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► **To cite this version:**

Christian Godon, Harinderbir Kaur, Jean-Marie Teulon, Shu-Wen W Chen, Thierry Desnos, et al.. Stiffening of the Plant Root Cell Wall Induced by a Metallic Stress. Malgorzata Lekka; Daniel Navajas; Manfred Radmacher; Alessandro Podestà. *Mechanics of Cells and Tissues in Diseases*, 2, De Gruyter, pp.333 - 342, 2023, Biomedical Applications, 978-3-11-099972-3. 10.1515/9783110989380-021 . hal-04103337

HAL Id: hal-04103337

<https://hal.science/hal-04103337>

Submitted on 23 May 2023

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6.11 Stiffening of the Plant Root Cell Wall Induced by a Metallic Stress

6.11.1 Introduction

Phosphate (Pi) is one of the main nutrients for plant growth, and Pi starvation has many physiological and developmental effects on crops. One of these responses is the inhibition of the primary root growth, correlated with a rapid decrease of cell elongation. We demonstrated that this inhibition occurs soon after the root-tip encounters substratum acidity, containing iron and low phosphate (–Pi). This condition is called low-Pi stress.

Genetic analysis in *Arabidopsis* unveiled several proteins whose mutation decrease or increase the root growth sensitivity to the low-Pi stress. These proteins belong to a putative functional pathway with two converging branches. In one branch of this pathway, the transcription factor STOP1 (SENSITIVE TO PROTON TOXICITY 1) directly activates the expression of *ALMT1* (ALUMINUM-ACTIVATED MALATE TRANSPORTER 1), coding a malate transporter of the plasma membrane. In seedlings exposed to a low external pH, Fe (as well as Al^{3+}) triggers the accumulation of STOP1 in the nucleus. RAE1 (RIBONUCLEIC ACID EXPORT protein 1), an E3 ubiquitin ligase, negatively regulates the stability of STOP1 in the nucleus. Thus, under iron deficiency, RAE1 promotes the degradation of STOP1, which can be prevented by the treatment with the proteasomal inhibitor, MG132. In the other branch of the pathway, *LPRI* (LOW PHOSPHATE ROOT 1) that codes for a cell wall located ferroxidase.

Acknowledgments: IBS acknowledges integration into the Interdisciplinary Research Institute of Grenoble (IRIG, CEA). This work was partly funded by the Agence Nationale de la Recherche (ANR-18-CE20-0023-03; ANR-09-BLAN-0118; ANR-12-ADAP-0019), CEA (APPTOX021401 and APPTOX021403), and Investissements d'avenir (DEMETERRES). This work acknowledges the AFM platform at the IBS. The authors acknowledge the support of the European Union's Horizon 2020 research and innovation program, under the Marie Skłodowska-Curie grant agreement no. 812772, project Phys2BioMed.

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Wild-type (WT, Col^{er105 1}) seedlings exposed to a low external pH, Fe (as well as Al³⁺) triggers the diminution of root elongation, while for the mutants *stop1* and *almt1*, the roots continue to grow. The current model postulates that exuded malate interacts with the apoplastic Fe. The mechanism inhibiting the cell elongation and cell wall modification is a matter of debate.

The primary cell wall (CW) is a 0.1–1 μm thick network of interconnected cellulose microfibrils and a matrix composed of hemicelluloses, pectins, and structural proteins (see Chapter 4.4). Located in between the two cell walls, the middle lamella primarily contains pectins. Cellulose microfibrils are made of highly crystalline domains (3–8 nm in diameter and several μm in length) linked together by amorphous regions with an interfibril spacing of 20–40 nm; they are very stable with negligible turnover (Cosgrove, 1997). Hemicelluloses, soluble only in strong alkali solutions, form a resilient and robust network with cellulose whereas pectins, soluble in hot aqueous buffer or diluted acids or with calcium chelators, form hydrated gels that push microfibrils apart, participate in wall thickness and wall porosity, and act as an adhesive layer between cells that are together in the middle lamella (Cosgrove, 2005). The major role of primary CW is to resist plant tensile stress while allowing a plant cell turgor-driven elongation. The cell wall is strong enough to support the cell turgor pressure (~1 MPa), which imposes a wall stress of about 10–100 MPa (Cosgrove, 1997).

The thickness, rigidity, and viscoelastic behavior of the cell wall determine the size, shape, morphology, and growth of a plant (Forouzesh et al., 2013). Studying plant biomechanics and mechanobiology extends our understanding of biological acclimation and adaptation of plants to changing physical environment (Moulia, 2013). Although biomechanical studies have been numerous for aerial parts of plants, there is much less study on roots due to the complexity of soils and the practical difficulties in visualizing roots in soils (Moulia, 2013).

