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# 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

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## Abstract

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

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

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

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

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

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

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

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

## 2. Material and methods

### 2.1. Plant material and growth conditions

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

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

The growth temperature was 22°C and the light intensity was approximately  $\pm 110 \mu\text{E.m}^{-2}.\text{s}^{-1}$ . For each treatment, at least ten 5-day-old seedlings were incubated in a 250 mL Erlenmeyer containing 20 mL of treatment solution composed of 0.5 mM  $\text{CaCl}_2$  and 0.6 mM Homo-PIPES (homopiperazine-1,4-bis(2-ethanesulfonic acid) adjusted to required pH, as previously performed (Graças et al., 2016). For the calcium treatment, only the  $\text{CaCl}_2$  concentration was changed. The solution was continuously gently aerated.

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

### 2.2. Evaluation of cell death in root tips

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

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

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

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

### 2.4. Manipulation of root cell wall tension with osmotic solutions

To decrease cellular turgor pressure, the seedlings were transferred from growth medium ( $\Psi_s$  of -0.032 MPa) to hyperosmotic (HYO) solutions composed of 0.5 mM  $\text{CaCl}_2$  and mannitol (150 mM) to reach water potential of -0.370 MPa  $\Psi_s$ , at pH 5.8 or pH 4.6. To increase the turgor pressure upon the CW, the seedlings were transferred from growth medium to solutions composed of 0.5 mM  $\text{CaCl}_2$  and different mannitol concentrations with water potential ranging from  $\Psi_s$  of -0.220 MPa to -0.470 MPa for 2 h. This transient HYO treatment was followed by a hypoosmotic treatment (HO) by transferring plants to new solutions at  $\Psi_s$  of -0.005 MPa pH 5.8 or pH 4.9 (mild low pH stress).

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

### 2.5. Evaluation of incipient plasmolysis

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

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

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

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

### 2.7. Evaluation of cell elongation in roots

Cell elongation was examined in the late EZ through the length of the first epidermal cell with visible root hair bulge (LEH). LEH was measured in these trichoblasts as described by Le *et al.* (2001) with minor modifications. After the treatment, roots were fixed in a modified Karnovsky solution (2.5 % glutaraldehyde, 2.0 % paraformaldehyde, 0.05 M cacodylate buffer, 0.001 M CaCl<sub>2</sub>) (Román *et al.*, 2004) overnight at 4°C. Then, CWs were stained with calcofluor white (0.1% w/v) for 5 min, and cells were imaged by epifluorescence microscopy as described above for evaluation of CW stiffness.

