Running head: Apoplastic pH and cell elongation in the Arabidopsis root.

Corresponding author:

Prof. Dr. Kris Vissenberg
University of Antwerp
Dept. Biology, Plant Growth and Development
Groenenborgerlaan 171
B-2020 Antwerpen, Belgium
Tel: +32-3-265.34.10
Fax: +32-3-265.34.17
kris.vissenberg@ua.ac.be

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Apoplastic alkalinisation is instrumental for the inhibition of cell elongation in the *Arabidopsis thaliana* root by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

Staal Marten\(^1\), De Cnodder Tinne\(^2\), Simon Damien\(^2\), Vandenbussche Filip\(^3\), Van Der Straeten Dominique\(^3\), Verbelen Jean-Pierre\(^2\), Elzenga Theo\(^1\), Vissenberg Kris\(^2,5\)

\(^1\) Center for Ecological and Evolutionary Studies, Laboratory of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA, Haren, The Netherlands
\(^2\) Department of Biology, Laboratory of Plant Growth and Development, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium
\(^3\) Department of Physiology, Laboratory of Functional Plant Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

\(^4\) Both authors contributed equally to this manuscript
Footnotes:

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To whom correspondence should be addressed: Prof. Dr. Kris Vissenberg, Fax: +32-3-265.34.17, E-mail: kris.vissenberg@ua.ac.be
Abstract

In Arabidopsis thaliana (Col-0) roots the so called zone of cell elongation comprises two clearly different domains: the transition zone, a post-meristematic region (approximately 200 to 450 μm proximal of the root tip) with a low rate of elongation, and a fast elongation zone, the adjacent proximal region (450 μm away from the root tip up to the first root hair) with a high rate of elongation. In this study, the surface pH was measured in both zones using the microelectrode ion flux estimation (MIFE) technique. The surface pH is highest in the apical part of the transition zone and is lowest at the basal part of the fast elongation zone. Fast cell elongation is inhibited within minutes by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and concomitantly apoplastic alkalinisation occurs in the affected root zone. Fusicoccin (FC), an activator of the plasma membrane (PM) H^+-ATPase, can partially rescue this inhibition of cell elongation, whereas the inhibitor N, N’-dicyclohexylcarbodiimide (DCCD) does not further reduce the maximal cell length. MIFE experiments with auxin-mutants lead to the final conclusion that control of the activity state of PM H^+-ATPases is one of the mechanisms by which ethylene, via auxin, affects the final cell length in the root.
**Introduction**

Expanding and elongating plant cells are characterized by their ability to undergo wall extension in acidic apoplastic conditions. The acid-growth theory indicates protons as the primary wall loosening factor causing cell expansion (Rayle and Cleland, 1970; 1992). Intensive research proved that a low apoplastic pH increases the activity of expansins in the wall, which probably break the hydrogen bonds between the cellulose chains and the cross-linking glycans (McQueen-Mason et al., 1992; Cosgrove 2000). The apoplastic pH is determined by the H⁺-efflux through the PM H⁺-ATPases and the H⁺-influx through H⁺-coupled symporters (Tanner and Caspari, 1996). Both hormonal signals such as auxin (Rayle and Cleland, 1992) and environmental cues can affect cell growth by inducing the cell to alter its wall pH through changes in the activity of PM H⁺-ATPases (Sze et al., 1999; Wu and Seliskar, 1998).

Kinematic growth analysis, with a high spatial and temporal resolution, indicated that elongation in the *Arabidopsis* root is not homogenous (Beemster and Baskin, 1998). From these results it became clear that the elongation zone can be divided into two domains with constant but distinct growth rates (van der Weele et al., 2003). Cells close to the meristem display a very slow elongation. Subsequently, in the second domain, there is a sudden switch to higher rates of elongation. The first domain is called the transition zone as was suggested for the root of maize (Baluška et al., 1996) and the adjacent zone is named the fast elongation zone. The subdivision in a so-called ‘transition’ and ‘fast elongation zone’ was affirmed by plotting the relative elemental growth rate along the root tip (see references in Verbelen et al., 2006). The fast elongation in the *Arabidopsis* root is instantaneously inhibited by applying the plant hormone ethylene or its precursor ACC (Le et al., 2001), and by different stresses, e.g. osmotic and salt stress (De Cnoddet al., unpublished results).

