University of São Paulo
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The cell wall is crucial for cellular sensitivity to low pH: the role of class III peroxidases and ethylene in cell death in *Arabidopsis thaliana* roots

Jonathas Pereira das Graças

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

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1. Acidez 2. Integridade de parede celular 3. Peroxidases de classe III 4. Etileno 5. Morte celular 6. Tensão de parede celular I. Título

DEDICATION

I dedicate this work to my parents, Antonio and Marta, and to my sister Pollyana, who I truly love.

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EPIGRAPH

“I can do all this through him
Who strengthens me”.

Holy Bible

TABLE OF CONTENTS

RESUMO

A parede celular é crucial para a sensibilidade celular ao baixo pH: o papel de peroxidases de classe III e etileno na morte celular em raízes de *Arabidopsis thaliana*

Evidencias recentes sugerem que a parede celular é um alvo direto do estresse por baixo pH em raízes. Estresse severo por baixo pH rapidamente causa a morte de células do ápice radicular, onde a parede é altamente dinâmica. Nossa hipótese é de que nessas células, o baixo pH cause mudanças na parede celular, como afrouxamento excessivo. Assim, a pressão de turgor sobre a parede deve ser necessária para causar danos que levam à morte das células. Neste trabalho, nós investigamos o papel da parede celular no estresse por baixo pH e na consequente morte de células radiculares. Além disso, tambem foi investigado o papel de peroxidases de classe III e sinalização por etileno, que promovem mudanças na parede celular as quais podem gerar sensibilidade diferenciada a baixo pH. Plântulas de *Arabidopsis thaliana* e mutantes no background de Col-0 foram crescidas em meio contendo ágar (0.8%) e metade da concentração dos nutirentes do meio de Hoagland. Plântulas com 5 dias de idade foram expostas a baixo pH em uma solução composta por 0.5 mM de $CaCl_2$ e 0.6 mM de tampão Homopipes. O tratamento de raízes a pH 4.6 causou morte em células da zona de transição (TZ) e zona meristemática (MZ). Entretanto, a morte celular foi negligível quando as plantas foram tratadas a pH 4.6 simultaneamente com a diminuição da tensão na parede celular, através de solução com potencial de - 0.37 MPa. Além disso, um choque repentino na pressão de tugor por intermédio de tratamento hiposmótico (HO) causou morte celular a pH 5.8 na TZ. A morte celular foi acelerada quando HO foi realizado em uma solução a baixo pH. A morte celular foi reduzida no mutante *wak-1* exposto a baixo pH. WAK-1 é um receptor de parede que atua no sistema de monitoramento de integridade da parede celular. A morte das células provavelmente ocorreu por meio de morte celular programada. Juntos, esses dados trazem evidências que a parede celular é crucial para percepção do estresse causado por baixo pH e essa percepção possivelmente está envolvida em repostas que causam a morte celular. Nós examinamos dados publicados procurando por peroxidases classe III possivelmente envolvidas com a morte celular devido baixo pH. O gene codante para AtPRX62 foi induzido 8.37 vezes em raízes expostas a baixo pH. O mutante KO *atprx62* foi menos sensível a baixo pH que raízes de Col-0. O mRNA de AtPRX62 acumulou-se na mesma zona de morte celular devido baixo pH em raízes de Col-0. Isso sugere que a atividade de AtPRX62 está relacionada com a morte celular devido baixo pH. Além disso, o pré-tratamento com etileno induziu tolerância de raízes à exposição subsequente a baixo pH. Esta indução foi dependente de sinalização via ETR1. No conjunto, nós mostramos que um estresse causado na parede celular pelo baixo pH causa a morte celular. Essa morte é em parte devido a atividade de AtPRX62 mas pode ser aliviada por etileno.

Palavras-chave: Acidez; Integridade de parede celular; Peroxidases de classe III; Etileno; Morte celular; Tensão de parede celular

ABSTRACT

The cell wall is crucial for cellular sensitivity to low pH: the role of class III peroxidases and ethylene in cell death in *Arabidopsis thaliana* roots

Evidence suggests that root cell walls are a target of low pH stress. Severe low pH stress causes cell death in the root tip. The walls of these cells are highly dynamic. Our hypothesis is that in these cells low pH causes stress in the cell wall due to excessive loosening. Thus, a certain level of turgor pressure should be required to cause cell death. Here, we aimed to investigate the role of the cell wall in low pH stress leading to cell death. We looked for the possible involvement of players such as class III peroxidases and ethylene signaling, which could promote changes in the cell wall and cause differential sensitivity to low pH. *Arabidopsis thaliana* and mutants in the genetic background of Col-0 were grown in a medium containing agar (0.8%) and half the concentration of Hoagland's nutrient medium. Five-day-old seedlings were exposed to low pH in a solution composed of 0.5 mM $CaCl_2$ and 0.6 mM Homopipes buffer. Treatment of roots at pH 4.6 caused death of cells in the transition zone (TZ) and meristematic zone (MZ). However, cell death was negligible when plants were treated at pH 4.6 in an hyperosmotic solution (Ψs = -0.37 MPa), thereby decreasing cell wall tension. Also, an hypoosmotic treatment (HO) caused cell death at pH 5.8 in TZ. Cell death was accelerated when HO was performed in a low pH solution. The mutant of a cell wall integrity sensor protein, *wak-1*, displayed reduced cell death when exposed to low pH. Also, cell death seems to occur through a programmed cell death mechanism. Thus, low-pH induced cell death appears to be triggered by perception of cell wall stress. We examined published data to search for class III peroxidases possibly involved in cell death due to low pH. The gene for AtPrx62 is induced 8.37-fold in low pH exposed roots. The *atprx62* KO mutant was less sensitive to low pH than Col-0 roots. The mRNA of *AtPRX62* accumulated in the same zone that cell death occurred due to low pH. This strongly suggests that AtPRX62 is positive regulator of low-pH induced cell death. Also, ethylene pretreatment induced subsequent tolerance of roots to low pH and this was dependent of its receptor ETR1. Together we show that a cell wall stress caused by low pH causes cell death. This death was in part due AtPRX62 activity and was also suppressed by ethylene.

Keywords: Acidity; Cell wall integrity; Class III peroxidases; Ethylene; Cell death; Cell wall tension

LIST OF ABBREVIATIONS AND ACRONYMS

Al	Aluminum
AtPrx	Arabidopsis thaliana peroxidase
CIII Prx	Class III peroxidases
EDDHA	Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
EZ	Elongation Zone
HO	Hypoosmotic
HPF	Hydroxyphenyl Fluorescein
HYO	Hyperosmotic
ISH	In situ Hybridization
KO	Knockout
KN	Knockdown
NBT	Nitroblue Tetrazolium Chloride
ROS	Reactive Oxygen Species
MZ	Meristematic Zone
PCD	Programmed Cell Death
Prx	Peroxidase
TZ	Transition Zone
WT	Wild Type

1. INTRODUCTION

Almost 40% of the world's agricultural areas are covered by acidic soils with a pH value below 5.0 (Vonuexkull and Mutert, 1995). In Brazil, there are about 500 million hectares of acid soils (Vitorello et al., 2005). These soils are characterized by low nutrient concentrations and solubilization of toxic forms of Al (Kochian, 1995). The combination of these factors is known as "acid soil syndrome" which results in inhibition of root development.

Many studies have focused on Al and soil nutrients. However, most of the toxic forms of Al to plants are only active at low pH. Thus, low pH has obvious implications for understanding of Al toxicity.

Indeed, low pH by itself can be toxic to the root system. The irreversible consequence of this toxicity is cell death that can happen within a short time of low pH exposure, such as half an hour (Koyama et al., 2001; Gracas et al., 2016).

Events induced by low pH in min have been previously described, such as changes in plasma membrane permeability and ion uptake (Bose et al., 2010). This can be crucial for nutrient acquisition and cellular homeostasis. However, it remains unclear whether such events are just merely effects of low pH, which within the time of exposure, might kill cells or might be part of a signaling that in ultimate is linked to the death of cells.

Evidence is emerging that the interaction between low pH and cell wall seems to be relevant for the sensitivity of cells to the stress (Bibikova et al., 1998; Monshausen et al., 2007; Horst et al., 2010; Lager et al., 2010; Gracas et al., 2016). The nature of this interaction and what this might change on cell wall is so far unknown.

Few works presented evidence that low pH can directly disturb cell wall integrity. The expansion of root hairs requires fine control of apoplast acidification. Expanding root hairs burst if maintained at pH 4.5 (Bibikova et al., 1998; Monshausen et al., 2007). However, root hairs that in which expansion had stopped did not burst at pH 4.5. One interpretation for this is that the root hairs probably burst due to cell wall failure caused by low-pH induced derangements, which are enhanced by turgor pressure. It is reasonable to suggest that others root cells can be affected by low pH in a similar mode, due to cell wall derangements.

It is known that root tip cells are the most sensitive cells to low pH (Koyama et al., 2001). A major challenge is to determine what special features of these cells cause this sensitivity to low pH. Indeed, these cells, which are situated in the MZ, TZ and EZ, possess remarkable differences concerning their development and responses to hormone signaling. One inevitable fact is that these cells, upon reaching the end of the EZ, will have become more than a hundred

times larger than their initial size in the MZ (Verbelen et al., 2006). In order to accommodate this growth, the cell wall must be highly dynamic, with large changes in its properties over time. The potential for errors appears to be great.

Cells have a system to monitor cell wall integrity allowing correct growth and preventing disturbances (Somssich et al., 2016). This system involves several players as cell wall integrity sensors, hormones, ROS and the activity of enzymes that physically change the wall (Ringli, 2010).

CIII Prx can promote cross-linkage between polysaccharides and proteins, important for cell wall formation and for physical change in cell wall such as loosening or tightening (Passardi et al., 2005). These last two remarkable opposite activities in the same structure make peroxidases important players for rapid response to biotic or abiotic constraints upon the wall.

An increase of H_2O_2 due to peroxidase activity triggered by Al stress was linked to the death of root cells (Simonovicova et al., 2004). Most probably, an uncontrolled ROS production caused damages to the cells. After wounding in roots, CIII Prx increased its activity and there was increased production of ROS (Minibayeva et al., 2009; Minibayeva et al., 2015). This may be useful to change cell wall properties for plant defense or might have a direct action upon pathogen infection. Also wounding response may invoke cell death dependent on ROS and peroxidases (Minibayeva et al., 2015). Application of SHAM, an inhibitor of CIII Prx activity greatly increased cell death in tomato roots treated with low pH (Gracas et al., 2016). Hence, CIII Prx activity and ROS seems to be responsive to cell wall stresses.

Plants under Pi starvation quickly inhibits the root growth through the activation of two noncoupled pathways involving peroxidases that stiffen cell walls in TZ and this results in the arrest the growth (Balzergue et al., 2017). One CIII Prx isoform, AtPRX71, was related to ROS production due to treatment with isoxaben that disrupts cell wall integrity (Raggi et al., 2015). This ROS production was interpreted as linked to responses for coordinated decrease in cell expansion under stress.

CIII Prx utilizes ROS and fine controls its metabolism due to their dangerous potential to the cells. Whether peroxidase activity may be involved in cell death or if are part of plant tightening to constraints, these enzymes, are clearly involved on different stresses responses. The role of individual isoforms must be investigated in low pH stress

Ethylene is well known as abiotic stress response hormone (Raja et al., 2017) and for signaling responses upon cell wall in the whole plant. In roots, application of the ethylene precursor ACC rapidly inhibits cell elongation (Le et al., 2001). This control upon elongation is in part achieved by ethylene due to a transcriptional control of cell wall-related enzymes (Markakis

et al., 2012). Among these enzymes are CIII Prx. These enzymes act upon cell wall changing its properties for stop of cell expansion. Thus, due its role in cell elongation and transcriptional control of peroxidases ethylene involvement in low pH response must be investigated

PCD is part of normal root development for events as xylogenesis, control of root cap cells and death of old root tissues (Bagniewska-Zadworna and Arasimowicz-Jelonek, 2016)). In roots, PCD is also induced by an increase in mitochondrial release of ROS induced by Al treatment (Huang et al., 2014). Al and low pH share commonalities but they might also have different targets in root cells. Hence, it would be discerning to investigate if the death of cells during low pH can be through a PCD mechanism.

Interesting, evolution has selected PCD as strategy for survival. Bacteria growing in colonies may face nutrient starvation. This threatens all individuals. A wave of PCD spreads through the colony, as if they were a single organism, reducing the population and allowing survival of the remaining avoiding nutrient competition (Engelberg-Kulka et al., 2006).

Since before the postulation of "acid growth theory" the interaction between low pH and cell wall is seem as relevant for understanding plant growth and development. Thus, understand the cell wall response due to low pH and determine players for this response is a central topic in plant biology. This work aimed to investigate the effect low pH toxicity in roots and its possible interaction with cell wall in the MZ, TZ and EZ.

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2. CELL DEATH IN LOW-pH TREATED ROOTS IS TRIGGERED AFTER A STRESS IN THE CELL WALL

ABSTRACT

Low pH can be detrimental to root development, causing the death of root tip cells. The events behind this cell death and the type of cell death are still not clear. Here, we examined the role of the cell wall on cell death due to low pH exposure in roots. Seedlings of *Arabidopsis thaliana* were exposed to low pH on different times and solution water potential. Roots exposed to pH 4.6 with reduced cell wall tension were tolerant to low pH compared to roots treated at pH 4.6 with normal cell wall tension. This clearly indicates that cell death caused by low pH is dependent of tension in cell wall. A suddenly increase on turgor pressure through a hypoosmotic treatment (HO), in solution at pH 5.8, caused cell death only in cells of the TZ. This death happened faster when this HO was performed in a solution at low pH. Also, the *wak-1* mutant of a cell wall integrity sensor was tolerant to low pH. Together, these demonstrate that cell wall is crucial for the occurrence of death due to low pH. The first dead cells in low pH treated roots unequivocally from TZ and then cell death spread towards MZ. The deformity in the nucleus of the dead cells revealed by DAPI staining, and, positive reaction for TUNEL, strongly suggest that the cell death due to low pH is through a programmed cell death mechanism. Whether this PCD is a plant adaptive response to acidic stress it remains to be investigated.

Keywords: Cell wall extensibility; Low pH; Cell death; Cell wall tension; Cell wall integrity

2.1. Introduction

Low pH has an unfavorable impact on root development. One irreversible consequence of the stress is the death of root tip cells (Koyama et al., 2001; Graças et al., 2016). Many events are probably behind this death, and the identification of cellular targets would be relevant to understand this process. Protons are involved in countless cellular processes. Thus, can be multiples the cellular targets for low pH. Whether necessary or disadvantageous, important interactions between protons and the plasma membrane, cytoplasm and cell wall have been reviewed by Shavrukov and Hirai (2016).

The increase in Ca^{2+} and borate greatly decreased negative effects of low pH upon root growth and cell mortality in roots (Koyama et al., 2001). Calcium and boron play a key role on cell wall and plasma membrane stabilization. However, they are synergistic on pectin matrix formation and stabilization (Koyama et al., 2001). This suggests that one of the primary targets of low pH might be due its interaction with the cell wall.

Direct evidence that cell wall interacts with low pH comes from studies of cell expansion. Root hairs undergoing elongation burst when treated with low pH (pH 4.5), but, root hairs that have ceased elongation did not burst (Bibikova et al., 1998; Monshausen et al., 2007).

The low pH might cause derangements in the wall, as excessive loosening, and, with constant turgor pressure and tension upon cell wall a failure may occur causing bursting. A similar process is likely to happen in root tip cells. Hence, to play with turgor pressure and cell wall tension should result on a different sensitivity of roots to low pH.

Cells from root tip are sensitive to low pH becoming dead from min into hours of exposure (Koyama et al., 2001; Graças et al., 2016). There is a need for understand if this sensitivity is due to negative impact of low pH direct in the cell wall, as in the case cited above for root hairs. The cell walls especially in MZ and TZ are high dynamic. They are ongoing synthesis and also being prepared for a rapid expansion in EZ (Verbelen et al., 2006; Somssich et al., 2016). This could probably be correlated with higher sensitivity to low pH and must be investigated.

Transcriptomic analysis of roots treated at low pH showed that among the cellular compartments, the cell wall was the one which gene expression has the greatest impact on change (Lager et al., 2010). Indeed, transcription factors and genes related to cell wall-modification have expression changed after 20 min of low pH treatment. Beyond merely a cellular response due to a change on pH of external medium, this might reflect the need of cells to modify its cell wall to promote a stress acclimation. Two polygalacturonase-inhibiting protein were direct regulated by STOP2 was proposed to stabilize cell wall during low pH exposure, decreasing cell sensitivity to the stress (Kobayashi et al., 2014).

Cell wall integrity sensors proteins are candidates to be investigated in low pH response. WAK-1 is expressed through root MZ and TZ (Anderson et al., 2001). Its role as a cell wall integrity sensor has been suggested (Anderson et al., 2001; Wagner and Kohorn, 2001; Liu et al., 2015). Also, COBRA-1 is required for correct expansion since the mutant displayed unusual cell expansion and cellulose microfibril poorly organized (Benfey et al., 1993; Roudier et al., 2002; Roudier et al., 2005). From the evidences presented above, low pH may directly affect cell wall. Thus, we speculate that mutants for these proteins might have differential sensitivity to low pH.

The ameliorative effect of Ca^{2+} for root growth in acid conditions was attributed to its capacity of displacement of Al^{3+} and H^{+} from the surface of plasma membrane (Kinraide, 2003; Kinraide et al., 2004). Also, low pH increased K^{+} efflux and caused membrane depolarization that indicates which membrane physiology is affected by H^{+} (Bose et al., 2010). This can pertub several cellular processes, but, if they are involved with cell due to low pH death is still unclear.

One orchestrated response for normal root development, as xylogenesis, or, several types of constraint situations is PCD (Bagniewska-Zadworna and Arasimowicz-Jelonek, 2016). It such type of cell death occurs during low pH stress it must be investigated.

We present here evidences that cell wall is crucial for sensitivity of root tip cells to low pH. Also we showed that death of cells occurs due to a plant response, after perception of cell wall stress. This death seems to be coordinated through a PCD mechanism.

2.2. Materials and Methods

2.2.1. Plant growth conditions

Seeds of *A. thaliana* were disinfected in commercial sodium hypochlorite solution (5%) for 10 min under stirring and then washed five times with distilled water. The seeds were then transferred to Petri dishes with 0.8% of agar and a modified Hoagland's solution at pH 5.8. Macronutrients consisted of 4 mM Ca $(NO_3)_2$, 6 mM KNO_3, 1 mM $MgSO_4$ and 2 mM $NH_4H_2PO_4$. Micronutrients were composed of 20 μM $MnSO_4$, 14 μM $ZnSO_4$, 20 μM H_3BO_3, 0.03 μM $NiSO_4$, 0.02 μM Na_2MoO_4, 0.02 μM $CuSO_4$, 0.02 μM $CoCl_2$ and 30 μM Fe-EDDHA as Libfer® SP.

The growth temperature was 22 °C and the light intensity approximately ± 120 $\mu E.m^{-2}.s^{-1}$. For all low pH treatments, at least ten five-day-old seedlings were incubated in 250 ml Erlenmeyer with 20 ml of treatment solution composed of $CaCl_2$ (0.5 mM) and homopipes buffer (homopiperazine-1,4-bis(2-ethanesulfonic acid) 0.6 mM. The solution was continuously aerated.

We used as WT seedlings of ecotype Columbia-0. In addition, also used the mutants *wak-1* (Germplasm stock: Salk_107175, Locus: AT1G21250.1) and *cobra-1* (Germplasm stock: CS8541, Locus: AT5G60920.1). The primers used to confirm the mutations are listed in supplementary information Table S1.

2.2.2. Evaluation of cell death in root tips

After the treatments, seedlings were stained in a 0.25% (w/v) solution of Evans blue for 15 min. Then they were washed three times for five min with distilled water. Immediately, bright field images were taken, or, the roots were frozen in liquid N and stored at - 20 °C for later quantification of Evans blue uptake.

For quantitative evaluation, the method described by Baker and Mock (1994) was used with minor modifications. We added 300 μl of DMSO (dimethyl sulfoxide) to segments of frozen excised root tips (0.2 cm) during 1 h at 24 °C. Then, they were centrifuged at 8.000 *g* for three

min, and only the liquid was transferred to a fresh plastic tube. This solution was used to quantify Evans blue uptake in spectrofluorometer, with excitation and emission wavelengths of 645 and 690 nm, respectively.

2.2.3. Manipulation of root cell wall tension with osmotic solutions

To decrease cellular turgor pressure, seedlings were transferred from growth medium (- 0.032 MPa) to hyperosmotic solutions composed of 0.5 mM $CaCl_2$ and mannitol to reach water potential of - 0.37 MPa, at pH 5.8 or pH 4.6.

For increase the turgor pressure upon the walls the plants were transferred from growth medium to solutions with water potential ranging from - 0.22MPa to - 0.47 MPa for 2 h. This transient hyperosmotic treatment was followed by a hypoosmotic treatment, by transferring plants to new solutions at - 0.005 MPa pH 5.8, or, at mild low pH stress at pH 4.9.

As control, plants were treated for the same amount of time for the duration of the whole experiment in solution at - 0.005 MPa at pH 5.8 or pH 4.9.

2.2.4. Evaluation of incipient plasmolysis

Roots treated at pH 5.8 - 0.005 MPa were placed for five min in mannitol solutions with a Ψs ranging from -0.74 MPa to -1.48 MPa. Roots treated at pH 5.8 - 0.42 MPa were exposed for five min to mannitol solutions ranging from - 0.99 MPa until - 1.48 MPa. Then, at exactly five min after, the epidermal cells from transition zone were imaged. The plasmolysis point was determined when approximately half of the observed cells were plasmolyzed. In each treatment, at least 20 epidermal cells of the transition zone were observed.

2.2.5. Evaluation of cell wall extensibility in epidermal cells of the transition zone of the roots

For evaluation of cell wall extensibility, we based on method described by Kierzkowski et al. (2012) with modifications. After treatment with pH 5.8 or 4.6 in solutions at - 0.005 MPa the root cell walls were stained with calcofluor white (0.1% w/v Sigma®) for 5 min and then mounted in a glass slide with a coverslip. Then, epidermal cells from transition zone were imaged with an epi-fluorescence microscopy (Leica LM 6000B, excitation 340/40, emission 470/40). 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 and after it was added a hyperosmotic solution with - 0.048 MPa (200 mM mannitol) in the roots with a pipette. After exactly 5 min of this mannitol treatment, the same interest cells were imaged again. All procedures were done watching the roots in the microscope to avoid damages and to prevent the roots from drying out.

