Ethylene Signaling Causing Tolerance of *Arabidopsis thaliana* Roots to Low pH Stress is Linked to Class III Peroxidase Activity

Jonathas Pereira Graças[1] · Joni Esrom Lima[3] · Lázaro Eustáquio Pereira Peres[1] · Elisabeth Jamet[4] · Christophe Dunand[4] · Victor Alexandre Vitorello[2] · Christian Chervin[5]

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 response to acidic pH that could possibly avoid cell mortality. Here, we have investigated the role of ethylene and class III peroxidases (CIII Prxs) activity on sensitivity to further low pH treatment. Seedlings of *Arabidopsis thaliana* were pretreated with ethylene, at various concentrations for various times, and then exposed to low pH. In contrast to non-treated roots, roots pretreated with ethylene for 3 h became tolerant to subsequent low pH, with negligible cell mortality in meristematic (MZ), transition (TZ), and early elongation (EZ) zones. This effect of ethylene was time dependent since it was achieved only when seedlings were pre-incubated with ethylene for at least 3 h. This tolerance induced by ethylene was not observed in the gain-of-function mutation *etr1-1* (*insensitive to ethylene 1–1*). Besides, it was prevented by salicylhydroxamic acid (SHAM) which is an inhibitor of CIII Prxs activity. In late EZ, the decrease in cell expansion due to low pH was dependent on both ethylene signaling and a SHAM-sensitive process. The responses mediated by ethylene signaling might involve CIII Prxs-dependent cell wall modifications, leading to tolerance to low pH and arrest in cell expansion during stress.

Keywords Acidity · Cell death · Cell wall stiffening · Ethylene signaling · Class III peroxidase · Root · Soil

Introduction

With acid rain, intensive agriculture, and pollution, soil acidification increases and is disadvantageous for crops growth, mainly, due to the inhibition of root growth (Kochian 1995). Root growth inhibition can intensify drought stress and decrease food productivity. Investigation of the cellular factors or signaling processes involved in this low pH-induced root growth inhibition is important for agricultural improvement and has been underrated so far. All studies cited below were performed in *Arabidopsis thaliana* unless otherwise specified.

One key event that causes this reduction in growth upon low pH, i.e., from 4.5 to 4.7, is the mortality of root tip cells as shown in *A. thaliana* and tomato plants (Koyama et al. 2001; Kobayashi et al. 2013; Graças et al. 2016). Root hairs ongoing tip growth burst upon low pH treatment (Bibikova et al. 1998) is most likely because of excessive loosening of their cell walls. In root tip tissues, increase in calcium and boron in treatment solution greatly alleviates cell death in response to low pH, probably, due to their roles in the

✉ Victor Alexandre Vitorello
victor@cena.usp.br

✉ Christian Chervin
christian.chervin@ensat.fr

Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo, São Paulo, Brazil

[2] Centro de Energia Nuclear Na Agricultura, University of São Paulo, São Paulo, Brazil

Departamento de Botânica, Federal University of Minas Gerais, Pampulha, Brazil

[4] Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Auzeville-Tolosane, France

Genomics and Biotechnology of Fruits, Université de Toulouse, Toulouse INP, INRA, Castanet-Tolosan, France

stabilization of pectin components (Koyama et al. 2001; Kobayashi et al. 2013). In fact, cell death in low pH-stressed roots was directly correlated with low pH-induced cell wall disturbances (Graças 2018). A massive change in the expression of genes related to cell wall polysaccharide remodeling was reported in roots in response to low pH (Lager et al. 2010). These results suggest that the cell wall plays a role in cellular sensitivity to low pH. Thus, to coordinate responses to avoid cell mortality, it is likely that plants can induce changes in the cell wall structure and composition supported by players such as hormones or cell wall-related enzymes/proteins.

In tomato, the reduction in overall root growth due to low pH was also suggested to be an active mechanism to avoid cell mortality (Graças et al. 2016), but the signals and the proteins controlling this mechanism are still elusive. The overall root growth is controlled by cell division, duration of cell cycle, and increase in cell size (Baluska et al. 1996; Verbelen et al. 2006). Cell division occurs in the meristematic zone (MZ) (Baluska et al. 1996). Then, the recently divided cells enter in the transition zone (TZ), an active site for hormone crosstalk (Kong et al. 2018) and also for changes in the cell wall properties (Verbelen et al. 2006). Next, the cells undergo anisotropic growth in the elongation zone (EZ) (Baluska et al. 1996; Verbelen et al. 2006). The root growth can be modulated in EZ by coordinated arrest of cell elongation (Le et al. 2001), in which ethylene plays a role (De Cnodder et al. 2005; Swarup et al. 2007). The activity of several cell wall-related enzymes is related to cell elongation in EZ (Somssich et al. 2016). Finally, when cells reach their final size, the epidermis cells start to differentiate and trichoblast cells form root hairs which undergo tip growth (Verbelen et al. 2006).

It was shown that tip-growing root hairs ceased growing when exogenous pH dropped below 5 (Bibikova et al. 1998). We speculate that this stop in root hair apical growth might result from a modification of the balance between cell wall loosening and stiffening (Passardi et al. 2004; Tsang et al. 2011). The pH-dependent growth arrest could be controlled by Class III peroxidases (CIII Prxs). Indeed, in *A. thaliana* roots, the ethylene-dependent cessation of cell expansion is in part mediated by the expression of genes related to cell wall modifications such as CIII Prxs (Markakis et al. 2012). CIII Prxs are key players for the control of reactive oxygen species (ROS) homeostasis required for normal root growth (Tsukagoshi et al. 2010). They can induce cell wall loosening by producing ROS in a spatio-temporal manner. They are also able to promote cross-linking of cell wall polymers by oxidizing aromatic compounds, thus causing the stiffening of the wall (Passardi et al. 2005; Francoz et al. 2015). Low pH-stressed roots of *A. thaliana* showed differential regulation of eight *AP2/EREBP* (*APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN*) and 13 *CIII Prxs* genes (Lager et al. 2010). Hence, there might be a connection between cell wall changes through CIII Prxs activity and ethylene signaling that could be relevant for the arrest in root growth and mortality of root cells due to low pH.

