Role of the *AtClC* genes in regulation of root elongation in Arabidopsis

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Abstract
The protein family of anion channel (ClC) constitute a family of transmembrane trnsporters that either function as anion channel or as H^+/anion exchanger. The expression of three genes of *AtClCa, AtClCb and AtClCd* in the model plant *Arabidopsis thaliana* was silenced by a T-DNA insertion. When the pH of the medium was slightly acidic the length of the primary root of plants with a disrupted *AtClCa* and *AtClCd* gene was reduced compared to the wild type and the plant with a disrupted *AtClCb* gene. The proton fluxes and pH were measured along the surface of the root at different positions, from root cap, through the transition zone, and up to the fast elongation zone, and at different pHs of the medium. A high proton influx was found in the apical part of the transition zone. Lower influxes or even small effluxes were found in the basal part of the elongation zone. At pH 6.2 the influx of protons in the apical part of the transition zone in the *Atclca* and *Atclcd* mutants was significantly lower than in wild type and the *Atclcb* mutant. This will suggest a model for the interaction between endomembrane anion/H^+ antiporters, plasma membrane proton fluxes and cell expansion in roots of Arabidopsis.

Key word: Proton, Root, Antiporter, Transition zone.

INTRODUCTION

Plant root cell elongation is sensitive to various endogenous and exogenous factors such as pH (Rayle and Cleland, 1970), ethylene (Le *et al*., 2001), auxin (Fujita and Syono, 1996), calcium (Kiegle *et al.*, 2000) and aluminium (Sivaguru *et al*., 2000). The primary cell wall of plants consists of long cellulose microfibrils embedded in a cross-linked matrix of polysacharides, largely pectin and glycans (Carpita and Gibeaut, 1993), and a small quantity of structural proteins (Showalter, 1993). The acid growth theory states that protons are the primary wall-loosening factor, causing the cleavage of load-bearing bonds in the cell (Rayle and Cleland, 1970; Royle and Cleland, 1992). Turgor will then cause a cell with a loosened cell wall to expand. In maize, the spatial profile of growth along the roots has been shown to coincide with the spatial profile of root-surface acidification (Pilet *et al*., 1983; Peters and Felle, 1999; Fan and Neuman, 2004). A more recent version of the acid-growth theory states that a low apoplastic pH (<5) activates expansions, cell wall-assiociated proteins that break the hydrogen bonds between the cellulose chains and the cross-linking glycans (Mc Queen-Mason *et al*., 1992; Cosgrove, 2000). The apoplastic pH is determined by the H^+-efflux through the plasma membrane H^+-ATPase and the H^+-influx through the H^+-coupled anion symporters (Taner and Caspari, 1996). Anion fluxes through anion channels may contribute to the maintenance and regulation of proton gradients across the different membrane compartments in plant cell. Based on transport studies and structure-function relationships the anion channel a and d in *Arabidopsis thaliana* (AtClCa and AtClCd proteins) are very likely to function as anion/proton antiporters (De Angeli *et al*., 2007; Lv *et al*., 2009). In combination

AtClCd and V-ATPase can support expansion growth of cells. This last result suggests more complex connections between ClC proteins and proton gradients (Scheel *et al*., 2005; De Angeli *et al*., 2006). As shown by GUS staining, the elongation and maturation zone of the root show high expression levels of *AtClCa* and *AtClCd* (Lv *et al*., 2009). Although there are enough indications that ClC proteins are essential in cell expansion in certain tissues and cell types, the functional relation between AtClC proteins and proton gradient development in expanding root cells is still unclear. In this study, we tried to elucidate the relationship between ClC transporter proteins and the proton flux in root cell elongation, by quantifying the fluxes along the root in wild type and the *Atclca*, *Atclcb* and *Atclcd* mutants at different external pHs.

