen resistance-associated gene expression in *Arabidopsis*
735 *thaliana*. BMC Plant Biol. 19, 17-34. <https://doi.org/10.1186/s12870-016-0959-1>.
- 736 Di Mambro, R., De Ruvo, M., Pacifici, E., Salvi, E., Sozzani, R., Benfey, P.N., Busch, W., Novak,
737 O., Ljung, K., Di Paola, L., Marée, A.F.M., Costantino, P., Grieneisen, V.A., Sabatini, S.,
738 2017. Auxin minimum triggers cell differentiation. Proc. Natl. Acad. Sci. U.S.A. 114,
739 E7641-E7649. <https://doi.org/10.1073/pnas.1705833114>.
- 740 Evered, C., Majevechia, B., Thompson, D.S., 2007. Cell wall water content has a direct effect on
741 extensibility in growing hypocotyls of sunflower (*Helianthus annuus* L.). J. Exp. Bot. 58,
742 3361-3371. <https://doi.org/10.1093/jxb/erm183>.
- 743 Fendrych, M., Akhmanova, M., Merrin, J., Glanc, M., Hagihara, S., Takahashi, K., Uchida, N.,
744 Torii, K.U., Friml, J., 2018. Rapid and reversible root growth inhibition by TIR1 auxin
745 signalling. Nat. Plants 4, 453-459. <https://doi.org/10.1038/s41477-018-0190-1>.
- 746 Feng, W., Kita, D., Peaucelle, A., Cartwright, H.N., Doan, V., Duan, Q., Ming-Che, L., Maman, J.,
747 Steinhorst, L., Schimits-Tom, I., Yvon, R., Kudla, J., Hen-Ming, W., Cheung, A.Y.,
748 Dinneny, J.R., 2018. The FERONIA receptor kinase maintains cell-wall integrity during salt
749 stress through Ca²⁺ signaling. Curr. Biol. 28, 666-675.
750 <https://doi.org/10.1016/j.cub.2018.01.023>.
- 751 Francoz, E., Ranocha, P., Nguyen-Kim, H., Jamet, E., Burlat, V., Dunand, C., 2015. Roles of cell
752 wall peroxidases in plant development. Phytochemistry 112, 15-21.
753 <https://doi.org/10.1016/j.phytochem.2014.07.020>.
- 754 Graças, J.P., Ruiz-Romero, R., Figueiredo, L.D., Mattiello, L., Peres, L.E.P., Vitorello, V.A., 2016.
755 Root growth restraint can be an acclimatory response to low pH and is associated with
756 reduced cell mortality: a possible role of class III peroxidases and NADPH oxidases. Plant
757 Biol. 18, 658-668. <https://doi.org/10.1111/plb.12443>.
- 758 Graças, J.P., Ranocha, P., Vitorello, V.A., Savelli, B., Jamet, E., Dunand, C., Burlat, V., 2020. The
759 class III peroxidase encoding gene *AtPrx62* positively and spatiotemporally regulates the
760 low pH-induced cell death in *Arabidopsis thaliana* roots. Int. J. Mol. Sci. 21, 7191.
761 <https://doi.org/10.3390/ijms21197191>.
- 762 Guan, B., Lin, Z., Liu, D., Li, C., Zhou, Z., Mei, F., Li, J., Deng, X., 2019. Effect of waterlogging-
763 induced autophagy on programmed cell death in *Arabidopsis* roots. Front. Plant Sci. 10,
764 468. <https://doi.org/10.3389/fpls.2019.00468>.
- 765 Hansen, S.L., Ray, P.M., Karlsson, A.O., Jorgensen, B., Borkhardt, B., Petersen B.L., Ulvskov P.,
766 2011. Mechanical properties of plant cell walls probed by relaxation spectra. Plant Physiol.
767 155, 246-258. <https://doi.org/10.1104/pp.110.166629>.
- 768 Hasenstein, K.H., and Evans M.L., 1988. The influence of calcium and pH on growth in primary
769 roots of *Zea mays*. Physiol. Plant. 72, 466-470. <https://doi.org/10.1111/j.1399-3054.1988.tb09152.x>.