Our hypothesis is that ethylene signaling can modulate cellular responses, through the regulation of *CIII Prxs* expression and activity, which in turn could control the ROS homeostasis and modify the pattern of cell mortality due to low pH stress. To examine this, different approaches can be used such as exogenous ethylene application, inhibition of ethylene synthesis, and examination of mutants impaired in ethylene signaling. We used the *A. thaliana etr1-1* (*insensitive to ethylene 1–1*) mutant in order to check if the ethylene signaling is important in plant response to low pH stress as *etr1-1* is a gain-of-function mutant which blocks ethylene signaling, making the plant ethylene-insensitive (Chang et al. 1993). We could show that ethylene promotes tolerance to low pH in *A. thaliana* root tissues. This tolerance was dependent on ethylene perception. Furthermore, downstream of ethylene perception, a salicylhydroxamic acid (SHAM)-sensitive process, most likely CIII Prxs activity, was important to promote tolerance to low pH and also decrease cell elongation in response to the stress.

Materials and Methods

Plant Material, Growth Conditions, pH Treatment, and Chemicals

Seeds of *A. thaliana* were disinfected in a 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 containing a ½ Hoagland's solution adjusted to pH 5.8 and 0.8% agar. Macronutrients consisted of 6 mM KNO_3, 4 mM Ca $(NO_3)_2$, 2 mM $NH_4H_2PO_4$, and 1 mM $MgSO_4$. Micronutrients were composed of 14 µM $ZnSO_4$, 20 µM H_3BO_3, 0.03 µM $NiSO_4$, 20 µM $MnSO_4$, 0.02 µM Na_2MoO_4, 0.02 µM $CoCl_2$, 0.02 µM $CuSO_4$, and 30 µM $FeSO_4$.

The growth temperature was constant at 22 °C, the day/night cycle was 16 h/8 h and the light intensity was approximately 120 µE m^{-2} s^{-1}. We used seedlings of the ecotype Columbia-0 (Col-0) as wild type. In addition, we used the *etr1-1* mutant (Germplasm stock: CS237, locus: AT1G66340, ABRC). We always grew wild type and the *etr1-1* mutant seedlings on separate growth media.

The duration of the pH treatments was variable and is specified in the figure legends. For each experiment, at least 10 five-day-old seedlings were carefully taken from the growth media and incubated in 250 mL Erlenmeyer with 20 mL of treatment solution. The treatment solution was composed of $CaCl_2$ (0.5 mM) and Homopipes buffer 0.6 mM

[homopiperazine-1,4-bis(2-ethanesulfonic acid)]. The final pH of the solution was adjusted by adding HCl. The solution was continuously aerated and kept at the same temperature as that used for growth.

All chemicals were purchased from Sigma-Aldrich (France or Brazil).

Evaluation of Cell Death in Root Tips

Seedlings treated at different pH were stained in a 0.25% (w/v) solution of Evans blue for 15 min (Baker and Mock 1994). Then, they were washed three times for 5 min with distilled water. Following this, bright-field images were taken with an inverted microscope equipped with a CCD color camera (Leica, Mannheim, Germany). Then, 0.2 cm of the root tips were excised, kept in plastic tubes, and frozen in liquid nitrogen prior to storage at – 20 °C for later quantification of Evans blue uptake. All the procedures were performed in glass Petri dishes to avoid damages or root dehydration.

For quantitative evaluation of Evans blue uptake, the method described by Baker and Mock (1994) was used with a few modifications. Indeed, 300 µL DMSO (dimethyl sulfoxide) was added to the segments of frozen excised root tips for 1 h at 24 °C. Then, they were centrifuged at 8000×*g* for 3 min, and the liquid was transferred to a fresh plastic tube and quantified with excitation and emission wavelengths at 645 and 690 nm, respectively.

We also obtained qualitative and quantitative data of cell viability from the same roots. This was helpful in experiments with a large number of treatments. For this purpose, the images showing Evans blue uptake were later analyzed using the ImageJ software (https://imagej.nih.gov/ij/docs/guide/index.html) to quantify the pixels corresponding to Evans blue staining. The contours of each root tip, reaching roughly 500 × 94 µm from the apex were drawn (see Fig. S1) and the pixels were counted in this region. The mean gray value of this region was calculated. From each value, the mean gray value of the background of the corresponding bright-field image was subtracted to eliminate the variation of light intensity between different images.

Ethylene and Aminoethoxyvinylglycine (AVG) Applications

For ethylene treatments, five-day-old seedlings were used as described above. We developed a system in square plastic Petri dishes for ethylene application to seedlings on the solid growth medium described above. The details of this system are shown in Fig. S2. The seedlings were grown in these modified Petri dishes. For each experiment, different ethylene concentrations (C_2H_4) were obtained freshly by mixing with air. An appropriate volume of these mixes was applied into the Petri dishes to achieve the ethylene final concentrations. Ethylene concentrations at various stages of the treatment were checked by analyzing gas samples with gas chromatography, as described previously (Trapet et al. 2016).

We also used ethephon (2-chloroethylphosphonic acid) 1 µM to release ethylene in treatment solutions, in 250 mL Erlenmeyers for the experiments performed to measure Length of the first Epidermal cell with root Hair bulge (LEH), see details below. This was performed during treatment at pH 5.8 or 4.8. We confirmed that the *etr1-1* mutant was ethylene-insensitive because ethephon 1 µM had no effect on hypocotyl elongation as in the wild type as reported by Bleecker et al. (1988). The treatment with 1 µM ethephon gave an ethylene concentration of 5 ± 2 ppm.

We examined the effects of inhibition of ethylene synthesis by AVG during low pH treatment on the cell viability of roots. Ten µM AVG were added to the treatment solution and then the final pH was adjusted to the appropriate level.

Evaluation of Total CIII Prxs Activity and Application of an Inhibitor

Guaiacol was used as a substrate to examine the total enzymatic activity of CIII Prxs in roots. Guaiacol (0.125% v/v) was diluted in 200 mM phosphate buffer (pH 6.1). Fresh H_2O_2 was added to the guaiacol solution to reach a final concentration of 1.65 mM and roots were incubated in the dark. After 5 min, the roots were washed with abundant water to stop the reaction and the brown tetra-guaiacol precipitate was immediately imaged in bright-field microscopy (Zeiss Axio Zoom.V16 stereomicroscope, Göttingen, Germany).

We applied the CIII Prxs activity inhibitor, SHAM, prior or during the low pH treatment. A stock solution of 250 mM SHAM was prepared in DMSO. The seedlings were transferred to the Hoagland growth medium as described at the beginning of the method paragraph, containing 0.50 mM SHAM and incubated for 3 h. For the simultaneous treatment with SHAM and low pH, we added 0.25 mM of SHAM to the treatment solution, 0.5 mM CaCl2, and 0.6 mM Homopipes, and then adjusted it to pH 4.8.