MATERIALS AND METHODS

Plant materials and culture conditions

The seeds of *Arabidopsis thaliana* (ecotype Columbia) were obtained from the SALK collection (*AtClCb*: SALK27349; *AtClCd*: SALK42895) and from the WiscDsLox T-DNA collection (*AtClCa: WiscDsLox 477-480I4)*. Seeds were surface sterilized with gaseous chlorine and sown in 90 mm petridishes containing half-strength Murashige and Skoog media (Duchefa, Haarlem, The Netherlands) with 0.8% (w/v) micro agar (Duchefa, Haarlem, The Netherlands). The dishes were sealed with surgical tape and incubated in the dark at 4°C for 3 days. Subsequently they were transferred to a growth chamber (set at a 16h/8h light/dark cycle, 20 ± 2°C temperature at 72% relative humidity) and placed on edge, 5 degrees off the vertical, such that the roots were growing down along the surface of the agar without penetrating it. About 4-6 day and 14 day old plants were used for MIFE (microelectrode ion flux estimation) and primary root measurement, respectively.

Ion flux experiments

Net fluxes of protons were measured non-invasively using vibrating H^+-selective microelectrodes with the MIFE technique (Shabala *et al*., 1997; Newman, 2001; Vreeburg *et al*., 2005; Lanfermeijer *et al.*, 2008). Micropipettes (diameter 50 µm) were pulled from borosilicate glass. The electrodes were silanized with tributylchlorosilane (Fluka 90974) and subsequently back-filled with 15 mM NaCl and 40 mM KH2PO4 and front filled with Hydrogen Ionophore II, Cocktail A (Fluka 95297). Only the 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. The electrodes were calibrated before and after use. Roots of five day-old Arabidopsis seedlings were mounted on glass capillary tubes with medical adhesive and placed in a measuring chamber with a transparent bottom, which was filled with BMS solution (1 mM KCl, 0.5 mM CaCl2, pH 5.8 for H^+ and Cl^- measurements). The whole chamber was placed on the stage of a Nikon TMS inverted microscope.

The H^+-microelectrode was mounted at an angle between 300 and 400 horizontally in a holder (MMT-5; Narishige) on a micromanipulator (PCT; Luigs and Neuman) driven by a computer-controlled motor (MO61-CE08). The electrode was positioned manually at a distance of 10 µm from the root. During the subsequent measurement, the distance between the electrode and the surface of the root was switched 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 H^+-flux and the pH were calculated.

The absolute pH value differred (± 0.1-1 pH units) between different MIFE experiments, but the overall pattern of the pH along the root stayed the same. The first measuring point was positioned at a root tip and the subsequent sampling points along the root were 75 µm apart. At each measuring point, the ion flux was recorded for 2 min. The last sampling point was chosen at the beginning of the root hair zone.

Screening for T-DNA insertion mutants

The T-DNA insertion disrupting *AtClCb* and *AtClCd* were identified in the database at the SALK Institute G-enome Analysis Laboratory (Salk-027349 for AtClCb, Salk-042895 for *AtClCd*) and Wiscd Siox 477-480i4 was identified in the WiscDsLox T-DNA collection for *AtClCa*. To obtain homozygous mutant lines, resistance to kanamycin was checked and PCR-based screens with the respective primers for each T-DNA were performed according to Salk and Wisc protocols (Table 1).

Expression analysis in roots

Total RNA was isolated from young root tips using a Nucleospin RNA plant kit (Macherey-Nagel). RNA was measured by the nanodrop machine. Total RNA (3 µg) was used as template for the first-strand cDNA synthesis using 200 U of RevertAid H-Minus M-MuLV reverse transcriptase (Fermentas, www.fermentas.com) and an Oligo (dT) primer. As a control for equal amounts of cDNA tubulin primers were included (Figure 1). PCR was performed at an annealing temperature of 55°C and 32 cycles were used for *AtClCa* and *AtClCd* and 35 cycles were used for *AtClCb*. Primers are given in Table 1.

Table 1. The PCR Primers for T-DNA confirmation and gene expression analysis, used in this study.