## 2.8. Evaluation of PCD in roots treated at low pH

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

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

## 2.9. Statistical analysis

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

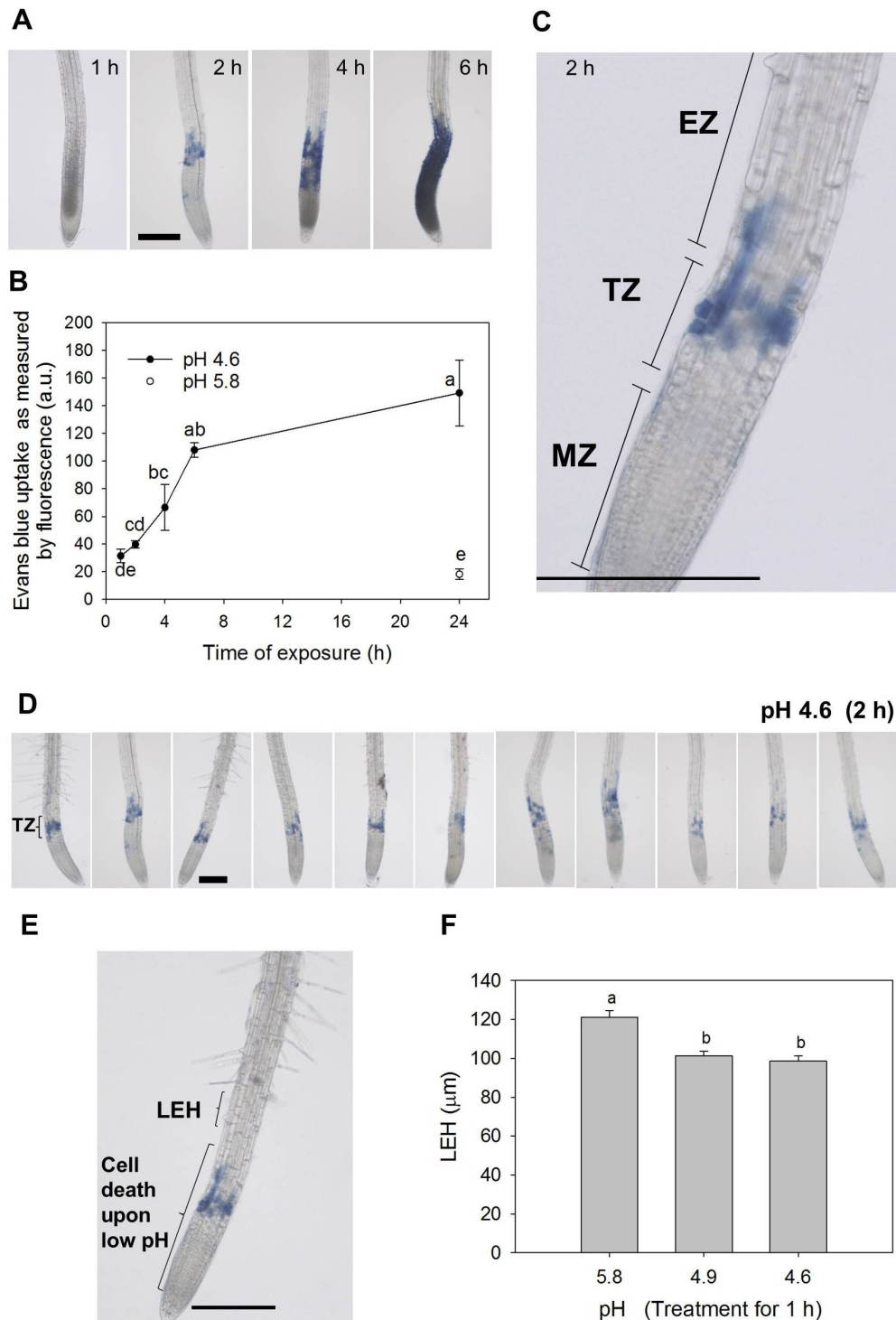
# 3. Results

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

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

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

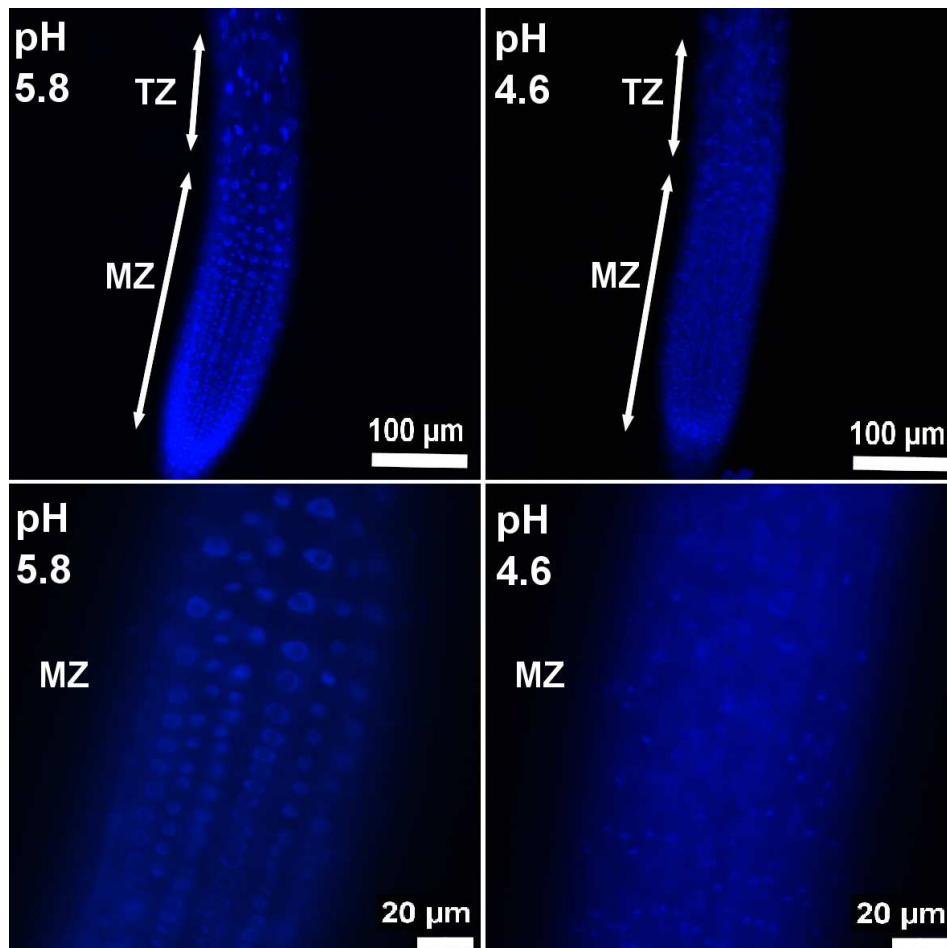


death occurred and the length of the first epidermal cell with visible root hair bulge (LEH) was measured in roots, based on all replicates from (A) and (B); (F) Analysis of cell elongation in roots by measurement of LEH after 1 h of treatment at different pH. The bars represent the standard errors of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