In maize plants, the spatial profile of growth along the roots has been shown to coincide with the spatial profile of root-surface acidification (Fan and Neumann, 2004; Peters and Felle, 1999). Peters (2004) identified the surface pH as a growth-related physiological phenomenon that indicates the transition from a slow to fast growth. In this study, the surface pH at the root surface was recorded along the growth zones of the *Arabidopsis* root during normal growth and during ACC-induced growth arrest. The role of plasma membrane H⁺-ATPases in both growth conditions was investigated as well. To our knowledge, this is the first study that correlates cell elongation and elongation arrest with changes in the surface pH in the *Arabidopsis* root.
Results and discussion

The surface pH and proton flux were measured along five-day-old *Arabidopsis* roots using the MIFE technique (Newman, 2001; Shabala et al., 1997). In this approach the proton flux density in or out of the root is determined by alternating the position of an H⁺-sensitive microelectrode between 10 and 50 µm perpendicular to the root surface. From the pH at those two positions, and taking into account the geometry of the root surface, the flux can then be calculated. The first measuring point was positioned at a distance of 125 µm from the root tip to avoid interference of the lateral root cap cells; the subsequent sampling points were 50 µm apart and the last sampling point was at the distal border of the root hair zone.

In Figure 1A the surface pH is plotted against the position along the root. For ease of interpretation the meristematic zone (MZ) and the two zones of elongation (transition zone, TZ and fast elongation zone, EZ) are also indicated on the figure. The highest pH occurred at a distance of 225 µm from the root tip. This pH maximum thus coincides with the apical limit of the transition zone (Verbelen et al., 2006). In this zone, the surface pH decreased over 0.2 units in a basipetal way, representing a change of 0.1 units per 100 µm distance along the root. In the fast elongation zone the surface pH further decreased but at a slower rate of only 0.1 units over a distance of 600 µm. The pH at a single point was stable in time (see inset in Fig. 1A). Also the shape of the pH profile along the root was stable in time as verified by recording the pH at different points repeatedly for a period of 3 h (results not shown). Based on these observations and the fact that these measurements are completely uninvasive (the electrode does not even touch the surface of the root) it is not expected that the measurements have an effect on root functioning.

The pH profile along the root was mirrored in the H⁺-flux density profile (Fig; 1B). The zone with the highest pH coincided with the zone along the root with the highest H⁺-influx.

Other studies have shown also a correlation between the maximal growth and the minimal pH occurring in the growing zone of wheat (Lundegårdh, 1942) and *Phleum pratense* L. roots (Monshausen et al., 1996). Simultaneous measurements of root surface pH and growth rate along the maize root indicated the highest pH in an area just apical from the zone of explosive growth (Felle, 1998; Peters and Felle, 1999). The latter authors found that the pH pattern along the maize root was independent of the pH of the medium (Peters and Felle, 1999). The pH range covered by the pH profile, however, was influenced by the pH of the medium, and increased with an increasing medium pH. It was hypothesized by Peters (2004) that this transient pH peak marked the transition of the root cells into an acid-growth-competent state, in which they burst...
into their adult size within a short period of time, a hypothesis that is clearly supported by our results.

Thus the transition zone is the region of the Arabidopsis root in which the surface pH decreases steeply towards a value (and the site) where fast cell elongation starts. A low apoplastic pH and the probable activation of expansins are a prerequisite for acid-induced cell expansion to occur (McQueen-Mason et al., 1992). Recent results indicate that also xyloglucan endotransglucosylase/hydrolase (XTH) proteins, capable of inducing cell wall loosening (Van Sandt et al., 2007), can be activated at more acidic pH values as isozymes with complementary pH activity profiles exist (Maris et al., 2009; 2010). From Vissenberg et al. (2005), Becnel et al. (2006) and Supplemental Figure 1 (based on the Arex database; Birnbaum et al., 2003; Brady et al., 2007) it is clear that several expansin and XTH genes are expressed in the transition and elongation zone of the root and that they are therefore candidates to be activated by this acidic environment.

