Dual role of Pectin Methyl Esterase activity for plant cell growth regulation.

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ABSTRACT

Acid-growth theory has been postulated in the 70s as a model to explain rapid elongation of plant stems in response to plant hormone auxin. More recently, it was demonstrated that hormones like auxin as well as brassinoesteroids (BR) induce cell elongation through the activation of proton ATPs pumps (H^+-ATPs) thereby inducing apoplastic acidification. However, the impact of this acidification on the mechanical properties of the cell wall remained largely unexplored. Here we use Atomic Force Microscopy (AFM) to demonstrate that acidification of the apoplast is sufficient and necessary to induce cell elongation through cell wall relaxation. Moreover, we demonstrate that a tight control of Pectin Methyl Esterase (PME) activity is required for proper hypocotyl elongation, and that PME can induce both cell walls softening or stiffening in extracellular calcium dependent-manner.

Key words: AFM, Hypocotyl, Auxin, Brassinoesteroid, Arabidopsis thaliana, cell elongation, cell wall, pectin

INTRODUCTION

Plants, as sessile organisms, developed exceptional plasticity of growth and development to adapt to their ever-changing environment. One of the most important mechanisms to ensure adjustability is a tightly controlled cell elongation. Plant cells grow by anisotropic expansion, which results from the interplay between turgor pressure and a fine-tuned local cell wall (CW) relaxation (Wolf et al., 2012; Braidwood et al., 2014).

Auxin was shown to induce rapid cell elongation in stem, coleoptiles, or hypocotyls segments within minutes (Rayle and Cleland, 1992; Fendrych et al., 2016). Current model of auxin-regulated cell expansion is based on the acid growth theory (reviewed in Mockaitis and Estelle, 2008; Perrot-rechenmann, 2010). In this model, auxin was shown to induce transcription of *SAUR* (*Small Auxin Upregulated genes*) genes, which indirectly activate the plasma membrane (PM)-H^+-ATPase, pumping protons into the extracellular space. Recently, it was also shown that auxin can activate PM-H^+-ATPase through yet another mechanism mediated by Receptor-like Transmembrane Kinase1 (TMK1) (Lin et al., 2021). The activation of the proton pump lowers the pH in the apoplast, activating CW loosening proteins such as expansins and xyloglucan endotransglycosylase/hydrolases. Activated CW remodeling enzymes make the CW relaxed for expansion (Cosgrove, 2018). In parallel to CW acidification, auxin in the nuclei enhances the transcription of genes including those encoding PM-ATPase, K^+ channels, expansins, and other CW remodeling enzymes. Other hormones, like brassinoesteroids, are also inducing stem elongation through activation of PM-H^+-ATPase, and at the same time also regulate expression of several cell wall remodeling enzymes (Minami et al., 2019).

Pectins are important components of the CW determining different aspects of tissue growth, such anisotropic growth of hypocotyl epidermal cells or meristem development (Peaucelle et al., 2011, 2015). Pectins are polymers composed by α-D-galactosyluronic acid (Gal-A) forming homogalacturonan (HG) linear chains. Pectins are by default transported in a methyl-esterified state to the apoplast where they can be de-esterified by Pectin Methyl-Esterases (PME) proteins, in a process that liberates protons. Methyl-esterified pectins have free carboxyl groups that are hypothesized to form crosslinks mediated by calcium ions (the so-called egg-box hypothesis), which might increase rigidity of cell wall (Hocq et al., 2017b). Based on super-resolution microscopy, it has been described that pectins possibly form nanofilaments that alter their quaternary structure upon de-methylesterification and calcium cross-linking, confirming the egg-box model (Haas et al., 2020).

In vitro assays showed that PME activity is attenuated at low pH and that interactions of PME with pectin methyl-esterase inhibitors (PMEIs) are stabilized (Hocq et al., 2017a). Despite their importance as components of the CW, there is still a gap of information on the effects of PME and PMEI on growth processes and contradictory results have been published on the effects of those proteins (Jonsson et al., 2022; Cosgrove, 2022). Whilst some authors reported increased stiffness and shorter cells in the hypocotyl of lines overexpressing pectin methylesterases (Bou Daher et al., 2018), others have shown decreased stiffness and increased cell size (Peaucelle et al., 2015). It has been hypothesized that blockwise demethylesterification might lead to stretches of demethylesterified Gal-A. On other hand, randomly or low-methylesterified HGs might be substrate for HG modifying enzymes (HGMEs) such as polygalacturonases (PGs) and pectin/pectate lyases (PLs/PNLs) that cleave demethylesterified

pectin to shorter HG chains and thereby might promote cell wall loosening. Lines overexpressing PMEI seems to have controversy of phenotypes too. AtPMEI3 overexpression resulted in shorter cells and loss of growth asymmetry. Inhibition of floral organ primordia formation (Peaucelle et al., 2011, 2008), and high PMEI activity also correlates with inhibition of growth in apical hook formation (Jonsson et al., 2021). However, increased root length was observed in Arabidopsis plants expressing a PMEI from cotton (Liu et al., 2018) and Arabidopsis plants with high levels of AtPMEI2 show enhanced growth and increased root length due to stronger cell elongation (Lionetti et al., 2007, 2010). Expression of AtPME1 in tobacco pollen tubes inhibited elongation, whereas AtPMEI2 expression led to an increase in their elongation rate (Röckel et al., 2008). Therefore, a developmental or cellular context needs to be taken into account to explain the dual role of PME and PMEI in growth processes, being able to correlate with both stiffening and softening of CW, and induce or inhibit growth.

Atomic Force Microscopy (AFM) techniques have recently arised as an important tool to monitor cell wall mechanical properties. Herein our study, we monitored the effect of several plant hormones in CW mechanical properties by AFM. We further applied this technique to characterize how modulation in PME activity affects cell wall mechanical properties in relation to calcium levels in the apoplast. We demonstrate a dual role of PME activity, indicating that a narrow window of pectin methylesterification is important for proper cell elongation.

