



**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-03437134v1>

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<sup>1,3,4\*</sup>, Mariana Belloti<sup>2</sup>, Joni Esrom Lima<sup>1</sup>, Lázaro Eustáquio Pereira**  
2 **Peres<sup>3</sup>, Vincent Burlat<sup>4</sup>, Elisabeth Jamet<sup>4</sup>, Victor Alexandre Vitorello<sup>2\*</sup>**

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

5 <sup>2</sup>Laboratory 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 <sup>3</sup>Laboratory of Hormonal Control of Plant Development, Escola Superior de Agricultura “Luiz de  
8 Queiroz”, University of São Paulo, Piracicaba, Brazil

9 <sup>4</sup>Laboratoire 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  $\mu$ 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  $\text{CaCl}_2$  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  $\text{CaCl}_2$  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.

158

### 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  $\mu$ 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 ( $\Psi_s$  of  
181  $-0.032$  MPa) to hyperosmotic (HYO) solutions composed of  $0.5$  mM  $\text{CaCl}_2$  and mannitol ( $150$  mM) to  
182 reach water potential of  $-0.370$  MPa  $\Psi_s$ , 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  $\text{CaCl}_2$  and different mannitol concentrations with water potential ranging from  $\Psi_s$  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  $\Psi_s$  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  $\Psi_s$  were placed for  $5$  min in mannitol solutions with a  $\Psi_s$   
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  $\Psi_s$  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  $\Psi_s$  of  $-0.005$  MPa, root CWs  
203 were stained with calcofluor white ( $0.1\%$  w/v Sigma<sup>®</sup>, 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  $\Psi_s$  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 ( $\Delta$   
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 CaCl<sub>2</sub>) (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.