The variation in the maximum length of the cells before and after this five min of mannitol treatment was calculated (Δ length %). The result is interpreted as extensibility of the wall in response to the hyperosmotic treatment.

2.2.6. Evaluation of the Length of the First Epidermal Cell with Root Hair Bulge

The length of the first epidermal cell with root hair bulge (LHE) was performed as described by Le et al. (2001) with minor modifications. After the treatment, roots were fixed in Karnovsky solution (2.5 % Glutaraldehyde, 2.0 % Paraformaldehyde, 0.05 M Cacodylate buffer, 0.001 M $CaCl_2$) overnight at 4 °C. Then, the cell walls were stained with calcofluor white (0.1% w/v) for 5 min, and the cells with bulge were imaged in epi- fluorescence microscopy (Leica LM 6000B, excitation 340/40, emission 470/40).

2.2.7. Evaluation of root meristem size

For the evaluation of meristem size, after the treatment seedlings were fixed overnight in Karnowski solution (2.5 % Glutaraldehyde, 2.0 % Paraformaldehyde, 0.05 M Cacodylate buffer, 0.001 M $CaCl_2$) at 4 °C overnight and then the roots were examined using DIC (Differential Interference Contrast Microscopy). Parenchymatic cells in a column from the stem cell niche to the first elongating cell were imaged (Casamitjana-Martinez et al., 2003). This was used to characterize the size of meristem that was expressed in length.

2.2.8. Evaluation of Programmed Cell Death (PCD) in roots treated at low pH

TUNEL (Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling) assays were performed to examine the occurrence of PCD. The DeadEnd™ Fluorometric TUNEL System (G3250) from Promega® was used. Roots were fixed overnight in 4 % paraformaldehyde diluted in phosphate buffer saline (PBS 1X). Then, roots 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.

2.2.9. Statistical analysis

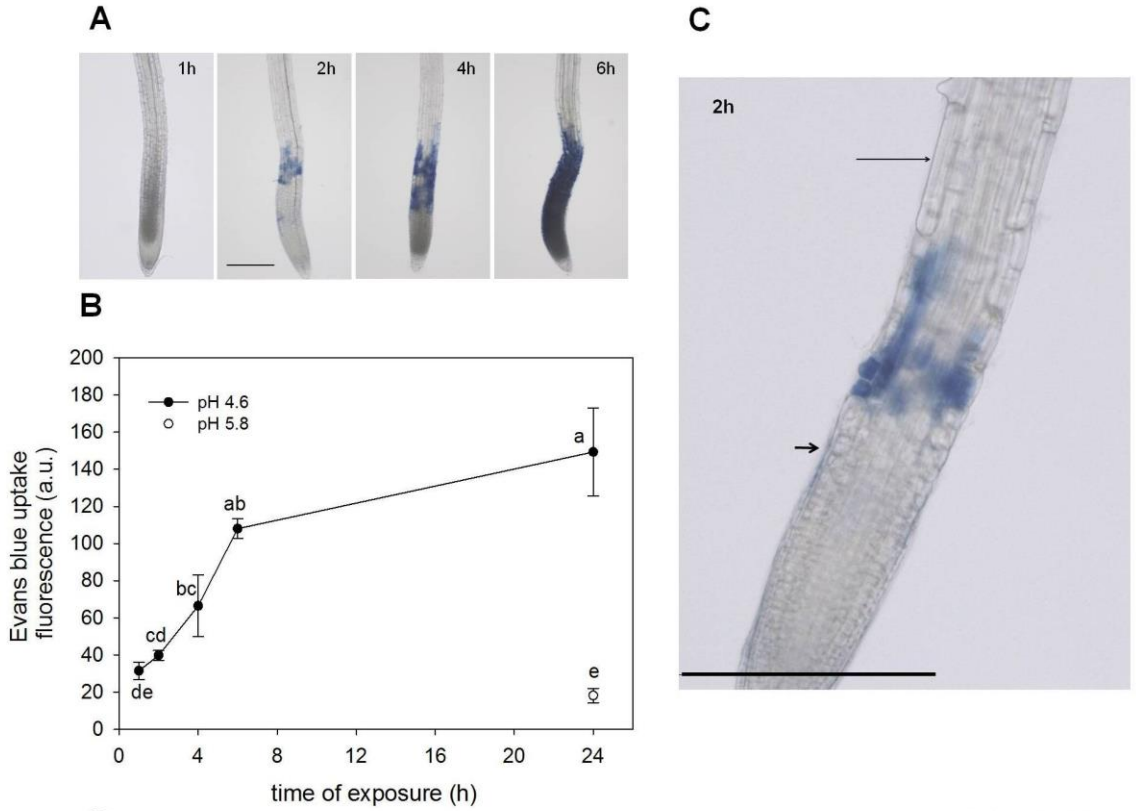
A completely randomized experimental design was used in all experiments. Each analysis was composed of at least three separate experiments. Each replicate was composed of at least ten plants. For the comparison of means, normal data were subjected to analysis of variance (ANOVA), followed by Duncan's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

2.3. Results

2.3.1. Spatiotemporal analysis of cell death in roots treated with low pH

Detailed knowledge about the location and progression of low-pH induced cell death within the root and the relative sensitivity of cells along the root tip is lacking. Thus, a spatiotemporal analysis of cell death was performed in roots exposed to severe acidity stress.

The first cells started to die after 2 h of exposure to pH 4.6 (Figure 1A) and were invariably located in the TZ (Figure 1 C, D, E). Loss of cell viability then progressed towards the apex after 4 h of exposure and, by 6 h of exposure, cell death extended over almost the entire MZ. Practically no further cell death was observed between 6 and 24 h of exposure (Figure 1 B; Supplementary Figure S2). Intriguingly, cell death did not advance much into more mature regions of the EZ, reaching only the distal EZ (Supplementary Figure S2).



E

Meristem Size (µm)			First cells sensitive to pH 4.6
	pH 5.8 2h	pH 4.6 2h	Distance from Quiescent Center
Mean	246±18	249 ±16	387 ±49

Figure 1. Temporal and spatial analysis of cell mortality in the root tips of *A. thaliana* (Col-0) exposed to severe acidity stress (pH 4.6). A, Bright field images of root tips exposed to pH 4.6 for different times and stained with Evans blue, indicative of cell death. Scale bars indicate 200 µm. B, Evans blue uptake in the root tips. As control roots were treated at pH 5.8 for 24 h. The increase in fluorescence (arbitrary units) is indicative of increased cell death. The bars are standard error of three experiments performed on different days. Statistical analysis was Duncan's test. C, Detail of the first sensitive cells in the root tips to strong acidity stress (pH 4.6). The first cells sensitive to low pH showed a marked penetration of Evans blue. They are above from meristematic zone (arrowhead) and bellow of the large expanded cells (arrow). Scale bars indicate 200 µm. D, The first sensitive cells to low pH are unequivocally located at the same region even in different roots. E, Root meristem size and spatial localization of the first sensitive cells to low pH. The position first sensitive cells in the roots were determined measuring its distance from the quiescent center.

2.3.2. Effect of low pH on cell wall extensibility in epidermal cells of the transition zone

The cells of the TZ were the most sensitive cells to low pH. Considering the "acid growth theory" and the fact that the cell wall is obviously affected in root hairs that burst at low pH (Bibikova et al., 1998; Monshausen et al., 2007), we examined whether, prior to cell death, low pH affected the wall extensibility of TZ cells. To evaluate this, we used a simple approach, similar to that of Kierzkowski et al. (2012), in which we measured changes in cell length in response to a decrease in turgor pressure caused by a HYO solution. We performed these measurements at 90 min of exposure to pH 4.6, before the occurrence of any cell death.

In roots previously treated at pH 4.6, the length of epidermal cells of the TZ decreased - 9 % after being placed in an HYO solution. However, in roots treated at pH 5.8, the length of epidermal cells of the TZ decreased less than - 1 % after being placed in an HYO solution (Figure 2). Thus, low pH increased wall extensibility of TZ cells when compared those of control roots.

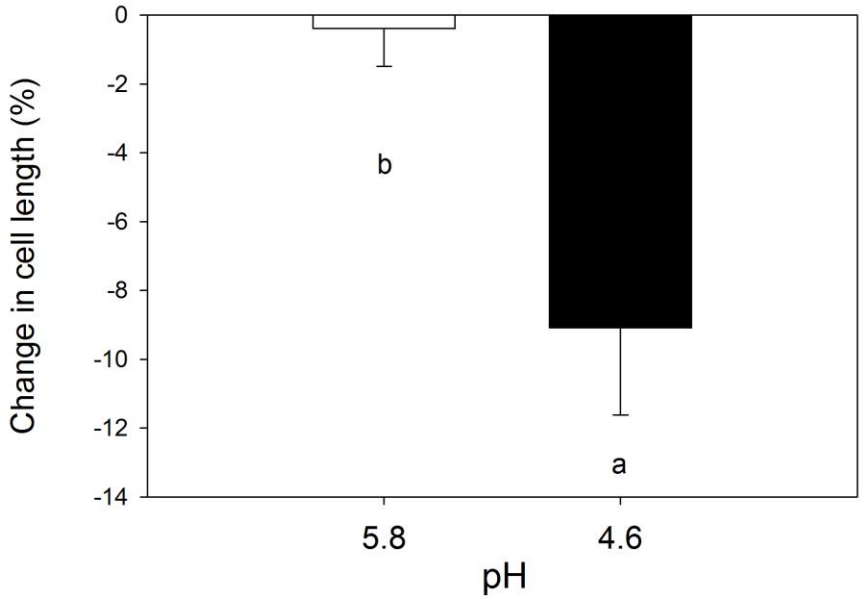


Figure 2. Cell wall extensibility of epidermal cells from transition in roots of *A. thaliana* (Col-0) in response to osmotic treatment after low pH stress. The roots were first treated at pH 5.8 or 4.6 for 1.40 h in a solution with - 0.005 MPa. After this treatment cell wall of roots were stained with calcofluor white (0.1 %) for 5 min and epidermal cells of interest at transition zone were photographed. Then, they were exposed to a solution with - 0.48 MPa by five min and immediately the same cells of interest were photographed again. The change (Δ) in maximum cell length was recorded as measure of cell wall extensibility. More than ten plants in each treatment were examined. In each plant ± four cells were imaged. Statistical analysis was Student *t*-test.

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

If low pH causes derangements in the cell wall through an excessive loosening, then, cell wall tension and turgor pressure should have an important effect on low pH stress. A key question is whether reduction of cell wall tension could prevent cell death induced by low pH. To test this, roots were exposed to pH 4.6 or 5.8 for 6 h in solutions supplemented or not with 150 mM of mannitol.

In roots exposed to pH 4.6 for 6 h in a - 0.005 MPa solution, cells died, as expected, with high Evans blue uptake (Figures 3 A, B, C). In sharp contrast, there was little cell death in roots treated at pH 4.6 for 6 h in a solution with a Ψs of - 0.37 MPa, similar to the high cell viability of control roots (pH 5.8) (Figure 3 A, B, C). Thus, the reduced pressure upon the walls prevented the death of cells exposed to severe acid stress.

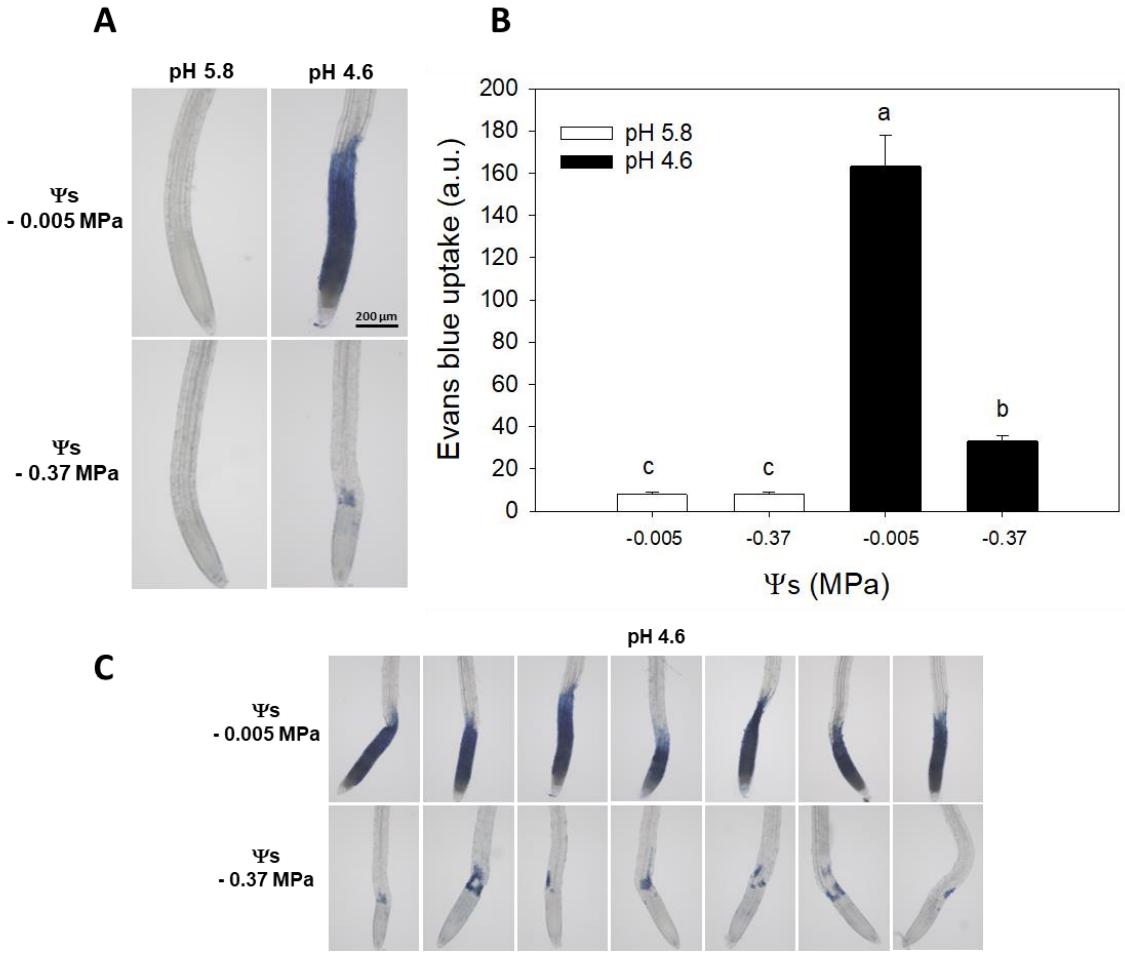


Figure 3. Cell mortality in root tips of *A. thaliana* (Col-0) exposed to strong acidity stress (pH 4.6) under reduced water potential (- 0.37 MPa) or at higher turgor pressure (- 0.005 MPa). A, Bright field images of Evans blue absorption in the root apex. The increase in blue color is indicative of cell death. Scale bar indicate 200 µm. B, Quantitative Evans blue uptake by a fluorimetric assay in root tips exposed to acidity at - 0.005 MPa or - 0.37 MPa. The increase in fluorescence is indicative of cell death. Statistical analysis was Duncan's test. C, Bright field images of Evans blue absorption in several root tips contrasting roots exposed to pH 4.6 at - 0.005 MPa compared to roots exposed to pH 4.6 with solution at - 0.37 MPa for 6h. The increase in fluorescence or blue color is indicative of cell death. Scale bars indicate 200 µm.

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

We next attempted to examine the effect of increased turgor pressure on cell death; by itself or in combination with low pH. To achieve this, roots were first subjected to a temporary HYO treatment in solutions ranging from - 0.22 to - 0.47 MPa at pH 5.8 for 2 h. The purpose of this pretreatment was to promote osmotic adjustment. Then, a HO treatment was applied by returning the roots to a solution with - 0.005 MPa for 1 h, at pH 5.8 or 4.9. Thus, HO treatments of differing magnitudes were applied by varying the Ψs of the pretreatment solution. The magnitude of these HO treatments was expressed as ΔΨs, the difference in Ψs between the HYO and HO solutions. For simplification, the Ψs of the HO solution was considered to be zero.

First, we verified whether a hyperosmotic pretreatment did indeed promote osmotic adjustment. This was evaluated by an analysis of incipient plasmolysis. In roots pretreated with a -0.42 MPa mannitol solution, approximately half of the cells plasmolyzed when transferred to a -1.0 MPa solution (Figure 4A). Whereas in control roots that were pretreated in a -0.005 MPa solution, half of the cells plasmolyzed in solutions of about -0.8 MPa. Thus, the -0.42 MPa hyperosmotic pretreatment decreased the Ψs of cells by approximately - 0.2 MPa.

Interestingly, an HO treatment at pH 5.8, following a HYO pretreatment, reduced cell viability (Figure 4 B, C) without any low pH treatment. An HO treatment with a ΔΨs of - 0.22 MPa affected the viability of cells only slighty. However, cell death was observed in root tips when the ΔΨs of the HO treatment was 0.32 MPa or greater and was quite pronounced at ΔΨs of 0.42 and 0.47 MPa (Figure 4 B, C). Importantly, as in low pH stress (Figure 1 C, D), the first cells to die were also those of the TZ. However, differently from low pH stress (Figure 1A, Supplementary Figure S2), cell death occurred only in cells of the TZ and advanced into the EZ, but not meristematic cells (Fig 4B). Another important difference from low pH stress is that cell death caused by HO treatment was much faster. Cell death was observable within 20 min of a HO treatment with a ΔΨs of 0.42 MPa and at pH 5.8 (Figure 4 E, F), in contrast to 2 h in low pH stress (Figure 1A).

We then examined the effect of HO treatment (ΔΨs = 0.42 MPa) at pH 4.9, a mild acid stress. Compared to pH 5.8, cell death due to HO treatment at pH 4.9 was faster and observable within 10 min (Figure 4 E, F).

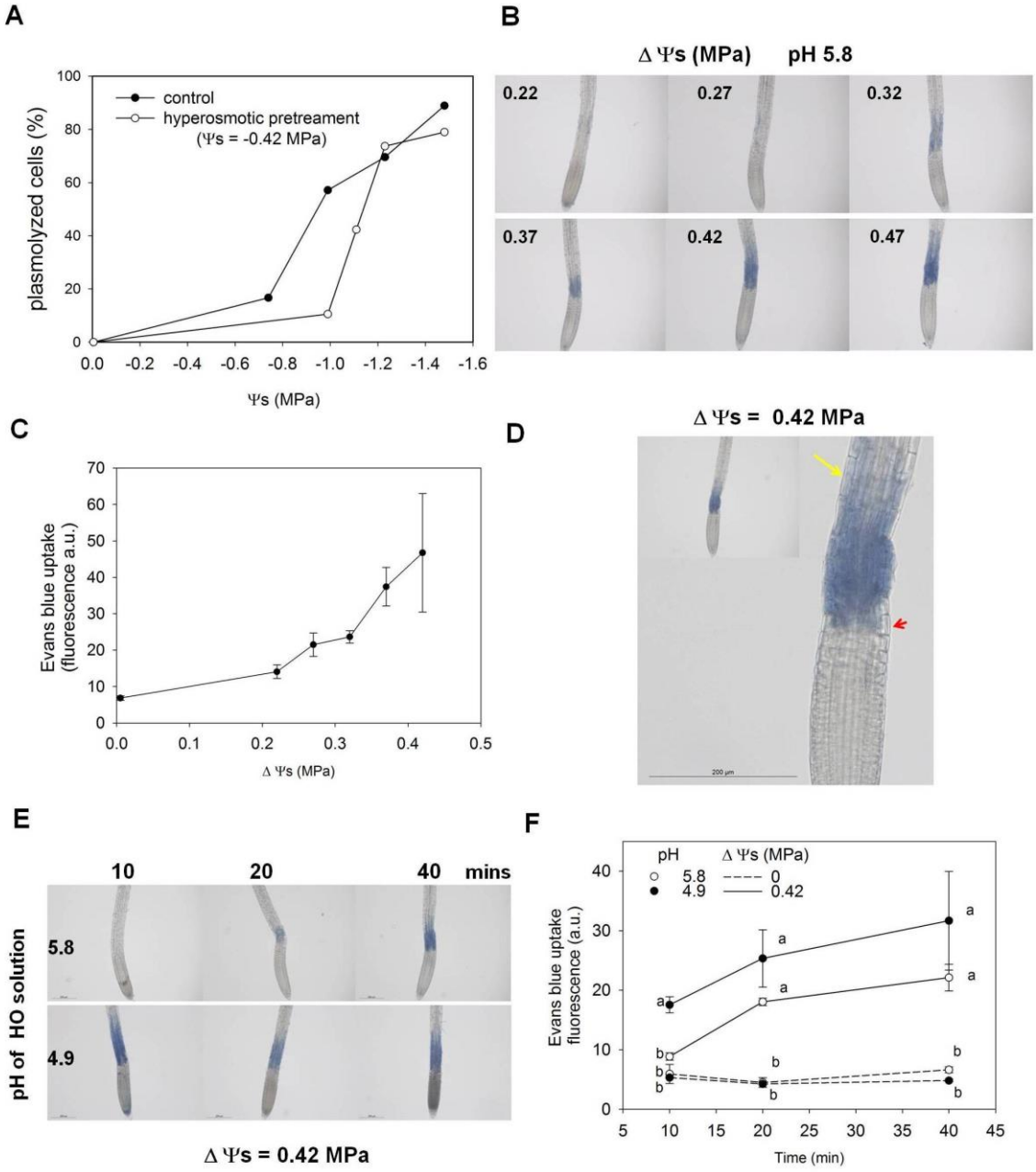


Figure 4. Cell mortality and turgor pressure changes in root tips of *A. thaliana* (Col-0) exposed to an increase in magnitude of hypoosmotic treatment combined or not with mild acidity. A, Measure of incipient plasmolysis in the cells of root tip with root hair bulge. The roots remained along 2 h in solution at -0.005 or -0.42 MPa and then transferred to solutions ranging from -0.74 until -1.48 MPa for five min and cells were quickly imaged in bright field. At least two cells were imaged for plant. Each treatment was composed by twelve plants. B, Bright field images of Evans blue absorption in the root tips exposed to an increase of magnitud of hypoosmotic treatment at pH 5.8. The increase in blue color is indicative of cell death. The roots remained two hours in solutions at pH 5.8 ranging from - 0.22 until -0.47 MPa, then, were transferred to a solution with - 0.005 MPa at pH 5.8 for one hour. Scale bars indicate 200 µm. C, Quantitative cell viability of the increase in magnitud of hypoosmotic treatment at pH 5.8. Measure of Evans blue uptake by a fluorometric assay. The increase in blue color is indicative of cell death. As control roots remained for three hours in solution at pH 5.8 at - 0.005 MPa. D, Only cells at transition zone and beginning of fast expansion zone shown sensitivity to increase on magnitude of hypoosmotic treatment at pH 5.8. The arrow indicates an expanding cell. The arrohead indicates the cell above the end of meristematic zone. Scale bar indicate 200 µm. E, Temporal analyse of cell viability in root tips after the hypoosmotic treatment combined or not with mild acidity (pH 4.9). The roots remained two hours in solutions with 0.42 MPa at pH 5.8, then, were transferred to a solution with - 0.005 MPa at pH 5.8 or 4.9 for ten, twenty or fourty min. Bright field images of Evans blue absorption in the root tips. The increase in fluorescence or blue color is indicative of cell death. The bars are standard error of three experiments performed in different days. Scale bar indicate 200 µm. F, Evans blue uptake in root tips after 10, 20 and 40 min of hypoosmotic treatment. As control roots remained for fourty min in solution at pH 5.8 or 4.9 with - 0.005 MPa. Statistical analysis was Duncan's test.