The images of CIII Prxs activity were analyzed using the ImageJ software. The pixels from CIII Prxs activity were counted in area of roughly 316 × 93 µm which included TZ and EZ (see Fig. S3). This area was chosen because CIII Prxs activity in roots mostly concentrates in these zones above MZ. The difference between the bright-field background and the roots was calculated to eliminate the differences in light intensity between each image.

Evaluation of the Length of the First Epidermal Cell with Root Hair Bulge (LEH)

We evaluated the LEH as described by Le et al. (2001) with some modifications. Changes in LEH size can be detected within minutes (Le et al. 2001) and thus it was not necessary to perform treatments for a long time such as 3 h. After 1 h of low pH or ethylene treatment, roots were fixed in Karnovsky solution (2.5% glutaraldehyde, 2.0% paraformaldehyde, 50 mM cacodylate buffer, and 1 mM CaCl2) (Román et al. 2004) 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, Wetzlar, Germany, excitation 340/40 bandwidth, emission 470/40 bandwidth). Details of LEH measurement are described in Fig. S4.

Statistical Analysis

The experimental design was completely randomized in all experiments. Each analysis was composed of at least 3 separate experiments. Biological replicates were composed of 10 plants each. The means were compared by analysis of variance (ANOVA) followed by Duncan's test. The difference between two means was compared by Student's *t* test at the 5% significance level.

Results

Cell Death Pattern Due to Low pH and Its Modulation by an Ethylene Pretreatment

The pattern of cell death due to low pH was initially observed in our experimental conditions (Graças 2018). In the whole study, the pH 5.8 is considered as a control. The tissues were stained with Evans blue to detect dead cells as described by Baker and Mock (1994). The Evans is excluded from live cells and stains only cells with altered membrane integrity. As expected, the treatment with low pH for 2.5 h resulted in cell death confined to MZ, TZ, and early EZ as indicated by the blue color in roots (Fig. 1A).

Then, we examined whether ethylene could change the sensitivity of roots to low pH, that is 4.6. Ethylene concentrations ranging from 3 to 1500 ppm (actually 1479 ppm after gas chromatography measurement) were applied to wild-type seedlings placed in a device containing growth media as described in Fig. S2. In control roots without ethylene pretreatment, cell death was markedly observed (Fig. 1B). The application of 3 ppm ethylene for 3 h prior to low pH treatment only slightly alleviated cell death after subsequent treatment at pH 4.6 (Fig. 1B). However, increasing ethylene concentration to 8, 131, or 1479 ppm resulted

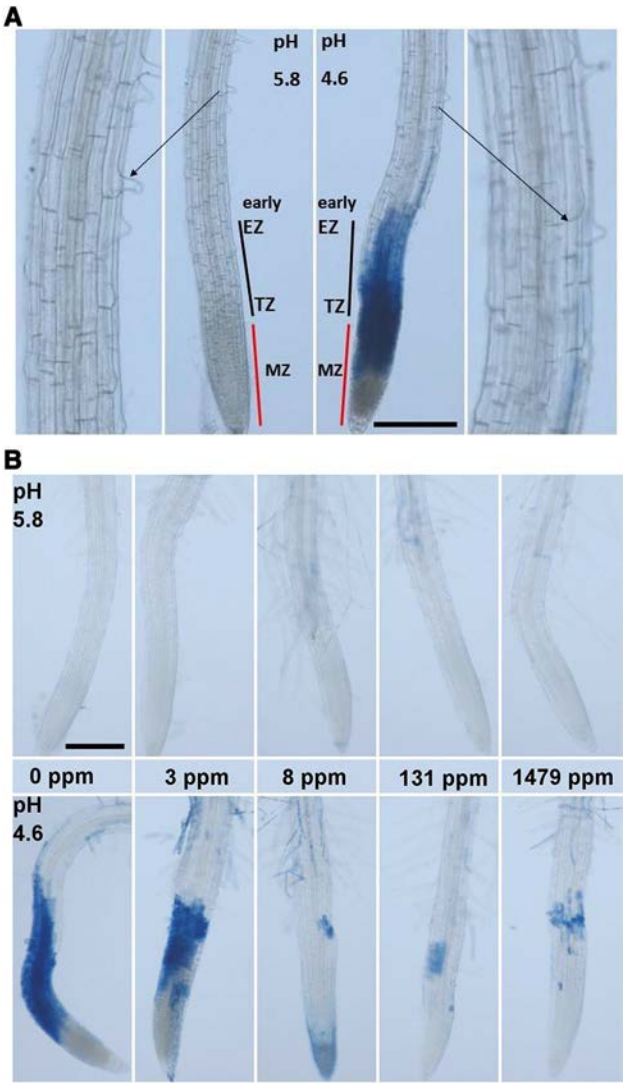

Fig. 1 Cell mortality in roots of *A. thaliana* (Col-0) due to low pH stress, modulated by a pretreatment with various ethylene concentrations for 3 h. **A** The blue color is indicative of cell death due to Evans blue uptake. The red line delimits MZ and the black line TZ and early EZ. As a characteristic of late EZ, the arrows show expanding root hairs magnified in the side boxes. The magnification was doubled in the left and right side images. **B** Seedlings were first pretreated with ethylene ranging from 0 to 1479 ppm for 3 h and then subjected to pH 5.8 (upper panel) or 4.6 (bottom panel) treatment for 2.5 h. Scale bars: 200 µm. Each image is representative of at least 15 roots from the same treatment (Color figure online)

in a sharp decrease in cell mortality. Based on these results, we chose 8 ppm ethylene for further experiments since no additional reduction in cell death occurred at higher concentrations and we wanted to reduce the possible side-effects of the ethylene treatment.

We next determined the shortest duration of pretreatment with 8 ppm ethylene, reducing death upon low pH. Pretreatment for 0.5 or 1 h only slightly decreased cell mortality, compared to the control without ethylene pretreatment

(Fig. 2A, B). Pretreatment for 2 h increased tolerance a bit further, but cell mortality clearly decreased after 3 h of ethylene pretreatment with only a few cells exhibiting death (Fig. 2A, B). The quantification of Evans blue staining showed some background level at pH 5.8 due to noise analysis by the ImageJ software (see "Materials and Methods" and Fig. S1), but these did not impede revealing significant differences due to the ethylene treatments at pH 4.6. Duncan's tests were run within one pH range only, so no comparison between pH 5.8 and 4.6 was performed.

Thus, we chose 3 h of 8 ppm ethylene pretreatment to induce tolerance to low pH stress for the subsequent analyses.