Application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Schaller and Kieber, 2002) immediately and significantly reduces root growth by the inhibition of the fast elongation of the root cells (Le et al., 2001). This effect thus occurs in the root zone where the surface pH has a value of about 5.4 (Fig. 1a) and slowly decreases further towards the basis of the root. To link the ACC-induced inhibition of fast elongation with a possible effect on wall pH, the surface pH was recorded during 2 h after ACC addition at a position between 400 and 500 µm of the root tip (i.e. the border between transition zone and fast elongation zone; Verbelen et al., 2006). In the period from 20 min to 60 min after application the surface pH of the root increased steeply with 0.2-0.25 pH units, and remained fairly constant afterwards (average ΔpH after 120 min was 0.23 ± 0.02, n=3) (Fig. 2). A blank addition of medium (i.e. without ACC) was found to have significantly less effect on the extracellular pH (average ΔpH after 120 min was 0.09 ± 0.01, n=3, student T-test p<0.005) (Fig. 2a). The effect of ACC on the root surface pH can be fully explained by the inhibition of the H⁺-efflux (Fig. 2b). In the control root a net H⁺-efflux is present (seen as a negative value for the H⁺-influx on figure 2b), while in the ACC-treated root the efflux is completely absent. Importantly, the ACC-induced arrest of fast elongation was not affected by keeping the root in a 10mM KCl solution in the experimental chamber (results not shown). This rules out that the KCl solution of the measuring chamber prevented the inhibitory effect of ACC on the root.

The ACC-induced apoplastic alkalinisation measured at the border between the transition and the fast elongation zone thus coincides in time and space with the inhibition of the fast cell
elongation. Cell wall loosening agents such as expansins that need more acidic environments now encounter a less favourable pH environment. It is even possible that the raise in pH renders the apoplastic environment more in favour of peroxidase activity, cross-linking specific components of the cell wall and counter-acting the cell wall loosening enzymes. In a previous study we indeed described cross-linking activity in the cell wall that was peroxidase-mediated and correlated with the inhibition of cell elongation (De Cnodder et al., 2005). Furthermore, besides isozymes that favour cell wall loosening (Van Sandt et al., 2007), it is described that some XTH proteins can strengthen the wall (Maris et al., 2009).

In a comparable study using the MIFE technique at a single point, water deficit caused by the addition of mannitol for 2 h to maize roots, resulted in an increase of the pH in the elongation zone (Shabala and Newman, 1998). Moreover, in maize roots under water deficit, induced by treatment for 48 h with polyethylene glycol (PEG 6000), the length of the zone of intense acidification extending behind the root tip was substantially shortened (Fan and Neumann, 2004). Our results fit with these studies. Together they clearly point to a correlation between the apoplastic H⁺-concentration and the cell’s ability to elongate.

Plasma membrane H⁺-ATPases govern the efflux of protons across the plasma membrane, providing the driving force for the uptake of ions and metabolites, and are thus required for cellular growth (Palmgren, 2001). The possible link between H⁺-ATPase activity and the alkalinisation of the cell wall in the ACC-growth response was investigated by modulating this enzyme activity and checking the effect on cell elongation. Fusicoccin (FC), initially identified as a fungal metabolite, has been shown to bind to a plasma membrane receptor complex that includes both an H⁺-ATPase and a 14-3-3 protein (Alsterfjord et al., 2004; Baunsgaard et al., 1998). FC promotes the activation of H⁺-ATPases, resulting in proton extrusion into the cell wall (Olivari et al., 1998). N, N’-dicyclohexylcarbodiimide (DCCD) is a well known inhibitor of H⁺-ATPase activity, which upon binding to the enzyme blocks the proton conductance across the plasma membrane (Nelson and Harvey, 1999).

Measuring the length of individual trichoblast cells at the onset of root hair formation, also known as the LEH (Length of the first Epidermal cell with visible root Hair bulge), is an easy and suitable manner to study cell elongation in Arabidopsis roots with high temporal and spatial resolution (Le et al., 2001). For reason of comparison, the LEH measurements were recalculated to a ‘growth extent’ (expressed in percent) with the length of trichoblasts in control plants set as 100% (Fig. 3). Addition of ACC reduced the growth extent from 100% to 34% (Schaller and Kieber, 2002), which was in agreement with previous findings by Le et al. (2001). However,
addition of 100 µM FC to ACC-treated plants resulted in a growth extent of 86%, thus partially rescuing cell elongation (Fig. 3), while in control plants it did not significantly affect cell elongation (102% versus 100% respectively). This implies that the observed block in cell elongation in ACC-treated roots was most probably in part due to the inactivation of H⁺-ATPase activity. Other elongation limiting factors described by De Cnodder et al. (2005), like HRGPs cross-linking by ROS and callose deposition in the cell wall, could be responsible for the non-reversible part of the inhibition by ACC (i.e. the missing 14% in the ACC + FC treatment). Application of DCCD (5 µM) to control plants inhibited cell elongation bringing the growth extent down to 53% after 3 h of treatment (Fig. 3). However, the growth extent in ACC-treated roots was not further decreased by DCCD, indicating that ACC had already minimized the H⁺-ATPase activity.