RESULTS

Apoplastic acidification is necessary and sufficient to induce cell elongation in hypocotyl.

Modulation of hypocotyl elongation growth is one of the prominent adaptive responses to changes in environmental conditions such as an increase in temperature or light changes. At the molecular level, hypocotyl growth is governed by the hormone auxin, which in concert with other hormonal pathways such as brassinosteroids (BR) or gibberellins (GA), promotes cell expansion. Acidification of the apoplast triggered by auxin is one of the essential regulatory steps in this process (Oh et al., 2014).

To test the impact of apoplastic acidification on hypocotyl elongation, we incubated segments of 3-day-old etiolated hypocotyls of *Arabidopsis thaliana* in acidic (pH 3,5) and alkaline (pH 9,0) buffers. Using the ApopH apoplastic pH sensor (Fendrych et al., 2016) we confirmed that incubation of hypocotyl segments in respective buffers for 2 hours resulted in expected changes of the apoplast pH in hypocotyl epidermal cells (Fig. S1A,B). Comparably to the incubation in acidic buffer, treatment with 10μM synthetic auxin 1-Naphthylacetic acid (NAA) for 2 hours led to acidification of the apoplast (Fig. S1A,B), while auxin was not able to trigger acidification of apoplast when pH9 buffer was used.

Measurements of hypocotyl elongation revealed that 3h of incubation in acidic medium was sufficient to trigger hypocotyl elongation, in similar range as hormone auxin or brassinoesteroid (epibrassinolide, eBL) at pH6. Alkaline buffer did not promote hypocotyl elongation, and interfered with hormone-induced elongation (Fig. 1A, B). Importantly, analysis of the auxin sensitive reporter R2D2 (Liao et al., 2015) demonstrated that auxin response is not affected by alkaline pH buffer itself (Fig. S1 C,D). To correlate hypocotyl elongation with individual cell elongation we measured individual cell

response to auxin treatment (Fig. S2). All cells of hypocotyl segments show same growth rate, confirming that whole tissue measures correlates with individual cell elongation.

These results show that the acidification of the apoplast is both sufficient and necessary for hypocotyl cell elongation.

Figure 1

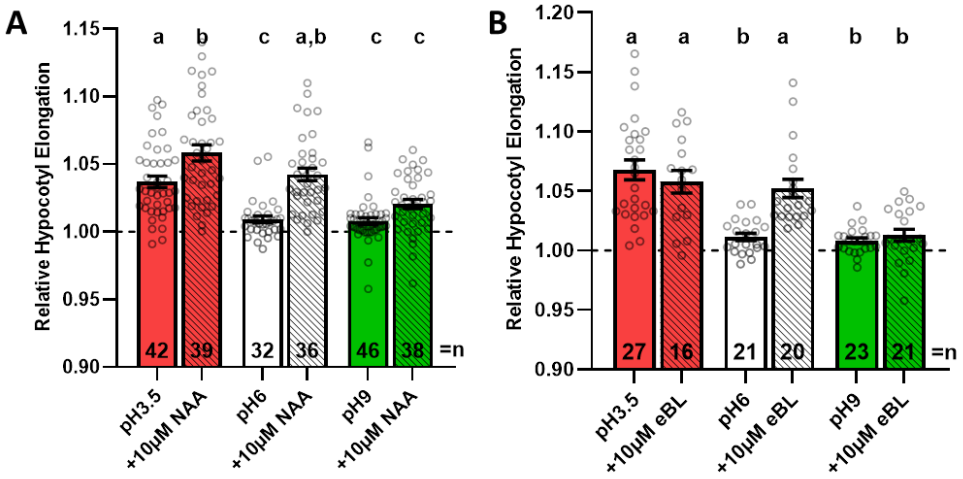


Figure 1. Acidification is required and sufficient to induce hypocotyl segments elongation. A and B. Relative hypocotyl segment length after 3h of indicated treatments. Hypocotyl segments do not elongate on just depletion medium (DM, pH6); auxin (10 µM NAA) and brassinoesteroid (10µM eBL) trigger segment elongation. Lower pH (pH3.5) also triggers elongation, but no treatment trigger elongation anymore in alkaline buffered media (pH9). Bars represent average ± SE, dots represents individual data points. N indicates total number of hypocotyls segments quantified. Tukey-Kramer test was performed and significant differences are shown with letters above each bar.

Acidification of the apoplast correlates with cell wall softening.

While the promoting/inducing effect of acidification on hypocotyl expansion is firmly established, its impact on cell wall bio-physical properties is still poorly understood. Old reports using extensometers had shown that stems seem to be able to stretch more after acidification (Cosgrove, 1993). However, latest results point that tensile stretching might not correlate with cell wall mechanical properties quantified by other techniques like Apparent Young Modulus (E_a) obtained from AFM (Zhang et al., 2019).

To establish and validate our AFM experimental platform for measurements of cell wall mechanical properties we inspected how cell wall rigidity of hypocotyl epidermal cells is altered in different growth regimes. Hence, we measured E_a in either long etiolated or short de-etiolated hypocotyls of Col-0 or hypocotyls of light insensitive mutants in phytochrome A and B (*phyAphyB*), which elongate despite growing in light conditions (Fig. S3B). We observed a strong correlation between cell wall mechanical properties and hypocotyl elongation. An average apparent E_a measured on longitudinal cell walls (parallel with hypocotyl growth axis) in etiolated hypocotyls was around 441 kPa ±87 *SE*. In light grown hypocotyls E_a increased to 3699 kPa ±670, while in the *phyAphyB* mutant (with intermediate hypocotyl elongation) intermediate E_a values of around 1544 kPa ±130 were detected (Fig. S3C). Those results are largely in agreement with previously published reports (Peaucelle et al., 2015), including measurements using other techniques such Brillouin scattering microscopy (Elsayad et al., 2016).