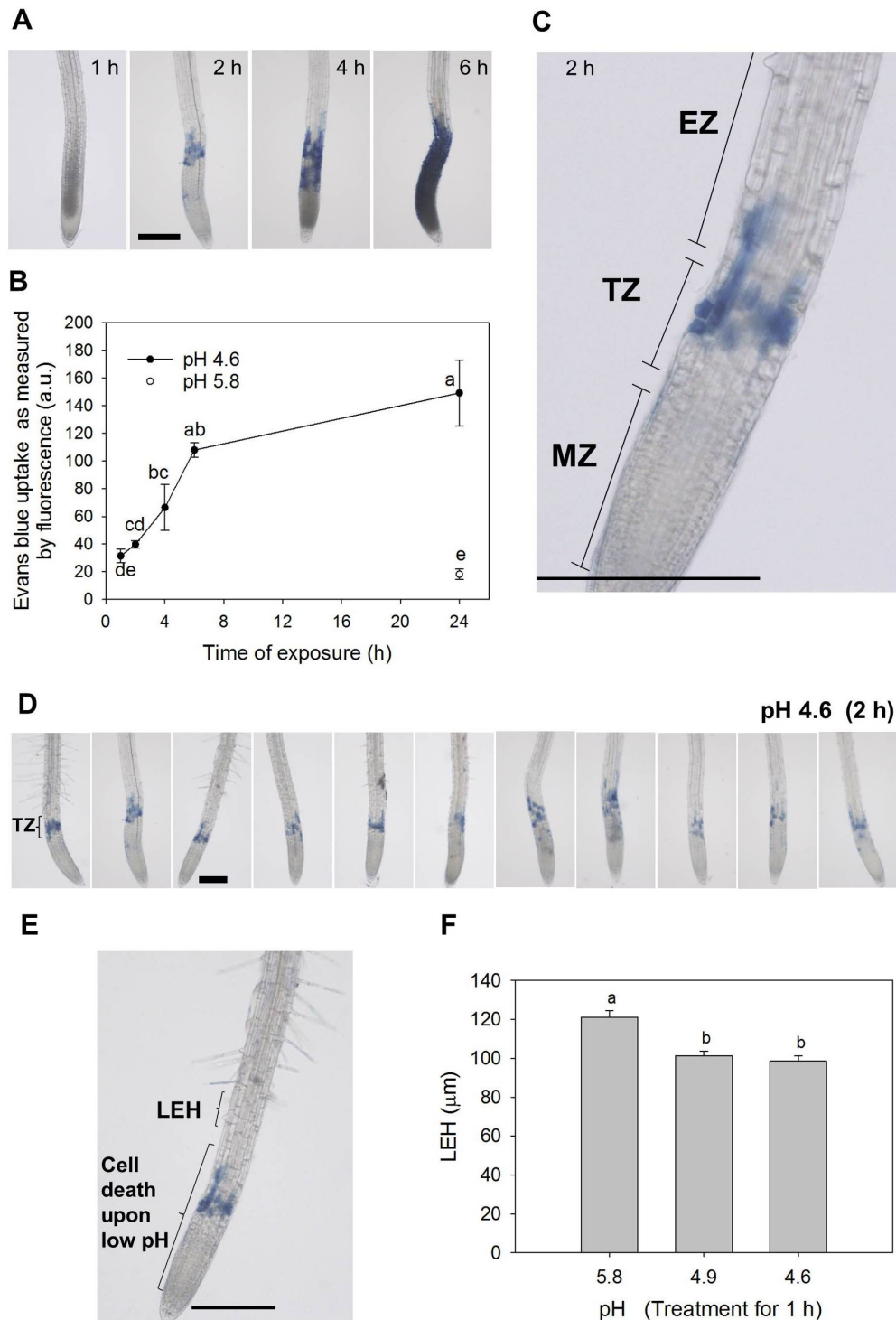
## 242 **3. Results**

### 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



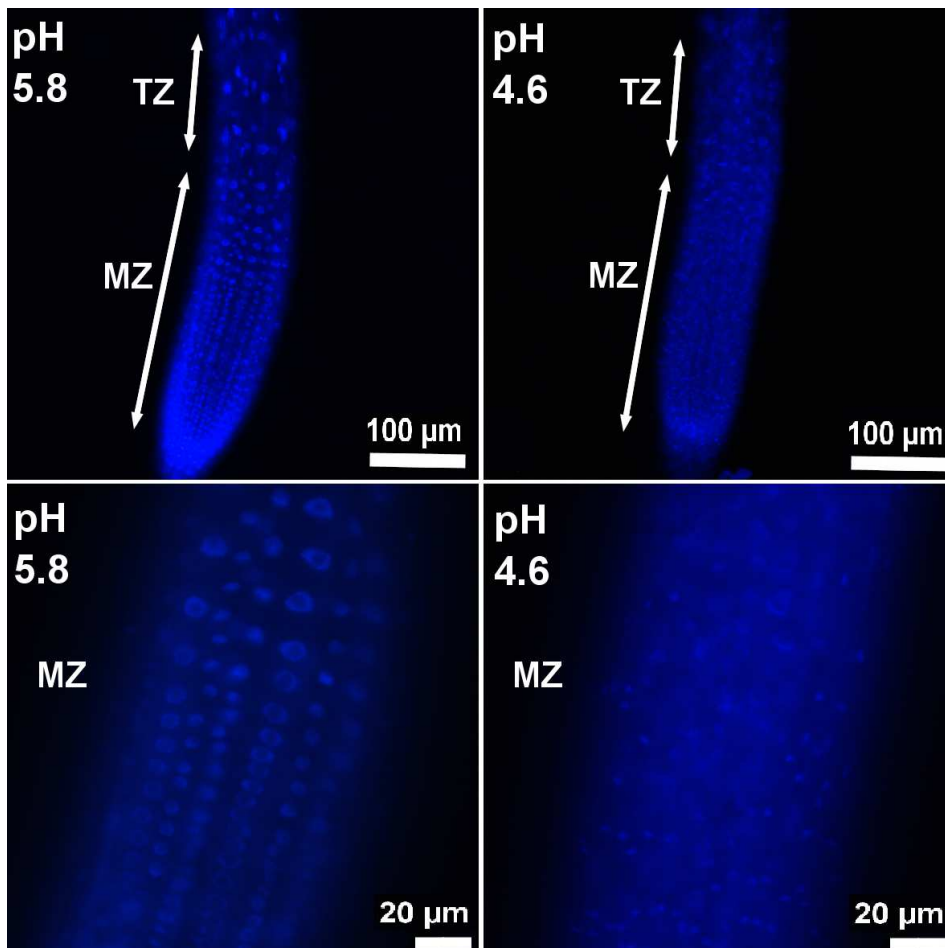
270 death occurred and the length of the first epidermal cell with visible root hair bulge (LEH) was measured in roots, based on all  
 271 replicates from (A) and (B); (F) Analysis of cell elongation in roots by measurement of LEH after 1 h of treatment at different pH. The  
 272 bars represent the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