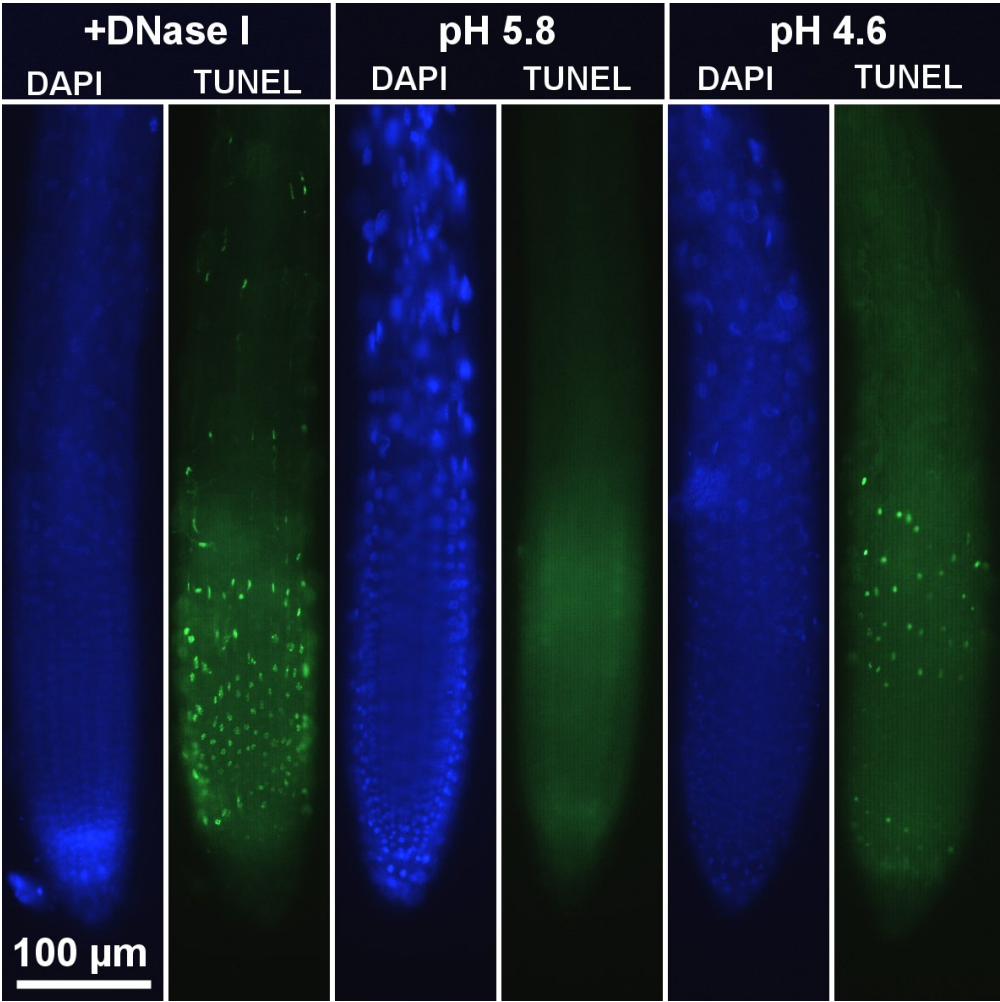
### 3.2. Analysis of PCD in roots treated at low pH

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

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



295 **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  
296 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  
297 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.  
298 The bottom row focuses on the distal part of MZ.



300 **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.**  
301 Nuclei stained with DAPI showing blue fluorescence. The markedly fluorescent green dots are indicative of nuclei positive for the  
302 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 We provided evidence above that TZ cells were highly sensitive to CD driven by low pH. This zone  
307 possesses a particular high CW dynamic (Verbelen et al., 2006; Somssich et al., 2016). Thus, to  
308 further investigate whether the low pH-induced CD could be related to CWDs in TZ, we measured  
309 CW mechanical properties at the cellular level. Briefly, we adapted a previously described method  
310 (Kierzkowski et al. 2012) that consists of measuring cell length before and after a decrease in turgor  
311 pressure caused by a hyperosmotic solution (HYO) ( $\Psi_s$  of -0.480 MPa). Therefore, a greater  
312 decrease in cell length would be indicative of CW loosening, meaning that stiffness decreased.