Target	Left primer	Right primer	Left Border primer
T-DNA insertion			
AtClCa	5'-CCAGATAAATCTTCAC TTTCTGATGG	5'-TGTCAATGCCATTAA GGTAAGC	5'-GCGTGGACCGCTTGC TGCAACT
AtClCb	5'-GGAGTTCTGTAGCCCC AGTTG	5'-GTAATCGGTGGAATT CTTGGG	5'-TGGTTCACGTAGTGG GCCATCG
AtClCd	5'-GGAACTGGATTAGCTG CTGTG	5'-CCTACCATGATGTGA CCTCCTC	5'-TGGTTCACGTAGTGG GCCATCG
Gene expression analysis			
AtClCa	5'-ACTGCATTTTGGGTCTTA	5'- GAAATGCTTGTAGAA TGTTA	
AtClCb	5'-ACTGCATCTTGGGGCTTT	5'-AGGCTTGTAGAATGT TGTAT	
AtClCd	5'-TTTACACATTAGCTGTAG	5'-TTGACTGTTGGAGTTC CACT	
β-Tubelin	5'-GAGCCTTACAACGCTACT CTGTCTGTC	5'-ACACCAGACATAGAGC AGAAATCAAG	

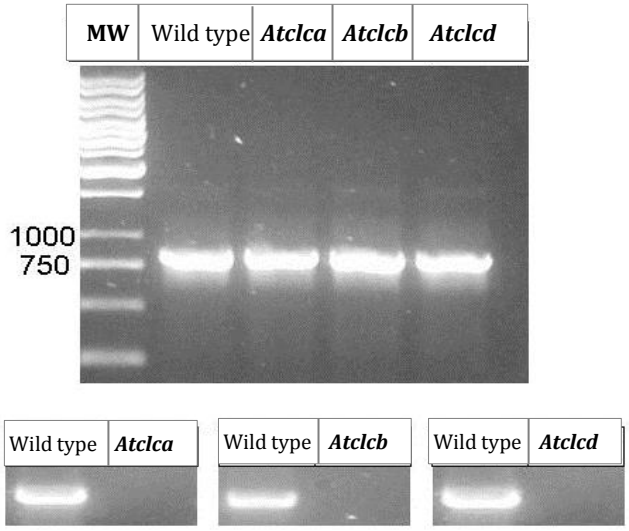

Figure 1. The absence of expression of the *AtClCa, AtClCb and AtClCd* genes in their respective T-DNA insertion lines. The four genotypes were analysed using the primers shown in Table 1. upper panel: the Tubulin transcript levels of the four genotypes are shown as a control. MW: lane with the molecular size marker, the size of the essential bands are shown on the left. Lower panels: expression levels of the *AtClCa* (left panel), *AtClCb*, (middle panel) and *AtClCd* (right panel) in wild type and the respective T-DNA insertion lines.

Cell imaging in primary root

Images were taken with a Nikon Coolpix 990 digital camera, mounted on an inverted optical microscope (CX41, Olympus, Tokyo, Japan) equipped with objectives of 20× and 40× magnification. After 7 and 14 days of growth on the vertical-placed plate the distance between the root tip and the first epidermal cell with visible root hair bulge (DFEH) and the root length was measured. At least 10 Arabidopsis wild type and mutant plants were measured for every condition in each experiment and each experiment was repeated 3 times.

RESULTS

Reverse transcript PCR analysis of gene expression

As shown in Figure 1, RT-PCR confirmed the expression of *AtClCa*, *AtClCb* and *AtClCd* genes in root tissues of wild type plants. This result is in agreement with an earlier study (Lv *et al.*, 2009) which showed a high expression in the roots of *AtClCa* and *AtClCd* and a moderate expression of *AtClCb*. In the homozygous mutant plants transcripts of the respective disrupted genes could not be detected (Figure 1).