Taken together, the majority of plasma membrane H⁺-ATPases at least in the epidermis of the elongation zone of control roots is probably in the high-activity state, whereas in ACC-treated roots they are probably locked in their low-activity state (Sze et al., 1999). This low-activity state of the H⁺-ATPases is instrumental for the measured alkalinisation of the wall pH and the concomitant inhibition of cell elongation in ACC-treated roots.

These observations were confirmed by a set of experiments in which the pH changes were measured at the border between the transition and the fast elongation zone in control situations, and in situations where FC and/or ACC were added (Fig. 4). From the graphs it is clear that FC indeed completely inhibits the alkalinisation imposed by ACC and that FC even results in a smaller pH change than in the control situation. Furthermore, FC was able to increase the LEH in the constitutive triple response mutant ctr1-1. In combination with the LEH measurements described above (Fig. 3) this confirms our conclusion that the activity of H⁺-ATPases is necessary in the control of cell elongation, but that additional factors indeed play a role in this process as well.

Given the reports that ethylene controls root growth by up-regulating auxin biosynthesis and transport-dependent auxin distribution (Ružička et al., 2007; Swarup et al., 2007), we recorded the pH changes at the border between the transition and the fast elongation zone in roots of wild type plants and known mutants in auxin transport (aux1-22, axr2-1 and axr3-1), both under normal conditions and after ACC addition (Fig. 5). The results show that the auxin influx carrier-mutant aux1-22 (Bennett et al., 1996) does not show a clear ACC-response and that the change in pH is similar to that observed in a wild type root grown under normal conditions. This mutant therefore seems ‘insensitive’ to the ACC application, indicating that auxin influx in cells is necessary for the ethylene-driven inhibition of cell elongation. Untreated axr2-1 and axr3-1
roots show very small pH changes that are comparable with the ones found in wild type plants. Addition of ACC to *axr2-1* roots induces a behaviour that is reminiscent of an untreated wild type root, i.e. no alkalinisation. As described in the literature, the dominant gain of function mutant in IAA7, *axr2-1*, confers auxin resistance probably by disruption of an early step in the auxin response pathway (Wilson et al., 1990; Timpte et al., 1994). In this test we nevertheless detect a small ACC effect, suggesting other IAAs or process might be involved. In *axr3-1* ACC leads to a hyper-alkalinisation of the root surroundings. This finding confirms the reports of a general increase in the amplitude of auxin responses (here evoked by the ethylene treatment) in the over-responsive gain of function mutant *axr3-1* (Leyser et al., 1996; Cline et al., 2001; Knox et al., 2003). AXR3/IAA17 is a member of the Aux/IAA protein family (Rouse et al., 1998) which generally are low abundance and labile transcription factors (Abel and Theologis, 1996). In Figure 6 the different interactions of inhibitors and mutants of auxin-related and auxin transport genes are depicted. The inter-relationship of auxin transport, cellular auxin levels, ethylene and H⁺ efflux allows the occurrence of positive and negative feedback loops. Ethylene enhances auxin biosynthesis and transport in the root tip (Stepanova et al., 2005; Ruzicka et al., 2007; Swarup et al., 2007), but the regulation by downstream factors has not been described. In this context it is interesting to note that cells and tissues that exhibit fast elongation, like pollen tubes (Holdaway-Clarke et al., 2003; Michard et al., 2008), root hairs (Monshausen et al., 2007) and root epidermal cells (Shabala and Knowles, 2002; Shabala, 2003) do show pronounced oscillations in H⁺ fluxes. Although oscillations were rarely observed in the present study, the complex interactions implied here could explain the reported oscillatory patterns in H⁺ fluxes in fast growing tissues.

This set of results provides further proof of the fact that ethylene (ACC) works by influencing the auxin content in specific cells in the treated roots. Our results strongly suggest that this increase in cellular auxin, resulting from modified transport and/or auxin biosynthesis (Swarup et al., 2007), in turn negatively modulates the activity of H⁺-ATPases which combined with changes in the apoplast (De Cnodder et al., 2005) results in the observed cell elongation phenotype.