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

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

**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 %

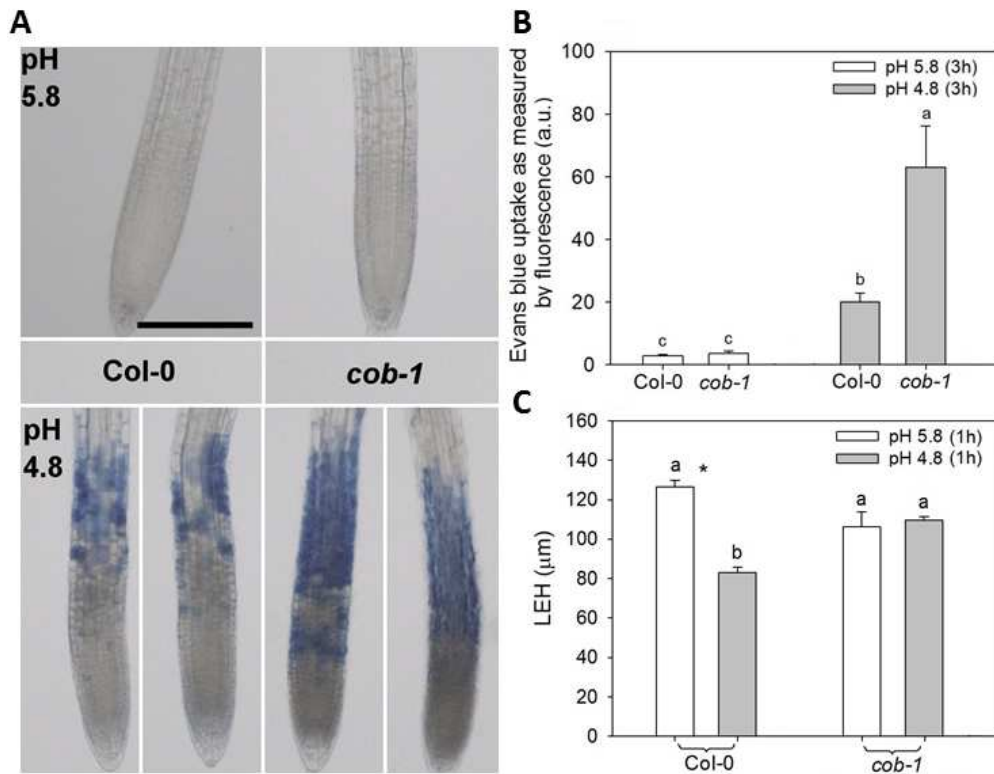
<sup>1</sup> Different letters indicate significant differences between treatments by Student *t*-test.

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

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.



**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 (pH 4.8).** The blue staining or increase in fluorescence is indicative of cell death. (A) Roots were stained with Evans blue. In upper 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  $\mu$ m; (B) Quantification of Evans blue uptake in root tips by a fluorometric assay; (C) Analysis of cell elongation in roots by measurement of LEH after 1 h of treatment at pH 4.8. Bars represent the standard errors of three independent experiments. Letters indicate significant differences between the treatments by Duncan's test. The asterisk indicates a significant difference for a given genotype by Student t-test.

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

Our data indicate that either CWDs or correct CW architecture were crucial for either CD or arrest in elongation upon low pH stress. Calcium has a role in pectin crosslinking (Willats et al., 2001), and low pH sensitive responses in roots are easily observed in solutions with low ionic strength and low calcium concentration ( $\leq 0.5$  mM  $\text{Ca}^{2+}$ ) (Koyama et al., 2001; Graças et al., 2016), simulating acidic soil stress. Therefore, we examined whether the increase of calcium concentration in the treatment solution (from 0.5 mM to 3.0 mM  $\text{CaCl}_2$ ) could modify the pattern of CD or cell elongation upon low pH treatment.

First, strong low pH stress was imposed on roots (pH 4.6 along 6 h) to reveal a possible protective effect of calcium upon cell viability of primary roots. Under 1.5 mM  $\text{CaCl}_2$ , we found a slightly alleviated CD due to strong low pH stress. However, 3.0 mM  $\text{CaCl}_2$  treatment reduced CD and had a protective effect to low pH stress (Fig. 5A). This concentration was chosen for comparison with 0.5 mM  $\text{CaCl}_2$  regarding cell elongation in roots upon intermediate low pH stress (pH 4.8), which was sufficient to cause the arrest of cell elongation in Col-0 as described above (Fig. 4C). Interestingly, roots treated at pH 4.8 in the presence of 0.5 mM  $\text{CaCl}_2$  exhibited a decrease in LEH upon low pH as compared to roots treated at pH 5.8, corresponding to an arrest of cell elongation of about 33 %. However, this prominent arrest did not occur in roots upon low pH in the presence of 3.0 mM  $\text{CaCl}_2$  (Fig. 5B). These results evidence the protective effect of calcium preventing the CD or arrest of elongation caused by low pH stress.