We also examined cell viability in roots for longer time as two hours after the HO treatment and there was no change in the standard of cell death, being always in the TZ and beginning of fast EZ (data not shown).

2.3.5. Sensitivity of *cob-1* and *wak-1* roots to low pH

We showed physiological evidence for the involvement of the cell wall with the cell death due to low pH. We next did a genetic approach to seek the involvement of cell wall-related proteins in these sensitivity responses. It was examined a mutant impaired in cell wall correct organization, *cob-1*, and a mutant of cell wall integrity sensing protein, *wak-1*, when exposed to low pH.

The mutants and WT were treated only for three hours at pH 4.8, an intermediary between mild and severe low pH stress (pH 4.6). The issue about severe stress is that only reveals very tolerant genotypes.

The *cob-1* roots showed high sensitivity due to low pH as indicated by the Evans blue uptake, three folds more than WT (Figure 5 A). Otherwise, *wak-1* was less sensitive to low pH compared to WT and *cob-1* mutant, resembling the Evans blue uptake of roots treated at pH 5.8 (Figure 5 A).

We also examined cell expansion after low pH treatment by measuring LEH. Roots from WT plants showed a decrease of 34 % in LEH after 1 h of treatment at pH 4.8. Otherwise, *cob-1* roots slight increased the LEH after treatment at pH 4.8. In *wak-1* roots there was no change in LEH after treatment at pH 4.8 for 1 h (Figure 5 B).

In summary, a disordered cell wall with triggered high sensitivity to low pH, while, the reduced perception of cell wall integrity increased the tolerance to the stress. In both cases, there were none reduction in cell expansion due to low pH treatment.

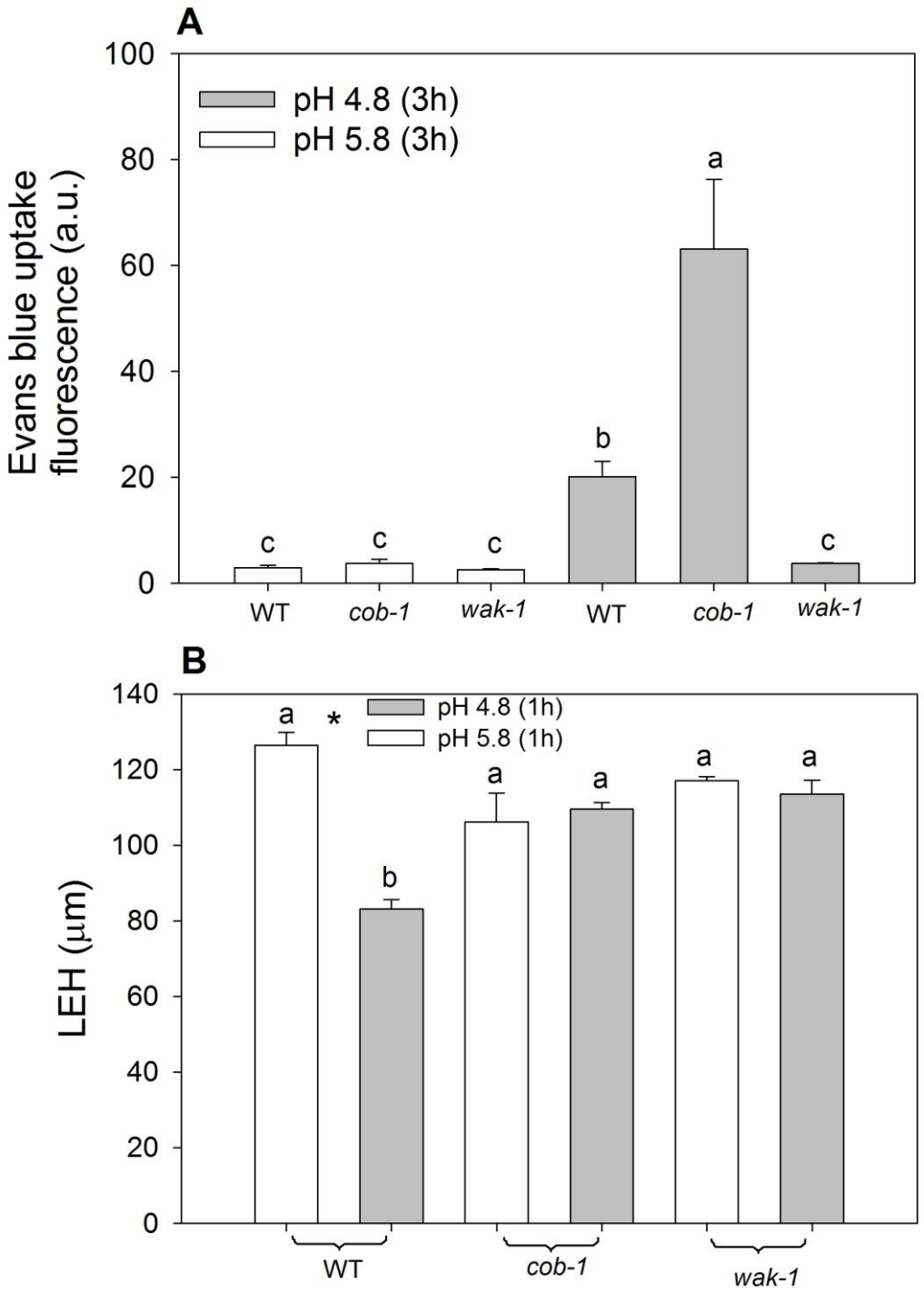


Figure 5. Cell mortality and LEH in root tips of WT of A. thaliana and *cob-1* and *wak-1* treated with low pH. A, Quantitative Evans blue uptake by a fluorometric assay in root tips. The increase on fluorescence is indicative of cell death. B, Length of first Epidermal cell with root Hair bulge (LEH) after 1h of low pH treatment. The bars are standard error of three separate experiments. Letters indicate significant difference between different treatments at the same pH by Duncan's test. On B, Asterisks indicate significant difference within the same treatment by Student *t*-test.

2.3.6. Analysis of PCD in roots treated at low pH

We observed a consistency in cell death in roots treated at low pH, always starting at TZ cells and then moving towards MZ. This behavior was wave-like. Thus, we examined the possibility of PCD.

The nucleus of root tip cells treated at pH 4.6 for 3 h showed deformity and irregular shape, compared to nucleus of cells treated at pH 5.8 (Figure 6). This irregular shape of nucleus seems ubiquitous in cells that died through a PCD. This morphology is in agreement with that one reported by Watanabe and Lam (2008) or Huang et al. (2014), where their evidences suggested PCD mechanism.

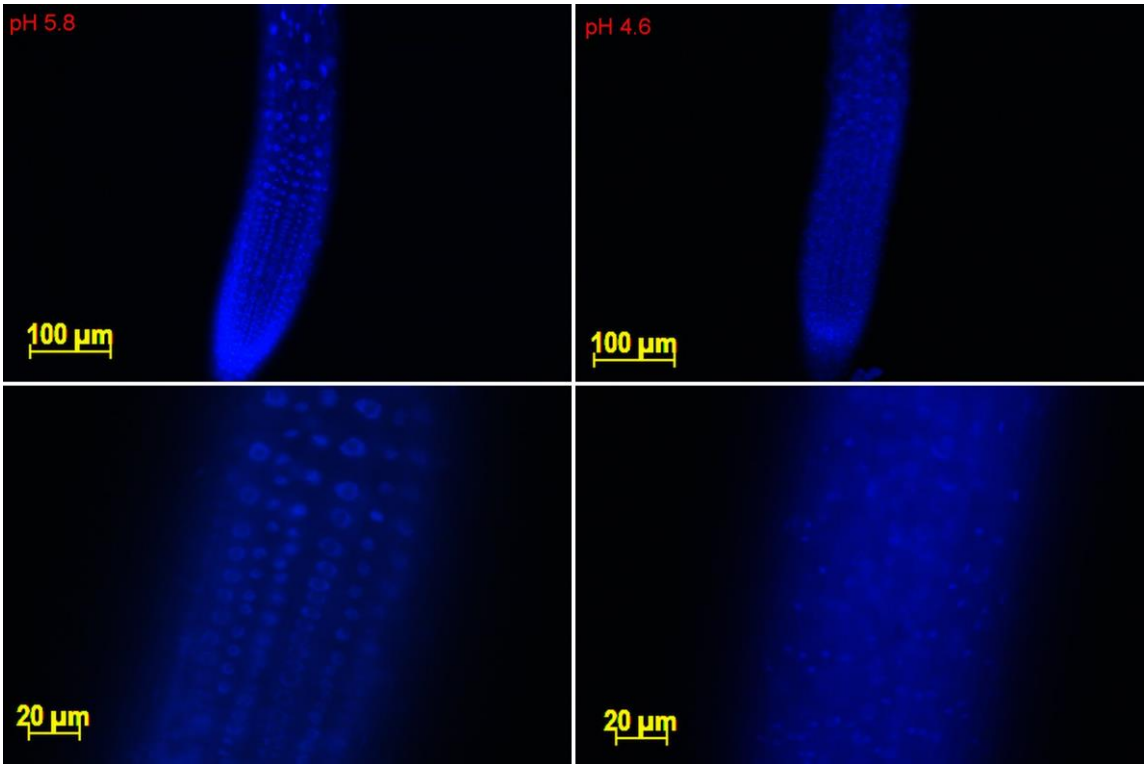


Figure 6. Characterization of nucleus morphology from root tip cells of *A. thaliana* (Col-0) assessed by DAPI staining. After the treatment at pH 4.6 pH 5.8 for four hours roots were fixed with Karnoswsk and then stained with DAPI by ten min. Controls cells (pH 5.8) shown clearly individual and oval nucleus morphology at the transition zone and distal part of meristem. Cells treated at severe low pH stress (pH 4.6) show disforme nucleus and apparently fragmented.

Also, we performed TUNEL reaction to find positive nuclei for PCD (Watanabe and Lam, 2008). Roots treated at pH 4.6 for 3 h showed positive reaction for TUNEL (Figure 7). This positive reaction was not found in roots treated at pH 5.8. TUNEL reaction labels DNA ends at 3'-OH by incorporation of fluorescein-12-dUTP. As positive control, roots treated at pH 5.8 were exposed to DNAse and showed positive reaction for TUNEL across the roots. Hence, this positive reaction in roots treated at pH 4.6 seems to be due DNA cleavage and not due to a false positive. Thus, cell death due to low pH seems to be coordinated by a PCD mechanism.

It is worth to mention that the signal of nucleus staining with DAPI is much better when this one is performed sole, without the steps for TUNEL reaction.

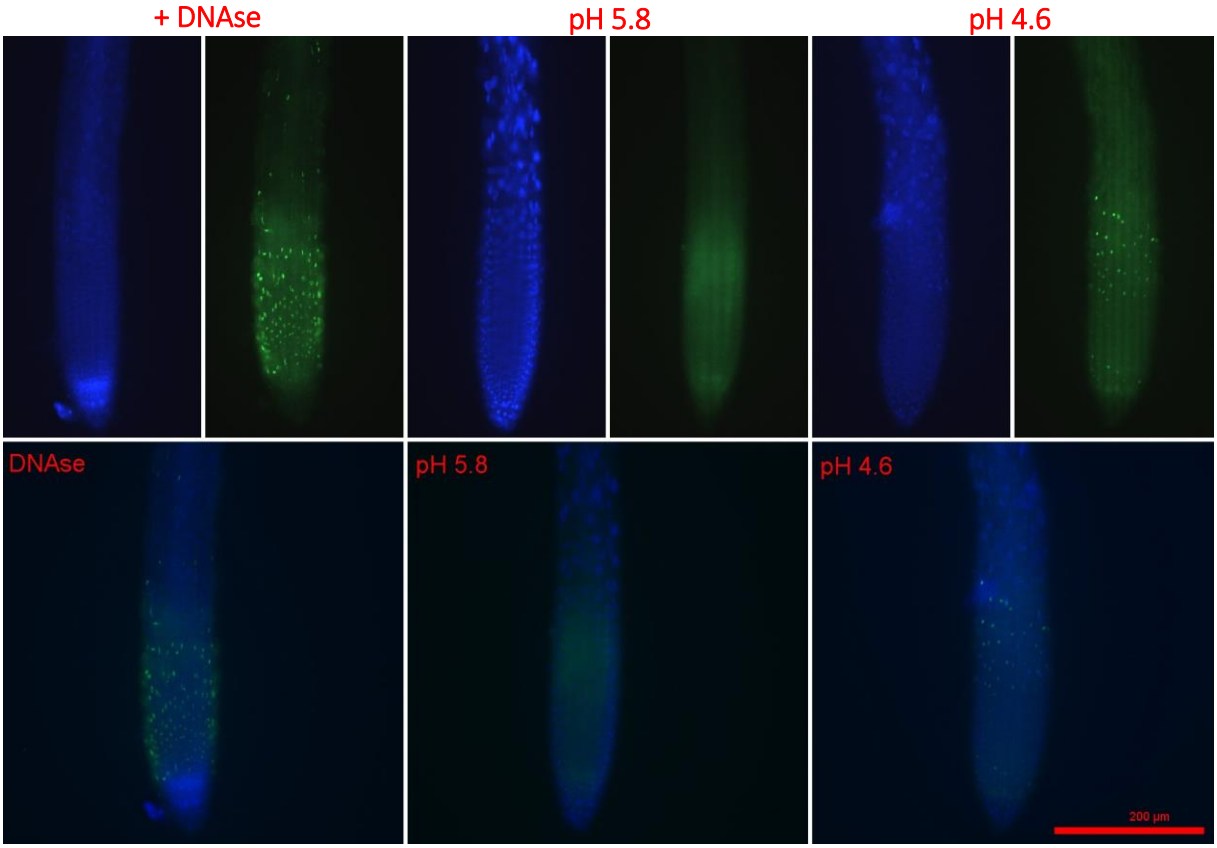


Figure 7. TUNEL reaction for detection of positive nucleus for Programmed Cell Death in roots of *A. thaliana* Col-0 treated at pH 5.8 or 4.6 for 3 h. Nucleus were stained with DAPI. As positive control roots taken directly of growth medium were treated with DNAse. In the upper part DAPI and TUNEL fluorescence in each treatment. In the bottom merged images with DAPI and TUNEL. The markedly fluorescent dots are indicative of positive nucleus for Programmed Cell Death.

2.4. Discussion

In this study, we performed a detailed spatial examination of the progression of low-pH induced cell death and show that this follows a defined, characteristic pattern. We then present a set of evidence which, building on previous literature, shows that the cell wall is a major actor in low pH toxicity and the ensuing cell death. Finally, our data indicate that this cell death occurs via a PCD mechanism which depends on perception of stress in the cell wall.

The first cells to die because of low pH stress were consistently those of the TZ and start of EZ (Figure 1, Supplementary Figure S2). Prior to the onset of cell death in these cells, our results indicate that treatment at pH 4.6 increased the extensibility of the cell wall of epidermal cells of this region (Figure 2). To test whether this increase in extensibility or some other cell wall

instability causes subsequent cell death, roots were treated simultaneously to low pH and at lower water potential (Ψw = - 0.37 MPa). A lower water potential reduces cell turgor pressure and thus the tension of the cell wall, compensating for an increase in cell wall extensibility. An increase in the osmoticum of the medium can also decrease the hydration of the cell wall which, in turn, reduces its extensibility (Evered et al., 2007; Hansen et al., 2011; Wolf et al., 2012). However, small molecules which can penetrate the pores of the cell wall are not expected to affect cell wall hydration (Passioura, 1994; Evered et al., 2007), but this cannot be ruled out. Either way, lowering of the water potential counteracts an increase in cell wall extensibility or instability.

Compared to low pH alone, cell death was greatly reduced in roots treated simultaneously to low pH and a lower water potential (-0.37 MPa)(Figure 3), demonstrating that a certain level of turgor pressure and cell wall tension is required for cell death to occur. Thus, an instability or excessive loosening of the cell wall seems to be the cause of cell death.

The manner in which low pH loosens the cell wall is still elusive. It may be caused by direct effects on cell wall structure since low pH can increase cell wall hydration and therefore increase its extensibility (Evered et al., 2007; Wolf et al., 2012). Low pH also increases the activity of expansins, which are known to promote loosening of the cell wall (Cosgrove, 2016).

Evidence is consistent with a scenario where low pH causes cell wall loosening or instability which then leads to a cellular counter response because these changes are excessive or simply to maintain homeostasis (Graças et al., 2016). This might explain why, in some classical "acid growth" studies, acid-induced wall loosening in the root EZ was found to be transient (Edwards and Scott, 1974; Evans, 1976; Winch and Pritchard, 1999). In actively growing root hairs, tip growth rate increased with decreasing pH, but then abruptly fell between pH 5.0 and 4.5. Growing root hairs treated to pH <4.5 either burst (25% of them) or ceased growth altogether (Bibikova et al., 1998). In Arabidopsis, low pH caused major changes in the expression of genes associated with cell wall modifications (Lager et al., 2010). Although it is not possible to know the net outcome of these changes, many of them indicate cell wall stiffening. For example, after exposure for 1 h to pH 4.5, four α-expansins and one β-expansin genes were strongly repressed; three of these were among the six most repressed genes by low pH (Lager et al., 2010). In addition, at least two polygalacturonases were repressed while two polygalacturonase-inhibiting proteins (PGIP) were induced. In our study, we found evidence for loosening of the cell wall of TZ cells whereas LEH in EZ was reduced, which is indicative of cell wall stiffening (Le et al., 2001; Tsang et al., 2011). These cells are rapidly expanding but it appears that they have mechanisms to quickly stiffen walls and reduce expansion. Importantly, we found that EZ cells farther from the root tip do not die.

Remarkably, we found that a HO treatment at pH 5.8 can also result in cell death. Similar to cell death induced by low pH alone, the first cells to die were those of the TZ and beginning of EZ. However, differently from low pH, cell death advanced partially into the EZ but did not affect the MZ. Furthermore, HO-induced cell death was much faster (20 min versus 2 h). Koyama et al. (2001) reported the beginning of cell death after 0.5 h of low pH treatment and after 2 h the cells of root tip including MZ were completely dead. They observed a fast stress than showed here, but, it was employed a solution with only 100 μM of $CaCl_2$. Here, we employed 500 μM of $CaCl_2$ that can be the major cause of this difference in sensitivity once Ca^{2+} and ionic strength are relevant for protons toxicity.

To perform this HO experiment, we pretreated the roots with a HYO solution to promote osmotic adjustment. Indeed, this reduced the cellular Ψs by roughly 0.2 MPa (Figure 4 A), meaning that turgor pressure was increased by this value when roots were placed back in a solution with a Ψs close to zero. However, a difference of 0.2 MPa in turgor pressure does not seem large enough to have such a drastic effect as death. In addition to osmotic adjustment, another probable effect of the HYO pretreatment is a loosening of the cell wall. Roots attempt to maintain growth under low water potentials by increasing the extensibility of the cell wall (Wu et al., 1994; Wu and Cosgrove, 2000), and this happens especially in the apical region of the EZ, which coincides with the HO-induced cell death observed here. Thus, besides an increase in turgor pressure, the effects of our HO experiments were also probably due to increased loosening of the cell during the HYO pretreatment.

Not surprisingly, low pH intensified the HO-induced cell death. When the HO treatment was performed with a mild acid stress, at pH 4.9, the onset of cell death was even faster (10 min) than at pH 5.8 (Figure 4 E, F). This again illustrates the relationship between low pH and the cell wall in cell death.

Cells of yeast displayed cell death due to HO shock (30 min) dependent of mechanosensitive channels that regulate cell volume in increased turgor pressure (Nakayama et al., 2012). Dead root cells due to HO treatment at pH 5.8 are located in a zone of high dynamic cell walls and the HYO pretreatment causes changes such as increase in wall extensibility (Wu and Cosgrove, 2000) before the HO treatment that. The death was accelerated by low pH. We do not exclude the possible participation of mechanosensitive channels as the case of yeast, but, the evidences here seems to be indicate major involvement of cell wall.

To further demonstrate the role of the cell wall in low-pH induced cell death, we also used a genetic approach with cell-wall related mutants. The *cob-1* roots showed higher cell mortality at pH 4.6 compared to WT (Figure 5). COB-1 is necessary for the correct organization

and deposition of cellulose microfibrils in the primary cell wall and is highly expressed above the meristematic zone (Roudier et al., 2005). Thus, cellulose organization is relevant to low pH sensitivity.

WAK-1 is a protein that is possibly involved in cell wall integrity perception (Anderson et al., 2001; Liu et al., 2015). Surprisingly, *wak-1* roots were tolerant to pH 4.6 (Figure 5). Among the five WAK proteins in *A. thaliana,* WAK-1 and WAK-2 are the most highly expressed (Anderson et al., 2001; Wagner and Kohorn, 2001). WAK-1 is expressed in root MZ and also in response to wall stress but expression in the EZ is not significant (Wagner and Kohorn, 2001). Importantly, WAK-1 is anchored in the membrane, has a cytoplasmic kinase domain and an N terminus that binds the cell wall in the pectin matrix (Anderson et al., 2001; Wagner and Kohorn, 2001; Liu et al., 2015). Thus, WAK-1 can communicate the apoplast with cytosol and also the sensing of cell wall tension has been attributed as one role of this protein. Hence, the tolerance of *wak-1* to low pH is actually in agreement with decreased cell mortality of WT roots due to decreased cell wall tension during low pH treatment (Figure 3).