Applying the same AFM set-up, we further analyzed impact of hormonal treatments on mechanical properties of cell walls. All treatments, which led to enhanced hypocotyl elongation such as auxin, BR and GA decreased cell wall stiffness in a similar rate (60% of E_a compared to non-treated stem; 0,52 ±0,08 *SE* for auxin, 0,72 ±0,05 for BR and 0,71 ±0,06 for GA; Fig. 2). As all these hormonal treatments can induce apoplastic acidification (Cosgrove, 2016; Chaiwanon et al., 2016), we inspected whether direct acidification of cell walls would induce similar changes in cell wall stiffness. Apoplastic acidification as result of either the PM-H^+-ATPase pump activation by fusicoccin drug (FC, Baunsgaard et al., 1998; Ballio et al., 1964), incubation of hypocotyls in acidic buffer (pH3.5) or overexpression of SAUR19 (Spartz et al., 2012) led to similar reduction of E_a when compared to wild-type control incubated in medium of pH6 (0,67±0,06 for pH3.5 buffer, 0,53±0,03 for FC, 0,59±0,04 for SAUR19). The incubation in alkaline buffer had no significant effect on hypocotyl E_a. Hence, we hypothesize that various hormonal treatments promoting hypocotyl elongation converge on regulation of mechanisms that control acidification of the apoplast and subsequently promote cell wall softening.

Figure 2

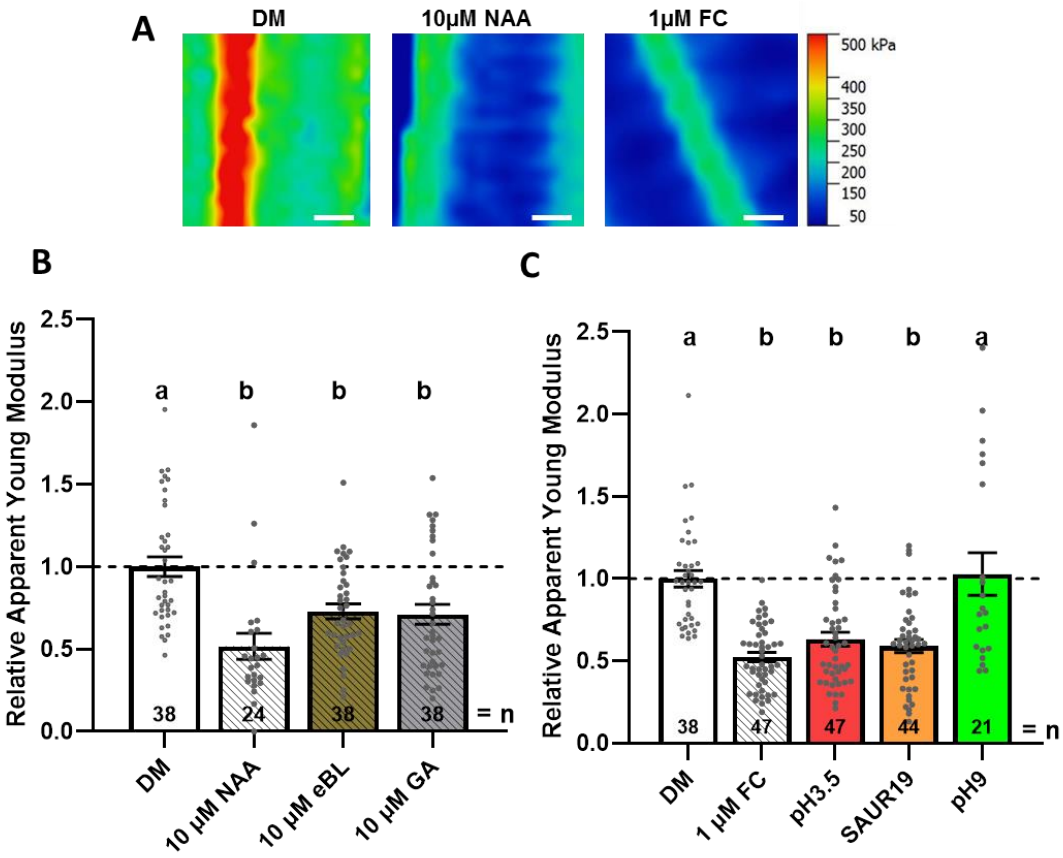


Figure 2. Several hormones and treatments induce similar decrease in E_a. A. Average E_a heat-maps for depletion media (DM), auxin or fusicoccin using 3day-old hypocotyl segments incubated for 1-2h with indicated treatment. B and C. Relative (to DM) E_a quantifications for hormonal treatments (B) or active acidification treatments (C). Bars represent average ± SE, dots represents individual data points. N indicates total number of scans performed and analyzed. Tukey-Kramer test was performed and significant differences are shown in the upper letters of each bar.

Auxin signaling is required to induce softening of cell wall.

Our results pointed out that hypocotyl elongation triggered by hormones like auxin or BR or by apoplast acidification correlate with cell wall softening. Published works have shown that intact signaling pathway is required for auxin-induced acidification and hypocotyl elongation (Fendrych et al., 2016), but that BR is able to activate PM-H^+-ATPs directly (Caesar et al., 2011; Minami et al., 2019). Using inhibitor of proteosynthesis cycloheximide (CHX) (Rose, 1974) we observed that whereas auxin mediated hypocotyl elongation requires protein biosynthesis, hypocotyl elongation triggered by incubation in acidic buffer (pH3.5) is not dependent on *de-novo* protein synthesis (Fig. 3A,B, middle and left columns). BR promoting effect on hypocotyl elongation is partially independent of *de novo* protein synthesis; although hypocotyls elongate more if proteosynthesis is not compromised (Fig. 3A, right columns). Next, we examined whether auxin-dependent hypocotyl elongation correlates with alterations in cell wall mechanical properties. Using heat inducible *HS::axr3-1* transgenic line (Knox et al., 2003), we found that accumulation of *axr3-1*, a dominant negative repressor of auxin signaling, interferes with auxin mediated softening of cell walls when compared to no-heat treated control (Fig. 3C). We conclude that auxin-induced elongation growth and cell wall softening requires *de-novo* protein biosynthesis activated through auxin transduction cascade.