Primary root growth of Atclca and Atclcd inhibition at high pH

Since anion transporters are involved in osmo-regulation and in cell expansion, we measured the primary root length of wild type and mutant plants growing on agar plates and exposed to different external pHs (5.8 to 6.8, buffered with 20 mM Mes). All genotypes showed the longest root when grown on the media with a pH of 5.8, while no differences could be observed between the genotypes (Figure 2). When the pH of the medium was raised root growth decreased

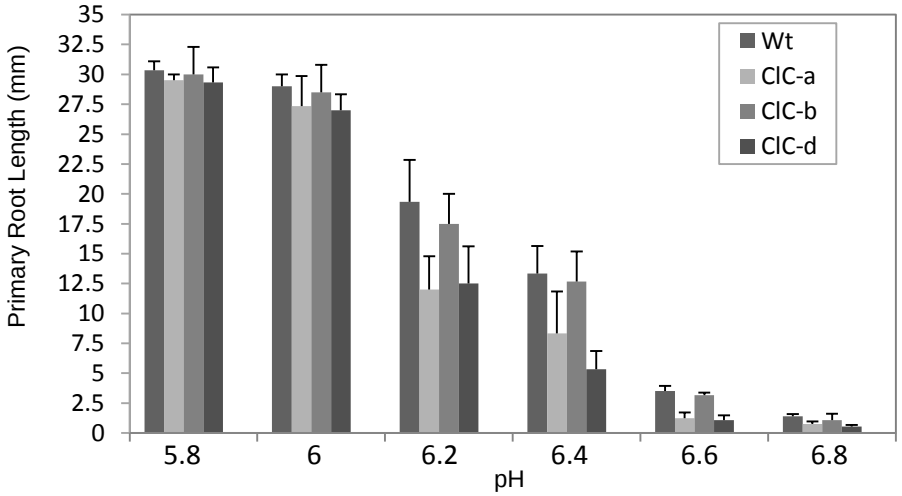

Figure 2. The effect of the pH of the growth medium on primary root length of wild type Arabidopsis and the three single mutant lines. Data points are the average of 4 experiments and the error bars indicate the standard deviation.

(Figure 2), however, compared to the wild type, root growth in Atclca and Atclcd was reduced more. *Atclcb* was not distinguishable from wild type at all pH values. In all, 8-day-old plants exposed to the highest pH (6.8) the roots of the seedlings had hardly grown and the leaves were yellowing.

Reduced proton flux on the growth zones of root of Atclca and Atclcd

To determine if the clear difference in root length between wild type plants and *Atclca* and Atclcd mutants at less acidic pHs, could be correlated to differences in cell wall acidification, the proton fluxes at the surface of the roots of 8-day-old plants were measured in wild type and mutant plants in the media with different pHs. The pH and H^+ flux profile along the root was recorded at 75 µm intervals. The last sampling point was chosen at the onset of the root hair zone. At pH 5.8 the largest influxes were recorded at a distance of 225 to 250 µm from the root tip, which is the border between the meristematic zone (MZ) and the transition zone (TZ) (Figure 3). In the transition zone the influx decreases steeply and remains relatively stable throughout the elongation zone. At pH 5.8 no differences were observed between wild type and mutants (Figures 3 and 4).

At higher pHs (in Figures 3 and 4 the results for pH 6.2 are shown) the largest net H^+ influx is shifted slightly basipetally to 300 µm from the root tip in wild type and Atclcb. However, more significant was the almost complete disappearance of the H^+ influx in the *Atclca* and *Atclcd* mutants.