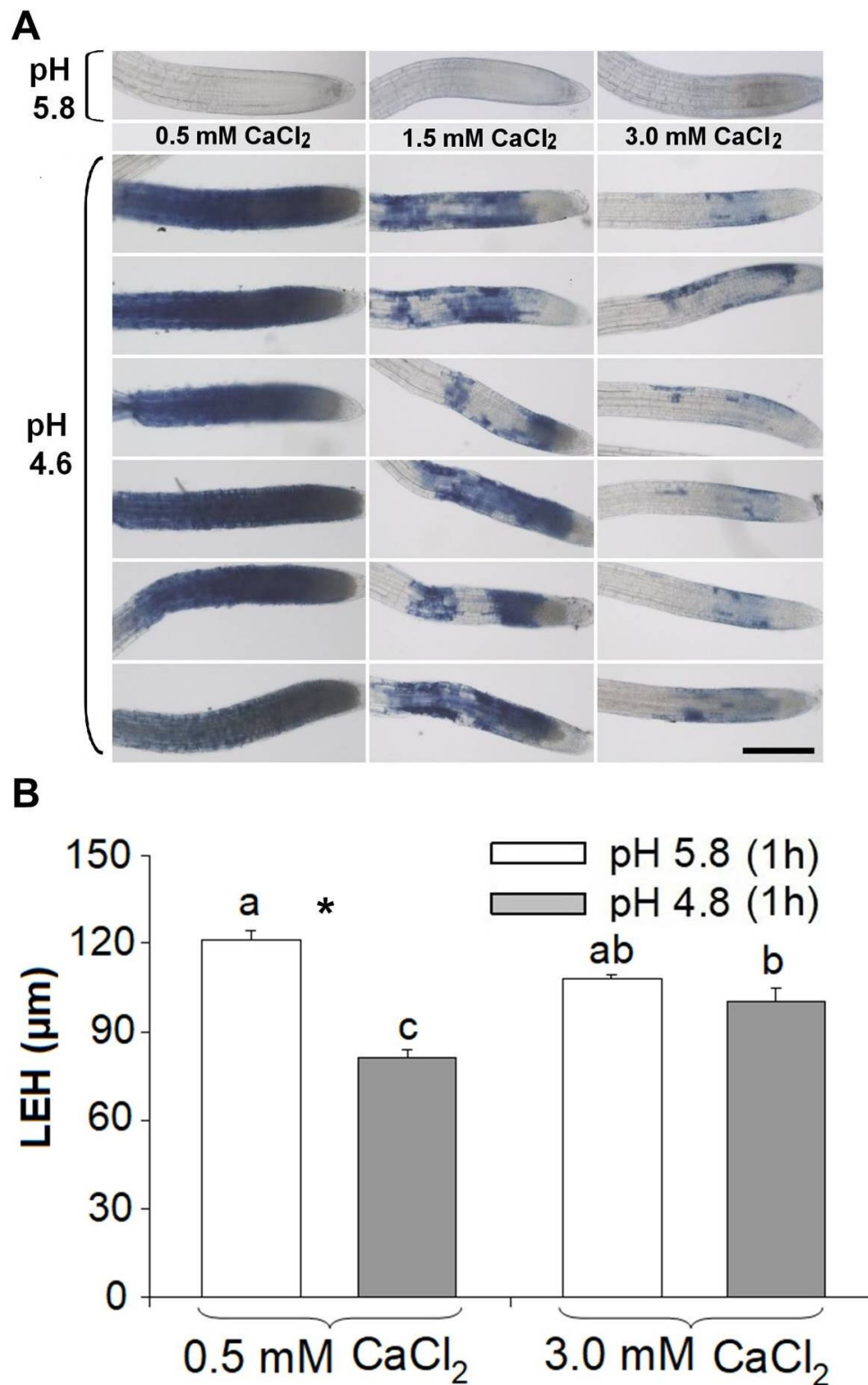


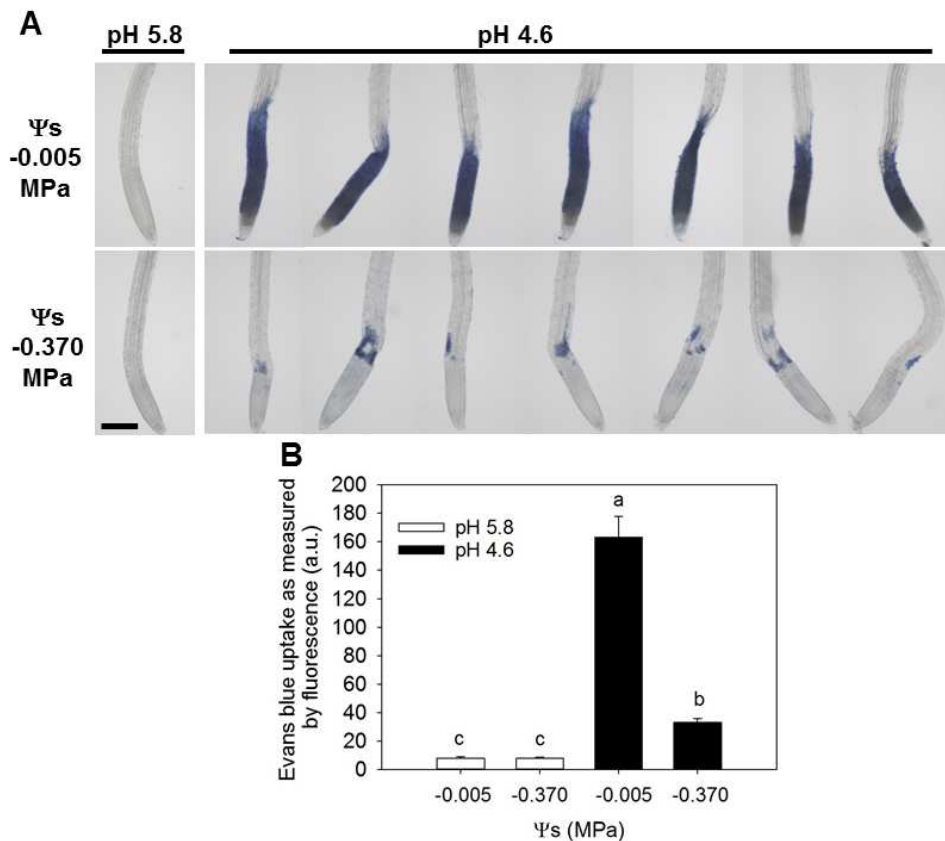
Fig. 5. Cell mortality and cell elongation in primary roots of *A. thaliana* (Col-0) exposed to low pH stress at different concentrations of  $\text{CaCl}_2$ . Blue color (Evans blue staining) is indicative of cell death. (A) Roots treated at pH 5.8 or pH 4.6 (strong low pH stress) for 6 h. Scale bar: 200  $\mu\text{m}$ . In each case, the images are representative of at least 15 roots. For simplification, a single image is shown for each treatment at pH 5.8, but there was no cell death in the roots of control replicates. Note that only 3.0 mM  $\text{CaCl}_2$  caused a clear 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 independent experiments. Letters indicate significant differences between the treatments by Duncan's test. The asterisk indicates a significant difference between treatments at pH 5.8 as compared to pH 4.8 for a given  $\text{CaCl}_2$  concentration by Student *t*-test.