- 771 Hauser, M., Morikami, A., Benfey, P.N., 1995. Conditional root expansion mutants of *Arabidopsis*.
772 Development 121, 1237-1252. doi: <https://dev.biologists.org/content/121/4/1237>.
- 773 Hocq, L., Pelloux, J., Lefebvre, V., 2017. Connecting homogalacturonan-type pectin remodeling to
774 acid growth. Trends Plant Sci. 22, 20-29. <https://doi.org/10.1016/j.tplants.2016.10.009>.
- 775 Hu, H., He, J., Zhao, J., Ou, X., Li, H., Ru, Z., 2018. Low pH stress responsive transcriptome of
776 seedlings roots in wheat (*Triticum aestivum* L.). Genes Genomics 40, 1199-1211.
777 <https://doi.org/10.1007/s13258-018-0680-6>.
- 778 Kierzkowski, D., Nakayama, N., Routier-Kierzkowska, A. L., Weber, A., Bayer, E., Schorderet, M.,
779 Reinhardt, D., Kuhlemeier, C., Smith, R.S., 2012. Elastic domains regulate growth and
780 organogenesis in the plant shoot apical meristem. Science 335, 1096-1099.
781 <https://doi.org/10.1126/science.1213100>.

- 782 Kobayashi, Y., Kobayashi, Y., Watanabe, T., Shaff, J.E., Ohta, H., Kochian, L.V., Wagatsuma, T.,
783 Kinraide, T.B., Koyama, H., 2013. Molecular and physiological analysis of Al³⁺ and H⁺
784 rhizotoxicities at moderately acidic conditions. *Plant Physiol.* 163, 180-192.
785 <https://doi.org/10.1104/pp.113.222893>.
- 786 Kobayashi, Y., Ohyama, Y., Ito, H., Iuchi, S., Fujita, M., Zhao, C. R., Tanveer, T., Ganesan, M.,
787 Kobayashi, M., Koyama, Y., 2014. STOP2 activates transcription of several genes for Al-
788 and low pH-tolerance that are regulated by STOP1 in *Arabidopsis*. *Mol Plant.* 7, 311-322.
789 <https://doi.org/10.1093/mp/sst116>.
- 790 Kong, X., Liu, G., Liu, J., Ding, Z.. 2018. The root transition zone: a hot spot for signal crosstalk.
791 *Trends Plant Sci.* 23, 403-409. <https://doi.org/10.1016/j.tplants.2018.02.004>.
- 792 Koyama, H., Toda, T., Hara, T., 2001. Brief exposure to low-pH stress causes irreversible damage to
793 the growing root in *Arabidopsis thaliana*: pectin-Ca interaction may play an important role
794 in proton rhizotoxicity. *J. Exp. Bot.* 52, 361-368. <https://doi.org/10.1093/jxb/52.355.361>.
- 795 Lager, I., Andreasson, O., Dunbar, T.L., Andreasson, E., Escobar, M.A., Rasmusson, A.G., 2010.
796 Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor
797 responses. *Plant Cell Environ.* 33, 1513-1528. <https://doi.org/10.1111/j.1365-3040.2010.02161.x>.
- 799 Le, J., Vandebussche, F., Van Der Straeten, D., Verbelen, J.P., 2001. In the early response of
800 *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled
801 from differentiation. *Plant Physiol.* 125, 519-522. <https://doi.org/10.1104/pp.125.2.519>.
- 802 Liu, Z.Y., Persson, S., Sanchez-Rodriguez, C., 2015. At the border: the plasma membrane-cell wall
803 continuum. *J. Exp. Bot.* 66, 1553-1563. <https://doi.org/10.1093/jxb/erv019>.
- 804 Ma, J.F., Shen, R., Nagao, S., Tanimoto, E., 2004. Aluminum targets elongating cells by reducing
805 cell wall extensibility in wheat roots. *Plant Cell Physiol.* 45, 583-589.
806 <https://doi.org/10.1093/pcp/pch060>.