One of the factors contributing to the complexity of the plant cell wall is the non-uniform spatial distribution of mechanical properties such as the elastic modulus (Yakubov et al., 2016). Several biophysical techniques have been used to study micro and nanomechanics on plant CW (Burgert and Keplinger, 2013) and several reviews on mechanical principles in plant growth, such as cell extension, growing CW, and CW architecture, can be found here (Cosgrove, 1997, Schopfer, 2006). Plant cell elongation theories are more than 100 years old, with a major account found in 1940 (Heyn, 1940), where it was emphasized that CW must be considered more as a living organ than a dead structure. To study the plant CW, the cellular force microscopy has been developed for large probes and high forces (up to mN), and revealed that

1 Col is the accession name that stands for Columbia (the city where this *Arabidopsis thaliana* strain has been found). *er*, stands for *erecta*, a mutation in the *ERECTA* gene. 105 is the allele (i.e., a specific mutation) number 105. See Torii et al. (1996).

stiffness experiments provide a convoluted property of CW elasticity, turgor pressure, indenter geometry, and history in indentation stress (Routier-Kierzkowska et al., 2012). By using the conventional atomic force microscopy (AFM) with a nanosized tip (~10 nm in radius), it is possible to perform nanoindentation experiments, with forces ranging from 0.1 to 100 nN and with a cantilever of <3 N/m spring constant. It is commonly assumed that with standard AFM, nanomechanical experiments probe only the CW, with an average indentation below 500 nm. It is also commonly accepted that an indentation depth of <5% of the size of the investigated object is a reasonable depth target for AFM experiments on plants (Braybrook, 2015).

Most of mechanical properties of plant cell wall studies by AFM have been obtained on isolated cells (Zhao et al., 2005, Peaucelle et al., 2012, Zdunek and Kurenda, 2013, Yakubov et al., 2016) or on sectioned plant materials (Arnould et al., 2017, Torode et al., 2018, Kozlova et al., 2019). Only a few nanomaterial studies in plant tissues have been performed (Milani et al., 2011, Peaucelle et al., 2011, Milani et al., 2014, Balzergue et al., 2017). It has been proposed that strain-stiffening limits growth and restricts organ bulging (Kierzkowski et al., 2012), but it has also been recently found that plant roots can become stiffer as early as 30 min after exposition to iron stress (Balzergue et al., 2017, Godon et al., 2019).

To understand how the $-Pi$ condition could rapidly inhibit root cell elongation, we reasoned that the mechanical properties of the CW could be modified. We explored the use of nanomechanical experiments with an AFM instrument to analyze the effect of the $-Pi$ stress on the *Arabidopsis* root tip. We focused the probed region on the root epidermis cells located in the transition zone. In this region, cells have ceased their divisions and are ready to elongate rapidly. Results showed that a CW stiffening occurs around 30 min after the onset of the $-Pi$ stress. This stiffening does not arise in the *almt1*, *stop1*, and *lpr1* mutants, and the WT grown on a medium with no added Fe. Using pharmacological drugs, we could show that peroxidases (probably the class III peroxidases that are abundant in the CW) are essential for this stiffening; they probably catalyze some covalent cross-links between macromolecular components of the cell wall. This is the first study showing plant CW stiffening, induced by stress. Most of the nanomechanical results with plant grown in $-Pi$ condition have been published previously (Balzergue et al., 2017).

6.11.2 Elasticity Measurements in the Elongation Zone of Living Plant Roots

All the related materials and methods are located in Chapter 5.4. First, we asked how low Pi ($-Pi$) inhibits cell elongation. In plants, one mechanism that could alter cell expansion is the modification of the mechanical properties of their surrounding cell wall. Cross-links between some polysaccharides or proteins can be at the origin

of this alteration of cell expansion. Thus, our hypothesis was that the cross-links in CW increase cell wall stiffness. To test the hypothesis that stiffness increases early after the onset of low Pi, we used a nanoindentation probe to measure cell surfaces stiffness on root plant seedlings. We measured the stiffness of the root surface in the transition zone, which is located between the root apical meristem (RAM) and the elongation zone (EZ) (Figure 6.11.1), where cells rapidly elongate when conditions are permissive. This region is localized at about 500 μm from the root tip.