Figure 3

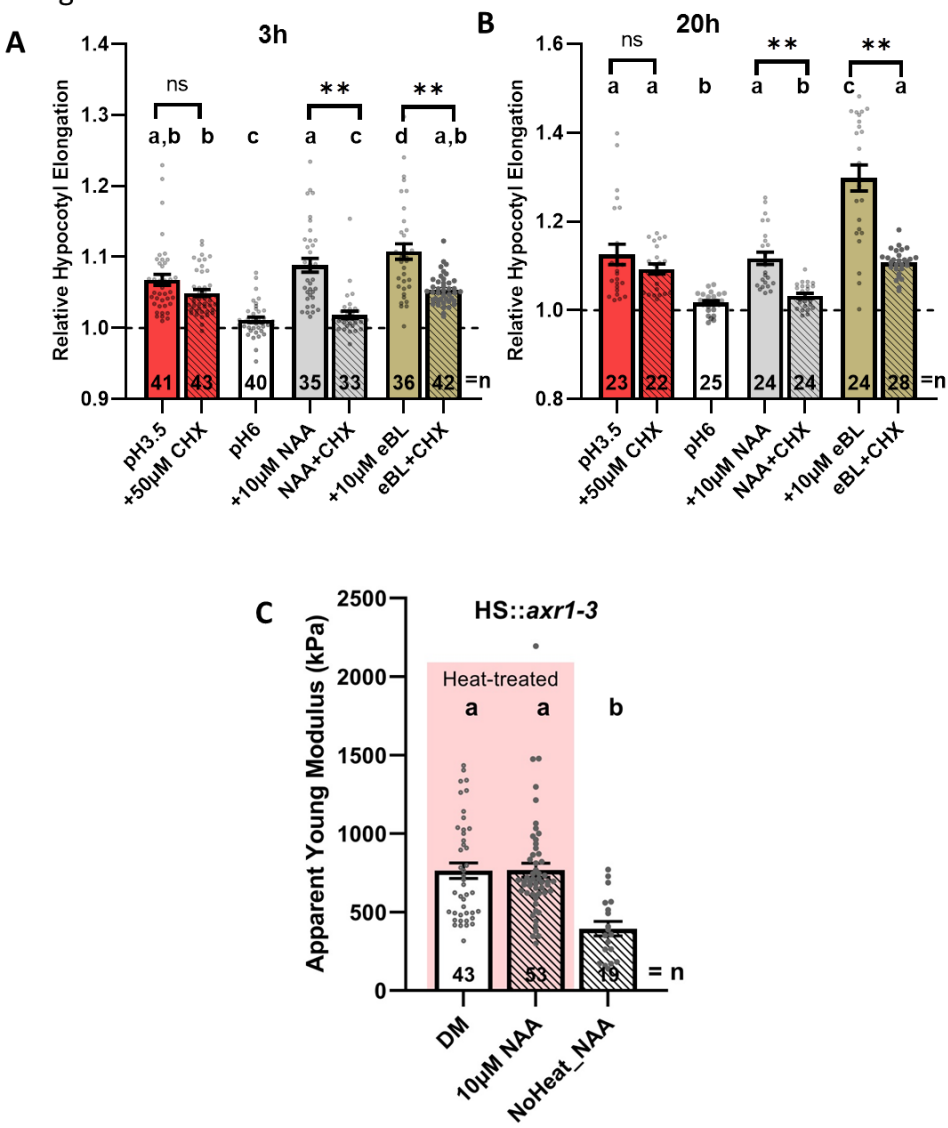


Figure 3. Auxin requires transcriptional machinery, but low pH and BR have a direct effect on hypocotyl segments elongation. A and B. Relative hypocotyl segment length after 3h (A) or 20h (B) of indicated treatments. The protein synthesis inhibitor cycloheximide (50 µM CHX) completely blocks auxin-induced elongation, but not pH or BR induced elongation. C. Average E_a quantification of cell walls of 3day-old hypocotyl segments incubated for 1-2h with indicated treatment for the dominant negative auxin line (HS::*axr1-3*). Bars represent average ± SE, dots represents individual data points. N indicates total number of hypocotyls segments quantified (A and B) or scans performed and analyzed (C). Tukey-Kramer test was performed and significant differences are shown in the upper letters of each bar. Direct pair significant differences are indicated as **P < 0.01 (t-test).

Balanced PME activity is necessary for fast hypocotyl elongation.

We showed that auxin (or hormonal) induced apoplastic acidification is necessary for fast hypocotyl elongation that correlates with cell wall softening. However, processes associated with re-structuring of cell wall that result in modulation of its biophysical properties are still poorly understood. Pectins, as important components of the primary cell wall, have been proposed as principal contributors of mechanical properties of the cell wall (Haas et al., 2021; Peaucelle et al., 2015). In particular, degree and pattern of methylesterification of homogalacturonan chains controlled by Pectin Methyl-Esterases (PME) and PME Inhibitors (PMEI) antagonizing their activities, might have a decisive impact on cell wall mechanics.