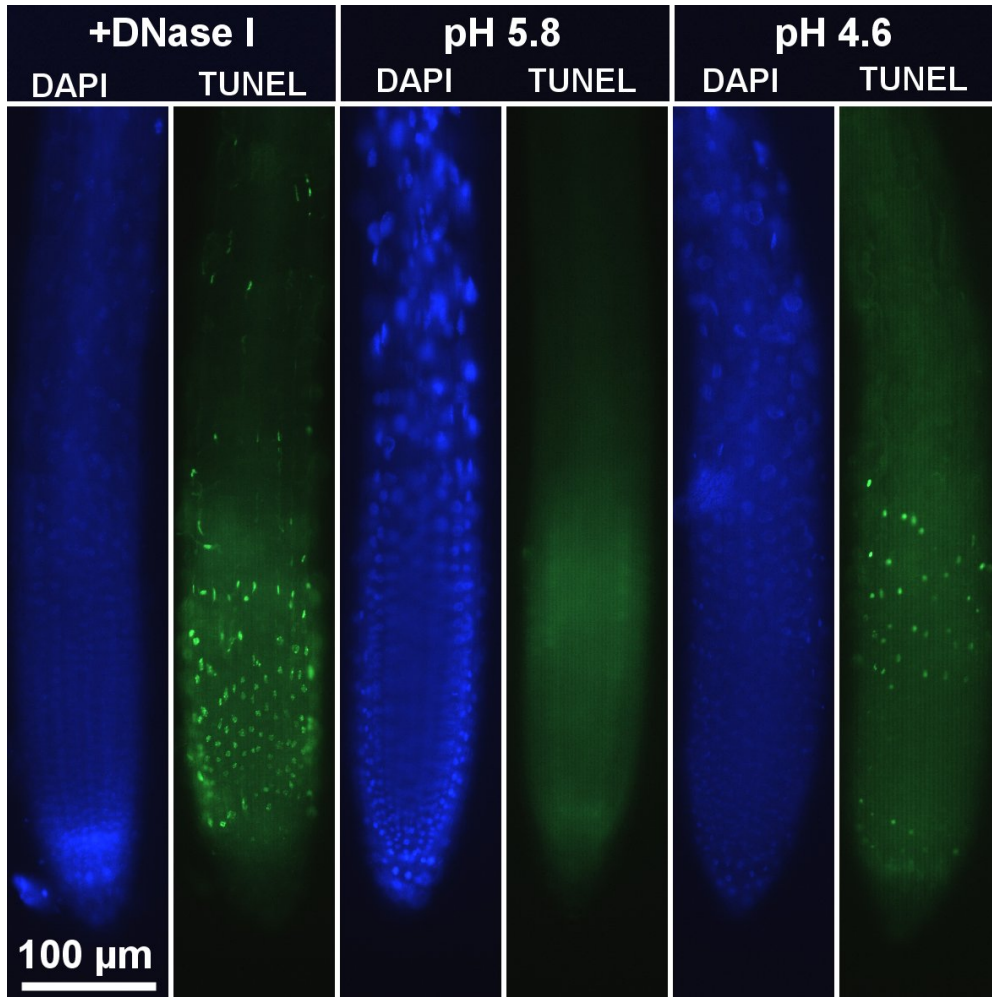
284 To characterize the CD mechanism, we performed a TUNEL assay which allows the labeling of  
 285 DNA ends at their 3'-OH end by incorporation of fluorescein-12-dUTP, *i.e.*, the coordinated DNA  
 286 fragmentation which is indicative of PCD (Reape et al., 2008). Finding positive nuclei would favor  
 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

**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.

305

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

306

307

308

309

310

311

312

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) ( $\Psi_s$  of  $-0.480$  MPa). Therefore, a greater decrease in cell length would be indicative of CW loosening, meaning that stiffness decreased.