WT roots showed a decrease in LEH after 1 h of treatment at pH 4.8 (Figure 5 B). Curiously, in *cob-1* roots LEH have a slight tendency to be increased compared to LEH of roots treated at pH 5.8. COB-1 is involved with correct deposition of cellulose (Roudier et al., 2002). Thus, cellulose architecture might be relevant for this decrease in expansion. Roots of *wak-1* treated at pH 4.8 didn't stopped expansion, thus, there was no reduction on LEH compared to roots treated at pH 5.8. This suggests a role of WAK-1 on signaling decreasing cell expansion under low pH.

Roots of *wak-1* and *cob-1* were tolerant and sensitive to low pH, respectively, and, both showed no reduction in LEH as WT roots did (Figure 5 B). These cells in which LEH was measured were not sensitive to low pH (Figure 2 C, arrow). Thus, there is none indication, that cells in MZ or TZ, will be died, or will survival to low pH, based in LEH behavior.

Part of low pH toxicity is interpreted in literature as negative interaction of H^+ directly with membrane, as the change in the electric potential (Bose et al., 2010). Here, the roots remained along 6 h in HYO solution to decrease the cell wall tension. During this time, the interaction between H^+ and membrane was still there, but, negligible cell death was observed (Figure 3). We did not ignore the effects of low pH on plasma membrane as electric potential changes and facilitated K^+ efflux (Bose et al., 2010) that can perturb several cellular processes. However, based in all evidences discussed above, we propose that this membrane disturbs could be not directly linked to cell death within a few hours of low pH exposure, as cell wall seems to be.

So far, our results and the above discussion might argue for cell death due to yielding of the cell wall, similar to the bursting of root hairs. However, some observations made us suspect this was not so. Although the time required for the onset of cell death was rather short (2 h), it was long compared to bursting of root hairs or cell death caused by HO treatment. Furthermore, the timing and manner in which cell death progressed into the meristem between 2 and 6 h of treatment also did not seem to favor this (Figure 1, Supplementary figure S2). Yielding of the cell wall would seem more likely in expanding cells of the EZ, and appears to be more consistent with HO-induced cell death. These considerations prompted us to investigate the possibility of low-pH induced PCD.

The changes in morphology of DAPI-stained nuclei of roots treated at pH 4.6 (Figure 6) and the positive reaction for TUNEL (Figure 7) strongly suggest that the cell death was due to PCD. These observations are similar to those reported by Watanabe et al. (2008) where PCD was investigated in roots. Furthermore, the tolerance of the *wak-1* mutant indicates the requirement of a signaling pathway for cell death to occur. WAK-1 is known to be a receptor for oligogalacturonides (Brutus et al., 2010). As far as we know, WAK-1 does not have other known functions that could explain why *wak-1* mutant displays less cell death. Importantly, low pH has been suggested to affect the pectin network of root cell walls (Koyama et al., 2001) and root hair tips, which are very sensitive to low pH, are rich in pectins (Wolf and Greiner, 2012). Pectins also have an important role in cell wall loosening and cell expansion (Wolf and Greiner, 2012; Chebli and Geitmann, 2017). Recently, the relationship between "acid growth" and pectin has been examined (Hocq et al., 2017).

The finding that low pH stress induces PCD in root cells opens several possibilities for investigation. Whether this is due to a disturbance that activates this pathway or whether this is a strategy for stress adaptation must be investigated. A barley mutant highly sensitive to saline stress displayed more PCD than wild-type plants (Huh et al., 2002). Accordingly, this helped the plants to eliminate the primary roots and to produce new roots more able to acclimate on a changed environment. In addition, we can also seek for PCD marker genes involved in different types of plant PCD (Olvera-Carrillo et al., 2015) that might also be activated in this low-pH triggered PCD.

Roots treated with Al displayed a mitochondrial-dependent ROS production that caused PCD (Huang et al., 2014). Although low pH data cannot be accessed alone in this work, most probably cells were subjected to a mild acidity stress. Thus, not significant cell death was observed in low pH treated roots.

In conclusion, we present experimental evidence that low pH affects the cell wall in the root tips and that this is highly correlated with occurrence of cell death during the stress. This brings insights for the fact that root cells which possess high dynamic cell walls, as those from TZ, are very sensitive to low pH. Also, reducing cell wall tension and preventing cell death addressed some insights for the role of cell wall as a target, rather than plasma membrane on cell death that happens within a few hours due to low pH. Whether PCD has an adaptive role in an acidic environment must be further investigated.

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Supplementary Material

Table S1. Primers used to confirm the mutants.

			Primers
Gene	**Locus**		**Sequence**
WAK-1	AT1G21250	F Lbb1.3	ATTTTGCCGATTTCGGAAC
		R	GGCCAGTTTCAATGTCCAAT
COB-1	AT5G60920	F LB3	TTCATAACCAATCTCGATACAC
		R COB	AGATTAGGCAGAGAAGAAGAAA

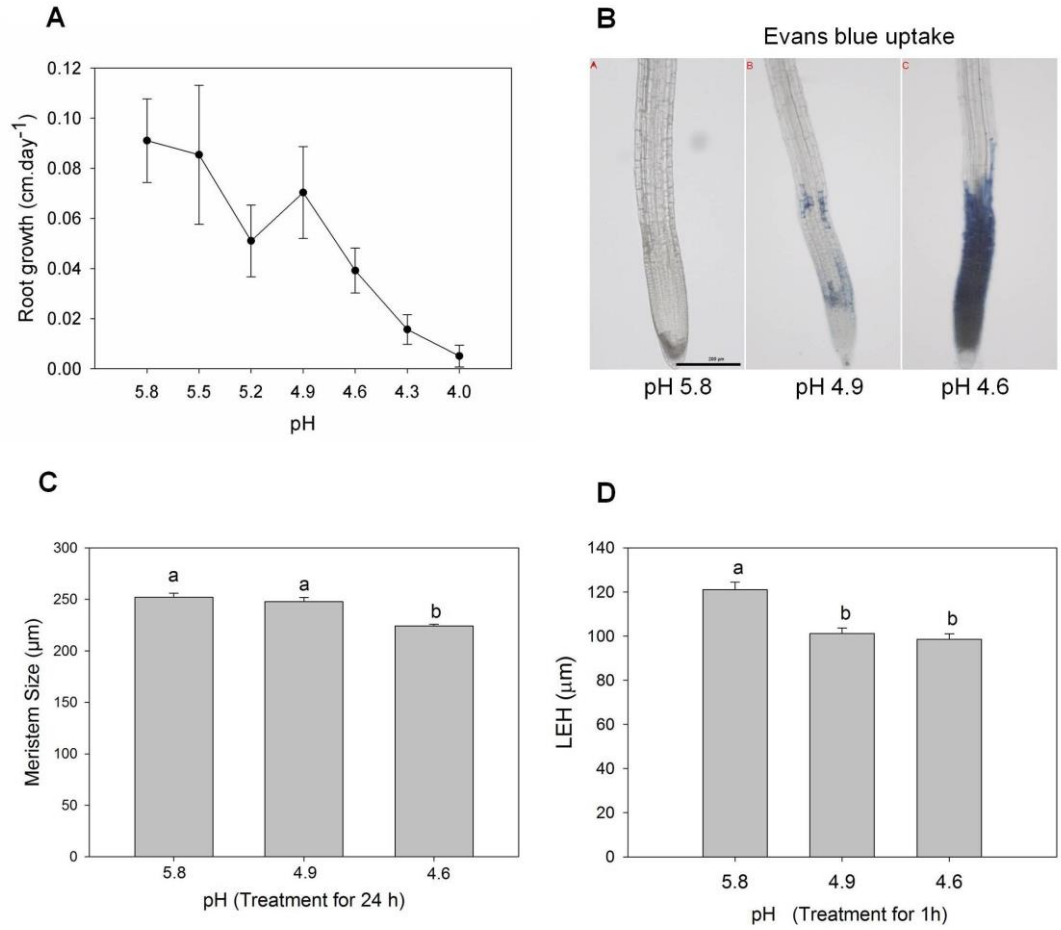

Figure S1. Growth, cell mortality and cell expansion in roots of A. thaliana (Col-0) exposed to an increase in solution acidity. The treatment solution in all experiments was composed by 0.5 mM of $CaCl_2$ and 0.6 mM of Homopipes buffer with aeration. A, root growth after 24 h on different acidic treatments. Ten plants per treatment were used in each experiment. The bars are standard error of five independent experiments. B, bright field images of cell viability in the root tips exposed to low pH for 6 h. From left to right are roots treated at pH 5.8, pH 4.9 and pH 4.6, respectively. The blue color is indicative of cell death due Evans blue penetration on cells. C, after the treatment for 24 h in pH 5.8, 4.9 or 4.6 the meristem size was evaluated. Ten plants per treatment were used in each experiment. The bars are standard error of three independent experiments. D, Length of the first Epidermal cell with root Hair bulge (LEH) after 1h of low pH treatment. Statistical analysis was Duncan's test.

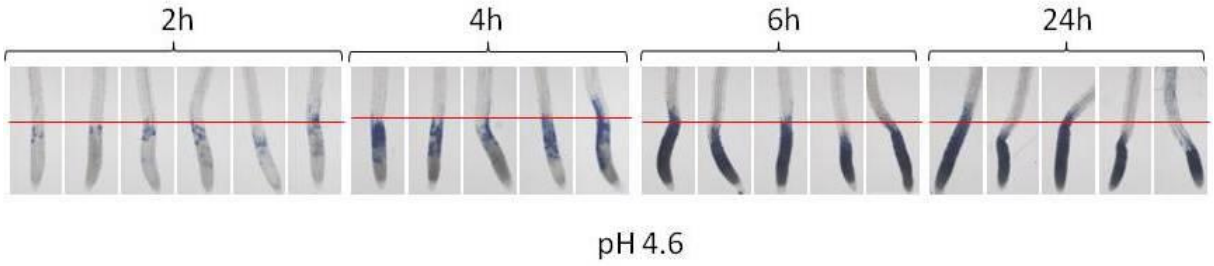

Figure S2. Time-course of cell mortality in roots of A. thaliana (Col-0) exposed to pH 4.6. The blue color is indicative of cell death due to Evans blue penetration in cells.

3. THE CLASS III PEROXIDASE AtPRX62 POSITIVELY REGULATES CELL DEATH DURING LOW-pH STRESS IN ARABIDOPSIS ROOTS

ABSTRACT

Low pH can cause the death of cells of the root tip. We have shown that the cell wall is crucial for the sensitivity of cells to low pH. The role of cell wall players, such as class III peroxidases (CIII Prx) and ROS in this sensitivity is still not clear. Here, we examined the role of CIII Prx and ROS in the cell death of *A. thaliana* roots due to low pH stress. Roots exposed to pH 4.6 displayed increased peroxidase activity in the same region that cell death occurred, in the transition and meristematic zone, and especially in the stele. In these root zones, there was a drastic decrease in $O_2^{\bullet-}$ levels but no changes were observed in H_2O_2 levels. Thus, excessive ROS levels were not a cause of cell death. Data mining of previously published transcript data revealed that AtPRX62, the expression of which was induced 8.4-fold in roots treated at low pH compared to control pH, was a good peroxidase candidate for further examination. An *atprx62* KO mutant was less sensitive to low pH compared to Col-0 roots with respect to cell death. The increase in peroxidase activity in the stele of MZ and TZ also did not occur in this KO mutant. *In situ* mRNA hybridization showed that *AtPRX62* was practically not expressed at pH 5.8, but in roots treated at pH 4.6, mRNA transcripts of *AtPRX62* accumulated in the MZ, TZ and EZ, coinciding approximately with the region where cell death occurred. Together, our data demonstrates that an increase in expression of *AtPRX62* at low pH positively regulates cell death.

Keywords: Low pH; Cell death; Class III peroxidase; ROS; Superoxide

3.1. Introduction

Plants growing in acid soils exhibit decreased root growth. This root growth inhibition is still not well understood, but, it was suggested to be an active plant response to decrease cell mortality during acid stress (Gracas et al., 2016). Root hairs burst when treated with low pH, but, only those undergoing expansion (Bibikova et al., 1998; Monshausen et al., 2007). This indicates that low pH may cause disorders in the cell wall that jointly with constant turgor pressure, might cause a cell wall failure and bursting. Damage to cell wall by low pH could be similar in others root cells and might prompt several players to generate a plant response.

Cell death has been reported to occur after only 30 min of low pH treatment and it is readily observed in root tip cells (Koyama et al., 2001). In these zones, cell wall synthesis and changes in cell wall physical properties are crucial for the expansion and consequent root growth (Somssich et al., 2016).

Most of CIII Prx (E.C.1.11.1.7) are secreted to the apoplast (Valerio et al., 2004). They play diverse roles including cell wall formation, cell wall modification, apoplastic ROS production, ROS metabolism and are responsive to biotic and abiotic constraints (Francoz et al., 2015; Podgorska et al., 2017). In roots of *A. thaliana* exposed to low pH one peroxidase gene was

early-induced (1 h), ten were late-induced (8 h) and only two were late-repressed (Lager et al., 2010). Indeed, mild or strong low pH stress increased CIII Prx activity in tomato roots (Gracas et al., 2016). This indicates that peroxidases are regulated either transcriptionally or post-transcriptionally under low pH stress. This is likely to have consequences for cell survival during the stress. Out of the 73 CIII Prx predicted in the genome of *A. thaliana*, 38 isoforms were found in a recent cell wall proteome analysis of roots (Nguyen-Kim et al., 2016). However, studies investigating the role of specific isoforms are significantly lacking.

ROS production by NADPH oxidases are crucial for formation and integrity of the cell wall and this has obvious implications for cell elongation (Foreman et al., 2003). The activity of CIII Prx can cause loosening or stiffening of the cell wall and uses H_2O_2 as an electron donor (Passardi et al., 2004). In addition, in the peroxidative cycle peroxidases consume H_2O_2 and superoxide to generate hydroxyl radical ($^{\bullet}OH$) which by itself can promote cell wall loosening (Chen and Schopfer, 1999). Thus, in concert with NADPH oxidases, CIII Prx produces and controls ROS levels. Also, the ratio between H_2O_2 and $O_2^{\bullet-}$ is finely adjusted and controlled by CIII Prx activity for maintenance of normal root growth (Dunand et al., 2007; Tsukagoshi et al., 2010).

Peanut roots treated with Al displayed increased ROS production causing cell death that was alleviated by addition of ROS scavengers (Huang et al., 2014). Inhibition of CIII Prx activity decreased ROS levels and sensitivity of barley roots to Al (Simonovicova et al., 2004). This data confirms that ROS levels must be highly controlled by several intracellular (Halliwell, 2006) and apoplastic actors (Podgorska et al., 2017) due to its potential toxicity to cells.

On the other hand, inhibition of NADPH oxidase and peroxidase activity in tomato roots greatly increased the sensitivity of cells to low pH (Gracas et al., 2016). It seems that depending on the type and magnitude of the stress, ROS and CIII Prx can increase or decrease cell sensitivity. This probably reflects the large number of peroxidase isoforms and how they work together. Interestingly, impairment of cell wall integrity with the cellulose synthase inhibitor, isoxaben, increased expression of AtPRX71 (Raggi et al., 2015) and its activity was linked to ROS production, presumably to strengthen cell walls in response to damage.

To investigate the role of specific CIII Prx isoforms during low pH stress, we performed a data mining of transcriptomic data generated by Lager et al. (2010). We found that AtPRX62 is positively linked to cell death in *A. thaliana* roots exposed to low pH.

3.2. Materials and Methods

3.2.1. Plant growth conditions

Seeds of *A. thaliana* were disinfected in commercial sodium hypochlorite solution (5%) for 10 min under stirring and then washed five times with distilled water. The seeds were then transferred to Petri dishes with 0.8% of agar and a modified Hoagland's solution at pH 5.8. Macronutrients consisted of 4 mM Ca $(NO_3)_2$, 6 mM KNO_3, 1 mM $MgSO_4$ and 2 mM $NH_4H_2PO_4$. Micronutrients were composed of 20 μM $MnSO_4$, 14 μM $ZnSO_4$, 20 μM H_3BO_3, 0.03 μM $NiSO_4$, 0.02 μM Na_2MoO_4, 0.02 μM $CuSO_4$, 0.02 μM $CoCl_2$ and 30 μM $FeSO_4$.

The growth temperature was 22 ° C and the light intensity was approximately ± 120 $\mu E.m^{-2}.s^{-1}$. For all low pH treatments, at least ten five-day-old seedlings were incubated in 250 ml Erlenmeyer with 20 ml of treatment solution composed of $CaCl_2$ (0.5 mM) and Homopipes buffer (homopiperazine-1,4-bis(2-ethanesulfonic acid) (0.6 mM). The solution was continuously aerated.

We used five-day-old seedlings of ecotype Columbia-0 as WT and the *atprx62* KO mutant (AT5G39580) (Germplasm: GK_287E07). The seeds were obtained from the laboratory collection of Dr. Christoph Dunand and Dr. Philippe Ranocha (Laboratoire de Recherché in Sciences Vegétales, CNRS, UPS, Toulouse, France).

3.2.2. Spatiotemporal evaluation of class III peroxidase activity and ROS distribution in roots exposed to low pH

Guaiacol was used as a substrate to examine the activity of CIII Prx in the roots. Guaiacol (0.125 % v/v) was diluted in 200 mM of phosphate buffer (pH 6.1). For the reaction, fresh H_2O_2 was added to the guaiacol solution to a final concentration of 1.65 mM and roots were stained in the dark. After five min the roots were washed with abundant water to stop the reaction and immediately imaged by bright-field microscopy.

Staining for detection of superoxide ($O_2^{\bullet-}$) was done using nitro blue tetrazolium chloride (NBT). A solution of NBT (2 mM) was prepared in 20 mM of phosphate buffer (pH 6.1). Roots were covered with this solution in the dark for 15 min and the reaction was stopped by adding water. The roots were imaged by bright-field microscopy.

Staining for detection of H_2O_2 was done using hydroxyphenyl fluorescein (HPF). The working concentration was 5 μM of HPF in 20 mM phosphate buffer pH 6.1. Staining was done

in the dark for 15 min. The reaction was stopped by washing the roots in 20 mM phosphate buffer (pH 6.1). The roots were imaged using a Zeiss Axio Zoom.V 16 stereomicroscope with a GFP long pass filter cube (excitation 485/12 nm and emission at >515 nm). The background of fluorescence in roots stained only with phosphate buffer was negligible.

3.2.3. Data mining for class III peroxidases involved in low pH response

We analyzed public transcriptomic data to search for potential class III peroxidases encoding genes potentially involved in low pH response. NASC 470 data from Lager et al (2010) was downloaded and edited. The mean of the $\log^2$ values for each condition (n = 3) was calculated as well as the ratio of absolute expression for low pH *vs* control pH. The 73 Class III peroxidases (http://peroxibase.toulouse.inra.fr/) were searched within the whole transcriptomics data. Absolute heat map was drawn for ratio of absolute expression (red to yellow) with an arbitrary threshold value set as 5.

3.2.4. Evaluation of cell death in root tips

Staining with Evans blue to assess cell viability was performed the same way as described in chapter 2. The images of Evans blue uptake were analyzed using ImageJ software. The contours of each root tip, reaching 500 µm from the apex, were drawn and the mean gray value (https://imagej.nih.gov/ij/docs/guide/index.html) of this region was calculated. From each of these values, the mean gray value of the background of the corresponding bright-field image was subtracted.

3.2.5. *In situ* mRNA hybridization

We performed *in situ* hybridization (ISH) to detect mRNA of AtPRX62 (AT5G39580) of *A. thaliana* in roots of the WT (Col-0) and in the *atprx62* KO mutant. All procedures were performed using whole roots of five-day-old *A. thaliana* seedlings.

We followed all the steps and solution preparation as described by Hejatko et al. (2006). Only minor modifications were introduced such as the use of Roti®Histo for sample permeabilization. As a positive control, we used a probe for *AtPRX42* (AT4G21960) the expression of which did not change in low pH treated roots according to our data mining done

on the public data from Lager et al. (2010). Information about the mRNA probes for *AtPRX62* and *AtPRX42* can be accessed with all details in the work of Francoz et al. (2016).

3.2.6. Statistical analysis

A completely randomized experimental design was used in all experiments. Each analysis was composed of at least three separate experiments. Each replicate was composed of at least ten plants. For the comparison of means, the analysis of variance (ANOVA) was followed by Duncan's test. When necessary, the difference between two means was evaluated by Student's t-test at the 5% significance level.

3.3. Results

3.3.1. Association between cell death, peroxidase activity and ROS distribution in WT roots exposed to low pH

Cell wall modifications are likely a part of the cell response to low pH. Peroxidases can use ROS to oxidize cell wall components to promote such modifications. In addition, changes in ROS concentration and localization can be part of a signaling process in roots during stress. We examined the association between cell viability, CIII Prx activity and ROS distribution in roots of Col-0 exposed to low pH.

In roots treated at pH 5.8 for 2 or 3 h, no dead cells were observed and guaiacol staining for peroxidase activity was observed along the entire root, except in the meristematic zone (Figure 1). Roots treated at pH 4.6 for 2 h showed dead cells at the transition zone and increased peroxidase activity in the transition and meristematic zone, as observed by a slight brown color in the root tip. Cell death, as previously observed, progressed toward the meristematic zone after 3h of low pH treatment and peroxidase activity increased strongly in the transition and meristematic zone (Figure 1). Noticeably, this increase in peroxidase activity was always strong in inner cells of the stele.

Roots treated at pH 5.8 for 2 or 3 h showed a strong blue staining for superoxide accumulation in the MZ, but, a decrease in the TZ and EZ (Figure 1). This was similar to the previously observed distribution of superoxide that strongly reacted with NBT in the MZ of *A. thaliana* (Dunand et al., 2007). In contrast, roots treated at pH 4.6 for 2 or 3 h showed decreased

staining for superoxide accumulation in MZ. Thus, under low pH stress, the normal superoxide distribution in the root tip was disrupted.

We also examined the H_2O_2 distribution in roots with HPF. Roots treated with pH 5.8 or 4.6 for 2 or 3 h did not show visible difference in H_2O_2 distribution (Figure 1). Also, we quantified H_2O_2 production by a highly sensitive fluorometric assay employing amplex® red (10-acetyl-3,7-dihydroxyphenoxazine; ampliflu™ red, Sigma) and found no difference between pH 5.8 and 4.6 during 1 h or 4 h of low pH treatment (data not shown).