To study early responses to transient increase of PME activity, we generated inducible PME lines. PME1 as an example of the auxin inducible homologue of PME family, and a previously studied PME5 were selected for further analyses. Several independent lines were obtained and selected based on expression levels determined by RT-qPCR and Western-Blot (Fig. S4A and B). Expression of PME1 as well as PME5 in seedlings exposed to β-estradiol (β-EST) for 48 hours affected seedling development and resulted in shorter root compared to non-induced seedlings (Fig. S4C), confirming that recombinant PME1-HA and PME5-HA proteins maintain their activities.

To examine how the observed growth defects correlate with cell wall mechanical properties we employed AFM. Monitoring of cell wall properties revealed surprising, time-dependent effects of PME activity on cell wall mechanics (Fig. 4B). Induction of PMEs expression for short time (~24h) resulted in stiffer CW, whilst PME expression persisting for about 3 days correlated with cell wall softening when compared to untreated control. Hence, softening of cell walls previously observed in seedlings with overexpression of PME5 (Peaucelle et al., 2015) might be indicative of very strong and/or prolonged PME activity.

Next, we analyzed whether enhanced activity of PME1 or PME5 affects auxin-induced elongation of hypocotyls. Intriguingly, auxin-induced elongation was hampered in hypocotyls in which PMEs expression was induced (24h of β-EST induction and 3h with auxin) when compared to non-induced controls, indicating that methyl-esterification of pectin interferes with auxin-triggered elongation of hypocotyls (Fig. 4A). As an enhancement of the PME expression interfered with auxin-induced hypocotyl elongation (results from Fig. 4A), we asked whether the PME inhibitory effect on auxin-mediated hypocotyl growth correlated with alteration of CW mechanical properties. AFM measurements revealed that PME expression attenuates auxin capacity to relax CW (Fig. 4C).

Altogether, these results suggest that methyl-esterification of pectin might be required for fast hypocotyl elongation in response to auxin, and that PME activity has a dual role, leading to both, softening or stiffening of cell wall depending on duration or intensity of PME activity.

Figure 4

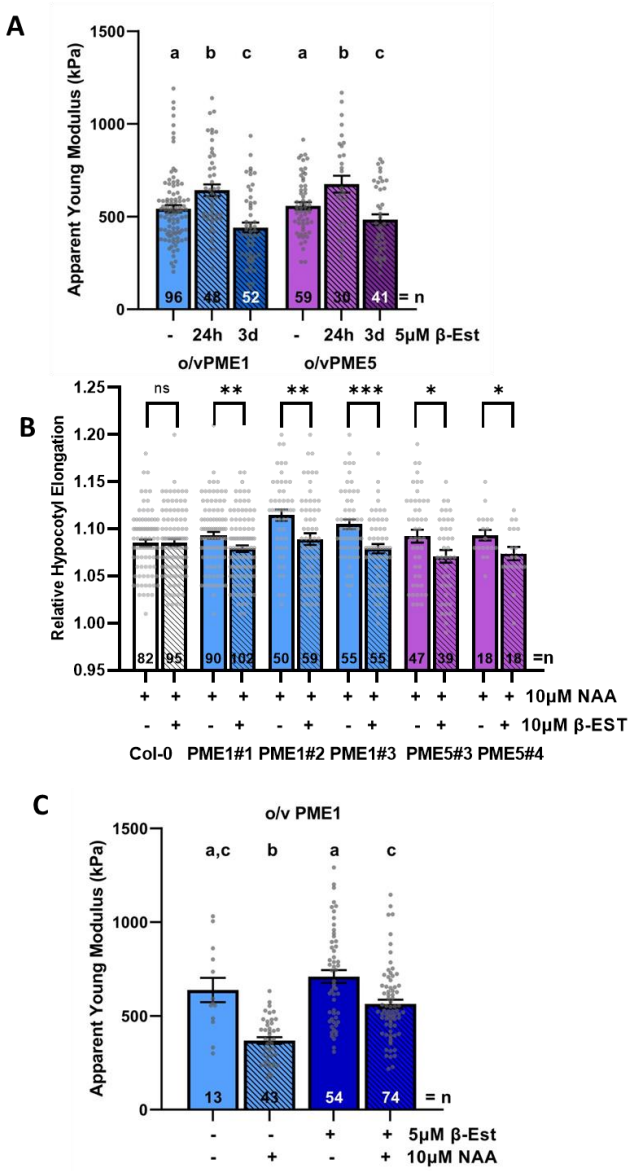
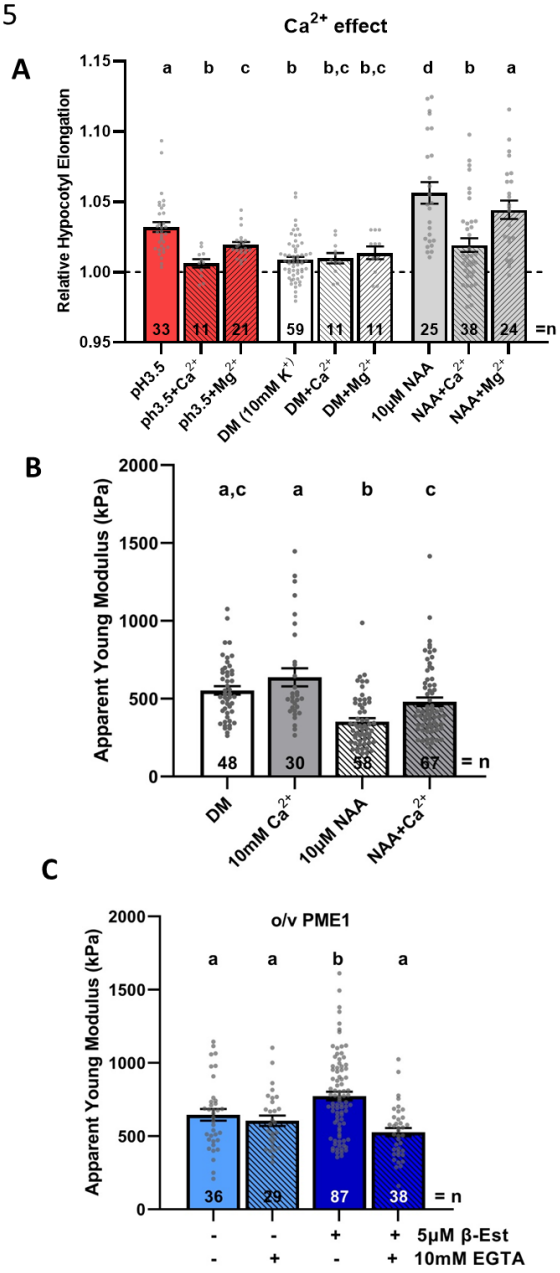