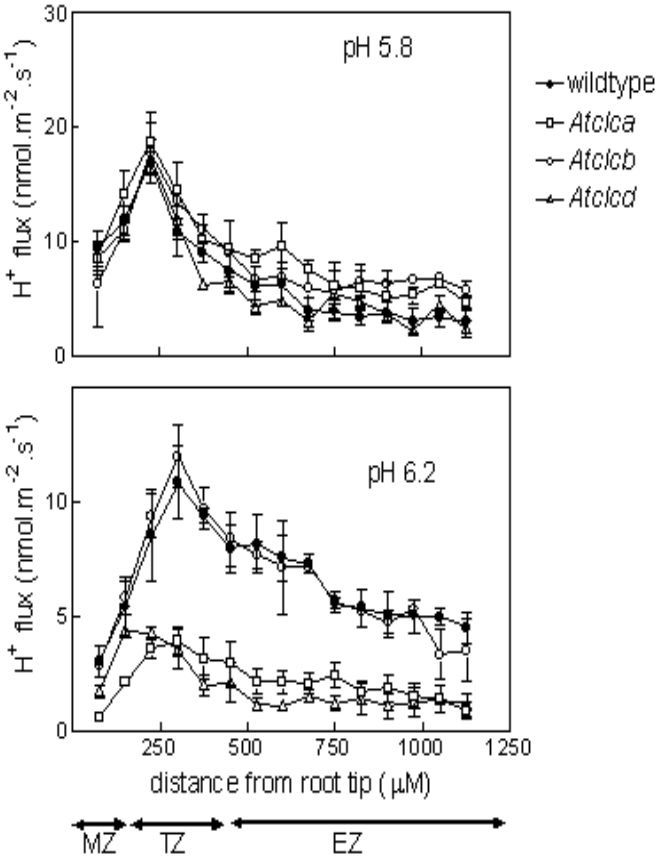

Figure 3. The proton flux profile along the roots of wild type and the three single mutants. Upper panel: proton fluxes measured at pH 5.8, lower panel: proton fluxes measured at pH 6.2. Indicated are the three zones of the growing root tip: the meristematic zone (MZ), the transition zone (TZ) and the elongation zone (EZ). Data points are the average of 4 experiments and the error bars indicate the standard deviation.

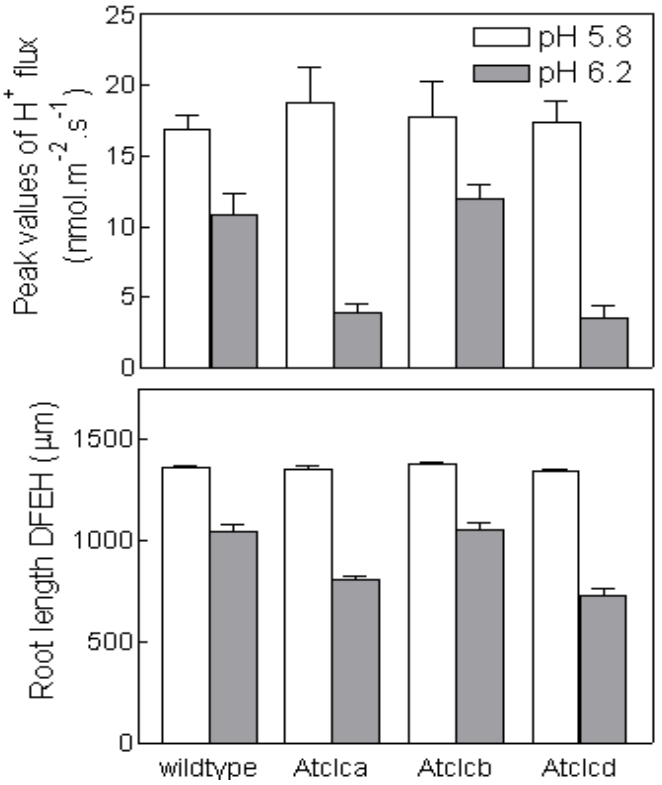

Figure 4. Comparison of the peak values of the proton-flux (upper panel) and the DFEH (lower panel) at two pH values. Upper panel: peak values are the fluxes measured around 250 µM from the root tip as shown in Figure 3. Lower panel: DFEH: the distance between root tip and first epidermal cell with visible root hair bulge. Data points are the average of 6 experiments and the error bars indicate the standard deviation.