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



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

Primary roots exposed to pH 4.6 for 6 h in a -0.005 MPa  $\Psi_s$  solution exhibited CD as shown by a high Evans blue uptake (Fig. 6A, B). In sharp contrast, there was a significant decrease in CD in roots 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 a reduced pressure/tension upon CWs prevented the death of root cells exposed to severe low pH stress.



**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 (-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. (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 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 uptake in root tips by a fluorometric assay. Bars represent the standard errors of three independent experiments. Letters indicate significant differences between the treatments by Duncan's test.

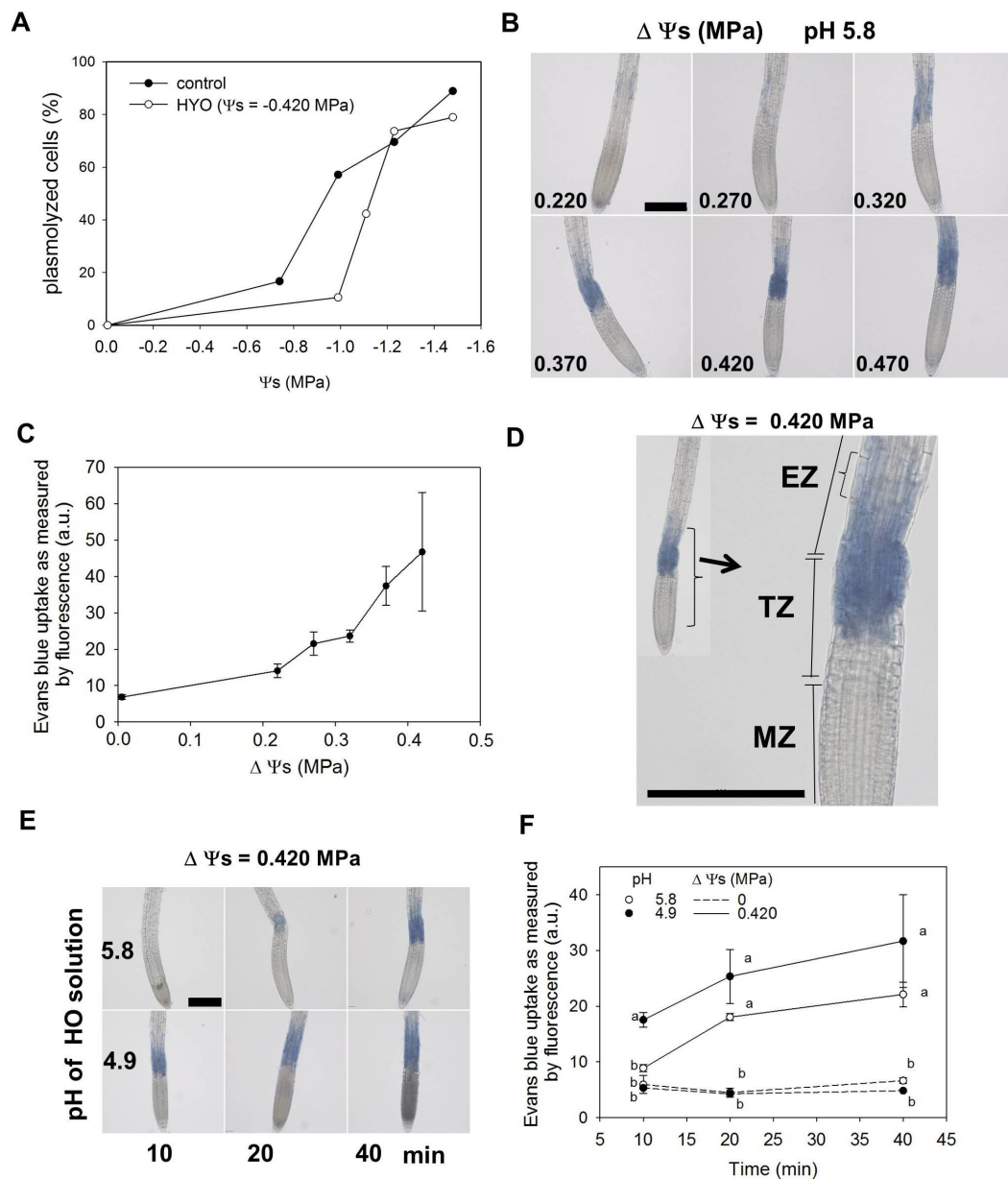
### 3.7. Effect of increased wall tension on death of root cells with or without simultaneous exposure to low pH