313

314

315

316

317

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  $\Psi$ 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  $\Psi$ s of -0.480 MPa (HYO) for exactly 5 min and the same cells were immediately imaged again. The change ( $\Delta$ ) 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 % <sup>b</sup>	1.10 %
4.6	-9.08 % <sup>a</sup>	2.53 %

329

<sup>1</sup>Different 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

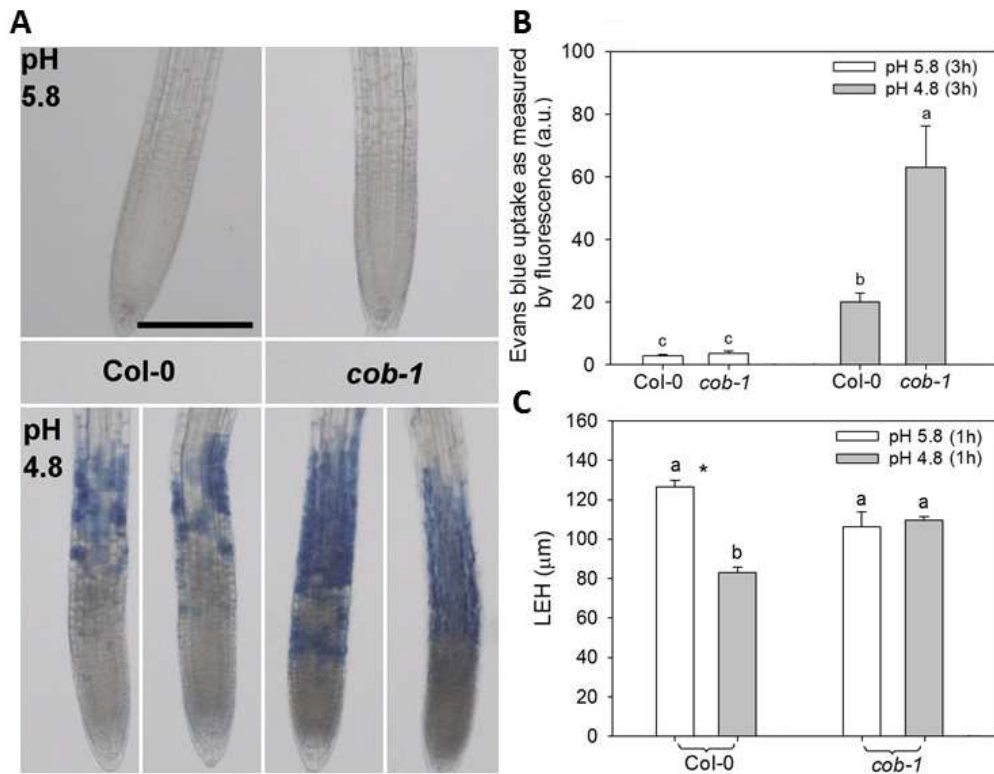
347

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.