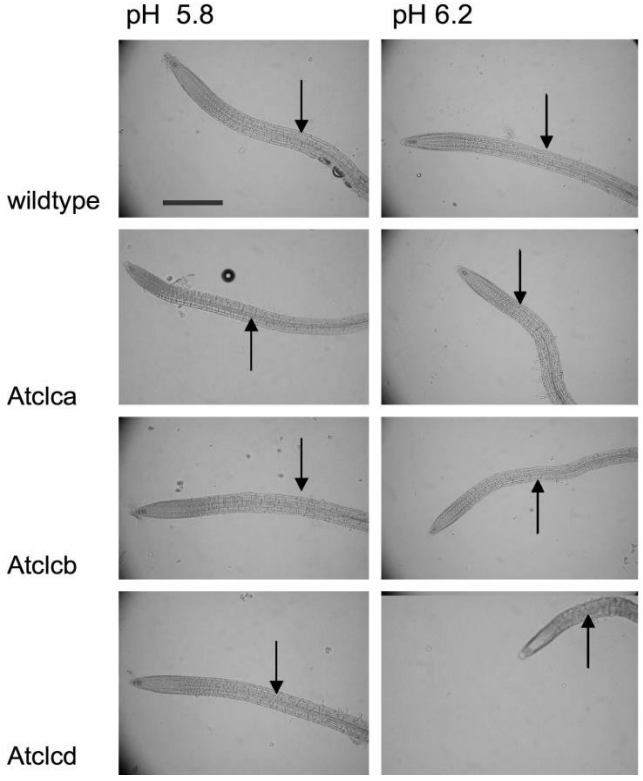

Figure 5. Phenotypes of the roots of the Arabidopsis wild type and the three single mutants when grown on media with different pH-values. The first epidermal cell with a visible root hair bulge is indicated by an arrow. This cell is used to measure the DFEH (distance between root tip and first epidermal cell with visible root hair bulge). The bar in the upper left photo indicates 0.5 millimeter.

Difference in DFEH between wild type and Atclc mutant plants

The acid growth theory predicts cell wall loosening and rapid cell elongation at low pH. In order to check the relation between different pHs and cell elongation in the primary root, we measured the distance between root tip and the first epidermal cell with visible root hair bulge (DFEH). In 8-day-old plants grown at pH 5.8 DFEH was 1355 ± 15 µm (Figures 4 and 5). At pH 6.2 DFEH decreased in all plants, but it was more significant in the *Atclca* and *Atclcd* mutants.

DISCUSSION

Expansion of cells is only possible when the yield threshold of the cell wall is low enough and the turgor, the pressure the cell exerts on the cell wall, is high enough. For both of these parameters a close interaction between transporter proteins is necessary. The apoplastic pH of root cells that reflects the pH of the medium, but is also determined by the H^+-efflux mediated by the plasma membrane H^+-ATPases and the H^+-influx through the H^+-coupled anion symporters (Tanner and Caspari, 1996). The plasmamembrane proton pumping ATPase activity is also one of the main regulators of the cytoplasmic pH state. An increase in cytoplasmic pH will necessarily result in down-regulation of the H^+-ATPase activity. Any transport process, also across endomembranes, that affects the cytoplasmic pH is likely to affect the net proton fluxes at the cell surface. Hence, the presence or absence of an anion/H^+ antiporter will have such an effect.

For the second requirement for cell expansion, the generation of sufficient turgor, the same anion/H^+ antiporter will also have a key role. Accumulation of solutes that have to provide the low osmotic potential to attract water to enter the cell will have to be balanced in all cellular compartments. Therefore, the vacuole, being the largest compartment has to be stocked with a

mixture of small organic molecules and nearly equal amounts of cations and anions. Under most conditions the accumulation factor between cytoplasm and vacuole found for anions can thermodynamically not be explained by simple diffusion down the electrical potential (positive inside the vacuole). The accumulation of anions is often so high that secondary active transport, mediated by anion/H^+ antiporters is essential. Also, plasma membrane anion channels play a central role in cytosolic pH regulation of plant cells (Johannes *et al*., 1998). The link between ClC anion transporters and H^+-pumping has been confirmed for the mammalian ClC3, ClC5 and ClC7 transporters (Jentsch *et al*., 2002). The prokaryotic ClC anion channel was shown to mediate a stoichiometrically fixed two anions/proton antiport activity (reviewed in Miller, 2006). In plants, the same function has been proposed for *AtClCa* and *AtClCd* (De Angeli *et al*., 2006 and De Angeli *et al*., 2007).