- 807 Markakis, M.N., De Cnodder T., Lewandowski, M., Simon, D., Boron, A., Balcerowicz, D.,
808 Doubo, T., Taconnat, L., Renou, J.P., Höfte, H., Verbelen, J.P., Vissenberg, K., 2012.
809 Identification of genes involved in the ACC-mediated control of root cell elongation in
810 *Arabidopsis thaliana*. *BMC Plant Biol.* 12, 208. <https://doi.org/10.1186/1471-2229-12-208>.
- 811 Monshausen, G.B., Bibikova, T.N., Messerli, M.A., Shi, C., Gilroy, S., 2007. Oscillations in
812 extracellular pH and reactive oxygen species modulate tip growth of *Arabidopsis* root hairs.
813 *Proc. Natl. Acad. Sci. U.S.A.* 104, 20996-21001. <https://doi.org/10.1073/pnas.0708586104>.
- 814 Mravec, J., Kracun, S.K., Rydahl M.G., Westereng, B., Pontiggia, D., De Lorenzo, G., Domozych,
815 D.S., Willats, W.G.T., 2017. An oligogalacturonide-derived molecular probe demonstrates
816 the dynamics of calcium-mediated pectin complexation in cell walls of tip-growing
817 structures. *Plant J.* 91, 534-546. <https://doi.org/10.1111/tpj.13574>.
- 818 Olvera-Carrillo, Y., Van Bel, M., Van Hautegeem, T., Fendrych, M., Huysmans, M., Simaskova, M.,
819 van Durme, M., Buscaill, P., Rivas, S., Coll, N.S., Coppens, F., Maere, S., Nowack, M.K.,
820 2015. A conserved core of programmed cell death indicator genes discriminates
821 developmentally and environmentally induced programmed cell death in plants. *Plant*
822 *Physiol.* 169, 2684-2699. <https://doi.org/10.1104/pp.15.00769>.
- 823 Pacifici, E., Di Mambro, R., Dello Ioio, R., Constantino, P., Sabatini, S., 2018. Acidic cell
824 elongation drives cell differentiation in the *Arabidopsis* root. *EMBO J.* 37, e99134.
825 <https://doi.org/10.15252/embj.201899134>.
- 826 Passioura, J.B., 1994. The physical-chemistry of the primary-cell wall - implications for the control
827 of expansion rate. *J. Exp. Bot.* 45, 1675-1682.
828 https://doi.org/10.1093/jxb/45.Special_Issue.1675.
- 829 Pavelescu, I., Vilarrasa-Blasi, J., Planas-Riverola, A., González-García, M-P., Caño-Delgado, A.,
830 Ibañes, M., 2018. A Sizer model for cell differentiation in *Arabidopsis thaliana* root growth.
831 *Mol Syst Biol.* 14, e7687. <https://doi.org/10.15252/msb.20177687>.

- 832 Peters, W.S., and Felle, H.H., 1999. The correlation of profiles of surface pH and elongation growth
833 in maize roots. *Plant Physiol.* 121, 905-912. <https://doi.org/10.1104/pp.121.3.905>.
- 834 Phyto, P., Gu, Y., Hong, M., 2019. Impact of acidic pH on plant cell wall polysaccharide structure
835 and dynamics: insights into the mechanism of acid growth in plants from solid-state NMR.
836 *Cellulose* 26, 291-304. <https://doi.org/10.1007/s10570-018-2094-7>.
- 837 Pritchard, J., Wyn Jones, R.G., Tomos, A.D., 1990. Measurement of yield threshold and cell wall
838 extensibility of intact wheat roots under different ionic, osmotic and temperature treatments.
839 *J. Exp. Bot.* 41, 669-675. <https://doi.org/10.1093/jxb/41.6.669>.
- 840 Rayle, D.L., and Cleland, R.E., 1992. The acid growth theory of auxin-induced cell elongation is
841 alive and well. *Plant Physiol.* 99, 1271-1274. <https://doi.org/10.1104/pp.99.4.1271>.