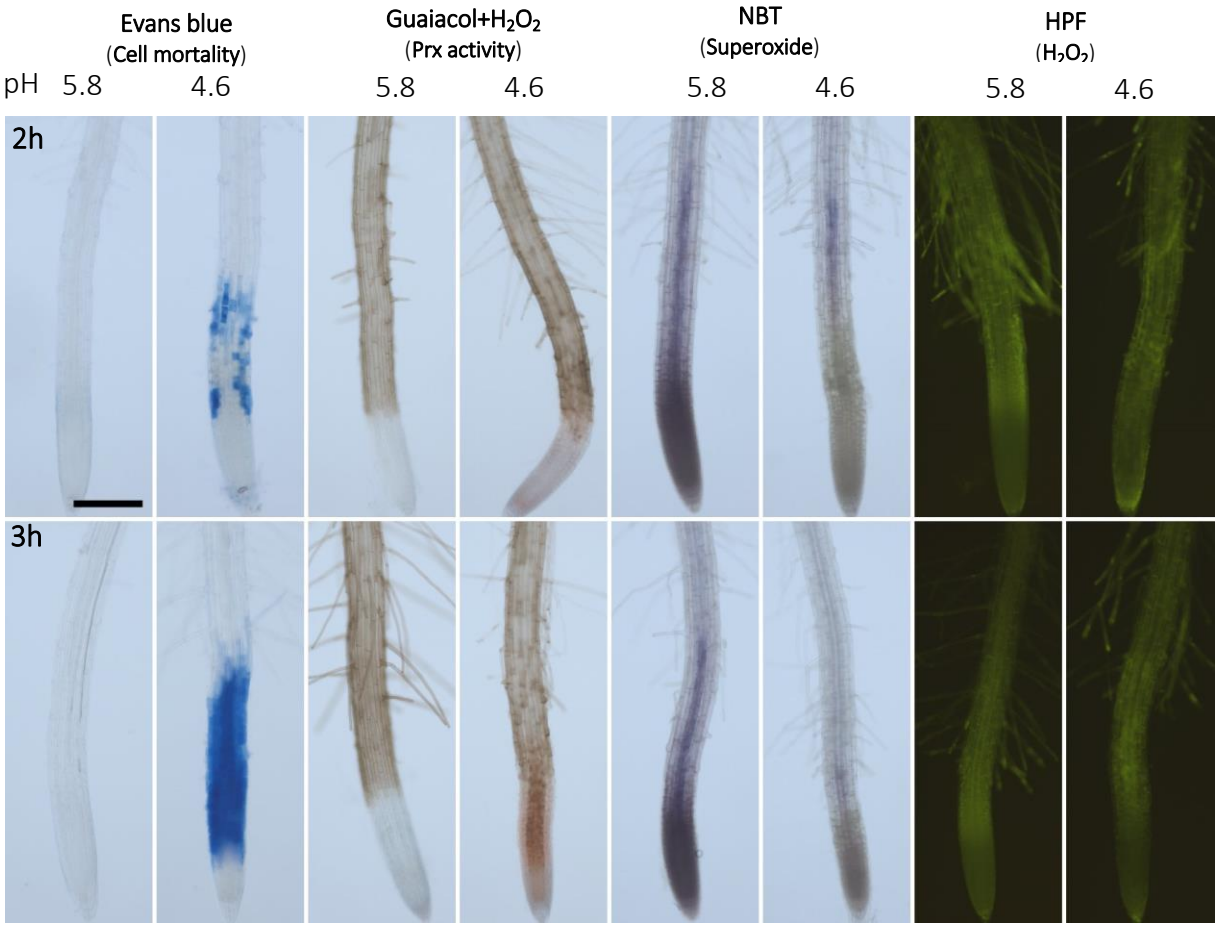

Figure 1. Characterization of cell mortality, peroxidase activity and ROS distribution in *A. thaliana* roots (Col-0) exposed to low pH. Cell mortality was examined by Evans blue, which only penetrates dead cells. Endogenous class III peroxidase activity was examined by addition of guaiacol and H_2O_2. The reaction for detection of superoxide ($O_2^{\bullet-}$) was performed using nitro blue tetrazolium chloride (NBT). The reaction for detection of H_2O_2 was done using hydroxyphenyl fluorescein (HPF). Scale bar indicate 200 µm. Three independent experiments were performed with similar results and representative images are shown.

3.3.2. Data mining of the literature for class III peroxidase isoforms related to low pH response

Our initial results showed a progression of cell death which coincided with an increase in peroxidase activity and change in ROS distribution in roots. Thus, we performed an absolute analysis on public transcriptomic data (NASC 470) from the work of Lager et al. (2010). We searched all class III peroxidases in the whole transcriptomic data looking for those which were most induced after 1 or 8 h of treatment. Among them, the peroxidase 62 (AT5G39580) gene was up-regulated (8.37 fold) after 8h of low pH treatment (Table 1). *AtPRX62* is also highly expressed in epidermal and stele cells at the beginning of the transition zone (Figure S1), where the first cells died after low pH treatment. Thus, we examined the sensitivity of the knockout mutant of AtPRX62 to low pH.

It must be noted that the low pH conditions used by Lager et al (2010) are different than the stress conditions used in our work. Although cell viability was not examined in Lager's work, our low pH stress seems to be more severe based on root growth data and the composition of the treatment solution. Indeed, the expression of *AtPRX62* was markedly induced only after 8 h, rather than 1 h of stress treatment (Table 1). Thus, we looked mostly at data from 8 h of treatment from Lager et al (2010).

Table 1. Data mining of NASC470 from Lager et al. (2010) that presented transcriptomic data for the response of *A. thaliana* roots to low pH. We calculated the ratio of absolute expression for roots treated at low pH (pH 4.5) vs roots of control treatment pH (pH 6.0). Genes which were down-regulated are shown in yellow and the most up-regulated are shown in red.

A. thaliana peroxidase	Locus	Probe Set ID	1 h Ratio	8 h Ratio
AtPrx25	AT2G41480	267101_at	1.34	1.85
AtPrx22 AtPrx23	AT2G38380 AT2G38390	267053_s_at	0.95	1.23
AtPrx16	AT2G18980	266941_at	0.78	0.36
AtPrx20	AT2G35380	266625_at	1.03	1.61
AtPrx24	AT2G39040	266191_at	0.66	0.12
AtPrx21	AT2G37130	265471_at	0.73	1.71
AtPrx07	AT1G30870	265102_at	0.84	0.99
AtPrx06	AT1G24110	264863_at	0.99	1.01
AtPrx01 AtPrx02	AT1G05240 AT1G05250	264567_s_at	0.95	1.03
AtPrx03	AT1G05260	264577_at	0.84	1.03
AtPrx17	AT2G22420	264001_at	1.05	1.60
AtPrx18	AT2G24800	263528_at	1.01	0.98
AtPrx14 AtPrx15	AT2G18140 AT2G18150	263063_s_at	1.15	1.08
AtPrx10	AT1G49570	261606_at	0.96	4.36
AtPrx12	AT1G71695	261518_at	0.87	1.49
AtPrx04	AT1G14540	261474_at	1.40	1.00
AtPrx05	AT1G14550	261475_at	2.81	0.76
AtPrx08	AT1G34510	261157_at	1.09	1.31
AtPrx09	AT1G44970	260941_at	0.61	1.77
AtPrx26	AT2G43480	260539_at	0.80	0.53
AtPrx11	AT1G68850	260035_at	1.04	1.78
AtPrx27	AT3G01190	259276_at	0.87	0.60
AtPrx28	AT3G03670	259197_at	0.64	1.02
AtPrx30	AT3G21770	257952_at	0.92	1.12
AtPrx29 AtPrx[P]29	AT3G17070 AT3G42570	257890_s_at	1.03	0.96
AtPrx19	AT2G34060	256713_at	0.83	0.68
AtPrx31	AT3G28200	256578_at	1.78	0.75
AtPrx37	AT4G08770	255110_at	0.99	2.25
AtPrx38	AT4G08780	255111_at	1.24	2.03
AtPrx39	AT4G11290	254914_at	0.75	1.51
AtPrx42	AT4G21960	254386_at	0.94	0.98
AtPrx43	AT4G25980	254036_at	1.03	0.88
AtPrx44	AT4G26010	253998_at	0.80	0.83
AtPrx45	AT4G30170	253667_at	0.90	1.32
AtPrx46	AT4G31760	253513_at	0.94	0.93
AtPrx47	AT4G33420	253332_at	1.23	1.73
AtPrx48	AT4G33870	253313_at	1.12	0.91

AtPrx50 AtPrx51	AT4G37520 AT4G37530	253099_s_at	0.96	1.14
AtPrx33 AtPrx34	AT3G49110 AT3G49120	252291_s_at	1.03	1.68
AtPrx35	AT3G49960	252238_at	1.22	3.16
AtPrx36	AT3G50990	252138_at	0.93	0.85
AtPrx52	AT5G05340	250798_at	0.76	2.00
AtPrx54	AT5G06730	250702_at	1.07	2.54
AtPrx53	AT5G06720	250646_at	1.02	1.48
AtPrx55	AT5G14130	250200_at	0.90	1.48
AtPrx56	AT5G15180	250157_at	0.85	0.56
AtPrx57	AT5G17820	250059_at	0.85	0.89
AtPrx60	AT5G22410	249934_at	1.46	1.04
AtPrx61	AT5G24070	249766_at	1.67	0.79
AtPrx62	AT5G39580	249459_at	1.82	8.37
AtPrx63	AT5G40150	249392_at	0.84	0.60
AtPrx64	AT5G42180	249227_at	0.78	1.35
AtPrx65	AT5G47000	248822_at	1.03	0.89
AtPrx66	AT5G51890	248382_at	1.00	0.96
AtPrx68	AT5G58400	247857_at	0.93	1.09
AtPrx67	AT5G58390	247812_at	0.97	1.02
AtPrx70	AT5G64110	247326_at	0.90	2.69
AtPrx71	AT5G64120	247327_at	0.87	3.23
AtPrx69	AT5G64100	247297_at	0.83	1.06
AtPrx72	AT5G66390	247091_at	0.99	1.92
AtPrx73	AT5G67400	246991_at	0.87	1.01
AtPrx49	AT4G36430	246228_at	0.95	2.25
AtPrx58	AT5G19880	246145_at	0.92	1.37
AtPrx59	AT5G19890	246149_at	1.04	2.53
AtPrx40	AT4G16270	245488_at	0.78	0.97
AtPrx41	AT4G17690	245376_at	0.96	0.90

3.3.3. Cell viability and peroxidase activity in knockout mutant of *AtPRX62* exposed to low pH

As expected, the Col-0 and the *atprx62* KO mutant treated at pH 5.8, showed viable root cells, with almost no Evans blue uptake (Figure 2 A, B, Figure 3). The Col-0 roots treated at pH 4.6 showed decreased cell viability as expected (Figure 2, J, Figure 3). However, the *atprx62* KO was less sensitive to pH 4.6 than Col-0 roots, indicated by decreased Evans blue uptake (Figure 2 K, Figure 3).

Peroxidase activity showed a similar behavior in Col-0 and KO *atprx62* mutant roots treated at pH 5.8 (Figure 2 C, D). The Col-0 roots showed an increase in peroxidase activity when treated at pH 4.6 for 2 h (Figure 3 B) with the marked strong color in the stele cells (Figure

2 L). In the *atprx62* KO there was also an increase in peroxidase activity in the meristematic and transition zone compared to roots treated at pH 5.8 (Figure 3 B). However, the strong activity signal in stele cells was not observable (Figure 2 M).

3.3.4. *In situ* mRNA hybridization of *AtPRX62* in roots exposed to low pH

The above results demonstrated that in roots of *atprx62* KO cell death was decreased in roots treated with low pH. Thus, we looked for AtPRX62 mRNA by whole mount *in situ* hybridization.

A weak signal for *AtPRX62* mRNA was detected in Col-0 roots treated at pH 5.8 and hybridized with antisense probe for *AtPRX62* (Figure 2 E). This signal was similar to the signal found in the Col-0 treated at pH 5.8 and hybridized with the antisense probe for *AtPRX62* (Figure 2 F). A, similar signal was also observed in the *atprx62* KO treated at pH 5.8 and hybridized with the antisense probe for *AtPRX62* (Figure 2 G). Thus, this in roots treated at pH 5.8 the staining was most likely background rather than mRNA for *AtPRX62*.

Roots of Col-0 treated at pH 4.6 showed an increased signal in the meristematic and transition zone for *AtPRX62* mRNA hybridized with the antisense probe (Figure 2 N). This signal corresponds to the same region of cell death (Figure 2 J). In contrast, roots of Col-0 treated at pH 4.6 but hybridized with the sense probe for *AtPRX62* showed a weak signal (Figure 2 O), resembling roots treated at pH 5.8. Roots of *atprx62* KO treated at pH 4.6 only showed a weak signal for *AtPRX62* mRNA, hybridized with the antisense probe for *AtPRX62* (Figure 2 P).

In all cases, the ratio of signal to background was high. Thus, the stronger staining in stressed Col-0 roots can be interpreted as *AtPRX62* mRNA accumulation during stress. Also, the lack of signal in the KO *atprx62* with antisense probe for *AtPRX62* indicates the specificity of the probe. As a positive control, we also used probes for mRNA of *AtPRX42* that showed constitutive expression in roots of Lager et al. (2010) treated at low pH (Table 1). The antisense probe showed a strong signal in WT roots treated at pH 5.8 or 4.6 (Figure 2 H, Q). As expected, the sense probe produced only background in roots treated at pH 5.8 or 4.6 (Figure 2 I, R).

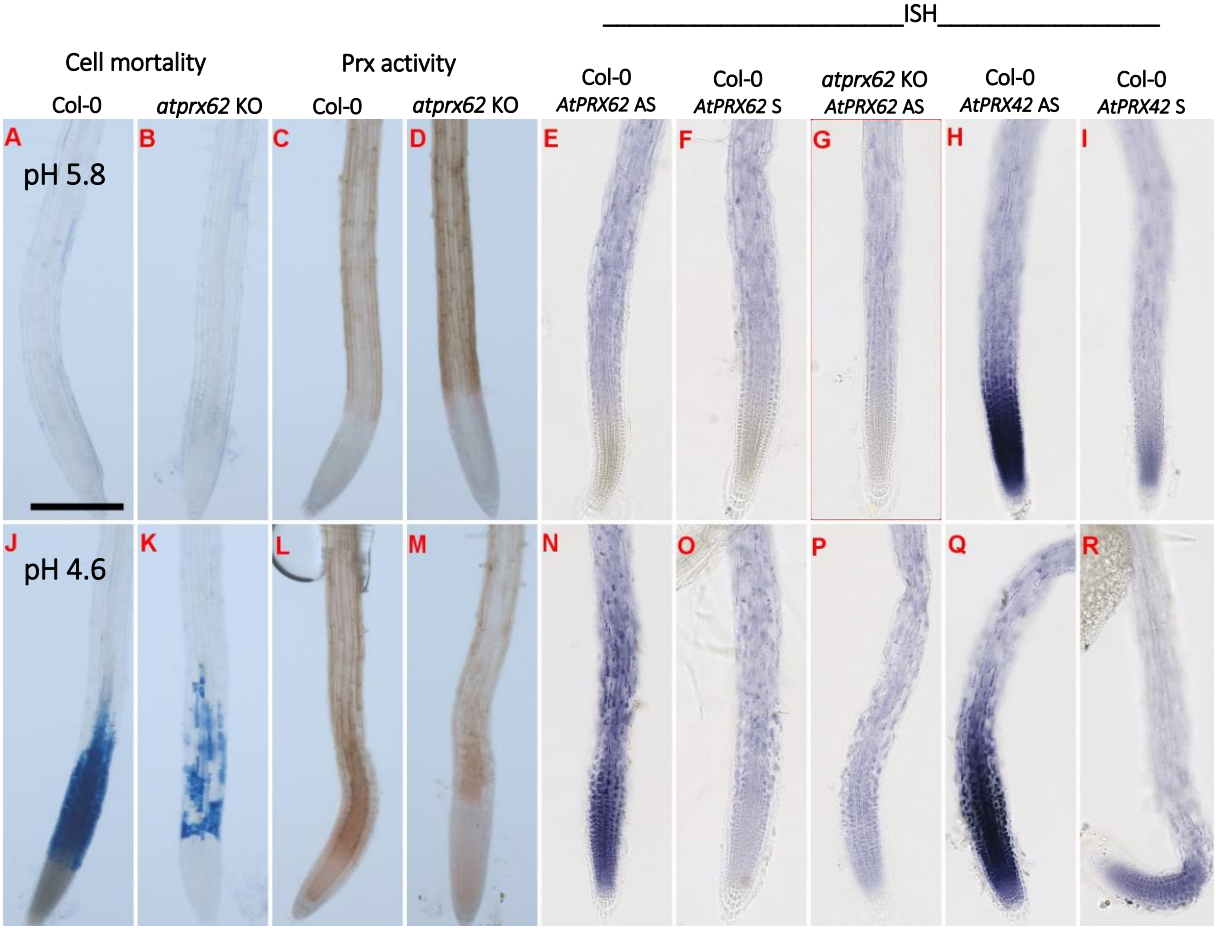
ISH
Cell mortality
Prx activity
Col-0
atprx62 KO
Col-0
atprx62 KO
Col-0 AtPRX62 AS
Col-0 AtPRX62 S
atprx62 KO AtPRX62 AS
Col-0 AtPRX42 AS
Col-0 AtPRX42 S
A
pH 5.8
B
C
D
E
F
G
H
I
J
pH 4.6
K
L
M
N
O
P
Q
R

Figure 2. Cell viability, peroxidase activity and *in situ* hybridization for *AtPRX62* mRNA in Col-0 and *atprx62* KO mutant treated at pH 4.6 or 5.8 for 2 h. A-I, Roots treated at pH 5.8 for 2h. A, Col-0 and B, KO *atprx62* stained with Evans blue. C, Col-0 and D, KO *atprx62* stained with guaiacol for peroxidase activity. E, Antisense probe and F, sense probe for *AtPRX62* in Col-0. G, Antisense probe for *AtPRX62* in KO *atprx62* mutant. H, Antisense probe and I, sense probe for *AtPRX42* in Col-0. J-R, Roots treated at pH 4.6 for 2h. J, Col-0 and K, KO *atprx62* stained with Evans blue. L, Col-0 and M, KO *atprx62* stained with guaiacol for class III peroxidase activity. N, Antisense probe and O, sense probe for *AtPRX62* in Col-0. P, Antisense probe for *AtPRX62* in KO *atprx62*. Q, Antisense probe and R, sense probe for *AtPRX42* in Col-0. Scale bar indicate 200 μm. Three independent experiments (A-D; J-M) were performed with similar results and representative images are shown. Two independent experiments (E-I; N-R), with 25 roots, were performed with similar results and representative images are shown.

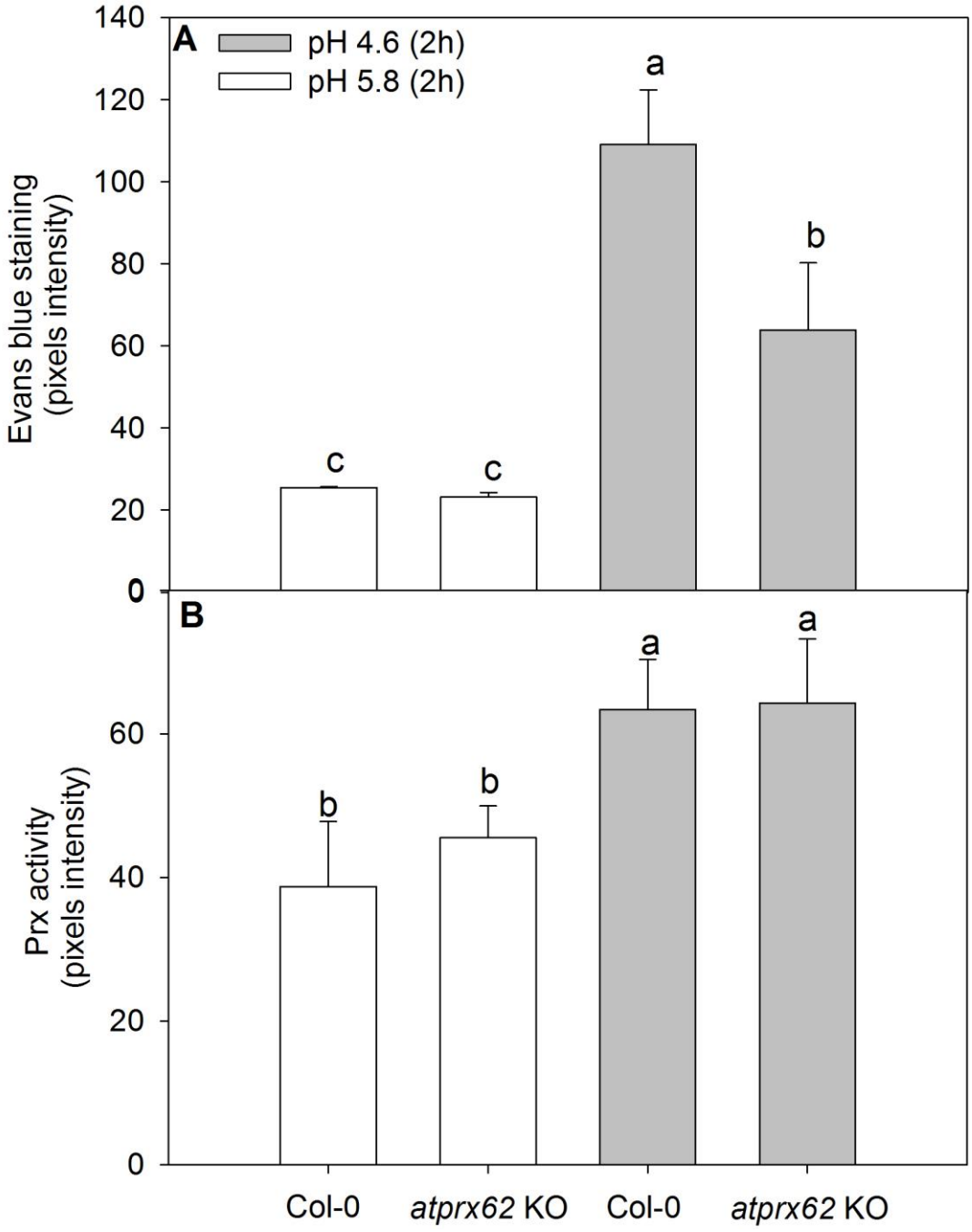

Figure 3. Evans blue staining as indicative of cell mortality and CIII peroxidase activity in roots of Col-0 or KO *atprx62* treated at pH 5.8 or 4.6 for 2 h. A, After images of Evans blue staining in roots were taken, the pixels were counted in ± 500 μm of root tips. The increase in pixels corresponds to an increase in cell death. The bars are standard error of three separate experiments. Statistical analysis was performed by Duncan's test. B, After images of peroxidase staining in roots were taken,

the pixels intensity were counted in ± 350 µm of root tips. The increase in pixels corresponds to an increase in peroxidase activity. The bars are standard deviation of twenty roots from two separate experiments. Statistical analysis was performed by Duncan's test.