**Materials and Methods**
Plant material and growth conditions

Growth conditions were as described by De Cnodder et al. (2005). Five-day-old Arabidopsis Col-0 seedlings and the aux1-22, axr2-1 and axr3-1 mutants were transferred to normal ½ MS media (as a control for transfer effects; Duchefa, The Netherlands) or to media supplemented with 5 µM 1-aminocyclopropane-1-carboxylic acid (ACC; Acros Organics, Belgium) or 5 µM N, N’-dicyclohexylcarbodiimide (DCCD) (dissolved in ethanol, Merck, Germany). Fusicoccin (FC) (final concentration 100 µM, stock dissolved in ethanol; Alexis Biochemicals, USA) was applied to the Arabidopsis seedlings in liquid ½ MS medium. Arabidopsis seedlings were placed in the liquid medium with the cotyledons above the liquid level and the hypocotyl and the root inside the liquid. The subsequent LEH measurements were performed as described by Le et al. (2001); here they were recalculated to a percentual growth extent to increase the ease of interpretation. Each experiment was repeated three times on at least 15 seedlings (n=15). To test for statistical significance a Student’s t-test with a probability of 95 % (P=0.05) was used.

Microelectrode ion flux estimation (MIFE) technique

Measurements of the surface pH were performed as described in detail by Shabala et al. (1997). Micropipettes (diameter 50 µm) were pulled from borosilicate glass (GC150-10, Harvard Apparatus LTD, UK). The electrodes were silanized with tributylchlorosilane (Sigma-Aldrich, USA) and subsequently back-filled with 15mM NaCl and 40 mm KH2PO4 and front-filled with Hydrogen Ionophore II, Cocktail A (Sigma-Aldrich, USA). Only electrodes with a response between 50 and 59 mV per pH unit and with a correlation coefficient between 0.999 and 1.000 (pH range 5.1-7.8) were used in experiments. A five to six-day-old Arabidopsis root from an intact seedling was mounted on a glass capillary tube with medical adhesive B (Aromando Medizintechnik, Germany) and placed in the experimental chamber which was filled with 1 ml of 10 mM KCl (pH 6.1). The seedling was left to recover from this manipulation for 10 minutes. The experimental chamber was placed on an inverted microscope (Nikon TMS-F, Uvikon, The Netherlands). The H+-microelectrode was mounted in a holder (MMT-5, Narishige, Japan) which was attached to a micromanipulator (PCT, Luigs & Neumann, Germany) driven by a computer-controlled motor (MO61-CE08, Superior Electric, USA). The electrode was positioned manually at a distance of 10 µm of the root surface. During the subsequent measurements, the distance between the probe and the surface of the root was altered between 10 µm and 50 µm at a frequency of 0.1 Hz. The chemical activity of H⁺ in solution at these two positions was recorded and from these data the surface pH and the H⁺-flux in or out of the root was calculated.
absolute pH value could differ between different MIFE experiments, but the overall pattern of the pH along the root stayed the same. For the sign of the H⁺-flux we adopted the convention by Newman (2001), a negative sign indicates an efflux and a positive sign an influx at the root surface. A final concentration of 5 µM ACC in the experimental chamber was attained by adding 1 µl of 5mM stock solution. As a control experiment, 1 µl of 10mM KCl was added to the chamber. Fusicoccin was added to a final concentration 100 µM from a stock dissolved in ethanol. In control experiments (data not shown) it was demonstrated that ethanol alone did not affect the LEH, the proton fluxes, nor the boundary layer pH.

Supplemental Material

Supplemental figure 1
Expression patterns of members of the cell wall modifying gene families expansins and xyloglucan endotransglucosylase/hydrolases (XTHs) in the Arabidopsis root based on the Arex database.

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**Figure legends**

**Figure 1A**
Plot of the surface pH along the *Arabidopsis* root. The surface pH along the *Arabidopsis* root is recorded using the MIFE system and sampled every 50 µm from 125 µm of the root tip off to the root hair zone (at 1050 µm). The extracellular pH reaches a maximum at a distance of 225 µm from the root tip and decreases towards the elongation zone. The pH values in one point are quite stable as seen in the inset of the figure. For each single point the average was taken from a MIFE recording lasting at least 50 seconds. MZ indicates the meristematic zone, TZ the transition zone and EZ the fast expansion zone.