- 842 Reape, T.J., Molony, E.M., McCabe, P.F., 2008. Programmed cell death in plants: distinguishing
843 between different modes. *J. Exp. Bot.* 59, 435-444. <https://doi.org/10.1093/jxb/erm258>.
- 844 Román, M.P., Cambra, M., Juárez, J., Moreno, P., Duran-Vila, N., Tanaka, F.A.O., Alves, E.,
845 Kitajima, E.W., Yamamoto, P.T., Bassanezi, R.B. Teixeira, D.C., Jesus Junior, W.C., Ayres,
846 A.J., Gimenes-Fernandes, N., Rabenstein, F., Girotto, L.F., Bové, J.M., 2004. Sudden death
847 of citrus in Brazil: A graft-transmissible bud union disease. *Plant Dis.* 88, 453-467.
848 <https://doi.org/10.1094/PDIS.2004.88.5.453>.
- 849 Roudier, F., Schindelman, G., DeSalle, R., Benfey, P.N., 2002. The COBRA family of putative
850 GPI-anchored proteins in *Arabidopsis*. A new fellowship in expansion. *Plant Physiol.* 130,
851 538-548. <https://doi.org/10.1104/pp.007468>.
- 852 Roudier, F., Fernandez, A.G., Fujita, M., Himmelspach, R., Borner, G.H.H., Schindelman, G.,
853 Song, S., Baskin, T.I., Dupree, P., Wasteneys, G.O., Benfey, P.N., 2005. COBRA, an
854 *Arabidopsis* extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically
855 controls highly anisotropic expansion through its involvement in cellulose microfibril
856 orientation. *Plant Cell* 17, 1749-1763. <https://doi.org/10.1105/tpc.105.031732>.
- 857 Růžička, K., Ljung, K., Vanneste, S., Podhorská, R., Beeckman, T., Friml, J., Benková, E., 2007.
858 Ethylene regulates root growth through effects on auxin biosynthesis and transport-
859 dependent auxin distribution. *Plant Cell* 19, 2197-2212.
860 <https://doi.org/10.1105/tpc.107.052126>.
- 861 Schindelman, G., Morikami, A., Jung, J., Baskin, T.I., Carpita, N.C., Derbyshire, P., McCan, M.C.,
862 Benfey, P.N., 2001. *COBRA* encodes a putative GPI-anchored protein, which is polarly
863 localized and necessary for oriented cell expansion in *Arabidopsis*. *Genes Dev.* 15, 1115-
864 1127. <https://doi.org/10.1101/gad.879101>.
- 865 Shavrukov, Y., and Hirai, Y., 2016. Good and bad protons: genetic aspects of acidity stress
866 responses in plants. *J. Exp. Bot.* 67, 15-30. <https://doi.org/10.1093/jxb/erv437>.
- 867 Somssich, M., Khan, G.A., Persson, S., 2016. Cell wall heterogeneity in root development of
868 *Arabidopsis*. *Front. Plant Sci.* 7, 1242. <https://doi.org/10.3389/fpls.2016.01242>.
- 869 Spartz, A.K., Ren, H., Park, M.Y., Grandt, K.N., Lee S.H., Murphy, A.S., Sussman, M.R.,
870 Overvoorde, P.J., Gray, W.M., 2014. SAUR inhibition of PP2C-D phosphatases activates
871 plasma membrane H⁺-ATPases to promote cell expansion in *Arabidopsis*. *Plant Cell* 26,
872 2129-2142. <https://doi.org/10.1105/tpc.114.126037>.
- 873 Tanimoto, E., Fujii, S., Yamamoto, R., Inanaga, S., 2000. Measurement of viscoelastic properties of
874 root cell walls affected by low pH in lateral roots of *Pisum sativum* L. *Plant Soil.* 226, 21-
875 28. <https://doi.org/10.1023/A:1026460308158>.
- 876 Tsang, D.L., Edmond, C., Harrington, J.L., Nühse, T.S., 2011. Cell wall integrity controls root
877 elongation via a general 1-aminocyclopropane-1-carboxylic acid-dependent, ethylene-
878 independent pathway. *Plant Physiol.* 156, 596-604. <https://doi.org/10.1104/pp.111.175372>.