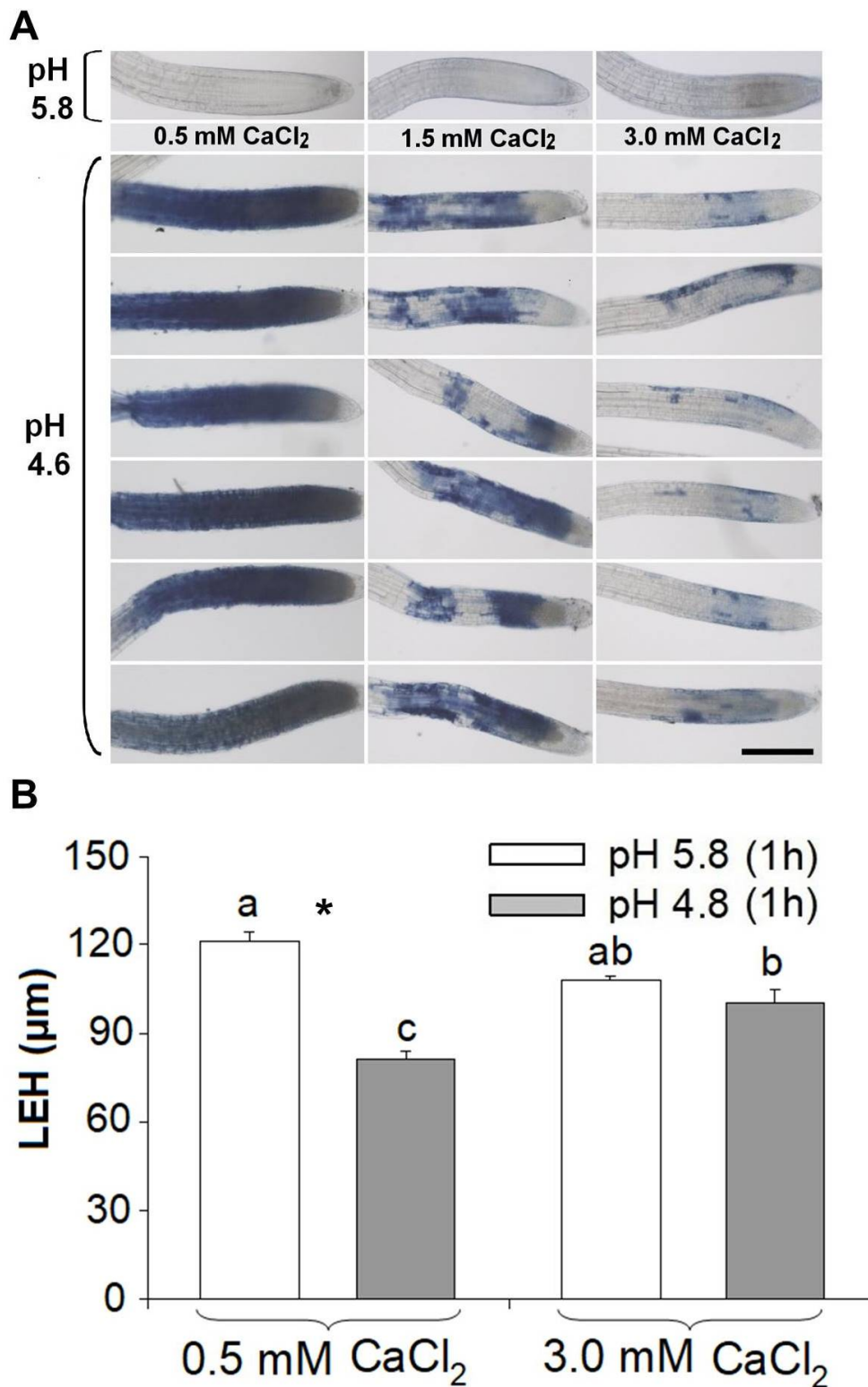


349  
 350 **Fig. 4. Cell mortality and cell elongation in primary roots of *A. thaliana* (Col-0) or *cob-1* seedlings treated at intermediate low pH stress**  
 351 **(pH 4.8).** The blue staining or increase in fluorescence is indicative of cell death. (A) Roots were stained with Evans blue. In upper  
 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 ( $\leq 0.5$  mM  $\text{Ca}^{2+}$ ) (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  $\text{CaCl}_2$ ) 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  $\text{CaCl}_2$ , we found a  
 368 slightly alleviated CD due to strong low pH stress. However, 3.0 mM  $\text{CaCl}_2$  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  $\text{CaCl}_2$  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  $\text{CaCl}_2$  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  $\text{CaCl}_2$  (Fig. 5B). These results evidence the protective effect of calcium  
 376 preventing the CD or arrest of elongation caused by low pH stress.  
 377