Figure 4. o/v PME interferes with auxin triggered elongation by increased E_a. A. Average E_a quantification of cell walls of 3day-old seedlings for o/vPMEs lines at indicated induction times. B. Relative hypocotyl segment length after 24h of β-EST and 3h of auxin treatments. C. Average E_a quantification of cell walls for 3day-old hypocotyl induced (or not) for 24h in β-EST and segments incubated (or not) for 1-2h with auxin treatment. Bars represent average ± SE, dots represents individual data points. N indicates total number of hypocotyls segments quantified (A and C) or scans performed and analyzed (B). Tukey-Kramer test was performed and significant differences are shown in the upper letters of each bar. Direct pair significant differences are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001 (t-test).

<u>Calcium availability affects the PME mediated cell wall stiffening</u>

Paradox of how PME activity might lead to two distinct effects on CW mechanics has been addressed by Hocq et al., (Hocq et al., 2017a) suggesting that degree of HGs demethylation might either promote Ca^{2+} cross-links resulting in higher stiffness of CW, or cleavage to small HGs chains and thus CW relaxation. Our AFM measurements in hypocotyls pointed that CW properties can be modified differently according to duration of PME activity. To assess a role of calcium in the modulation of hypocotyl elongation and CW mechanical properties, we performed elongation assays with supplemented calcium. Adding calcium, including or not potassium into the depletion media, attenuated auxin- as well as acidification-triggered elongation of hypocotyls (Fig. 5A and S4). These results support a scenario in which extracellular calcium might constrain hypocotyl elongation through promoting pectin cross-linking and increasing cell wall stiffness. In line with this hypothesis, AFM measurements of hypocotyls segments revealed significantly increased stiffness of apoplast after auxin treatment at presence of calcium when compared to control hypocotyls exposed to auxin without co-treatment with calcium (Fig. 5B). To further examine whether higher CW stiffness as result of PME expression involves calcium mediated crosslinking of HGs we incubated hypocotyls of PME5 expressing line in medium supplemented with calcium chelator EGTA (Feng et al., 2018). Reduction of CW stiffness to levels comparable to these detected in control hypocotyls with non-induced PMEs expression (Fig. 5C) suggests that calcium in the apoplastic region plays a critical role in HG cross-linking shortly after activity of PME is increased.

Figure 5



Altogether, this data support the model in which a short-term PME activity generates blockwise de-methylation pattern of HGs, which is prone to cross-linking by Ca^{2+} that promotes stiffening of cell walls.

Figure 5. Calcium interferes with auxin triggered elongation by increased Ea. A. Relative hypocotyl segment length after 3h of indicated treatments. B. Average E_a quantification of cell walls at indicated conditions for 3day-old hypocotyl segments incubated for 1-2h with indicated treatments. C. Average E_a quantification of cell walls for 3 day-old hypocotyl induced for 24h in β-EST and segments incubated for 1-2h with indicated treatment. Bars represent average ± SE, dots represents individual data points. N indicates total number of hypocotyls segments quantified (A) or scans performed and analyzed (B and C). Tukey-Kramer test was performed and significant differences are shown in the upper letters of each bar. Tukey-Kramer test was performed and significant differences are shown in the upper letters of each bar.

DISCUSSION:

The relation between CW composition and its mechanical properties is still a big unsolved enigma. Cell wall remodeling is a dynamic process that might depend on tissue and time scale, being a complex result of cross-talk between several hormones, regulating remodeling enzymes, apoplastic calcium ions and pH. Plant hormones are changing expression and activity of components that regulate cell wall synthesis and structure, but how many of those mechanisms are shared or hormone specific is still unclear (Nemhauser et al., 2006).

Here, we show that acidification is a first necessary step for CW relaxation in hypocotyl cells. When acidification by hormones is compromised by alkaline environment softening of cell walls and growth is significantly attenuated, indicating that it is a shared mechanism among several plant hormones that promote hypocotyl elongation.

Pectin is an important component of cell wall. Recent report using 3D-STORM microscopy applied on cotyledon pavement cell revealed that degree of methylation of HGs might determine quaternary structure that defines intrinsic pectin swelling properties. These observations were summarized in the “expanding beam model” proposing that local HG demethylesterification leads to nanofilament radial

swelling, which is caused by conversion between quaternary structures with different packaging (Haas et al., 2020, 2021). The author showed that demethylesterification of HG alone is sufficient to induce tissue expansion in cotyledon pavement cells. In such context, the activity of PME and PMEI enzymes should be a key factor regulating cell elongation. Previous reports have demonstrated that PME and PMEI are important for asymmetrical cell growth (Peaucelle et al., 2015; Jonsson et al., 2021). Our results reveal novel aspects about how PME activity might lead to completely different outputs depending on time and/or intensity. Noteworthy, PME activity is liberating protons, but once certain level of apoplastic acidification is reached, PMEs are barely active and PMEI are strongly blocking their activity, forming a negative feedback loop that might protect cells from PME hyperactivity (Hocq et al., 2017b). These suggest that demethylesterification of HGs is an essential determiner of cell expansion and tissue growth and that a narrow window of PME activity is required for proper cell elongation.