3.4. Discussion

Class III peroxidases play a central role in the formation and modification of the cell wall. Hence, we aimed to investigate the role of CIII Prx in cell sensitivity to low pH. Here, we demonstrate that peroxidase activity increased upon low pH treatment and this coincided spatially and temporally with cell death. Importantly, AtPRX62 displayed positive regulation of low-pH induced cell death. These findings are a step forward to understand the role of peroxidases isoforms in root responses to low pH.

Cell death progressed from TZ towards MZ between 2 to 3 h of treatment at pH 4.6. Interestingly, peroxidase activity increased first in the damaged TZ and also in the MZ at 2 h of low pH exposure before these cells became dead (Figure 1). Thus, an increase in peroxidase activity coincided with cell death. Notably, high peroxidase activity was observed after 3 h within the stele (Figure 1). Indeed, peroxidase activity was a good marker for low pH stress in roots of tomato (Gracas et al., 2016).

The increase in total peroxidase activity can be interpreted as having either direct involvement in cell death or involvement in the alleviation of cell damage. In the first scenario, damages in TZ would be a consequence of increased peroxidase activity in TZ and MZ. In the second, the increase in peroxidase activity would be an attempt to prevent further damages, but if the stress was overwhelming and root cells were not able to respond they would die. Indeed, peroxidases have been reported to be involved in the alleviation of cell damage, as a wound-response enzyme to several stresses (Minibayeva et al., 2015). However, as discussed below, this does not seem to be the case here.

We searched for peroxidases induced by low pH treatment examining transcriptomic data of Lager et al. (2010). Among the 73 genes coding for CIII Prx, *AtPRX62* displayed the highest ratio of induction (8.37 fold) in pH 4.5 compared to pH 6.0 (Table 1).

In the absence of functional AtPRX62 (AT5G39580) protein, conferred by a KO mutation, roots showed less cell death due to pH 4.6 compared to Col-0 roots. Thus, AtPRX62 is likely positively involved in regulation of cell death during the stress and not in alleviation of cell damages. This was evident from less Evans blue uptake in *atprx62* KO (Figure 2 K, Figure 3). We

also examined a *atprx62* KN (50 %) that suffered more cell death than *atprx62* KO, but, slightly less than Col-0 roots (data not shown).

It was rather remarkable to observe such a drastic decrease in peroxidase activity in roots of the *atprx62* KO treated at pH 4.6 (Figure 2 M, Figure 3) since, so far, 38 peroxidase isoforms were identified in *A. thaliana* roots by proteomic analysis (Nguyen-Kim et al., 2016) and it would seem likely that these peroxidase isoforms could play overlapping and redundant roles.

It is also interesting that the KO of AtPRX62 decreased the signal of peroxidase activity only in the stele of TZ and MZ of roots treated with low pH. The *atprx62* KO did not decrease total peroxidase activity in roots (Figure 2 D, M, Figure 3). Thus, the signal of peroxidase activity present in stele cells in MZ and TZ observed in WT roots at pH 4.6 seems to be linked to activity of AtPRX62 because it was lost in *atprx62* KO treated at pH 4.6 (Figure 2 L, M). Furthermore, the expression map for *AtPRX62* obtained from the literature shows a signal in stele cells at the TZ and beginning of EZ (Figure S1). Thus, the peroxidase activity in stele cells of MZ under low pH stress was most likely due to activity of AtPRX62.

The ISH showed a correlation between the zone of cell death due to low pH and expression of *AtPRX62*. The mRNA for *AtPRX62* accumulated highly in the TZ and MZ where cells died due low pH exposure (Figure 2 J, N). This corroborates the possible role of this protein in positive regulation of cell death due to low pH.

Although ISH is not quantitative, the signal from the antisense probe for *AtPRX62* at pH 5.8 was most probably background (Figure 2 F). The value for expression of *AtPRX62* at pH 6.0 was 7.76 (Table S1), close to the background set up as five. Importantly, this corroborates the induction of *AtPRX62* (8.37-fold) specifically at low pH and its positive link to cell death, confirmed by analysis of the *atprx62* KO in low pH treatment.

AtPRX42 showed no change in expression at both pH 6.0 or 4.5 with the ratio of 0.94 and 0.98 folds at 1 and 8 h of treatment, respectively. We took this into consideration to choose *AtPRX42* as a positive control for ISH, but, mostly, because we noticed that *AtPRX42* had absolute expression level at pH 6.0 of 13.10 folds, being the highest value of expression among all CIII Prx at both 1 and 8 h of treatment (Table S1). Although these are not data obtained by RT-qPCR we speculate that AtPRX42 could be abundant in roots. Indeed, a similar strong signal of *AtPRX42* was observed in roots treated with both pH 5.8 or 4.6 (Figure 2 H, Q). These information obtained by examining the original data of Lager et al. (2010), were very important to find this a candidate peroxidase isoform to play a role in low pH stress among the 73 CIII Prx isoforms (Valerio et al., 2004).

The *AtPRX10* and *AtPRX71* were also highly up-regulated after 8 h of low-pH treatment with the ratio of induction in pH 4.5 compared to pH 6.0 of 4.36 and 3.23 folds, respectively. The expression of AtPRX71 was induced by impairment of cell wall integrity due to treatment with isoxaben (Raggi et al., 2015).

Despite the involvement of AtPRX62 in cell death, we do not yet know if during low pH stress AtPRX62 activity in the apoplast merely induced detrimental changes in the cell wall, such as the loosening of the cell wall which we discussed in chapter II. Alternatively, the activity might be part of an orchestrated network for cell death, as a signaling protein.

Curiously, AtPRX62 does not seem to be regulated by STOP1 (Sawaki et al., 2009), a transcription factor involved in low pH and Al tolerance, which has been intensively studied in recent years (Iuchi et al., 2007; Sawaki et al., 2009; Kobayashi et al., 2013). In chapter II, we demonstrate that reduced cell wall integrity perception in the *wak-1* mutant reduced cell death caused by low pH. It could be that *AtPRX62* is regulated by a WAK-1 signaling pathway. Intriguingly, WAK-1 expression appears to be affected in *stop1* relative to WT under low pH stress (Sawaki et al., 2009).

AtPRX62 is also not a direct target of UPBEAT1, a transcription factor that regulates the expression of many peroxidase isoforms and the balance between H_2O_2 and $O_2^{\bullet-}$ controlling root growth (Tsukagoshi et al., 2010). However, AtPRX62 was up-regulated in *A. thaliana* roots after 6 h of Al treatment (Kumari et al., 2008).

Unfolded proteins of the secretory pathway can accumulate in the endoplasmic reticulum causing stress that disturbs the most vital cellular functions and also can activate programmed cell death (Urade, 2007). Interesting, the AtPRX62 is an up-regulated gene during endoplasmic reticulum stress (Martinez and Chrispeels, 2003). Also, oligogalacturonides can trigger plant immune response leading to cell death. Suspension-cultured cells of *A. thaliana* treated with oligogalacturonides showed down-regulation of AtPRX62 (Moscatiello et al., 2006). Indeed, AtPRX62 expression is negatively regulated during hypoxia stress by ethylene responsive factor ERF73/HRE1 (Yang et al., 2011). Thus, this protein seems involved in responses to various stresses that cause cell death.

ROS distribution in roots is crucial for root development. ROS can be produced by class III peroxidases that also use it to oxidize cell wall components and physically change the cell wall properties (Passardi et al., 2005; Dunand et al., 2007). A balance between $O_2^{\bullet-}$ and H_2O_2 levels is finely adjusted in the root tip and apparently coordinates cell production and root growth (Tsukagoshi et al., 2010).

The signal for superoxide distribution in roots treated at pH 5.8 was strong in the MZ and beginning of TZ, and was similar to the superoxide distribution reported by Dunand et al. (2007). Low pH caused a marked decrease in superoxide levels in the MZ and beginning of TZ within 2 h of treatment and apparently preceded both the increase in peroxidase activity and cell death (Figure 1). This result was not a direct effect of pH on NBT staining since the reaction was performed after low pH treatment in phosphate buffer at pH 6.1.

It was reported that, *in vitro*, horseradish peroxidase can use H_2O_2 and $O_2^{\cdot-}$ in the hydroxylic cycle (Chen and Schopfer, 1999). Although we did not examine $O_2^{\cdot-}$ distribution and peroxidase activity in stressed roots before 2 h of stress, peroxidase activity could be a possible explanation for the observed decrease on $O_2^{\cdot-}$ in low pH stressed roots. However, it is not clear in the literature whether peroxidases can perform this reaction *in vivo*.

Naturally, we asked whether $O_2^{\bullet-}$ distribution was altered in the *atprx62* KO mutant when compared to the WT, especially in roots treated at pH 4.6. However, there was no difference (Figure S3), indicating that this Prx isoform is not involved in the reduction of superoxide levels during low pH stress.

The control of ROS production is critical for survival of root cells undergoing stress. Peanut roots treated with Al showed increased cell death that was alleviated by addition of ROS scavengers and antioxidative enzymes (Huang et al., 2014b). The mitochondria-dependent release of ROS was interpreted as a trigger for PCD (Huang et al., 2014a; Huang et al., 2014b).

Barley roots displayed cell death with 48 h of Al treatment and this was linked to an increased H_2O_2 production (Simonovicova et al., 2004). Also, it was found that inhibition of CIII Prx with SHAM decreased H_2O_2 production. Thus, the increase in production of H_2O_2 by peroxidase was linked to cell death in Al-treated roots. Although the responses to low pH alone cannot be evaluated in the work cited above because of a lack of control at higher pH (>5.5), their results regarding ROS production and cell death seems to be different from what was found here.

In low-pH stressed roots, we found increased peroxidase activity, decreased superoxide levels and no changes in H_2O_2 levels. Thus, excessive ROS levels were not a cause of cell death. Whether the drastic decrease of superoxide in root tips was itself involved in cell death it remains to be investigated. Given the importance of the balance of ROS for root development (Tsukagoshi et al., 2010), this possibility seems quite plausible.

In conclusion, we demonstrate that AtPRX62 is a positive regulator of low-pH induced cell death in root tip cells. This information further establishes low pH stress and associated cell death as a cell wall process. This study also illustrates the importance of reverse genetic studies to

uncover the function of peroxidases (Francoz et al., 2015). The increase in peroxidase activity, especially of *AtPRX62*, and the decrease of superoxide in the MZ and TZ may be part of a cell death signaling network.

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Supplementary Material

Table S1. Data mining of transcriptomic data from Lager et al. (2010) for response of A. thaliana roots to low pH. The ratio of absolute expression for roots treated at low pH (pH 4.5) vs roots of control treatment pH (pH 6.0). Also the relative mean values of expression at pH 4.5 or 6.0 at 8 h of treatment are showed.

		mean 8 h (n = 3)		
		pH 4.5	**pH 6**	8 h Ratio
AT5G39580	*AtPRX62*	**10.82**	**7.76**	8.37
AT4G21960	*AtPRX42*	**13.10**	**13.13**	0.98

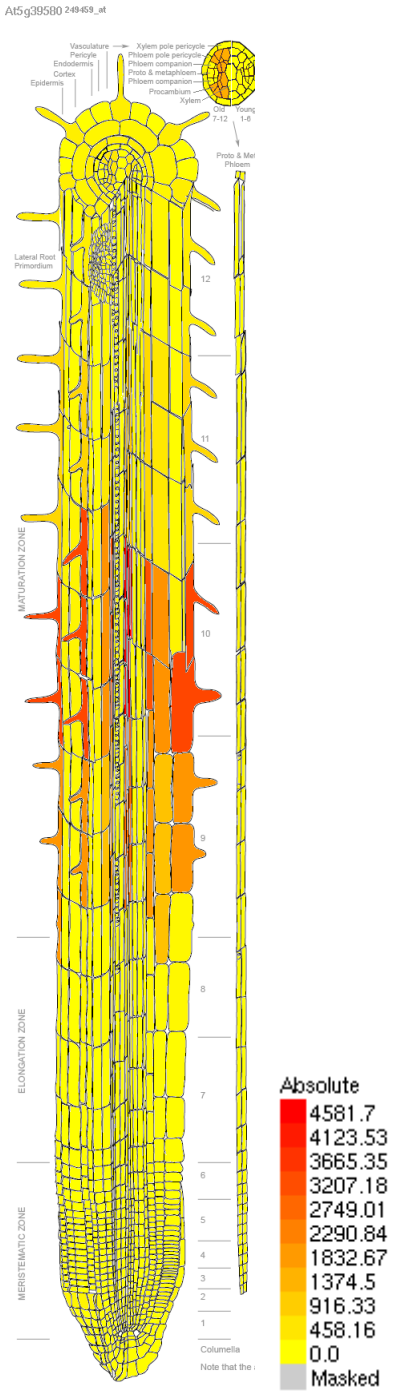

Figure S1. Absolute expression map for Arabidopsis thaliana class III peroxidase 62 (AT5G39580, probe 249459_at) in roots. The colors are from yellow to red in accord with expression level. This map can be accessed in http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi.

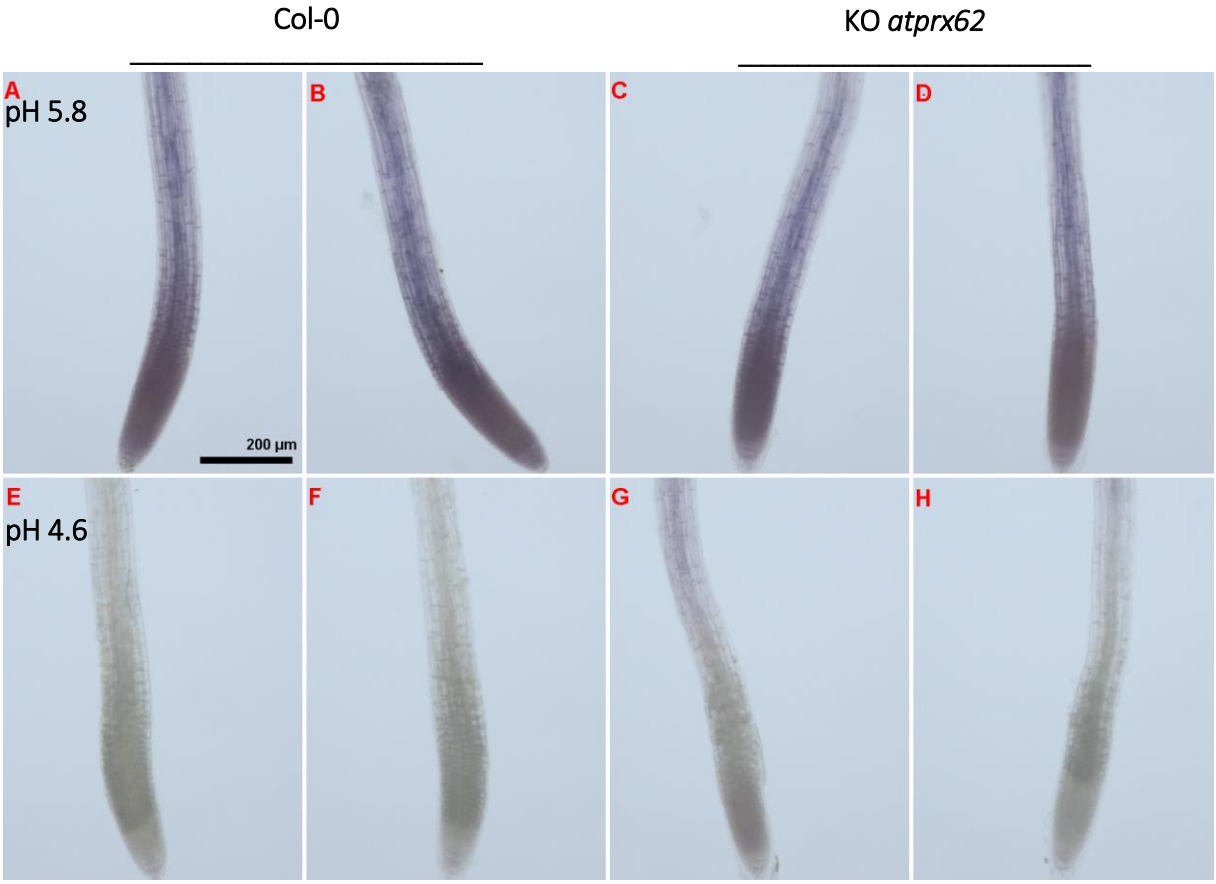

Figure S2. NBT reaction showing superoxide distribution in roots of *A. thaliana* (Col-0) and KO *atprx62* treated at pH 5.8 or pH 4.6 for 2 h. A and B, roots of Col-0 treated at pH 5.8 for 2 h. C and D, roots of Col-0 treated at pH 4.6 for 2 h. E and F, roots of KO *atprx62* treated at pH 5.8 for 2 h. G and H, roots of KO *atprx62* treated at pH 4.68 for 2 h. The images are representative of at least 15 roots of each treatment.

4. ETHYLENE DECREASES CELL MORTALITY DUE TO LOW pH: THE ROLE OF CLASS III PEROXIDASES AND ETR1 SIGNALING

ABSTRACT

One irreversible consequence of acidic pH for roots is cell death. Growing evidence suggests the role of hormones and cell-wall-related enzymes in responses that possible could avoid cell mortality. Here, we investigated the role of ethylene pretreatment and class III peroxidase activity on sensitivity to further low pH treatment. Seedlings of *Arabidopsis thaliana* were pretreated with ethylene and then exposed to low pH for 2.5 h. In contrast to roots without ethylene treatment, roots pretreated with 3, 8, 131 and 1479 ppm of ethylene for 3 h become tolerant to subsequent low pH, with negligible cell mortality. This effect of ethylene was time-dependent since it was achieved only when seedlings were preincubated with ethylene for 3 h. This tolerance induced by ethylene was dependent on ETR1, since *etr1-1* suffered high cell mortality when exposed at low pH similar with ethylene pretreatment. Also, *etr1-1* displayed increased cell mortality due to mild acidic low pH. These data indicates that ethylene signaling through ETR1 invokes cellular changes that trigger tolerance to low pH. We added SHAM to inhibit CIII Prx during ethylene incubation. The addition of SHAM abolished the tolerance to low pH induced by ethylene and this was similar in *etr1-1*. Thus, a SHAM-sensitive process, most likely CIII Prx activity may play a central role in this tolerance to low pH induced by ethylene treatment.

Keywords: Low pH; Ethylene application; Class III peroxidases; Cell mortality

4.1. Introduction

Acid soils are disadvantageous for many cultivated plants due to inhibition of root growth. One irreversible consequence of low pH stress is the death of cells from root tip cells (Koyama et al., 2001). Investigation of the cellular players involved with this low pH-induced cell death has received little attention in the last years. Evidences are growing that cell wall modification and hormone signaling are likely involved in a plant response that ultimately might generate tolerance to stress (Lager et al., 2010; Kobayashi et al., 2014).

A compelling evidence that low pH affects cell wall is that root hairs burst at low pH but only those that are elongating (Bibikova et al., 1998). Indeed, low pH treatment promoted major changes in expression of genes related to cell wall in comparison to other cellular compartments (Lager et al., 2010).

Besides a direct toxic effect of low pH, root growth reduction during low pH stress can be an active plant mechanism to avoid cell mortality (Graças et al., 2016). Plants can stop root growth quickly by inhibiting expansion and ethylene has an important role in this inhibition (De Cnodder et al., 2005; Swarup et al., 2007). The application of ACC rapidly stops cell expansion in EZ (Le et al., 2001).

In *A. thaliana* roots, this ethylene-dependent cessation of cell expansion is in part mediated by expression of genes related to cell wall modifications (Markakis et al., 2012). Among these genes are those coding for class III peroxidases (CIII Prx). CIII Prx can cross-link cell wall components and promote stiffening of the wall (Passardi et al., 2004), contributing to reduction in root growth. Indeed, *A. thaliana* roots treated with low pH showed differential regulation of eight AP2/EREBPs (APETALA2/ethylene-responsive element binding proteins), seven of them being early-induced and one early-repressed (1 h) (Lager et al., 2010). Also, it was reported that one CIII Prx gene was early-induced, one early-repressed, but eight were late-induced (8 h).

Roots treated with Al^{3+} showed increased ethylene production and decreased root growth that was dependent on ethylene disruption of auxin transport and distribution (Sun et al., 2010). The role of this root growth inhibition in Al^{3+} toxicity is not clear. It is possible that low pH by itself is important for the ethylene-dependent responses observed during Al^{3+} treatment. However, this could not be appropriately evaluated since this study, as many other Al^{3+} studies, lacked a control at higher pH.

We hypothesize that ethylene can promote cellular responses, especially cell wall modifications, which can ensure cell viability in low pH stress. Also, CIII Prx activity may be a downstream response to ethylene signaling that is required for tolerance. To examine this, we used different approaches: exogenous ethylene application, inhibition of ethylene synthesis and a genetic approach with a mutant of an ethylene receptor (ETR1). Among the five ethylene receptors in *A. thaliana*, ETR1 plays a predominant role in the signaling of this hormone (McDaniel and Binder, 2012).

Here, we assembled evidence showing that ethylene promotes tolerance to low pH. This tolerance caused by ethylene was dependent on ETR1 signaling and a SHAM-sensitive process, most likely CIII Prx activity.

4.2. Materials and Methods

4.2.1. Plant growth conditions

Seeds of *A. thaliana* were disinfected in commercial sodium hypochlorite solution (5%) for 10 min under stirring and then washed five times with distilled water. The seeds were then transferred to Petri dishes with 0.8% of agar and a modified Hoagland's solution at pH 5.8. Macronutrients consisted of 4 mM Ca $(NO_3)_2$, 6 mM KNO_3, 1 mM $MgSO_4$ and 2 mM

$NH_4H_2PO_4$. Micronutrients were composed of 20 µM $MnSO_4$, 14 µM $ZnSO_4$, 20 µM H_3BO_3, 0.03 µM $NiSO_4$, 0.02 µM Na_2MoO_4, 0.02 µM $CuSO_4$, 0.02 µM $CoCl_2$ and 30 µM $FeSO_4$.