To examine the effect on CD of increased turgor pressure alone or in combination with low pH, 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 h, to promote osmotic adjustment. Following, we subjected these same roots to a hypoosmotic (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 (mild low pH stress). Thus, HO treatments of different magnitudes were applied in primary roots by varying the  $\Psi_s$  of the pre-treatment solution. The magnitude of these HO treatments was expressed as  $\Delta\Psi$ , i.e., the difference in  $\Psi_s$  between HYO and HO solutions. For simplification, the  $\Psi_s$  of the HO solution was considered to be zero.

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

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

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



**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. Each treatment was composed of 12 plants and at least two cells were imaged per plant. 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.

## 4. Discussion



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

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

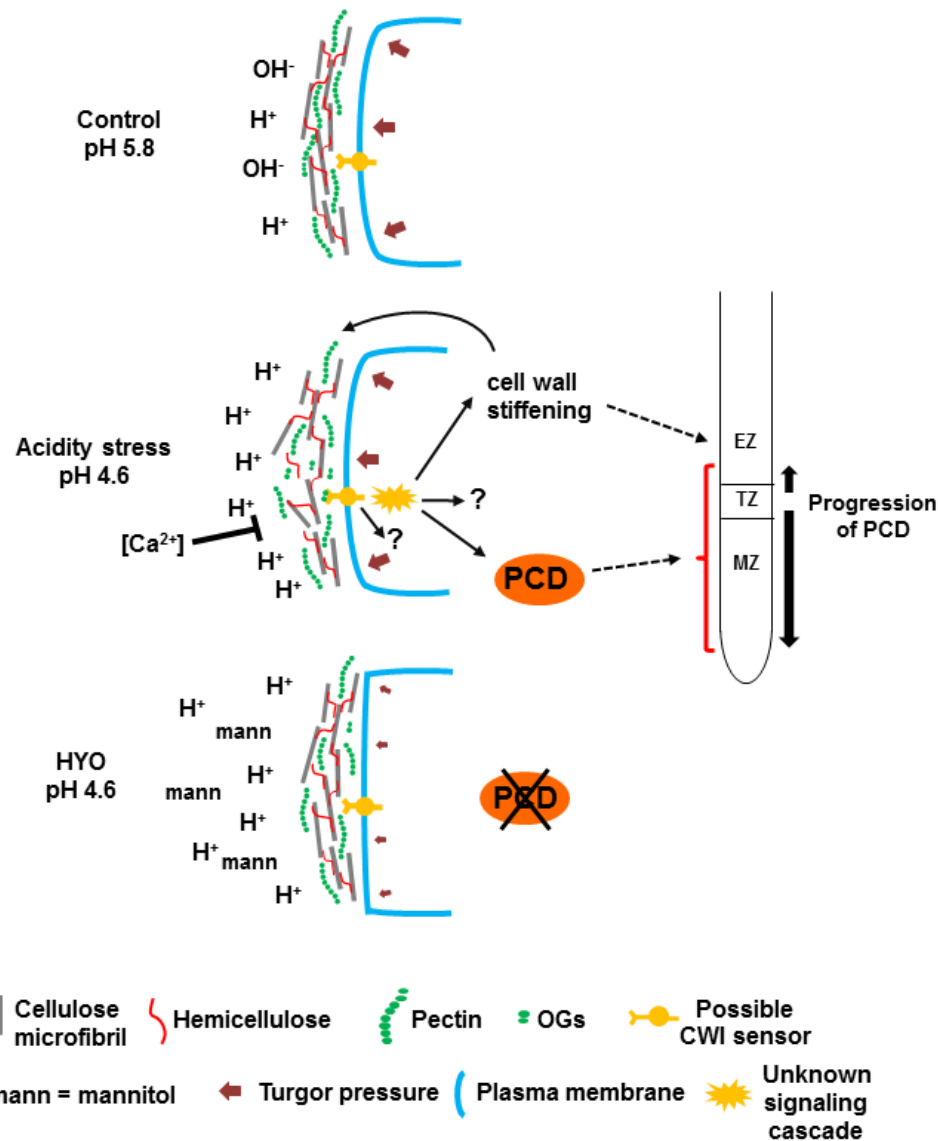
We suspected first that CD due to exogenous low pH stress in the root tip could be similar to that of the bursting observed in root hairs upon low pH (Bibikova et al., 1998), which is likely due to instantaneous loss of CWI. Alternatively, the CD could be due to a sum of several cellular damages leading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