**Figure 1B**
Plot of the H⁺-flux (mean ± s.d., n=4) along the Arabidopsis root. The flux was measured with the MIFE system as described in Figure 1a. MZ: meristematic zone, TZ: transition zone and EZ: fast elongation zone

**Figure 2A**
Changes in pH (ΔpH, mean ± s.d. n=3) at a distance between 400 and 500 µm from the *Arabidopsis* root tip measured during 120 min after the addition of ACC (+ ACC, final concentration 5 µM) and after the addition of KCl as a control. The traces were recorded with a time interval of 10 seconds between data points. For clarity symbols indicating the mean and standard deviation bars were placed at 5 minutes intervals only. ACC addition causes an alkalinisation in the zone of the root that marks the transition from slow to fast elongating cells. The addition of 5 µM ACC did not significantly alter the pH of the medium in the experimental chamber (results not shown). Before ACC addition, the sampling points at this position displayed a steady influx and remained at a constant pH (results not shown).

**Figure 2B**
Effect of addition of ACC or KCl (control) on the H⁺-flux (mean ± s.d., n=3) measured with the MIFE system at a distance of 400-500 µm from the root tip. Treatments were as described in figure 2A.

**Figure 3**
Modulation of the H⁺-ATPase activity by the activator fusicoccin (FC) and the inhibitor N, N’-dicyclohexylcarbodiimide (DCCD) and its effect on the *Arabidopsis* root growth extent (in percent) in control roots and in roots in which the ethylene content is increased by ACC. FC can partially rescue the inhibition of cell elongation imposed by ACC, whereas DCCD reduces cell lengths only in control conditions, but not in the presence of ACC. Treatments with different lower case characters above the bars are statistically significant different at the p<0.01 level.

**Figure 4**

Changes in pH (ΔpH, mean ± s.d. n=3) at a distance between 400 and 500 µm from the *Arabidopsis* root tip measured during 120 min in control conditions (circles) and after the addition of ACC (filled symbols) and/or FC (triangles) and after the addition of KCl as a control (open symbols). ACC addition causes an alkalinisation in this point along the root, whereas FC reduces the changes in pH to a minimum, regardless of the presence of ACC.

**Figure 5**

Changes in pH (ΔpH, mean, n=3) at a distance between 400 and 500 µm from the root tip in *Arabidopsis* auxin transport mutants measured during 120 min in control conditions (open symbols) and after the addition of ACC (filled symbols). ACC has no clear effect on the pH changes in *aux1-22* (circles), whereas the effect on pH in *axr2-1* (triangles) restores the behaviour of a wild type plant under normal conditions. *Axr3-1* (squares) exhibits an hyper-alkalinisation response after the addition of ACC. For clarity, the standard deviation bars are omitted (in general the standard deviations were comparable with those found in figure 4).

**Figure 6**

Model of interactions of ethylene with different auxin response mutations (axr3-1 and axr2-1), the auxin transporter mutant (*aux1-22*), the H⁺-pumping ATPase inhibitor N, N’-dicyclohexylcarbodiimide (DCCD) and the H⁺-pumping ATPase activator fusicoccin (FC). In the cartoon on the left of the root tip the disruption of auxin transport from the vascular bundle into the root tip and from there to the cortical cells in the elongation zone by the mutation in the *aux1-22* gene, is depicted. The box on the right shows the regulation of the proton pumping ATPase in the cells in the elongation zone. The mutation in the *aux1-22* gene prevents the accumulation of auxin in the cell to ATPase-inhibiting levels. The gain of function mutation axr3-1 increases the auxin responses while axr2-1 induces auxin-insensitivity. In this model ethylene is assumed to interfere with auxin transport and biosynthesis, increasing the cellular auxin concentration to a
level that is inhibitory to the H⁺-pumping ATPase activity. From a previous study it is known that addition of ethylene increases ROS production and leads to HRGP cross-linking in the cell wall.

**Supplemental figure 1**

Expression patterns of members of the cell wall modifying gene families expansins and xyloglucan endotransglucosylase/hydrolases (XTHs) in the Arabidopsis root based on the Arex database. EXPA = expansin A, EXPB = expansin B, EXPLA = expansin-like A, EXPLB = expansin-like B, XTH = xyloglucan endotransglucosylase/hydrolase