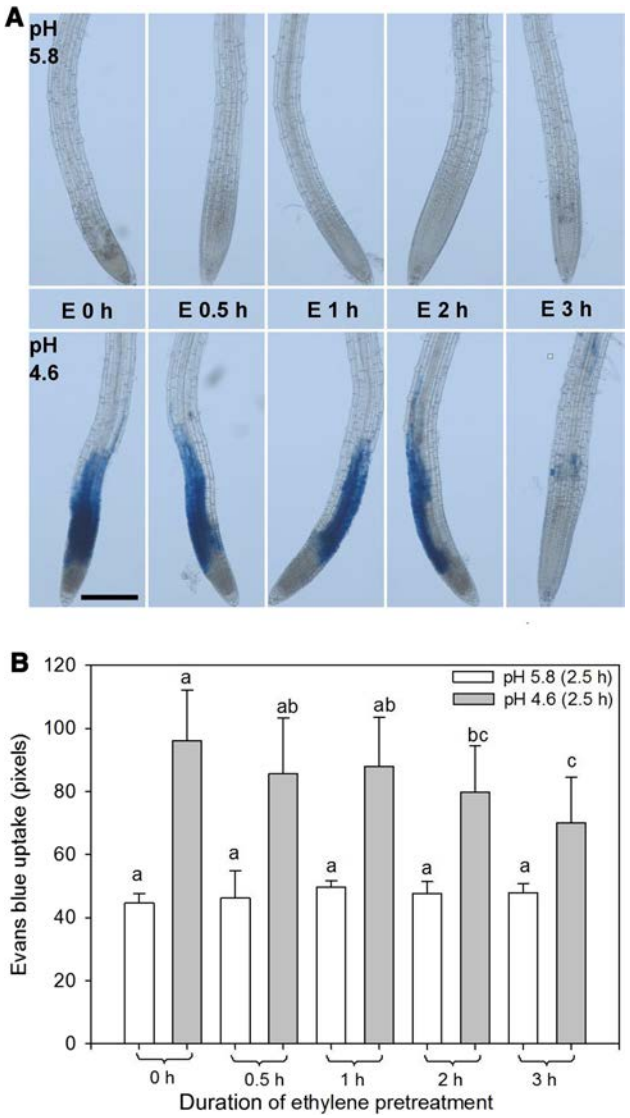

Fig. 2 Cell mortality in roots of *A. thaliana* (Col-0) upon low pH is modulated by a pretreatment with 8 ppm ethylene over different durations. The blue color is indicative of cell death due to Evans blue uptake. **A** The seedlings were first pretreated with 8 ppm ethylene (E) for 0, 0.5, 1, 2, or 3 h and then subjected to pH 5.8 (upper panel) or 4.6 (bottom panel) treatment without ethylene for 2.5 h. Scale bar: 200 µm. Each image is representative of at least 15 roots from the same treatment. **B** Quantification of cell mortality by measuring the pixels of Evans blue staining in root tips. The bars are SD of 10 roots from two independent experiments. Letters indicate significant differences between different durations of ethylene incubation at the same pH by Duncan's test (Color figure online)

Effects of Ethylene and SHAM Applications on Cell Mortality Upon Low pH

To examine whether the low pH tolerance triggered by ethylene application was correlated with CIII Prxs activity, we treated the seedlings with ethylene alone or together with SHAM, a CIII Prx activity inhibitor (Marmion et al. 2004). So far, 38 isoforms of CIII Prxs have been identified in the roots of *A. thaliana* by cell wall proteomics (Nguyen-Kim et al. 2016). SHAM (0.50 mM) was added in the growth media and seedlings were placed onto the growth medium surface being in contact with SHAM and also with ethylene applied as a gas.

As observed previously, the treatment of wild-type seedlings with 8 ppm ethylene for 3 h restrained cell death observed at pH 4.6, in comparison to the control without ethylene (Fig. 3A, B). This strong stress at pH 4.6 was very important to evidence the ethylene-induced tolerance. Conversely, the seedlings placed in growth media with SHAM and treated with 8 ppm ethylene did not show tolerance to pH 4.6. The roots of seedlings placed in growth media with only SHAM for 3 h and then exposed to pH 4.6 displayed cell death similar to control roots in growth media without SHAM. The seedlings placed in growth media with only SHAM over 3 h and then treated at pH 5.8 showed no loss in root cell viability (Fig. 3A, B), which indicated that SHAM was not toxic when added in the growth media. The 8 ppm ethylene treatment alone increased CIII Prxs activity in root TZ and EZ compared to control without ethylene treatment, but the activity was decreased when the roots were placed in growth media with SHAM and ethylene (Fig. S3). For comparisons, in the roots of *A. thaliana* plantlets grown in vitro during 7 days in the presence of 50 mM SHAM, total CIII Prxs activity is reduced by about 40% (Dunand et al. 2007), whereas the elongation of maize roots was reduced by 25% or 50% after a 3-h treatment with 0.3 or 1.0 mM SHAM, respectively (Liszkay et al. 2004).

It is important to mention that we also tried to inhibit CIII Prxs activity with SHAM for a period longer than 1 h directly in the treatment solution instead of growth media prior to the low pH treatment as described above. However, after 3 h in treatment solution at pH 5.8, SHAM was toxic mainly to late EZ cells and root hairs (Fig. S5). Such a high sensitivity to SHAM was not observed previously in *Solanum lycopersicum* roots (Graças et al. 2016).