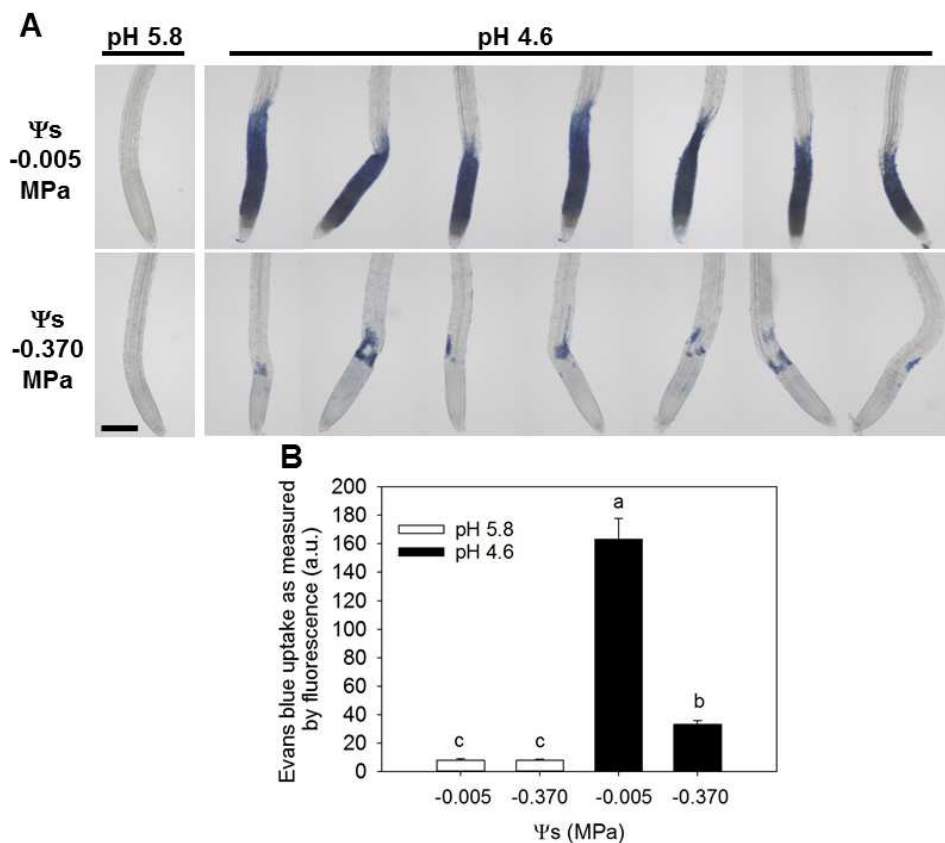
378  
379  
380 Fig. 5. Cell mortality and cell elongation in primary roots of *A. thaliana* (Col-0) exposed to low pH stress at different concentrations of  
381 CaCl<sub>2</sub>. Blue color (Evans blue staining) is indicative of cell death. (A) Roots treated at pH 5.8 or pH 4.6 (strong low pH stress) 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 CaCl<sub>2</sub> 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 CaCl<sub>2</sub> 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  $\Psi_s$  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  $\Psi_s$  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  $\Psi_s$  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  $\Psi_s$ . For simplification, one  
 402 representative image is shown for each condition of  $\Psi_s$  upon pH 5.8 (control). Scale bar: 200  $\mu$ 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.