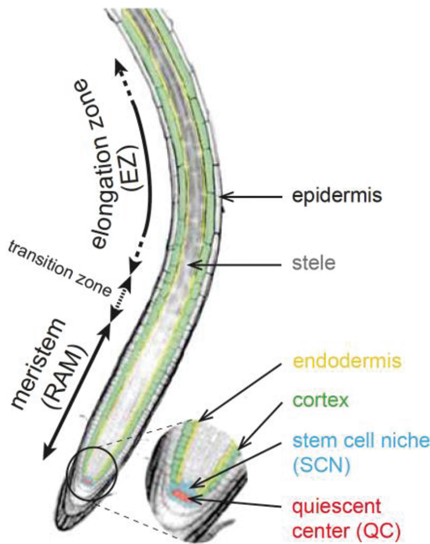


Figure 6.11.1: Scheme depicting different cell types of the primary root tip. Figure adapted from Balzergue et al. (2017).

AFM measurements were performed on WT seedlings that grew on a +Pi medium and transferred to a -Pi medium for up to 2 h. We discovered that cell wall stiffness increased as early as 30 min after transfer to -Pi and continued to increase in later time points (Figure 6.11.2). All the experimental details for these experiments could be found in Chapter 5.4.

Three different mutants *lpr1*, *stop1*, and *almt1*, in which root cell expansion is not restrained under -Pi and isolated in our laboratory (Balzergue et al., 2017), were tested under -Pi condition (Figure 6.11.3²).

² *Col^{er105}* is the wild-type seedling whereas *lpr1,lpr2* is a double mutant in the *lpr* genes. *Stop1⁴⁸* and *almt1³²* are mutants in *STOP1* and *ALMT1* genes. All the mutants *lpr*, *stop1*, and *almt1* are insensitive to the reduction of phosphate concentration in the environment (or the increase of iron concentration in the environment).

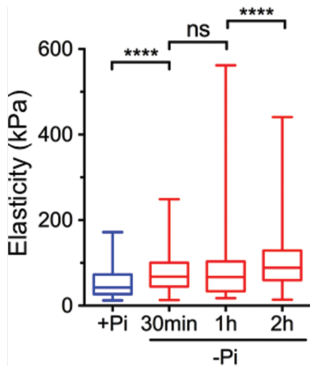


Figure 6.11.2: WT (*Col^{er105}*) seedlings were transferred to $-Pi$ or $+Pi$ medium for the indicated time, prior to measuring by AFM the stiffness of the cell surface in the transition zone of the primary root (See Methods, Chapter 5.4) (median \pm interquartile, Mann–Whitney’s test. **** $P < 0.0001$; ns, not significant ($P > 0.05$)). $-Pi$ indicates the absence of added Pi in the culture medium. Figure adapted from Balzergue et al. (2017).

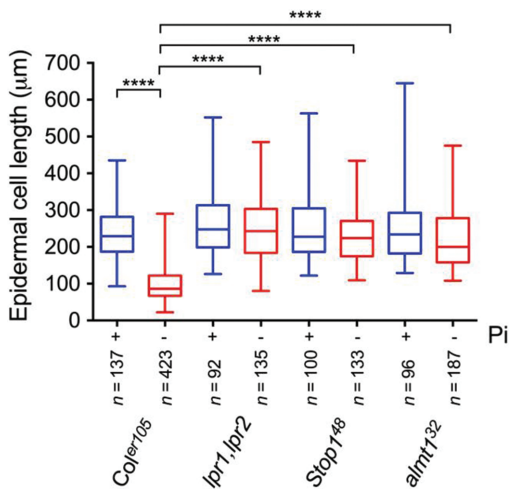


Figure 6.11.3: Seven-day-old seedlings of the indicated genotype were transferred to $+Pi$ or $-Pi$ medium for 24 h before measuring the final length of root epidermal cells (median \pm interquartile; Tukey’s whiskers; Mann–Whitney’s test: **** $P < 0.0001$; n, number of cells). Figure adapted from Balzergue et al. (2017).

We observed that the higher cell wall stiffness, measured for the WT, was decreased for the four *stop1*, *almt1*, and *lpr1,lpr2* mutants (Figure 6.11.4). For the first time, using AFM/nanoindentation probe, our results reveal a negative reciprocal relation between cell wall stiffness in the root transition zone and the final epidermal

cell length. This suggests that low Pi triggers cell wall stiffening of pre-elongated cells to restrict their elongation.