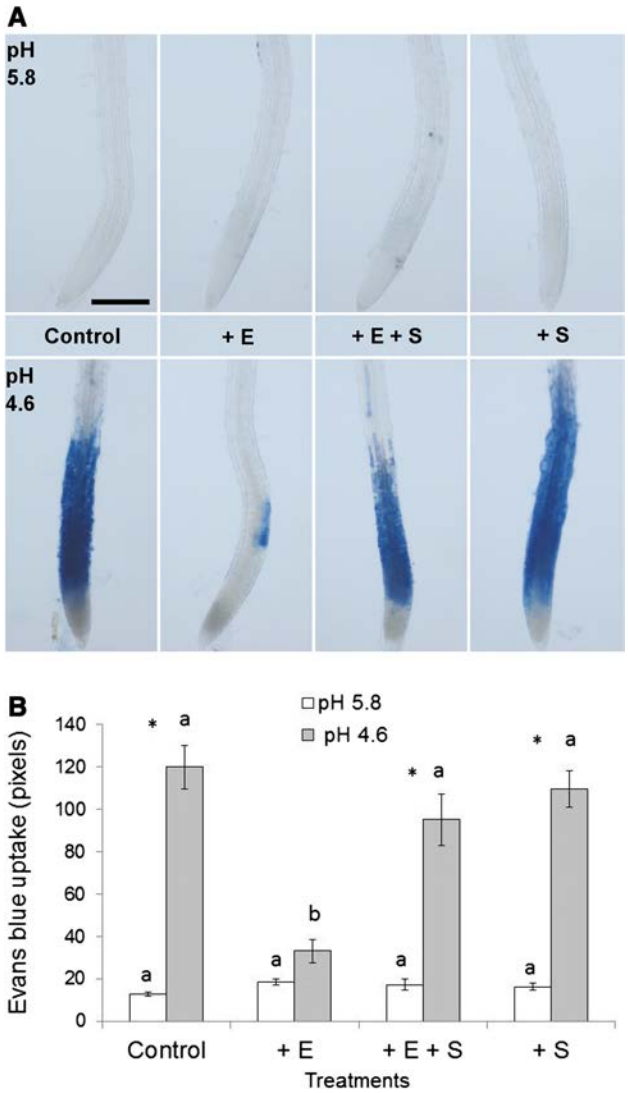

Fig. 3 Effects of the SHAM CIII Prxs activity inhibitor on cell mortality in roots of *A. thaliana* (Col-0) due to low pH stress, as modulated by a pretreatment with 8 ppm ethylene over 3 h. **A** The blue color is indicative of cell death due to Evans blue uptake. The seedlings were treated for 3 h with 8 ppm ethylene (+E), 8 ppm ethylene with 0.50 mM SHAM (+E +S), or only SHAM (+S) and then subjected to pH 5.8 (upper panel) or 4.6 (bottom panel) for 2.5 h without ethylene or SHAM. In the control, the seedlings were kept in growth medium and then were treated at pH 5.8 or 4.6. Scale bar: 200 µm. **B** Quantification of cell mortality by counting the pixels of Evans blue staining in root tips in an area extending 500×94 µm from the apex until early EZ. Letters indicate significant differences between different treatments at the same pH by Duncan's test. Asterisk indicates significant differences between pH 5.8 and 4.6 from the same treatment by *t* test (Color figure online)

In summary, our results have shown that the low pH tolerance triggered by an ethylene pretreatment depends on the induction of CIII Prxs activity.

Effects of Ethylene Synthesis Inhibition and Effects of Insensitivity to Ethylene Perception on Cell Mortality Due to Mild Low pH Stress

The above results have shown that ethylene pretreatment allowed tolerance to low pH. It was necessary to further investigate whether the impairment of ethylene signaling or synthesis would make plants more sensitive to low pH. We inhibited ethylene synthesis with AVG (Even-Chen et al. 1982) and we examined the consequences of the insensitivity to ethylene in the *etr1-1* mutant.

The stress at pH 4.6 for 2.5 h was quite overwhelming for the wild-type seedlings, causing cell death in almost all MZ, TZ, and early EZ cells (Fig. 1A). This would have made difficult distinguishing between them and mutant seedlings showing enhanced sensitivity to low pH. Thus, we chose a milder stress. After preliminary experiments, we chose to apply a pH 4.8 solution for 3 h. Then, the *etr1-1* mutant was more sensitive than wild type to this mild stress (Fig. 4A). The *etr1-1* roots displayed twofold increase in cell mortality compared to wild type as shown by Evans blue uptake (Fig. 4B).

The wild-type roots treated with AVG and low pH showed a 66% increase in cell death compared to those without AVG. This compound inhibits aminocyclopropane-1-carboxylic acid (ACC) synthesis, the immediate precursor of ethylene. It is noticeable that there was a slight increase in Evans blue uptake in roots at pH 5.8 treated with AVG compared to wild type or *etr1-1* (Fig. 4B), though not being significant. This point deserves further study. In any case, these results cannot be interpreted as AVG toxicity because this compound has been used at similar µmolar concentrations for decades on *A. thaliana* seedlings without showing any cell toxicity (Schaller and Binder 2017).

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

Effects of Ethephon and SHAM Application on Root Cell Elongation Upon Low pH Treatment

The Length of the first Epidermal cell with root Hair bulge (LEH) is representative of cell elongation in roots and in normal conditions of cell division and cell cycle, and has been shown to be modified by ethylene (Le et al. 2001). However, unlike the overall root growth, any change in LEH can be easily detected within minutes (Le et al. 2001) We examined the effect of ethephon or SHAM on LEH upon low pH treatment and we measured LEH after 1 h of low pH treatment. We applied ethephon, a compound that releases ethylene when entering cells and we measured LEH.