The growth temperature was 22 ° C and the light intensity was approximately ± 120 $\mu E.m^{-2}.s^{-1}$. For all low pH treatments, at least ten five-day-old seedlings were incubated in 250 ml Erlenmeyer with 20 ml of treatment solution composed of $CaCl_2$ (0.5 mM) and Homopipes buffer (homopiperazine-1,4-bis(2-ethanesulfonic acid) 0.6 mM. The solution was continuously aerated.

We used seedlings of the ecotype Columbia-0 (Col-0) as WT. In addition, we used the mutant for ethylene receptor *etr1-1* (Germplasm stock: CS237, Locus: AT1G66340). We grew Col-0 and *etr1-1* mutant on separate growth medium and applied ethephon (Ethrel®; 1 µM) to examine the elongation of hypocotyls. The seeds were kept in the dark for three days. The mutant was totally insensitive to ethylene since there was no inhibition of elongation. In contrast, this ethephon concentration inhibited hypocotyl elongation by 60 % in the Col-0 seedlings (data not shown).

4.2.2. Evaluation of cell death in root tips

Staining with Evans blue to assess cell viability was performed the same way as described in chapter 2. The images of Evans blue uptake were analyzed using ImageJ software. The contours of each root tip, reaching 500 µm from the apex, were drawn and the mean gray value (https://imagej.nih.gov/ij/docs/guide/index.html) of this region was calculated. From each of these values, the mean gray value of the background of the corresponding bright-field image was subtracted.

4.2.3. Evaluation of class III peroxidase activity and application of peroxidase inhibitor

Guaiacol was used as a substrate to examine the activity of CIII Prx in the roots. Guaiacol (0.125 % v/v) was diluted in 200 mM of phosphate buffer (pH 6.1). For the reaction, fresh H_2O_2 was added to the guaiacol solution to a final concentration of 1.65 mM and roots were stained in the dark. After five min the roots were washed with abundant water to stop the reaction and immediately imaged by bright-field microscopy.

We applied the peroxidase activity inhibitor, SHAM, prior to or during the exposure of roots to low pH. A stock solution of 250 mM SHAM (salicylhydroxamic acid) was prepared in DMSO. Five-day-old seedlings were transferred to the growth medium containing 0.5 mM SHAM and were incubated for 3h. For the simultaneous treatment with SHAM and low pH, we added 0.25 mM of SHAM to the treatment solution and then adjusted the pH 4.8.

The pixels from peroxidase activity were counted in area of ± 316 x 94 μm in the TZ and beginning of EZ. The results were the mean gray value. The difference in pixels between the bright field background and the roots was calculated. The images of peroxidase activity were analyzed using ImageJ software. The contours of each root tip, reaching 316 x 94 μm in the TZ and beginning of EZ, were drawn and the mean gray value of this region was calculated. From each of these values, the mean gray value of the background of the corresponding bright-field image was subtracted.

4.2.4. Ethylene and AVG application

Because of the limitations to gas diffusion in liquids, we developed a system for ethylene application to seedlings on growth medium in square plastic Petri dishes. We introduced a latex septum by perforation of the Petri dish lids to facilitate gas application and sampling. To the Petri dishes, 17 ml of growth medium was added producing a void volume of 156 cm^3. We sealed the Petri dishes with two layers of Dow Saran: Barrier food wrap® and two layers of Parafilm "M" for laboratory®. Bottles with different ethylene concentrations (C_2H_4) were prepared fresh for each experiment. From these, we applied 1 ml of ethylene to the Petri dishes, through the septum, to achieve different final concentrations.

After zero and three hours, we sampled ethylene from the Petri dish system and measured its concentration by gas chromatography. The ethylene concentration was very stable for 3 h in this system with practically no decrease in concentration (data not shown).

We also examined the effects of inhibition of ethylene biosynthesis during low pH treatment on the cell viability of roots. We added 10 μM of AVG (Aminoetoxyvinylglycine) to the treatment solution and then adjusted the final pH.

In addition, we also used ethephon, 2-chloroeethylphosphonic acid (Ethrel ®) to release ethylene in seedlings. Seedlings were simultaneously treated with different pH solutions and 1 μM of ethephon.

4.2.5. Evaluation of the length of the first epidermal cell with root hair bulge (LEH)

We evaluated the length of the first epidermal cell with root hair bulge (LHE) as described by Le et al. (2001) with some modifications. After low pH or ethylene treatment, roots were fixed in Karnovsky solution (2.5 % Glutaraldehyde, 2.0 % Paraformaldehyde, 0.05 M Cacodylat buffer, 0.001 M $CaCl_2$) overnight at 4 °C. Then, the cell walls were stained with calcofluor white (0.1% w/v) for 5 min and the cells with bulge were imaged by epi- fluorescence microscopy (Leica LM 6000B, excitation 340/40, emission 470/40).

4.2.6. Statistical analysis

A completely randomized experimental design was used in all experiments. Each analysis was composed of at least three separate experiments. Each replicate was composed of at least ten plants. For the comparison of means, the analysis of variance (ANOVA) was followed by Duncan's test. When necessary, the difference between two means was evaluated by Student's t-test at the 5% significance level.

4.3. Results

4.3.1. Cell death due to low pH modulated by concentration and duration of ethylene pretreatment

We pretreated roots with ethylene concentrations ranging from 3 to 1400 ppm to examine the effect on subsequent sensitivity to low pH. The application of 3 ppm ethylene for 3 h only slightly alleviated cell death after subsequent treatment at pH 4.6, as judged by Evans blue staining in comparison to the control without ethylene pretreatment (Figure 1). However, increasing ethylene concentration to 8, 131 and 1449 ppm resulted in a sharp decrease in cell mortality. Based on these results, we chose 8 ppm of ethylene for further experiments since most of the reduction in cell death already occurred with this concentration.

We next determined the shortest duration of pretreatment with 8 ppm ethylene that would guarantee little cell death due to low pH. Pretreatment for 0.5 or 1 h only slightly decreased cell mortality due to pH 4.6, compared to the control without ethylene pretreatment (Figure 2 A, B). Pretreatment for 2 h increased tolerance a bit further, but cell mortality due to low pH only clearly decreased with 3 h of ethylene pretreatment, where only a few cells exhibited death (Figure 2 A, B).

Thus, we chose 3 h of 8 ppm ethylene pretreatment as a minimum time to guarantee tolerance to further low pH stress.

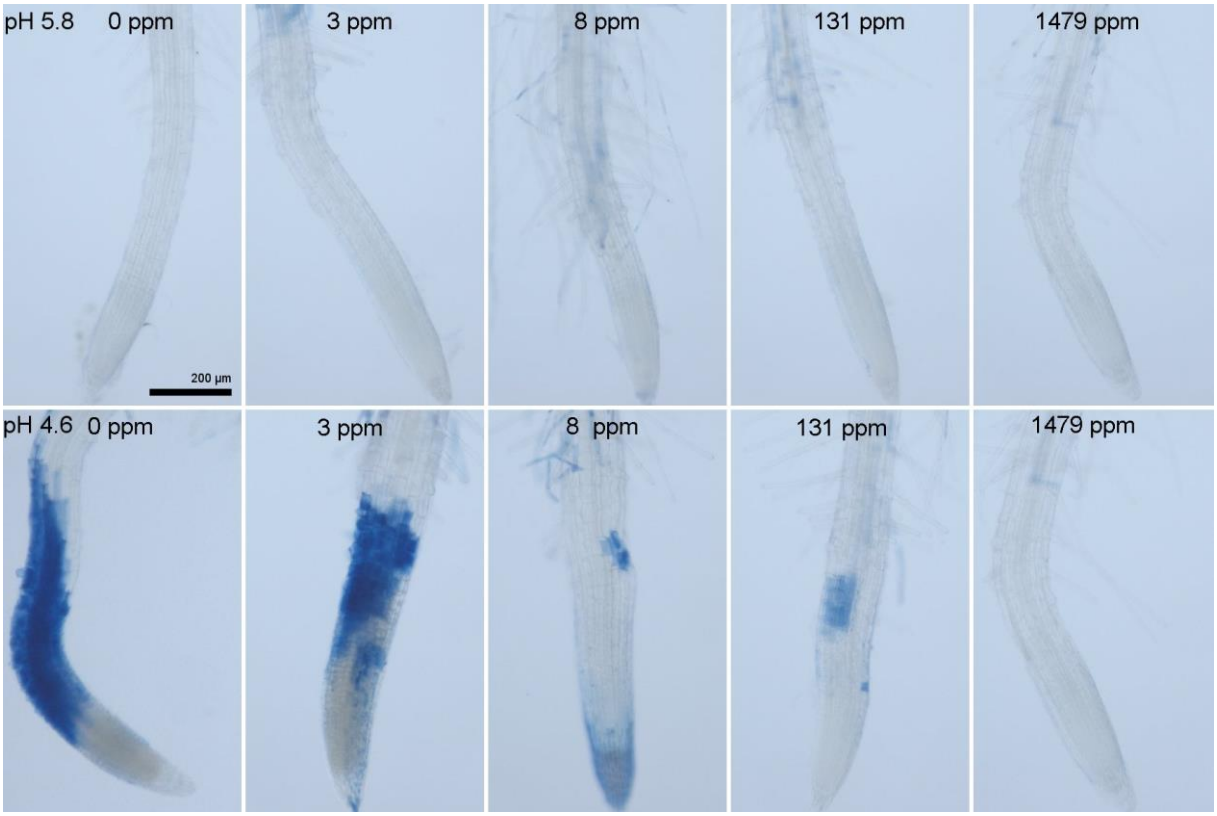

Figure 1. Cell mortality in roots due to low pH stress, modulated by ethylene pretreatment (3 h) at different concentrations. The blue color is indicative of cell death due to Evans blue uptake. Seedlings of Col-0 were first pretreated with ethylene (C_2H_4) ranging from 0 to 1479 ppm for 3 h and then subjected to pH 5.8 (upper) or 4.6 (bottom) for 2.5 h. Scale bar indicate 200 µm. Each image is representative of at least fifteen roots from the same treatment.

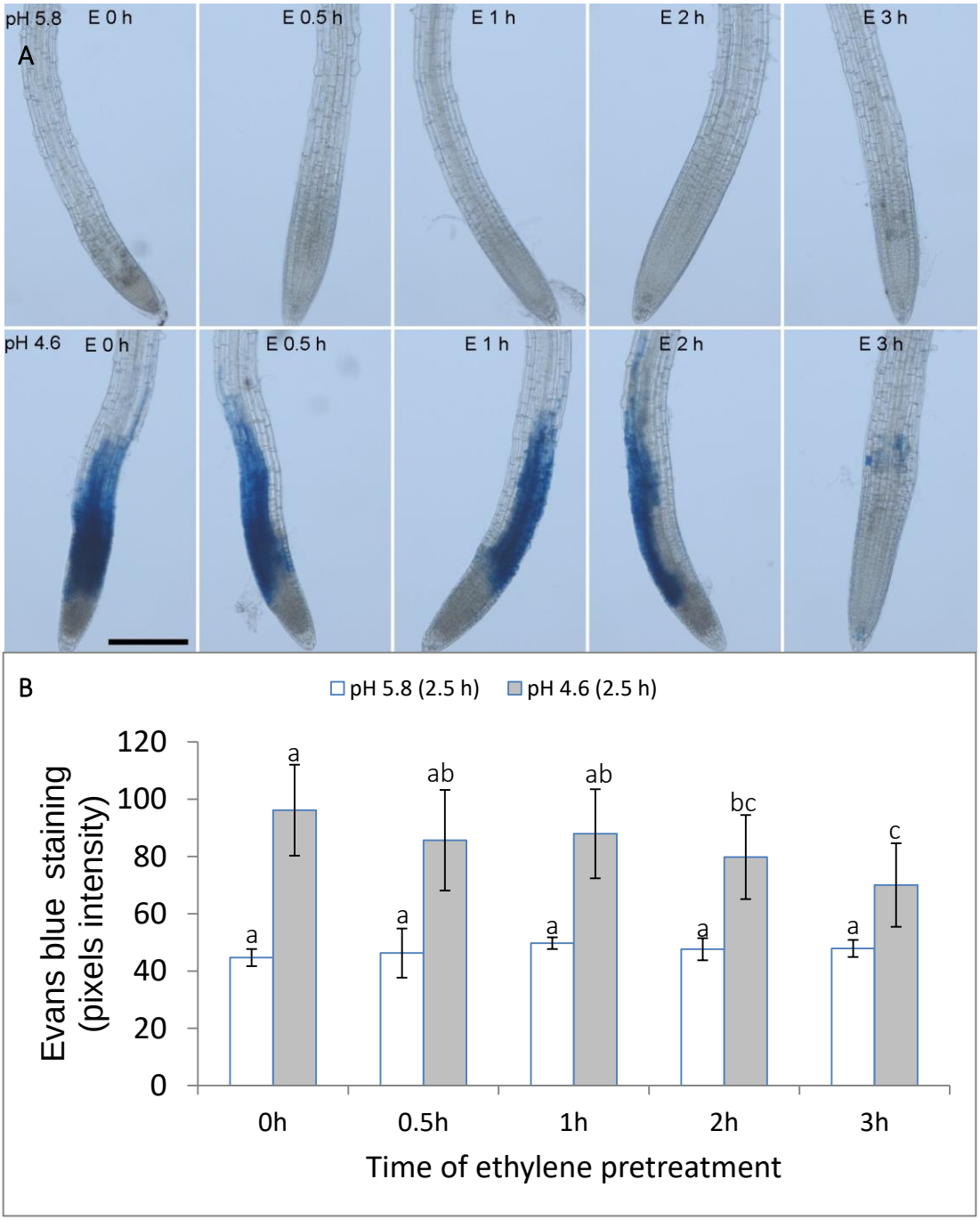

Figure 2. Cell mortality in roots due to low pH modulated by 8 ppm of ethylene over different times of incubation. The blue color is indicative of cell death due to Evans blue uptake. The seedlings of Col-0 were first pretreated with 8 ppm of ethylene (E) for 0, 0.5, 1, 2 or 3 h and then subjected to pH 5.8 (upper) or 4.6 (bottom) without ethylene for 2.5 h. Scale bar indicate 200 μm. Each image is representative of at least fifteen roots from the same treatment. B, Quantification of Evans blue uptake by pixel intensity analysis of 500 μm of root tips. The bars are SD of ten roots from two separate experiments. Letters indicate significant difference between different treatments at the same pH by Duncan's test.

4.3.2. Effects of ethylene synthesis inhibition and lack of ETR1 on cell mortality due to mild low pH stress

The above results show that ethylene pretreatment causes tolerance to low pH. To probe for an endogenous role of ethylene, we inhibited its synthesis with AVG and examined the consequences of lack of ETR1 in the sensitivity of cells to 3 h of mild stress at pH 4.8.

The *etr1-1* mutant was more sensitive than Col-0 to mild acidity stress at pH 4.8; roots displayed higher cell mortality as indicated by Evans blue uptake (Figure 3). Furthermore, Col-0 roots treated with AVG, during low pH exposure, showed increased cell death compared to Col-0 roots without AVG at pH 4.8. Treatment of Col-0 roots with AVG at pH 5.8 slightly increased cell death, but this was observed mostly in larger expanded cells (data not shown).

Together, our data indicate that blocking of ethylene biosynthesis with AVG increased cell death due to low pH in a similar manner to the lack of ethylene signaling conferred by *etr1-1*.

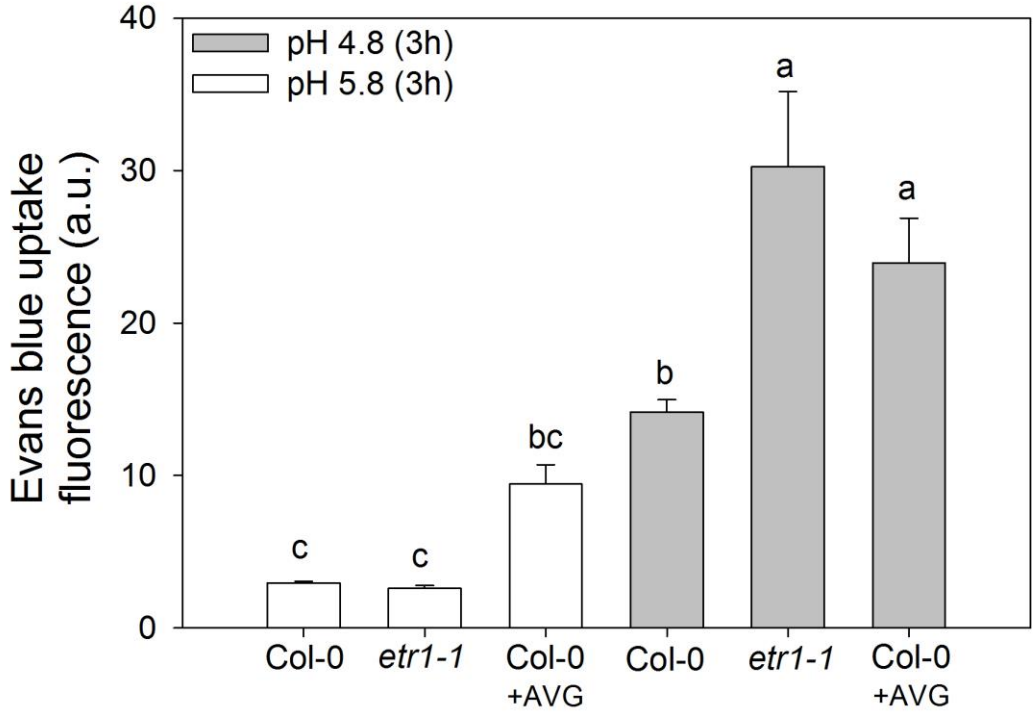

Figure 3. Effects of an ethylene receptor mutation (*etr1-1*) and an ethylene biosynthesis inhibitor (AVG) on cell mortality due to low pH stress in roots. The increase in Evans blue fluorescence is indicative of cell death. AVG (10 μM) was added to treatment solutions. The bars are standard error of three independent experiments. Letters indicate significant difference between treatments by Duncan's test.

4.3.3. Effects of ethylene and SHAM application upon peroxidase activity and cell mortality due to low pH

To examine if the low pH tolerance triggered by ethylene application is correlated with peroxidase activity, we treated seedlings with ethylene and ethylene simultaneously with 0.5 mM of SHAM. Since there are 38 isoforms of peroxidases that have been so far identified in roots of *A. thaliana* by proteomics (Nguyen-Kim et al., 2016) we used SHAM, a broad inhibitor of peroxidase activity, to circumvent redundancy among them.

Treatment of seedlings with 8 ppm ethylene for 3 h increased peroxidase activity in roots compared to control without ethylene treatment (Figure 4 A, B, D). In contrast, there was a decrease in peroxidase activity when ethylene was applied simultaneously with SHAM (Figure 4 C, D).

As expected, treatment of Col-0 seedlings with 3 ppm of ethylene for 3 h restrained cell death caused by pH 4.6. This was judged from the low Evans blue staining in comparison to the control treated with 0 ppm of ethylene and then subjected to pH 4.6 (Figure 5). Conversely, the treatment of Col-0 roots with 8 ppm of ethylene in the presence of SHAM did not cause tolerance to pH 4.6. The roots of Col-0 treated for 3 h only with SHAM and then exposed to pH 4.6 displayed cell death similar to control roots without SHAM treatment (Figure 5). SHAM was added to the growth medium and showed no effect on cell viability of roots over 3 h.

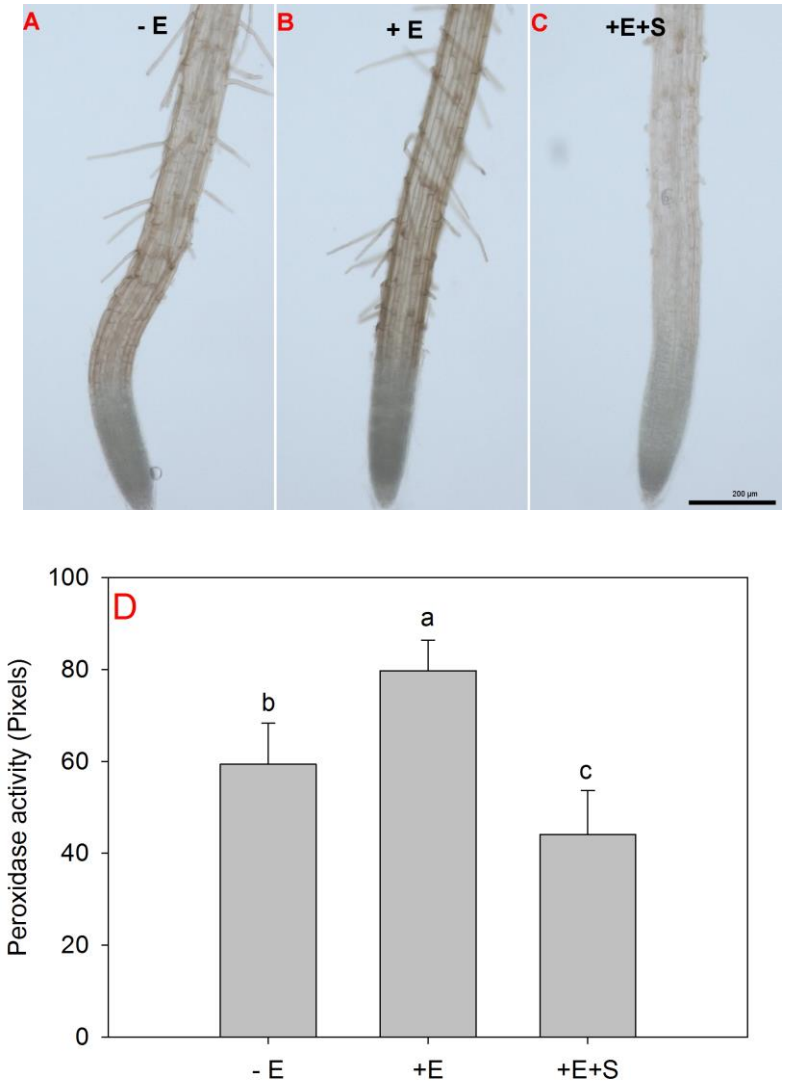

Figure 4. Activity of peroxidase class III in the roots of *A. thaliana* (Col-0) modulated by ethylene (+E) or SHAM (+S) pretreatment for 3 h. Seedlings were pretreated with 0 (A) or 8 ppm of ethylene (B), or 8 ppm of ethylene plus 500 µM of SHAM (C) for 3 h. Then, roots were stained with guaiacol and H_2O_2 to examine endogenous class III peroxidase activity, indicated by a brown color. SHAM was not added to the reaction. Scale bar indicate 200 µm. Each image is representative of at least fifteen roots from the same treatment. D, quantification of pixels of roots stained for peroxidase activity. Bars are SD of pixel quantification in ten roots. Statistical analysis was Duncan's test.