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

In the graphical model (Fig. 8), we summarized the results obtained here and postulated that exogenous low pH disturbs the normal CW structure, thus threatening CWI. The direct evidence was that before the onset of CD, low pH decreased CW stiffness of TZ epidermal cells, *i.e.*, the most sensitive cells to low pH-induced CD. Prominently, the CD decreased in roots treated simultaneously with low pH and mannitol (Fig. 6). The simplest interpretation of this result is that a certain level of turgor pressure, and therefore, CW tension is required for low pH to cause CWDs and then a signaling cascade would be activated for PCD. An additional explanation is that an increased concentration of osmoticum in the solution could also decrease the level of hydration of the CW, which in turn could increase its stiffness (Evered et al., 2007; Hansen et al., 2011; Wolf et al., 2012). However, small molecules such as mannitol, which can penetrate the pores of the CW, are not expected to affect the CW hydration state (Passioura, 1994; Evered et al., 2007), but this assumption cannot be ruled out. Either way, the effect of mannitol supports the idea that low pH-induced CD is caused by CWDs. Supporting this notion, calcium is a key cation that stabilizes homogalacturonan crosslinking (Willats et al., 2001; Mravec et al., 2017) within 20 min of  $\text{CaCl}_2$

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

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



**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 decrease in cell wall (CW) stiffness that could be due to weakening of pectin-cellulose interactions, as previously proposed (Phyo et al., 2019). These CWDs are likely perceived by yet to be identified cell wall integrity (CWI) sensor(s) type(s). Activation of CWI receptor(s) triggers a signaling pathway and a cellular response dependent on cell type. In transition zone (TZ) cells, perception and signaling of CWDs result in programmed cell death (PCD), perhaps by mimicking pathogen attack. Signals are relayed to meristematic zone (MZ) and early elongation zone (EZ) cells, sequentially triggering PCD. In contrast, in expanding trichoblasts of late EZ, perception of CWDs does not lead to CD but rather activates a CW remodeling system that is likely already organized and ready to promote the arrest of elongation (not illustrated here). The coordinated elongation arrest requires correct CW architecture and is most likely triggered due to rapid CW stiffening, as indicated by decreased LEH upon low pH. Spatial separation between PCD and elongation arrest may indicate that they are not activated by the same signaling cascade. Given the previously reported involvement of pectin in low pH-responses, oligogalacturonides (OGs) may be one of the primary CWD signals for these sensitive responses or even the decrease in CW stiffening that we measured here in TZ cells. Low pH-induced CWDs that trigger PCD require a certain level of CW tension (conditioned by turgor pressure). Low pH-induced CWDs can be alleviated by increasing concentrations of  $\text{Ca}^{2+}$  which stabilize homogalacturonan crosslinks and therefore prevents a signaling cascade (s) triggering PCD in MZ/TZ/early EZ and arrest of elongation in late EZ (cell wall stiffening).

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

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

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

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

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

The low pH-induced elongation arrest in roots was prevented upon increased calcium concentration in the treatment solution (Fig. 5). *Z. mays* roots were able to maintain growth at pH 4.5 only when they were formerly soaked in a high calcium concentration solution (10 mM  $\text{CaCl}_2$ ) (Hasenstein and Evans, 1988). Accordingly, upon low pH and low calcium concentration in solution treatment (1 mM  $\text{CaCl}_2$ ), low pH triggered the release of calcium from the tip of *Z. mays* roots resulting in reduced root growth. Indeed, low pH reduces the cation exchange capacity of pectin homogalacturonans since protons can displace cations from their binding sites (White, 2012). The decrease in interaction between galacturonic acid residues and calcium can cause the collapse of the homogalacturonan network upon low pH (Phyo et al., 2019). Increasing calcium concentration restored the overall root growth upon low pH stress, likely due to pectin stabilization (Koyama et al., 2001). Thus, increased calcium concentration in the low pH stress treatment most likely counteracts the low pH-induced CWDs. Given the known role of calcium in the maintenance of CWI (Voxeur and Höfte, 2016), the calcium treatment supports the idea that low pH-induced CWDs may activate a yet to be identified signaling cascade that triggers a coordinated elongation arrest in late EZ. EZ cells possess several mechanisms to tightly control elongation. Root elongation can be rapidly inhibited via transcriptional (Růžicka et al., 2007) or non-transcriptional mechanisms that perceive endogenous signals (Fendrych et al., 2018). Ethylene (Le et al., 2001; Markakis et al., 2012), auxin

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

## 5. Conclusions

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

## Declaration of Competing Interest

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

## Authorship Statement

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 M.B.; validation, J.P.G., V.B. and M.B.; formal analysis, J.P.G.; investigation, J.P.G., M.B.; resources, J.P.G., V.A.V. and M.B.; data curation, J.P.G., V.A.V. and V.B.; writing—original draft preparation, 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, J.P.G.; supervision, V.A.V., E.J. and L.E.P.P.; project administration, V.A.V.; funding acquisition, V.A.V. and E.J. All authors have read and agreed to the final version of the manuscript.

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