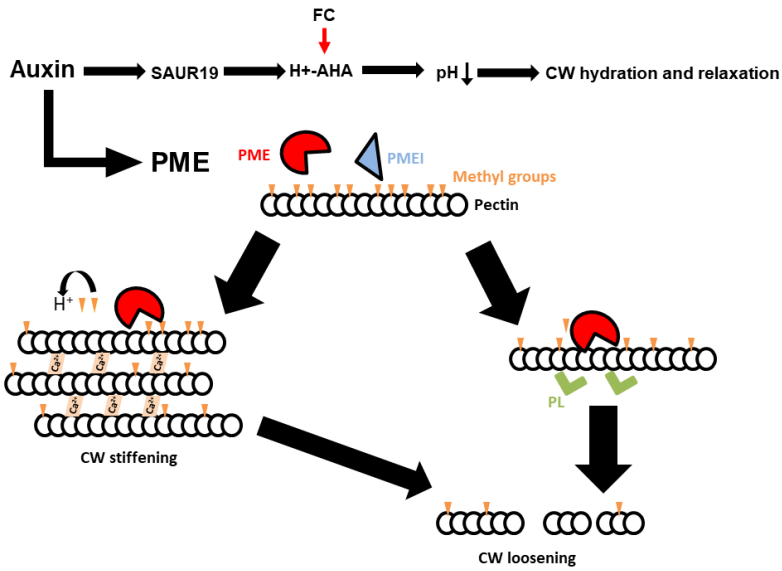


Figure 6. Model for dual role of PME activity. Hormonal treatments converge in apoplastic acidification, which trigger a CW relaxation. In parallel, PME activation might promote increased CW rigidity by calcium-mediated pectin cross-linking, but if PME activity is too strong or too long in time, it might induce pectin degradation and CW softening. Partially adapted from Hocq et al., 2017b.

Our experiments corroborate previous hypothesis proposing differences in short and long term effects of PME (Fig. 6) (Hocq et al., 2017b). PME long-term effect, or high activity, induces softening of cell walls most probably because loss of methyl groups allows pectin lyases and other degradation enzymes to break pectin fibers, whilst short term or middle activity induces CW stiffening mediated by pectin calcium cross-links. Noteworthy, the reported softening effects of PME5 overexpression lines might be explained by specific phenotypic selection of transgenic lines exhibiting strong elongation defects as described by the authors (Peaucelle et al., 2015). In our work we are presenting results from random chosen seedlings, with no selection based on phenotype, and therefore our results might be a mixture of strong and mild expressing seedlings.

Noteworthy is also the fact that hypocotyl elongation assays are typically performed under calcium depletion conditions, which might create an artificial condition under which pectin is not able to form new calcium cross-links due to strong depletion of calcium ions. Indeed our results confirm that adding calcium might interfere with elongation processes. Another important aspect of calcium interference is found in the usage of 2F4 antibody (specific for low methyl-esterified pectin) to evaluate pectin calcium crosslinking. This antibody is used in buffers containing high concentrations of calcium, and therefore might not capture egg-box cross-links as at endogenous condition, but might produce an

overestimation of it. Also noteworthy, it is proposed that acidification and cell wall relaxation is correlated with calcium import (Cho et al., 2012; Conn et al., 2011) and cell expansion might stretch the plasma membrane and open stretch-activated calcium channels. The resulting increase in cytosolic calcium might inhibit the H^+-ATPase and open H^+ channels, leading to the alkalization of the apoplast, forming a negative feedback loop. In that aspect, it is important to note that our AFM results are performed under plasmolyzing conditions, impeding any calcium signaling effect that might be introduced due to changes in cytoplasmic calcium.

Overall, we have demonstrated that acidification and growth processes mediated by several hormones converge on a cell wall loosening process and that regulation of the PME activity tightly linked with calcium levels are essential factors to determining CW mechanical properties. Further studies characterizing the effects of expansin proteins on cell wall composition and mechanical properties might shed light on the complex network of CW remodeling in growth processes.

Material and Methods

Plant material

Arabidopsis thaliana plants were grown in a growth chamber at 21°C under white light (W), which was provided by blue and red LEDs (70-100 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation). The transgenic lines have been described elsewhere: HS::*axr3-1* (Knox et al., 2003), 35S::GFP-SAUR19 (Spartz et al., 2012). The HS::*axr3-1* plants were heat-shocked at 37°C for 40 min in aluminum-wrapped petri dishes; experiments were started 1.5hr after the end of the heat shock.

Growth conditions

Seeds were sterilized in 5% bleach for 10 min and rinsed with sterile water before plating on half-strength Murashige and Skoog (MS) medium (Duchefa) with 1% sucrose, 1% agar (pH 5.7). Seeds were stratified for 3-4 days at 4°C, exposed to light for 2-4 hours at 21°C, and cultivated in the growth chamber under appropriated light conditions at 21°C (wrapped in aluminum foil and disposed in a cardboard box for dark growth, or into light in the same chamber).

Elongation assays.

Hypocotyl elongation assays were performed as described elsewhere (Fendrych et al., 2016) with minor modifications. Hypocotyls were obtained from etiolated seedlings ~72h-old (starting after transferring plates into growth-room), by removing the hook and the half lower part of the hypocotyl (and root) using a razor blade in a dark space (with the help of a green LED light). The hypocotyl segments were placed on the surface of the Depletion Medium (DM, 10 mM KCl, 1 mM MES, pH 6 using KOH, 1.5% phytagel) and kept in darkness for at least 20 min. Then the segments were transferred to a new plate with depletion medium and the supplemented hormones or inhibitors as specified in the figures. The samples were placed on a flatbed scanner (Epson perfection V370) and imaged through the layer of phytagel, a black filter paper was placed above the dishes to improve the contrast of the images. Samples were scanned immediately after transfer (used as initial length) and, unless other specification, after 3h of incubation in the respective treatment.