The treatment of *etr1-1* with ethylene did not trigger tolerance to subsequent treatment at pH 4.6, as observed with this same treatment of Col-0. Cell death in root tips of *etr1-1* was similar to the control without ethylene treatment. Also, simultaneous SHAM and ethylene treatment or treatment with SHAM alone in *etr1-1* did not prevent subsequent cell death by pH 4.6 (Figure 5).

In summary, our results show that the low-pH tolerance triggered by ethylene pretreatment is dependent on perception of ethylene by ETR1 receptor. Also, during ethylene treatment a SHAM-sensitive process plays a central role to trigger subsequent tolerance to low pH.

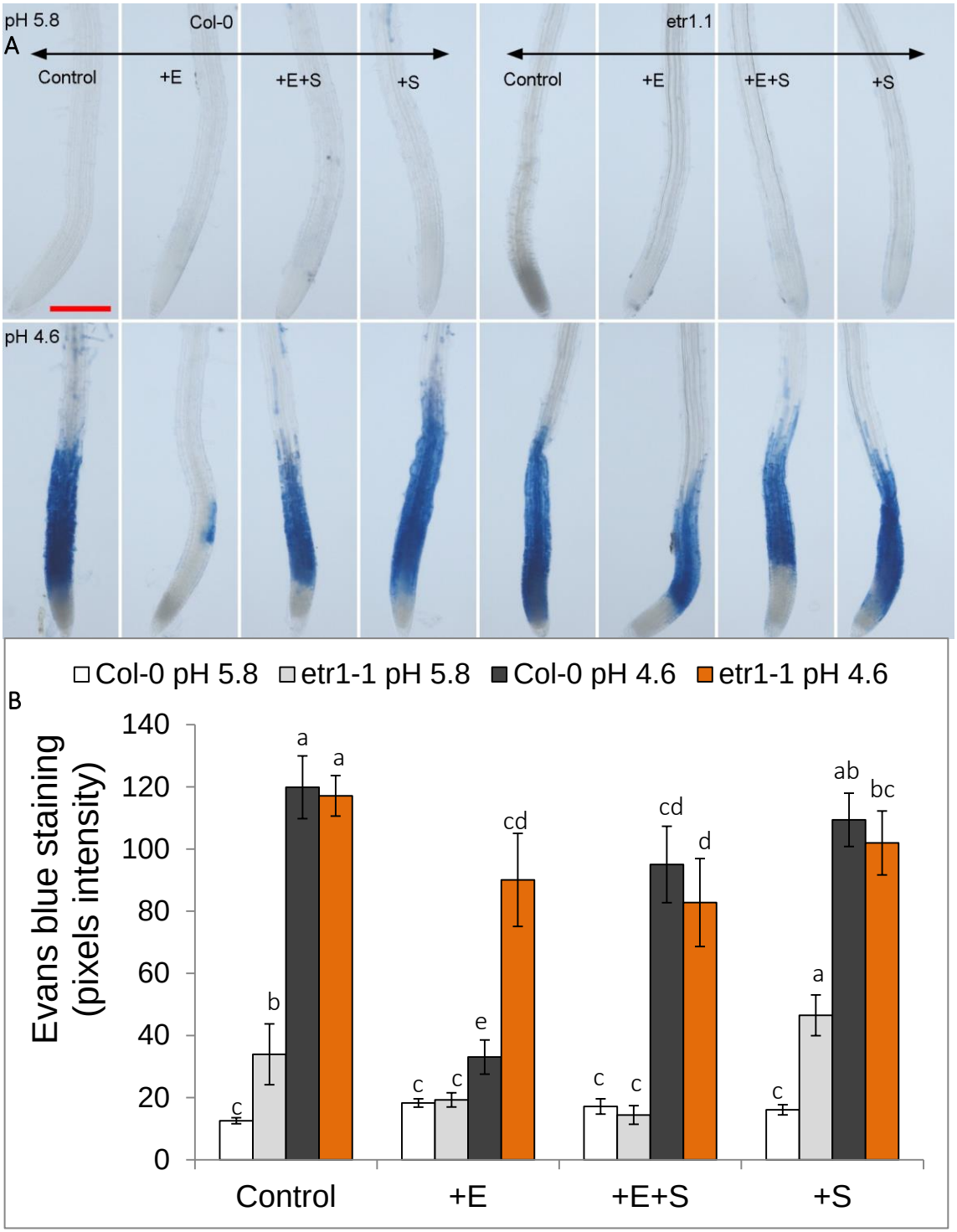

Figure 5. Effects of ethylene receptor (ETR1) or inhibition of peroxidase activity on cell mortality in roots due to low pH stress, modulated by treatment with 8 ppm of ethylene over 3 h. The blue color is indicative of cell death due to Evans blue uptake. A, seedlings of Col-0 and ethylene receptor mutant *etr1-1* were treated for three hours with 8 ppm of ethylene (+E) or with 8 ppm of ethylene with SHAM (+E+S) or only SHAM (+S) and then subjected to pH 5.8 (upper) or 4.6 (bottom) without ethylene or SHAM for 2.5 h. In the control treatment the seedlings were kept in growth medium and then were treated at pH 5.8 or 4.5. Scale bar indicate 200 μm. Each image is representative of at least fifteen roots from the same treatment. B, Evans blue staining measured by accounting of pixels intensity in the first 500 μm of root tips. The treatments are the same as described in A. Bars are SD of 10 roots from two separate experiments. Letters indicate significant difference within the same pH. Statistical analysis was performed with Duncan's test.

4.3.4. Effects of ethephon and SHAM application in cell expansion during low pH treatment

We examined the modulation of cell expansion during low pH treatment by application of ethephon or SHAM. In Col-0 roots, LEH decreased by 37 % after 1 h of treatment at pH 4.8 compared to roots treated at pH 5.8 (Figure 6). As expected, application of 1 μm of ethephon during treatment at pH 5.8 decreased LEH by about 45 %. A similar decrease in LEH was also observed with ethephon application during treatment at pH 4.8. SHAM application had no effect on LEH of roots treated at pH 5.8. However, the LEH of Col-0 roots decreased only 8 % when roots were treated at pH 4.8 with SHAM for 1 h. Simultaneous application of ethephon with SHAM did not cause marked reduction in LEH at pH 5.8. When roots were treated at pH 4.8 with ethephon and SHAM there was a decrease in LEH of only 11 % (Figure 6). Altogether, a SHAM-sensitive process caused reduction in LEH at pH 4.8. Furthermore, the remarkable decrease in LEH due to ethephon treatment was restrained by application of SHAM.

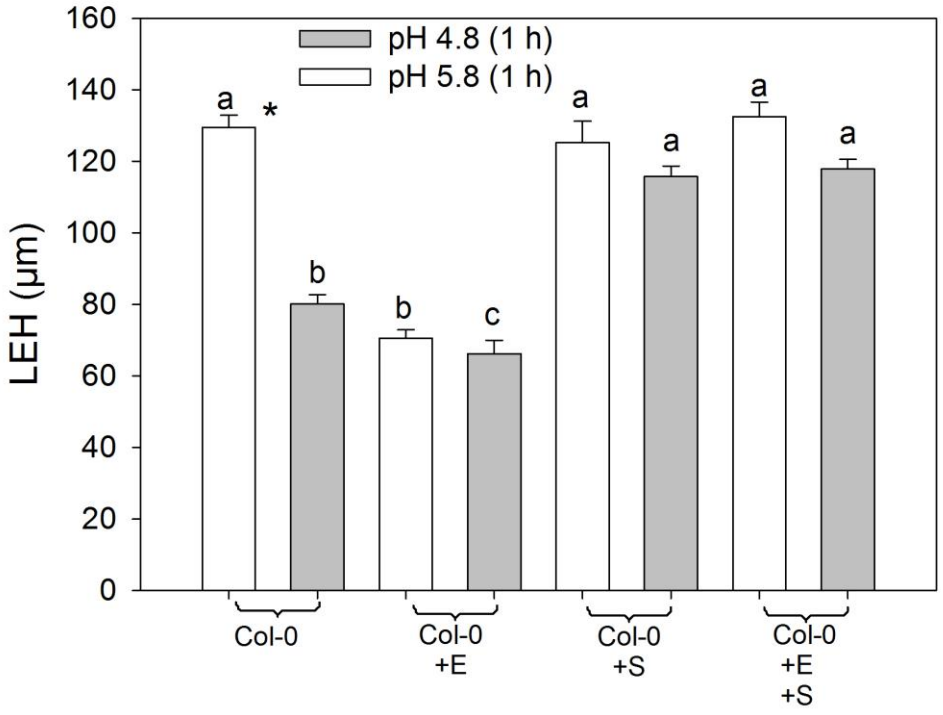

Figure 6. Length of first Epidermal cell with root Hair bulge (LEH) due to low pH treatment for 1 h modulated by ethephon application and inhibition of class III peroxidase activity by SHAM. Ethephon (+E) (1 μM) or SHAM (250 μM) was added to the treatment solution. The bars are standard error of three separate experiments. Letters indicate significant difference between different treatments at the same pH by Duncan's test. Asterisks indicate significant difference within the same treatment by Student t-test.

4.4. Discussion

Ethylene plays important role in root growth and development and is involved in responses to biotic and abiotic stresses (Le et al., 2001; Raja et al., 2017). Here, we show that ethylene promotes tolerance of *A. thaliana* roots to low pH stress by reducing cell death. We first showed this by exogenous application of ethylene and then demonstrated an endogenous role of ethylene by inhibition of its synthesis and from the response of the ethylene receptor mutant *etr1-1*.

Previous transcriptomic analysis of roots treated with low pH showed that eight transcription factors of the AP2/EREBPs (APETALA2/ethylene-responsive element binding proteins) family are differentially expressed in response to low pH (Lager et al., 2010). Seven of them were early induced and one early repressed (1 h). Thus, part of early events for the response to low pH seems to be ethylene-related.

Ethylene pretreatment promoted tolerance of root cells to subsequent low pH stress. This tolerance was dependent on the concentration and the duration of ethylene pretreatment. Tolerance to pH 4.6 increased with prior application of 8, 131 or 1479 ppm of ethylene, as indicated by negligible Evans blue staining (Figure 1). Tolerance to low pH was evident when plants were pretreated with 8 ppm of ethylene for more than 2 h (Figure 2).

Evidence that ACC treatment quickly (0.5 h) decreases cell expansion (Le et al., 2001; Ruzicka et al., 2007) suggest rapid changes in cell wall properties, especially increased stiffness. Treatment of roots with ACC for 3 h resulted in 240 differentially expressed genes (Markakis et al., 2012). Notably, the 10 most up or down-regulated are mostly coding for cell wall-related proteins. Also, two CIII Prx were up-regulated and two expansins down-regulated. Peroxidases can promote cross-linking of cell wall components and therefore cell wall stiffening. The repression of expansins, known for cell wall loosening, corroborates for this.

We are aware that a decrease in cell expansion caused by ethylene happens in the EZ where cells normally do not die significantly due to low pH (Figure S1). However, we propose that cellular events in root tips mediated by ethylene signaling, most likely cell wall changes or cell wall-related enzyme activity, are involved in tolerance to low pH.

We examined if the responses to ethylene application involve a signaling pathway through the ethylene receptor ETR1. The roots of *etr1-1* were not tolerant to low pH when pretreated with ethylene, in contrast to roots of Col-0 (Figure 5). This is direct evidence that after ethylene application ETR1 signaling is involved in events that makes cells tolerant to low pH.

There are overlapping roles among the five isoforms of ethylene receptors in *A. thaliana*, but ETR1 is predominant for ethylene responses and its loss greatly reduces silver effects, a strong ethylene signaling inhibitor (McDaniel and Binder, 2012).

To examine an endogenous role of ethylene in low pH toxicity, we inhibited ethylene biosynthesis during low pH treatment with AVG and also examined the sensitivity of the *etr1-1* mutant to low pH.

The inhibition of ethylene biosynthesis increased cell mortality during low pH exposure. Treatment of roots with AVG simultaneous with pH 4.6 increased cell death compared to Col-0 roots treated only with pH 4.6. The application of AVG only slightly increased cell death in roots treated at pH 5.8 as indicated by increased Evans blue uptake (Figure 3). The same effect was observed when we applied $AgNO_3$ at pH 5.8 a widely-used ethylene action inhibitor (data not shown).

The stress at pH 4.6 was quite severe, with death of almost all cells in MZ and TZ in both Col-0 and *etr1-1* with only 2.5 h of treatment (Figure 5). Thus, to discern any difference between these genotypes, we used a less overwhelming stress at pH 4.8. This mild stress revealed that roots of *etr1-1* were more sensitive to low pH than Col-0 roots treated at pH 4.8 for 3 h, as observed by higher Evans blue uptake (Figure 3). Interestingly, ETR1 expression in roots is concentrated in MZ, TZ and beginning of EZ (Grefen et al., 2008). Low-pH induced cell death occurred within these zones (Figure 1, Supplementary Figure S1).

The response of the *etr1-1* mutant to low pH and the effect of AVG treatment on cell sensitivity to low pH indicate that ETR1-mediated ethylene signaling has an endogenous role triggering responses to avoid cell death during low pH stress. We did not examine ethylene production during treatment at pH 4.6. Nevertheless, AVG application strong increased cell death in Col-0 roots treated at low pH.

It was reported that Al treatment increased ethylene evolution that inhibited auxin polar transport resulting in arrest of root growth (Sun et al., 2010). The role of this ethylene-mediated root growth inhibition is unclear. Information on ethylene production induced solely by low pH was not addressed in the above cited work due to lack of a control at higher pH (pH >5.5), as unfortunately often happens in studies of Al toxicity. Thus, data concerning Al and ethylene in roots without controls at higher pH must be carefully interpreted.

We also searched for a role of peroxidase in tolerance to low pH induced by ethylene. Roots of Col-0 pretreated with 8 ppm of ethylene and SHAM, a peroxidase inhibitor, were not tolerant to subsequent pH 4.6 treatment in contrast to roots pretreated only with ethylene (Figure 5). The SHAM pretreatment had no effect on roots treated at pH 5.8. In addition, pretreatment with SHAM did not alter subsequent sensitivity to low pH.

Class III peroxidase activity was responsive to ethylene treatment. Activity increased with 8 ppm of ethylene treatment but was reduced in the presence of SHAM (Figure 4). Together

these data indicate that a SHAM-sensitive process, most likely peroxidase activity is involved in tolerance to low pH acquired during ethylene pretreatment. We also tried to inhibit peroxidase activity with SHAM for a longer period than 1 h during low pH stress. However, after 1 h at pH 5.8, even SHAM concentrations as low as 100 μM were toxic to root cells of *A. thaliana* (data not shown).

Possibly, peroxidases are acting downstream of ETR1 and causing cell tolerance to low pH. Interesting, most of transcription factors responsive to ethylene were early-induced (1h) by low pH treatment (Lager et al., 2010), whereas it was reported that only one CIII Prx gene was early-induced (1 h) but eight were late-induced (8 h). We speculate that this separation between changes in expression of ethylene-responsive transcription factors and changes in expression of peroxidase genes might be due to transcriptional modulation of CIII Prx by ethylene during low pH stress. Indeed, the expression of *AtPRX59* (AT5G19890) and *AtPRX10* (AT1G49570) were induced by ACC application (Markakis et al., 2012) and showed late-induced expression (8 h) by low pH (Lager et al., 2010).

So far, a proteomic approach revealed 38 CIII Prx isoforms in roots (Nguyen-Kim et al., 2016). We employed SHAM, an inhibitor of CIII Prx activity to circumvent redundancy of peroxidase isoforms. SHAM also inhibits urease, lipoxygenase and alternative oxidase (Rich et al., 1978; Marmion et al., 2004). However, we suggest that these other enzymes are not as likely as CIII Prx to be involved in ethylene response. Transcriptomic data reported up-regulation of two peroxidase genes after ACC treatment of roots (Markakis et al., 2012). Application of 8 ppm of ethylene caused increase in CIII Prx activity except when SHAM was added jointly with ethylene (Figure 4). A decrease in cell expansion is indicative of cell wall stiffening, an event modulated by peroxidases (Passardi et al., 2005). There was no decrease in cell expansion at low pH when SHAM was added in the treatment solution (Figure 6). This most probably reflects peroxidase activity rather than other enzymes.

Overall root growth rate is highly correlated with LEH (Le et al., 2001) and LEH reflects the behavior of root cell expansion. Application of ethephon in the presence of SHAM at pH 5.8 or 4.8 for 1 h did not cause as marked a decrease in LEH as the application ethephon alone, which strongly decreased LEH (Figure 6). Hence, peroxidases are involved in ethylene-mediated reduction of cell death and reduction in cell expansion caused by low pH.

In conclusion, we show that ethylene promotes tolerance of *A. thaliana* roots to low pH stress by reducing cell death. This was most evident with exogenous application of ethylene. However, ETR1-mediated ethylene signaling appears to have an endogenous role in triggering responses to avoid cell death during low pH stress. The possible role of CIII Prx in the ethylene-

mediated tolerance to low pH suggests that changes in cell wall properties are likely involved in this tolerance.

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Supplementary Material

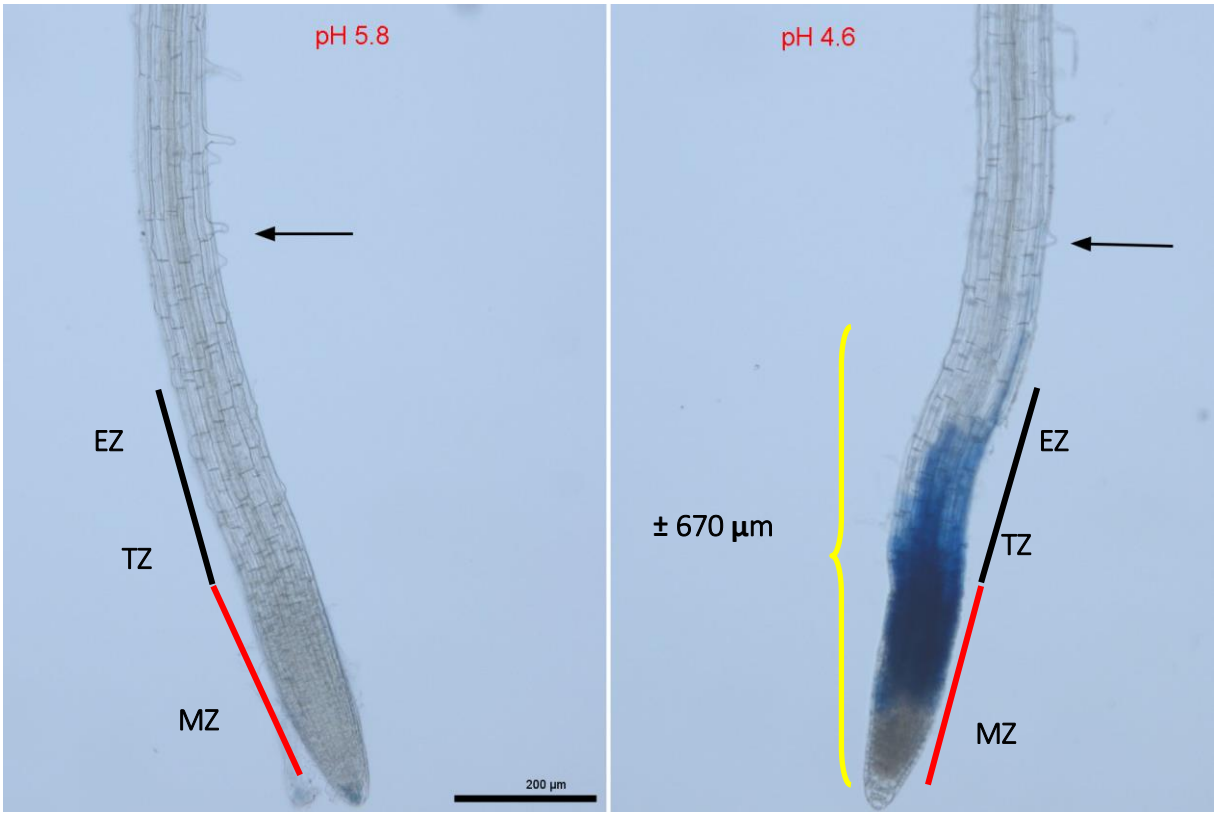

Figure S1. Cell death zones in *A. thaliana* (Col-0) roots treated at pH 5.8 or 4.6 for 2.5 h. The blue color is indicative of cell death due Evans blue staining. The arrows indicate expanding root hairs. The yellow key delimits the average for zone of cell death in root tips treated with low pH. The red line delimits meristematic zone (MZ). The black line indicates the transition zone (TZ) and beginning of elongation zone (EZ).

5. FINAL CONSIDERATIONS

We have provided evidence that, taken together, demonstrates that the cell wall has a crucial role in the death of root tip cells caused by low pH stress. A considerable part of the response to low pH appears to be due to the activity of class III peroxidases. Cell death also appeared to be partially dependent on ETR1 signaling and could be reversed with ethylene pretreatment.

Our first results showed that low pH increased cell wall extensibility in epidermal cells of the TZ. This event or some other cell wall instability due to low pH was required for the onset of cell death. Thus, the cell wall is a target of low pH stress. However, cell death did not appear to be a direct consequence of cell wall disorders, rather it appeared to be triggered by perception of cell wall integrity and activation of a signaling pathway leading to programmed cell death. Considering this, it is perhaps not surprising that gene expression responses to low pH resembled those in response to pathogens and their elicitors (Lager et al. 2010). Understanding the cell wall integrity system is a central theme in plant biology. Thus, knowledge of the dynamics of the cell wall under low pH may contribute to this field of research.

The investigation of players involved in low pH-induced cell death appears to be complex. Nonetheless, it was remarkable that a single isoform of peroxidase, AtPRX62, could have such a pronounced effect on low-pH induced cell death. Our results with ethylene, however, suggest that this peroxidase may be just one link, among others, of a signaling network for cell death. Elucidation of such a network, as well as the function and role of CIII Prx will require the combination of diverse approaches such as those discussed by Francoz et al. (2015).

Finally, cell death due to low pH as an orchestrated response through PCD opened several questions. It would be interesting to investigate, for example, if PCD confers any advantage for plant adaptation to an acidic environment.