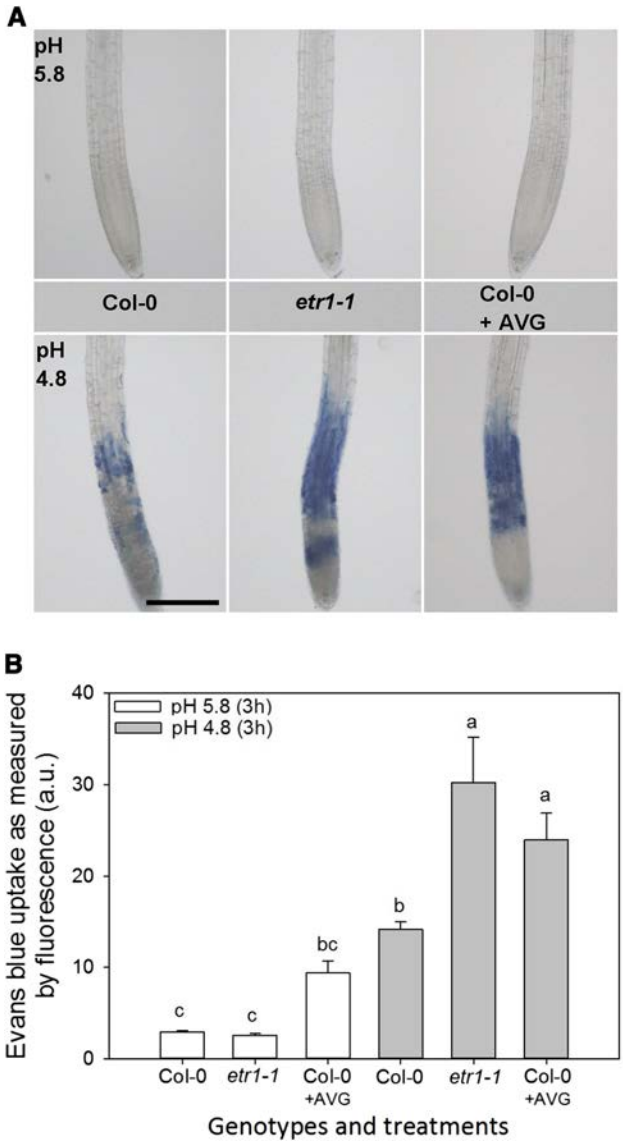

Fig. 4 Effects of ethylene insensitivity (*etr1-1*) and ethylene biosynthesis inhibitor (AVG) on cell mortality in roots upon low pH stress. **A** The blue color is indicative of cell death due to Evans blue uptake. AVG (10 µM) was added in treatment solution. Scale bar: 200 µm. **B** Quantification of cell mortality by measuring the pixels of Evans blue staining in root tips. Bars are standard errors of 3 independent experiments. Letters indicate significant differences between treatments by Duncan's test (Color figure online)

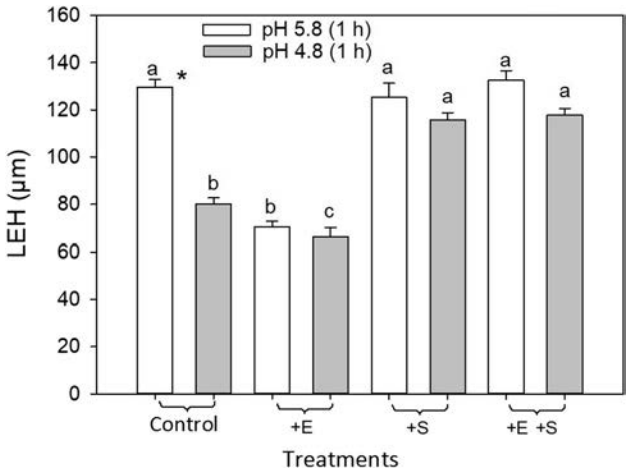

Fig. 5 Length of first Epidermal cell with root Hair bulge (LEH) upon low pH treatment for 1 h modulated by the application of ethephon and/or SHAM. Ethephon (+E) (1 µM) or 0.25 mM SHAM (+S) was added directly in the treatment solution. Bars are standard errors of 3 independent experiments. Letters indicate significant differences between different treatments at the same pH by Duncan's test. The asterisk indicates a significant difference between pH 5.8 and 4.8 for the same ethylene or SHAM treatment by *t* test

In wild-type roots, LEH decreased by 37% after 1 h of treatment at pH 4.8 compared to pH 5.8 (Fig. 5). The simultaneous treatment with SHAM and pH 5.8 had no effect on LEH. However, LEH decreased only by 8% when roots were treated at pH 4.8 with SHAM for 1 h (Fig. 5).

As expected, the application of 1 µM ethephon during treatment at pH 5.8 decreased LEH by about 45%. A similar decrease in LEH was also observed with the application of ethephon upon pH 4.8. The simultaneous application of ethephon and SHAM did not cause marked reduction in LEH at pH 5.8, but a decrease in LEH of 11% at pH 4.8 (Fig. 5).

Altogether, the remarkable decrease in LEH due to ethephon application upon pH 5.8 or 4.8 was restrained by the simultaneous application of SHAM. Besides, the stop in LEH increase solely at pH 4.8 was dependent on a SHAM-sensitive process.

Discussion

Signaling Through ETRs is Crucial for the Occurrence of Low pH-Induced Cell Death

Low pH stress causes mortality of cells of root tip tissues within minutes following the exposure to stress (Koyama et al. 2001). Our hypothesis is that a counter-response, to prevent such a drastic and irreversible event, is mediated through hormones such as ethylene. Our results indicate that ethylene plays an antagonist role in the progression of cell death due to low pH in the root MZ, TZ, and early EZ (Fig. 1A). This is suggested by our observations: (i) an ethylene pretreatment limited cell death due to low pH stress (Figs. 1, 2); (ii) the *etr1-1* mutant which is insensitive to ethylene displayed increased cell mortality due low pH stress compared to wild-type roots (Fig. 4); (iii) inhibiting ethylene biosynthesis with AVG in wild-type roots increased cell mortality due to low pH stress (Fig. 4).

The cell death reported here is due to a strong pH effect. Indeed, a 2.5 h treatment at pH 4.6 affected most cells in the

root tip (Fig. 1A). This result is consistent with the observed cell death in *A. thaliana* root tips upon 2 h of low pH exposure as reported by Koyama et al. (2001). However, the strength of this stress allowed to clearly show the protection generated by an ethylene treatment. A mild low pH stress (pH 4.8) showed that the *etr1-1* roots were more sensitive to low pH than wild-type roots (Fig. 3), thus bringing an additional proof that tolerance to low pH needs an active ethylene pathway. *etr1-1* is a gain-of-function mutant which did not display any symptom in response to ethylene application (Chang et al. 1993; Bleecker et al. 1988). There are five ethylene receptors in *A. thaliana:* ETR1, ETR2, ERS1, ERS2, and EIN4. However, *etr1-1* is a dominant mutant blocking ethylene perception, even if the other receptors are active (Chang et al. 1993). Importantly, the five *A. thaliana* ethylene receptor genes are expressed within the root tissues sensitive to low pH in distal MZ, TZ, and EZ (Grefen et al. 2008, Fig. S6). In these zones, low pH-induced cell death occurred and was accentuated in *etr1-1* as reported here. Thus, we could not predict whether some ETRs are more important than others in the low pH-sensitivity. In other words, we were not able to state if there is sub-functionalization at the ETR level regarding cell mortality due to low pH.

Upon low pH treatment, eight genes encoding transcription factors of the *AP2/EREBP* (*APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN*) family are differentially expressed in *A. thaliana* roots (Lager et al. 2010). Seven of them were induced within 1 h after treatment, while one was repressed. This further supports the fact that the ethylene signaling pathway is activated upon low pH stress.