Chemicals used

IAA, 1-naphthaleneacetic acid (NAA), epibrassinolide (eBL), Gibberellic acid (GA), Fusicoccin (FC) and β-Estradiol (β-Est) and EGTA were ordered from Sigma-Aldrich. NAA and eBL were used at 10mM, GA was dissolved at 100mM and FC at 1mM in EtOH. β-Est was dissolved in water at 10mM. EGTA was prepared in stock solution at 0.5M in water.

Confocal imaging

Confocal laser-scanning micrographs were obtained with a Zeiss LSM800 with a 488-nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Using a 20x air objective, confocal scans were performed with the pinhole at 1 Airy unit. Localization was examined by confocal z-sectioning. Each image represents either a single focal plane or a projection of individual images taken as a z-series. Z-stacking was performed collecting images through cortex and epidermal layers. Full z-stack confocal images were 3D-projected using ImageJ software. At least five seedlings were analyzed per treatment. Images were processed in ImageJ.

AFM measurements and Apparent Young's Modulus Calculations

The AFM data were collected and analyzed as described elsewhere with minor changes (Peaucelle et al., 2015; Hurný et al., 2020; Velasquez et al., 2021). To examine extracellular matrix properties we suppressed turgor pressure by immersion of the seedlings in a hypertonic solution (10% mannitol) for at least 20min before examination. Three day-old seedlings grown in darkness (in normal AM plate or indicated treatment) or stem segments treated as described for elongation assays were placed in microscopy slides and immobilized using double-glued side tape. We focused on the periclinal cell walls (parallel to growth axis, but perpendicular to the organ surface), and its extracellular matrix. To ensure proper indentations, especially on the regions in the bottom of the doom shape between two adjacent cells, we used cantilevers with long pyramidal tip (14-16µm of pyramidal height, AppNano ACST-10), with a spring constant of 7.8 N/m. The instrument used was a JPK Nano-Wizard 4.0 and indentations were kept to <10% of cell height (typically indentations of 100-200nm depth and 500nN force). Three scan-maps per sample were taken over an intermediate region of the hypocotyls, using a square area of 25 x 25 µm, with 16 x 16 measurements, resulting in 1792 force-indentation experiments per sample. The lateral deflection of the cantilever was monitored and in case of any abnormal increase the entire data set was not used for analysis. The apparent Young's modulus (E_A) for each force-indentation experiment was calculated using the approach curve (to avoid any adhesion interference) with the JPK Data Processing software (JPK Instruments AG, Germany), based on Herz model adjusted to pyramidal tip geometry. To calculate the average E_A for each periclinal wall, the E_A was measured over the total length of the extracellular region using masks with Gwyddion 2.45 software (at least 20 points were taken in account). The pixels corresponding to the extracellular matrix were chosen based on both topography and Young Modulus maps. For topographical reconstructions, the height of each point was determined by the point-of-contact from the force-indentation curve. A standard t-test was applied to test for differences between genotypes/treatments. Total number of scans performed is indicated in the figures (n).

Expression analysis by RT-qPCR

Around 60 seedlings (72h old dark grown) were harvested and frozen in liquid nitrogen. Tissue was ground using a ball mill (model MM400; Retsch) with 4-mm diameter balls in a 2-ml Eppendorf (Hamburg, Germany). Total RNA was isolated using Monarch kit (New England Biolabs) according to manufacturer protocol (DNase treatment was included during extraction protocol). cDNA was prepared from 1 µg of total RNA with the iScript cDNA Synthesis Kit (Biorad), diluted 10 times and 1 µl was used in 5-µl PCR reaction on a LightCycler 480 (Roche Diagnostics) with Luna Master Mix (New England Biolabs) according to the manufacturer's instructions. As control, non-RT-treated samples were included to test the purity of the cDNA. All experiments were done with three technical replicates and three biological samples. The PP2A gene (At1g69960) was used as a control for normalizations. Primer sequences can be found in Table S1.

Western-blot analysis

Around 60 seedlings (72h old dark grown) were harvested and frozen in liquid nitrogen. Frozen tissue was ground with stainless steel 4-mm diameter balls in a 2-ml Eppendorf (Hamburg, Germany) tube using a ball mill (model MM400; Retsch). Extraction buffer [100 mM Tris–HCl (pH 7.5), 25% (w/w, 0.81 M) sucrose, 5% (v/v) glycerol, 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 10 mM ethyleneglycoltetraacetic acid (EGTA pH 8.0), 5 mM KCl, and 1 mM 1,4-dithiothreitol (DTT); Abas and Luschnig, 2010] was added to the frozen tissue. After Bradford quantification, 20 µg of protein were diluted in 20µl of buffer and prepared for electrophoresis by adding 5µl of loading buffer (5xSDS) and incubating at 45°C for 5min. Run was performed in a commercial 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) at 35mA. Transference was done with semidry system with a Trans-blot turbo transfer pack PVDF (Bio-Rad). The blot was washed in TBST buffer with 5% Milk Powder and 0.05% Tween20, and blocked over/night in the same buffer at 4°C. Hybridization was done with anti-HA-HRP antibody (monoclonal antibody from Sigma, dilution 1:7000) in TBST for 2h at room temperature. The blot was washed three times in TBST and one in water, previous the visualization by SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) and exposure to Amersham Imager 600 (GE Healthcare).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At1g53840 (*PME1*), At5g47500 (*PME5*).

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Author contributions

Conceived and designed the experiments: MG, EH and EB. Performed the experiments: MG; JMCL contributed with western-blot and immuno assays and discussion of whole manuscript; NZ performed western-blot and elongations assays; JP and PS contributed in AFM experiments. Analyzed the data: MG, EH and EB. Wrote the manuscript: MG and EB with inputs from all authors.

Competing interests statement

The authors declare no competing financial interests.

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