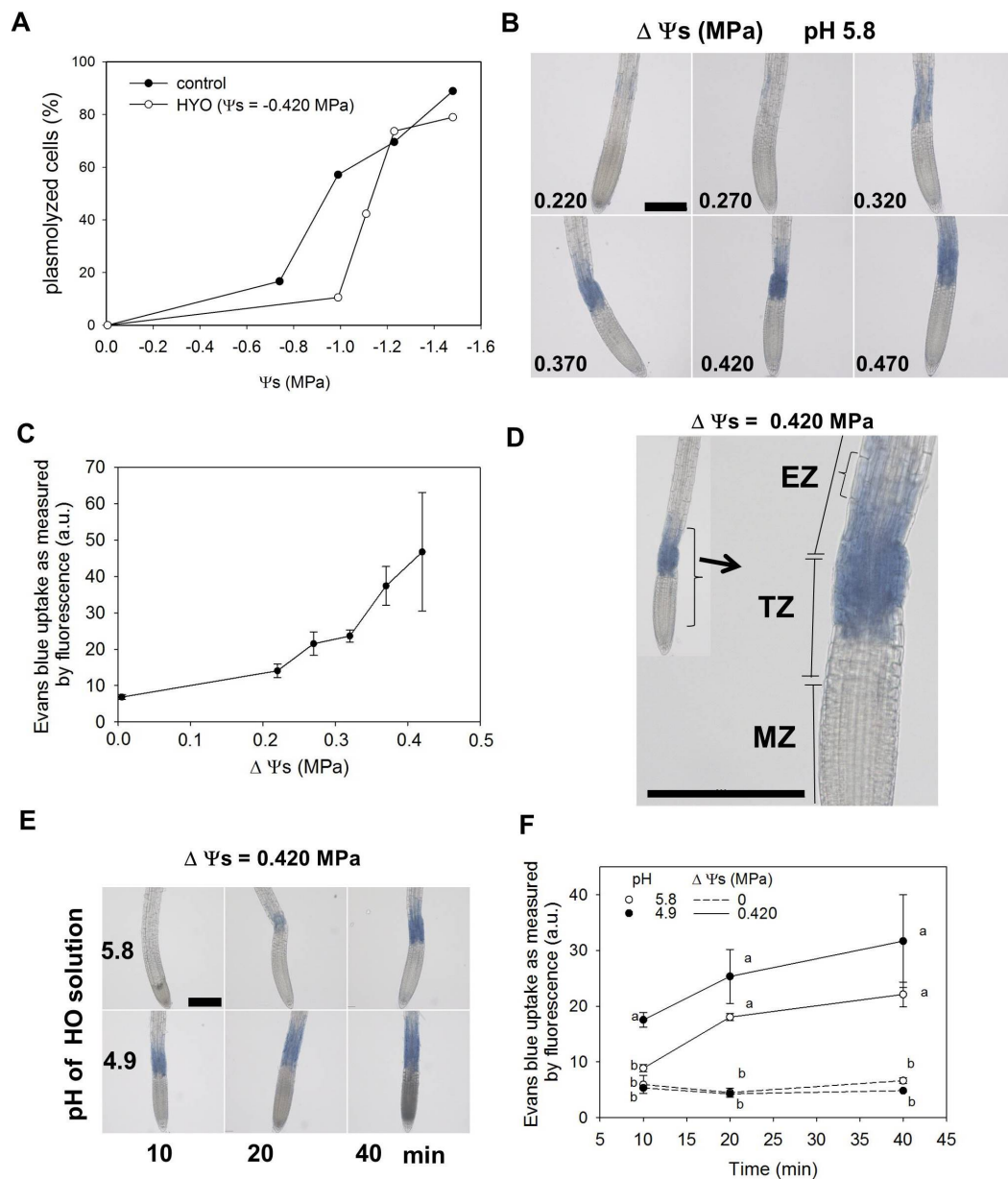
### 3.7. Effect of increased wall tension on death of root cells with or without simultaneous exposure to low pH

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

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  $\Psi_s$   
418 mannitol solution, approximately half of the cells plasmolyzed after transfer to a  $-1.100$  MPa  $\Psi_s$   
419 solution (Fig. 7A). Conversely, in control roots pre-treated in a  $-0.005$  MPa  $\Psi_s$  solution,  
420 approximately half of the cells plasmolyzed in solutions of a  $\Psi_s$  of  $-0.900$  MPa. Thus, the  $-0.420$   
421 MPa  $\Psi_s$  HYO pre-treatment decreased the  $\Psi_s$  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  $\Psi_s$  of -0.005 (control) or -0.420 MPa (HYO) and were then transferred to solutions ranging from a  $\Psi_s$  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  $\Psi_s$  of -0.220 until -0.470 MPa and following were transferred to HO solution at a  $\Psi_s$  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  $\Psi_s$  of -0.420 MPa at pH 5.8 (HYO) and were then transferred to a HO solution at a  $\Psi_s$  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  $\Psi_s$  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  $\mu$ 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 CaCl<sub>2</sub>

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  $\Psi_s$  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  $\Psi_s$  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



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  $\alpha$ -  
 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 CaCl<sub>2</sub>)  
635 (Hasenstein and Evans, 1988). Accordingly, upon low pH and low calcium concentration in solution  
636 treatment (1 mM CaCl<sub>2</sub>), 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 Ca<sup>2+</sup> 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 Al<sup>3+</sup> and H<sup>+</sup>  
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](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<sup>+</sup>-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