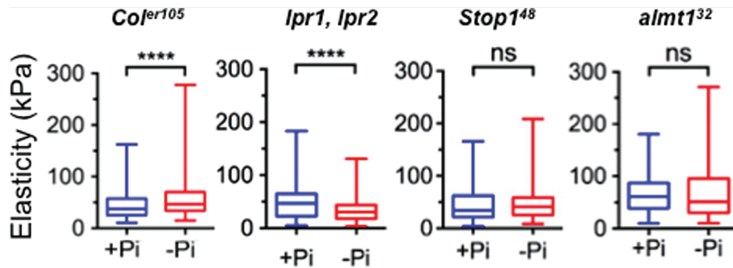


Figure 6.11.4: Seedlings of the indicated genotype were transferred to +Pi or -i medium for 30 min prior to measuring by AFM the stiffness of the cell surface in the transition zone of the primary root (median +/- interquartile, Mann-Whitney's test; **** $P < 0.0001$; ns, not significant ($P > 0.05$)). Figure adapted from Balzergue et al. (2017).

Class III peroxidases are thought to inhibit cell expansion by catalyzing cross-links between some polysaccharides or proteins, thereby tightening the cell wall (Passardi et al., 2004, Wolf et al., 2012). To test the hypothesis that -Pi induced peroxidase activity would cause cell wall stiffening at the root tips, we used a pharmacological approach to inhibit peroxidase activity. Salicylhydroxamic acid (SHAM), a peroxidase inhibitors (Rich et al., 1978, Balazs et al., 1986), treatment restored the WT root growth under -Pi condition and significantly increased the root epidermal cell length (Figure 6.11.5A). Consistent with the cell wall elongation restoration, we discovered that SHAM strongly decreased cell wall stiffness in -Pi (Figure 6.11.5B). These observations support the view that peroxidase activity catalyzes crosslinks in some cell wall components, thereby reducing cell expansion by chemical modification in the cell wall.

Recently, we demonstrated that in growth conditions with limited Pi allowing to distinguish the effect of Fe from the -Pi condition, Fe triggers the accumulation of STOP1 in the nucleus and increases the expression of *ALMT1* (Godon et al., 2019). In our previous work (Balzergue et al., 2017), we showed that in -Pi condition without Fe addition, root cell expansion is not restrained, and the root growth is comparable to seedlings growing on +Pi (Figure 6.11.6). Cell wall stiffness was measured in -Pi condition, with or without Fe addition, and results reveal a reciprocal relation between increased cell stiffness in the root transition zone and increased iron quantity in the medium. This suggests that Fe triggers cell wall stiffening to restrict cell elongation.

All the above experiments demonstrated that after the transfer of seedlings from the condition for which the Fe had no effect on root cell elongation (+Pi + Fe or -Pi-Fe) to a condition for which the Fe has an effect (-Pi + Fe), the cell stiffness increased rapidly (Balzergue et al., 2017). To evaluate the operational impact of our protocol (Azimzadeh et al., 1992, Godon et al., 2017, Teulon et al., 2019), it was decided to

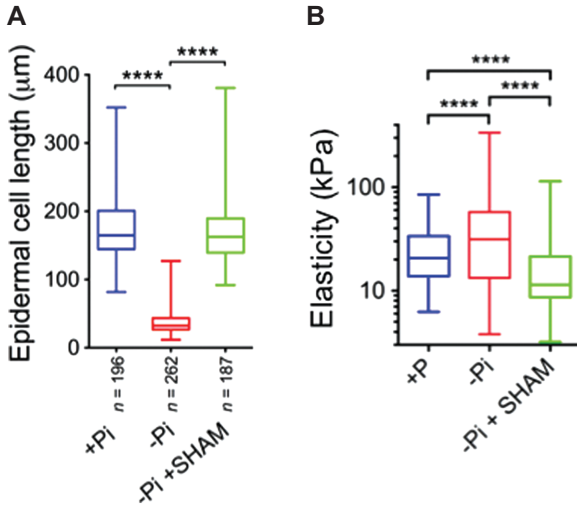


Figure 6.11.5: Effect of SHAM on root epidermal cell length. (A) Three-day-old WT seedlings were transferred for 7 days to +Pi or –Pi medium with or without 15 μM SHAM prior measuring the root epidermal cell length. (B) Three-day-old WT seedlings were transferred for 30 min to +Pi or –Pi medium with or without 15 μM SHAM prior to measuring by AFM the stiffness of the cell surface in the transition zone of the primary root (median \pm interquartile; Tukey’s whiskers, Mann–Whitney’s test: **** $P < 0.0001$, n = number of cells). Figure adapted from Balzergue et al. (2017).