After confirmation that the *Atclca*, -b and -d mutants were homozygous, and lacked a full transcript (Figure 1) we used them to elucidate the role of endomembrane H^+/anion antiporters in Arabidopsis root elongation.

Proton fluxes and root cell elongation

Changing the pH from 5.8 to 6.2 reveals three differences between Atclca and Atclcd on the one hand and wild type and Atclcb on the other. In Atclca and Atclcd increasing the pH leads to 1) a most drastic decrease in H^+ influx, 2) a stronger inhibition of primary root growth and 3) to a shortening of the root expansion zone.

From these results we conclude that the *AtClCa* and *AtClCd* transporter proteins are involved in primary root expansion growth. This conclusion is based on the following considerations:

1) For the different genotypes exposed to a higher pH, the length of the primary root correlates with the distance between root tip and first epidermal cell with visible root hair bulge (DFEH) and this indicates that specifically cell expansion is reduced in the *Atclca* and *Atclcb* mutant plants. The length of the first epidermal cell with a visible root hair bulge (LEH) was previously defined as a parameter to study root development and the control of elongation on cell level (Le *et al*., 2001). This parameter is less useful when cell size measurements are more difficult to perform, which for instance is the case when the root tips are swollen and have accumulated pigments, and the epidermal cell walls are obscured. Measuring the DFEH is easier since it only involves the recognition of the first root hair bulge and the root tip. Since the epidermal cell exhibiting the first root hair marks the end of the fast elongation zone and the onset of the differentiation zone in Arabidopsis root (De Cnodder *et al*., 2006) the DFEH should therefore give a fairly accurate reflection of the expansion rate in the distal part of the primary root. This implies that in *Atclca* and *Atclcb* mutants the expansion rate is reduced compared with wild type and *Atclcb* (Figures 4 and 5).

2) The zone of highest expression of the ClC genes, that show a reduced elongation rate when mutated, coincides with the elongation zone. By using GUS staining in the root Lv *et al*. (2009) showed that *AtClCa* and *AtClCd* have the highest expression in the elongation and maturation zones, but that they are absent in the division zone.

3) A function of *AtClCd* in cell expansion in root growth has been proposed earlier. Atclcd mutant plants exhibit a reduction in root growth when compared to wild type at elevated pHs of the medium, which is also attributed to low cell expansion rates (Fecht-Bartenbach *et al*. 2007). The *AtClCd* protein is essential for normal cell expansion of hypocotyl cells in which the V-type ATPase is inhibited or only partly functional (Fecht-Bartenbach *et al*., 2007).

The result that the mutants with a stronger reduced H^+ influx, are most severely inhibited in root growth, is not immediately consistent with the accepted acid growth theory for expansion growth. Normally, the reduction of H^+ influx would result in a lowering of the apoplastic pH and, consequently, it would be expected that the expansion growth is stimulated in this situation. In the literature the indications that lowering the pH induces cell elongation are many (Rayle and Cleland, 1992). For instance, part of elongation zone growth is regulated by acid growth phenomena associated with cellular control over the cell wall pH (Edwards and Scott, 1974; Buntemeyer *et al*., 1998; Peters and felle, 1999). Under normal conditions the surface pH along Arabidopsis roots, is highest in the transition zone, and lower in the adjacent to the fast elongation zone (De Cnodder *et al*., 2006). Our results thus do not fit with this general model. We found that faster elongation correlates with higher influx, not with higher efflux. We hypothesize that for maintaining root growth at a higher pH, functional *AtClCa* and *AtClCd* proteins are necessary to drive the accumulation of anions in intracellular compartments, specifically the vacuole and/or acidic vesicles, and to generate sufficient turgor. This hypothesis would fit with our results: 1) the increased proton efflux in wild type is possibly the result of sustained anion/H^+ co-transporter activity in the plasma membrane and 2) the reduced root length phenotype of the mutants is only obvious at higher pH values. At these higher pHs cell wall elasticity is lower

and elongation will only be possible by higher turgor values.

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