Class III Prxs Activity Seems to be Involved in the Ethylene-Induced Tolerance to Low pH Stress and in the Modulation of Cell Elongation Upon This Stress

The application of SHAM, an inhibitor of CIII Prxs activity, together with ethylene, prevented tolerance to low pH (Fig. 5). CIII Prxs activity was also stimulated by the ethylene treatment in TZ and EZ, except in the presence of SHAM (Fig. S3). This strongly suggests that the stimulation of CIII Prxs activity by ethylene is involved in tolerance to low pH stress. Cell death upon low pH occurred in MZ, TZ, and early EZ. In late EZ, there was no cell death reported upon low pH, but a decrease in LEH was reported(Fig. 5). LEH can be used directly as indicative of root cell elongation (Le et al. 2001). The arrest in LEH increase was accentuated by ethylene at pH 5.8, and upon low pH it was blocked by the SHAM treatment, thus suggesting that it was dependent on active CIII Prxs (Fig. 5). These data indicate that a CIII Prxs-dependent process, most likely downstream of ethylene signaling, is involved in the tolerance to low pH and in the decrease in cell elongation upon low pH stress.

SHAM also inhibits urease, lipoxygenase, and alternative oxidase (Rich et al. 1978; Marmion et al. 2004). However, the SHAM concentration used here (0.50 mM) is not supposed to affect alternative oxidase (Spreen Brouwer et al. 1986). Besides, the genes encoding these three kinds of enzymes were not among the most induced or repressed after treatment with ACC, a precursor of ethylene (Markakis et al. 2012). It has been shown that the arrest in cell elongation due to the ACC treatment was correlated with cross-linking of cell wall components (De Cnodder et al. 2005), most likely causing cell wall stiffening. CIII Prxs are known to contribute to cell wall stiffening, which could in turn arrest cell elongation (Passardi et al. 2005; Francoz et al. 2015), as reported here. Hence, our results are in favor of a SHAM-sensitive process, most likely related to CIII Prxs activity. However, we cannot exclude the involvement of the other enzymes inhibited by SHAM. Previous reports also corroborate our model in which ethylene signaling stimulates CIII Prxs activity causing changes in cell walls that could trigger tolerance to low pH. The ACC treatment decreased cell expansion within minutes (Le et al. 2001; Ruzicka et al. 2007). As shown before, among the 240 differentially expressed genes upon ACC treatment, the 10 most induced or repressed encode cell wall-related proteins/enzymes, including two upregulated CIII Prxs and two downregulated expansins (Markakis et al. 2012). The repression of expansin genes, known to be involved in cell wall loosening upon cell growth (Cosgrove 2015), is consistent with the observation of cell elongation arrest. Additionally, most of the transcription factors responsive to ethylene were found to be induced early upon low pH treatment (1 h) (Lager et al. 2010), when *CIII Prx* genes were induced within a few hours range. This temporal separation between changes in transcript levels of ethylene-responsive transcription factors genes and those of *CIII Prxs* genes suggest that the latters are downstream of ethylene signaling during the plant's response to low pH stress. Indeed, the expression of *AtPrx59* (*AT5G19890*) and *AtPrx10* (*AT1G49570*) was induced by ACC application (3 h) (Markakis et al. 2012) and showed late-induced expression (8 h) upon low pH (Lager et al. 2010).

Conclusions

In this study we have shown that ethylene promotes the tolerance of *A. thaliana* roots to low pH stress. A CIII Prxs-dependent process is crucial for the tolerance to low pH, triggered by the ethylene pretreatment, and also for the decrease in cell elongation. Thus, players such as ethylene and cell wall-related enzymes, among which CIII Prxs,

seem essential for the plant-coordinated responses to low pH stress.

Acknowledgements We are thankful to CAPES for the support to J.P.G. through the Programa de Doutorado-Sanduíche no Exterior - Brazilian CAPES (Grant # 88881.135425/2016-01). Also, we thank the Paul Sabatier-Toulouse 3 University, CNRS, and the GBF laboratory for experimental support. Finally, we thank the FR AIB imaging platform in Auzeville-Tolosane, particularly Dr Alain Jauneau, Yves Martinez, and Aurélie Le Ru for their great help with microscopy. Finally, we thank the science editor and the anonymous reviewer for their time and a fruitful series of comments.

Author Contributions JPG performed all experiments. JEL participated in the experiments examining cell expansion. CC designed a system for ethylene application and experimental trials. CD examined experiments regarding CIII Prx. LEPP contributed with suggestions for the project. JPG and VAV wrote the manuscript with major contributions of EJ and CC.

Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any conflict of interest.

References

Baker CJ, Mock NM (1994) An improved method for monitoring cell-death in cell-suspension and leaf disc assays using evans blue. Plant Cell, Tissue Organ Cult 39:7–12. https://doi.org/10.1007/BF00037585

Baluska F, Volkmann D, Barlow PW (1996) Specialized zones of development in roots: view from cellular level. Plant Physiol 112:3–4. https://doi.org/10.1104/pp.109.3.725

Bibikova TN, Jacob T, Dahse I, Gilroy S (1998) Localized changes in apoplastic and cytoplasmic pH are associated with root hair development in *Arabidopsis thaliana*. Development 125:2925–2934

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a mutation in *Arabidopsis thaliana*. Science 241:1086–1089. https://doi.org/10.1126/science.241.4869.1086

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science 262:539–544. https://doi.org/10.1126/science.8211181

Cosgrove DJ (2015) Plant cell wall extensibility: connecting plant cell growth with cell wall structure, mechanics, and the action of wall-modifying enzymes. J Exp Bot 67:463–476. https://doi.org/10.1093/jxb/erv511

De Cnodder T, Vissenberg K, Van Der Straeten D, Verbelen JP (2005) Regulation of cell length in the *Arabidopsis thaliana* root by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid: a matter of apoplastic reactions. New Phytol 168:541–550. https://doi.org/10.1111/j.1469-8137.2005.01540.x

Dunand C, Crevecoeur M, Penel C (2007) Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their influence on root development: possible interaction with peroxidases. New Phytol 174:332–341. https://doi.org/10.1111/j.1469-8137.2007.01995.x

Even-Chen Z, Mattoo AK, Goren R (1982) Inhibition of ethylene biosynthesis by aminoethoxyvinylglycine and by polyamines shunts label from 3,4-[^{14}C] methionine into spermide in aged orange peel discs. Plant Physiol 69:385–388. https://doi.org/10.1104/pp.69.2.385

Francoz E, Ranocha P, Nguyen-Kim H, Jamet E, Burlat V, Dunand C (2015) Roles of cell wall peroxidases in plant development. Phytochemistry 112:15–21. https://doi.org/10.1016/j.phytochem.2014.07.020

Graças JP (2018). 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. Tese de Doutorado, Escola Superior de Agricultura Luiz de Queiroz. https://doi.org/10.11606/T.11.2018.tde-25072018-164614