- 879 Tsukagoshi, H., Busch, W., Benfey, P.N., 2010. Transcriptional regulation of ROS controls
880 transition from proliferation to differentiation in the root. *Cell* 143, 606-616.
881 <https://doi.org/10.1016/j.cell.2010.10.020>.

- 882 Vaahtera, L., Schulz, J., Hamann, T., 2019. Cell wall integrity maintenance during plant
883 development and interaction with the environment. *Nat. Plants* 5, 924-932
884 <https://doi.org/10.1038/s41477-019-0502-0>.
- 885 Verbelen, J-P., Cnodder, T., Le, J., Visseberg, K., Baluska, F., 2006. The root apex of
886 *Arabidopsis thaliana* consists of four distinct zones of growth activities. *Plant Signal Behav.*
887 6, 296-304. <https://doi.org/10.4161/psb.1.6.3511>.
- 888 Von Uexküll, and H.R., Mutert, E., 1995. Global extent, development and economic-impact of acid
889 soils. *Plant Soil.* 171, 1-15. <https://doi.org/10.1007/BF00009558>.
- 890 Voxeur, A., Hofte, H., 2016. Cell wall integrity signaling in plants: “To grow or not to grow that’s
891 the question”. *Glycobiology* 26, 950-960. <https://doi.org/10.1093/glycob/cww029>.
- 892 Wagner, T.A., Kohorn, B.D., 2001. Wall-associated kinases are expressed throughout plant
893 development and are required for cell expansion. *Plant Cell* 13, 303-318. <https://doi.org/10.1105/tpc.13.2.303>.
- 894
- 895 Watanabe, N., and Lam, E., 2008. BAX inhibitor-1 modulates endoplasmic reticulum stress-
896 mediated programmed cell death in *Arabidopsis*. *J. Biol. Chem.* 283, 3200-3210.
897 <https://doi.org/10.1074/jbc.M706659200>.
- 898 White, P.J., 2012. Ion uptake mechanisms of individual cells and roots: short-distance transport. In:
899 Marschner, P. (Ed.), *Marschner’s mineral nutrition of higher plants*. Academic Press,
900 London, 7-47. <https://doi.org/10.1016/B978-0-12-384905-2.00002-9>
- 901 Willats, W.G.T., McCartney, L., Mackie, W., Knox, J.P., 2001. Pectin: cell biology and prospects
902 for functional analysis. *Plant Mol. Biol.* 47, 9-27. <https://doi.org/10.1023/A:101066291>.
- 903 Wolf, S., and Greiner, S., 2012. Growth control by cell wall pectins. *Protoplasma* 249, 169-175.
904 <https://doi.org/10.1007/s00709-011-0371-5>.
- 905 Wolf, S., Hematy, K., Hofte, H., 2012. Growth control and cell wall signaling in plants. *Annu. Rev.*
906 *Plant Biol.* 63, 381-407. <https://doi.org/10.1146/annurev-arplant-042811-105449>.
- 907 Wu, Y.J., Spollen, W.G., Sharp, R.E., Hetherington, P.R., Fry, S.C., 1994. Root-growth
908 maintenance at low water potentials - increased activity of xyloglucan endotransglycosylase
909 and its possible regulation by abscisic-acid. *Plant Physiol.* 106, 607-615. <https://doi.org/10.1104/pp.106.2.607>.
- 910
- 911 Wu, Y.J., and Cosgrove, D.J., 2000. Adaptation of roots to low water potentials by changes in cell
912 wall extensibility and cell wall proteins. *J. Exp. Bot.* 51, 1543-1553. <https://doi.org/10.1093/jexbot/51.350.1543>.
- 913
- 914 Xi, X., Kim, S.H., Tittmann, B., 2015. Atomic force microscopy based nanoindentation study of
915 onion abaxial epidermis walls in aqueous environment. *J Appl Phys.* 117, 024703.
916 <https://doi.org/10.1063/1.4906094>.
- 917