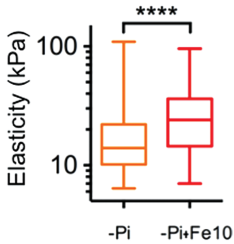


Figure 6.11.6: WT (*Col^{er105}*) seedlings were transferred to –Pi or –Pi + Fe (10 μM FeCl_2) medium for 30 min prior to measuring by AFM the stiffness of the cell surface in the transition zone of the primary root (median \pm interquartile, Mann–Whitney’s test. **** $P < 0.0001$; ns, not significant ($P > 0.05$)). The experiment was performed twice with consistent results, and one representative experiment is shown. Fe10 means the presence of 10 μM FeCl_2 . –Pi indicates the absence of added Pi to the culture medium. Figure adapted from Balzergue et al. (2017).

measure the stiffness on seedlings that have grown continuously in the (–Pi + Fe) medium, and not after a transfer. WT seeds were sown on –Pi plates containing 0, 8, 10, or 12 μM Fe, and they were grown for 4 or 7 days. We observed that after 4 days on plates containing 10 or 12 μM Fe, the primary root growth was strongly reduced, compared to lower concentrations of Fe, whereas the seedlings on 8 μM Fe were as long as those on the control plate without Fe (Figure 6.11.7A). Interestingly,

all the seedlings grown for 4 days with Fe showed the same increased stiffness, compared to the control 0 Fe (Figure 6.11.7B). By letting seedlings grow for 3 more days (7-day-old) we observe that those under 8 μM Fe condition are shorter than the control 0 Fe, although not as short as those on 10 and 12 μM Fe. This shows that a Fe-triggered stiffening of root cell surface occurs even without an immediate reduced growth. These observations suggest that either the stiffening of internal cell walls (not accessible with our AFM setup) is lower at 8 μM Fe than at 10 and 12 μM , thus allowing the root to grow longer, or the stiffening is necessary to prevent root growth and there is another Fe-dependent reaction inhibiting the growth.

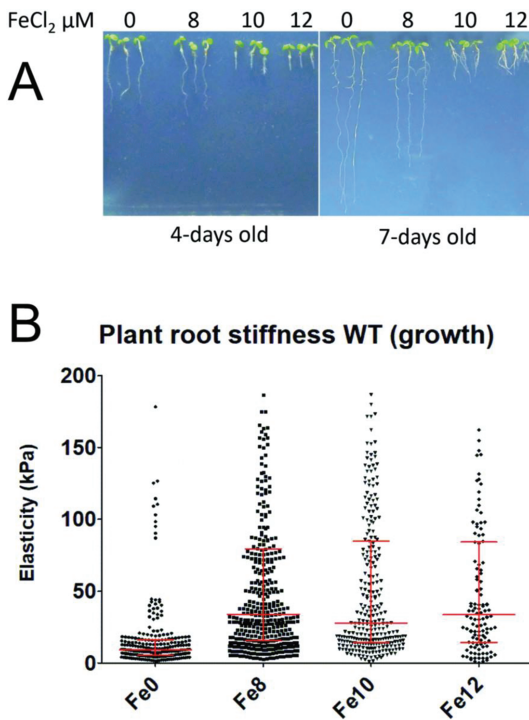


Figure 6.11.7: (A) WT seeds were sown on $-\text{Pi}$ agar medium containing 0, 8, 10, or 12 μM Fe, and grown for four or seven days before taking the picture. (B) Stiffness of the root transition zone surface from seedlings grown 4 days in conditions as in (A). The minimum and maximum scale of red bar shows the interquartile region showing 50% of the data, whereas the middle red line signifies the median. Y-axis shows the stiffness (kPa) and X-axis shows the different iron concentrations. Sixty-one points are scattered outside the visible Y-scale.

In conclusion, a similar stiffening of plant roots has been observed, whether plants were growing on iron-free medium (then transferred to Fe-rich medium before nanomechanical measurements) or on iron-containing medium until nanomechanical measurements. The impact of nanomechanical measurements (implying a probing

at the nm scale) with AFM is demonstrated by the ability to detect very early events in plant root physiology, even before an observable phenotype. However, plants growing continuously in the presence of iron show a relatively small growth in time, whereas plants grown in the absence of Fe in agar and then transferred to a Fe-rich agar show a strong growth arrest (data not shown). This indicates that plants behave differently when they are sown under a stress condition, and they tend to counteract and develop their own signaling pathway to overcome the stress.

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