Graças JP, Ruiz-Romero R, Figueiredo LD, Mattiello L, Peres LEP, Vitorello VA (2016) Root growth restraint can be an acclimatory response to low pH and is associated with reduced cell mortality: a possible role of class III peroxidases and NADPH oxidases. Plant Biol 18:658–668. https://doi.org/10.1111/plb.12443

Grefen C, Stadele K, Ruzicka K, Obrdlik P, Harter K, Horak J (2008) Subcellular localization and in vivo interactions of the *Arabidopsis thaliana* ethylene receptor family members. Mol Plant 1:308–320. https://doi.org/10.1093/mp/ssm015

Kobayashi Y, Kobayashi Y, Watanabe T, Shaff JE, Ohta H, Kochian LV, Wagatsuma T, Kinraide TB, Koyama H (2013) Molecular and physiological analysis of Al^{3+} and H^+ rhizotoxicities at moderately acidic conditions. Plant Physiol 163:180–192. https://doi.org/10.1104/pp.113.222893

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46:237–260. https://doi.org/10.1146/annurev.pp.46.060195.001321

Kong X, Liu G, Liu J, Ding Z (2018) The root transition zone: a hot spot for signal crosstalk. Trends Plant Sci 23:403–409. https://doi.org/10.1016/j.tplants.2018.02.004

Koyama H, Toda T, Hara T (2001) Brief exposure to low-pH stress causes irreversible damage to the growing root in *Arabidopsis thaliana*: pectin-Ca interaction may play an important role in proton rhizotoxicity. J Exp Bot 52:361–368. https://doi.org/10.1093/jxb/52.355.361

Lager I, Andreasson O, Dunbar TL, Andreasson E, Escobar MA, Rasmusson AG (2010) Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. Plant Cell Environ 33:1513–1528. https://doi.org/10.1111/j.1365-3040.2010.02161.x

Le J, Vandenbussche F, Van Der Straeten D, Verbelen JP (2001) In the early response of arabidopsis roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiol 125:519–522. https://doi.org/10.1104/pp.125.2.519

Liszkay A, van der Zalm E, Schopfer P (2004) Production of reactive oxygen intermediates (O2˙−, H2O2, and ˙OH) by maize roots and their role in wall loosening and elongation growth. Plant Physiol 136:3114–3123. https://doi.org/10.1104/pp.104.044784

Markakis MN, De Cnodder T, Lewandowski M, Simon D, Boron A, Balcerowicz D, Doubbo T, Taconnat L, Renou JP, Höfte H, Verbelen JP, Vissenberg K (2012) Identification of genes involved in the ACC-mediated control of root cell elongation in *Arabidopsis thaliana*. BMC Plant Biol. https://doi.org/10.1186/1471-2229-12-208

Marmion CJ, Griffith D, Nolan KB (2004) Hydroxamic acids—an intriguing family of enzyme inhibitors and biomedical ligands. Eur J Inorg Chem. https://doi.org/10.1002/ejic.200400221

Nguyen-Kim H, Clemente HS, Balliau T, Zivy M, Dunand C, Albenne C, Jamet E (2016) *Arabidopsis thaliana* root cell wall proteomics: increasing the proteome coverage using a combinatorial peptide ligand library and description of unexpected Hyp in peroxidase amino acid sequences. Proteomics 16:491–503. https://doi.org/10.1002/pmic.201500129

Passardi F, Penel C, Dunand C (2004) Performing the paradoxical: how plant peroxidases modify the cell wall. Trends Plant Sci 9:534–540. https://doi.org/10.1016/j.tplants.2004.09.002

Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. Plant Cell Rep 24:255–265. https://doi.org/10.1007/s00299-005-0972-6

Rich PR, Wiegand NK, Blum H, Moore AL, Bonner WD (1978) Studies on mechanism of inhibition of redox enzymes by substituted hydroxamic acids. Biochem Biophys Acta 525:325–337. https://doi.org/10.1016/0005-2744(78)90227-9

Román MP, Cambra M, Juárez J, Moreno P, Duran-Vila N, Tanaka FAO, Alves E, Kitajima EW, Yamamoto PT, Bassanezi RB, Teixeira DC, Jesus Junior WC, Ayres AJ, Gimenez-Fernandes N, Rabenstein F, Girotto LF, Bové JM (2004) Sudden death of citrus in Brazil: a graft-transmissible bud union disease. Plant Dis 88:453–467. https://doi.org/10.1094/PDIS.2004.88.5.453

Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell 19:2197–2212. https://doi.org/10.1105/tpc.107.052126

Schaller GE, Binder BM (2017) Inhibitors of ethylene biosynthesis and signaling. Methods Mol Biol 1573:223–235. https://doi.org/10.1007/978-1-4939-6854-1_15

Somssich M, Khan GA, Persson S (2016) Cell wall heterogeneity in root development of *Arabidopsis*. Front Plant Sci 7:1242. https://doi.org/10.3389/fpls.2016.01242

Spreen Brouwer K, van Valen T, Day DA, Lambers H (1986) Hydroxamate-stimulated O_2 uptake in roots of *Pisum sativum* and *Zea mays*, mediated by a peroxidase. Plant Physiol 82:236–240. https://doi.org/10.1104/pp.82.1.236

Swarup R, Perry P, Hagenbeek D, Van Der Straeten D, Beemster GTS, Sandberg G, Bhalerao R, Ljung K, Bennett MJ (2007) Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. Plant Cell 19:2186–2196. https://doi.org/10.1105/tpc.107.052100

Trapet P, Avoscan L, Klinguer A, Pateyron S, Citerne S, Chervin C, Mazurier S, Lemanceau P, Wendehenne D, Besson-Bard A (2016) The *Pseudomonas fluorescens* siderophore pyoverdine weakens *Arabidopsis thaliana* defense in favor of growth in iron-deficient conditions. Plant Physiol 171:675–693. https://doi.org/10.1104/pp.15.01537

Tsang DL, Edmond C, Harrington JL, Nühse TS (2011) Cell wall integrity controls root elongation via a general 1-aminocyclopropane-1-carboxylic acid-dependent, ethylene-independent pathway. Plant Physiol 156:596–604. https://doi.org/10.1104/pp.111.175372

Tsukagoshi H, Busch W, Benfey PN (2010) Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 143:606–616. https://doi.org/10.1016/j.cell.2010.10.020

Verbelen J-P, Cnodder T, Le J, Vissemberg K, Baluska F (2006) The root apex of *Arabidopsis thaliana* consists of four distinct zones of growth activities. Plant Signal Behav 6:296–304. https://doi.org/10.4161/psb.1.6.3511