Chapter 4

Anion Channels and Root Elongation in *Arabidopsis thaliana*

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ABSTRACT

Anion transporting proteins belonging to the chloride channel (ClC) family are involved in anion homeostasis in a variety of organisms. Progress in the understanding of their biological functions is limited by the small number of genes identified so far. Seven chloride channel members could be identified in the Arabidopsis genome, amongst which AtClCa, AtClCb, and AtClCd are more closely related to each other than to the other plant ClCs in same subclass. Chloride channels from Arabidopsis have been shown to participate in nitrate accumulation and storage. In this study, the physiological role of AtClCa, AtClCb and AtClCd proteins was investigated. Disruption of the AtClCa, AtClCb and AtClCd gene by a T-DNA insertion did not yield a phenotype that was different from wildtype under normal conditions, however, when the pH of the medium was slightly less acidic (raised from 5.8 to 6.2) the length of the primary root of plants with a disrupted AtClCa and AtClCd gene was reduced compared to wildtype 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 pH’s 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 at 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 wildtype and the Atclcb mutant. Measurement of the distance between root tip and first epidermal cell with visible root hair bulge indicate that the mutants that are affected in the H⁺ flux, the Atclca and Atclcd mutants, also have a reduced cell expansion. A model for the interaction between endomembrane anion/H⁺ antiporters, plasma membrane proton fluxes and cell expansion is discussed.
INTRODUCTION

Root development is determined by cell division, differentiation and expansion. Each of these processes is under the control of an intrinsic developmental program and of external biotic and abiotic factors (Lynch, 1995). A wealth of information is available on the cellular organization, the differentiation and genetic background of developmental processes in the root of Arabidopsis, the model system of choice for plants (Dolan et al. 1993; Schiefelbein et al., 1997; Scheres et al., 2002; Park et al., 2008). Several groups have explored the mechanisms of cell expansion in the elongation zone, the zone of maximal cell growth rate (Befey et al., 1993; Hauser et al., 1995; Verbelen et al., 2001; Swarup et al., 2007; Cnodder et al., 2006). Cell elongation in roots 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 (Fan and Neuman, 2004; Peters and Felle, 1999; Pilet et al., 1983). A more recent version of the acid-growth theory states that a low apoplastic pH (<5) activates expansins, cell wall-associated 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 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 (Angeli et al., 2006; Jennifer et al., 2007 and Scheel et al., 2005). 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 CIC proteins are essential in cell expansion in certain tissues and cell types, the functional relation between AtCIC proteins and proton gradient development in expanding root cells is still unclear. In this study, we tried to elucidate the relationship between CIC transporter proteins and the proton flux in root cell elongation, by quantifying the fluxes along the root in wildtype and the Atclca, Atclcb and Atclcd mutants at different external pH’s.
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: WiscDsLox477-480I4). Seeds were surface sterilized with gaseous chlorine and sown in 90 mm petridishes containing with 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 days and 14 days old plants were used for MIFE and primary root measurement, respectively.

Ion flux experiments

Net fluxes of protons were measured non-invasively using vibrating H⁺-selective microelectrodes with the MIFE (microelectrode ion flux estimation) 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 rang 5.1-7.8) were used. The electrodes were calibrated before and after use. Roots of five days 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 30° and 40° with the horizontal 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 could be calculated.

The absolute pH value could differ (± 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**

Homozygous mutants lines were identified by resistance to kanamycin and by a PCR-based screen with the left border primer (LB) according to the Salk protocol and the respective primers (has been described in chapter 2)

**Expression analysis in roots**

Total RNA were isolated from roots using a Nucleospin RNA plant kit (Macherey-Nagel). RNA was measured by the nanodrop machine. Total RNA (3 μg) were used as template for first-strand cDNA synthesis using 200U 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 for *AtClCb*. Primers are given in table1 chapter 2.

**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 of chapter 2. **upper panel:** the *Tubulin* transcript levels of the four genotypes are shown as a loading control. MW: lane with the molecular marker, the size of the essential bands is shown on the left. **lower panels:** expression levels of the *AtClCa* (left panel), *AtClCb*, (middle panel) and *AtClCd* (right panel) in wildtype and the respective T-DNA insertion lines.
Cell imaging in primary root

Images were taken with a Nikon Coolpix 990 digital camera, which was 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 were measured. At least 10 Arabidopsis wildtype and mutant plants were measured for every condition in each experiment and each experiment was repeated 3 times.
RESULTS

Isolation of homozygous knockout lines

From the seven members of the CIC transporter protein family in Arabidopsis thaliana tree genes (AtClCa, b and d) that are phylogenetically more closely related to each other than to any of the other members were selected. Plant CIC proteins are grouped into two distinct subclasses with significant divergence (Lv et al., 2009). In subclass 1 AtClCa and AtClCb are the most closely related, while AtClCd although in another branch of the same subclass, is also rather similar. Of the other proteins in this family, AtClCc and AtClCg, also belong to subclass 1, while AtClCe and atClCf, belong to subclass 2 and are more distantly related. Homozygous T-DNA insertion lines in the Columbia ecotype were selected from Salk and the WiscDsLox T-DNA collections.

Reverse transcript PCR analysis of gene expression

As shown in figure 1, RT-PCR confirmed high expression levels of AtClCa, AtClCb and AtClCd in root tissue of wild type plants. This result is in agreement with an earlier study (Lv et al. 2009) with showed high expression in the root of AtClCa and AtClCd and 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 wildtype and mutant plants growing on agar plates and exposed to different external pH’s (5.8 till 6.8, buffered with 20 mM Mes). All genotypes showed the longest root when grown on 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), however, compared to the wildtype, root growth in Atclca and Atclcd was more reduced. Atclcb was not distinguishable from wildtype at all pH values. In all 8-days-old plants exposed to the highest pH (6.8) the roots of the seedlings had hardly grown at all and the leaves were yellowing.
Reduced proton flux on the growth zones of root Atclca and Atclcd

To determine if the clear difference in root length between wildtype plants and Atclca and Atclcd mutants at less acidic pH’s, could be correlated to differences in cell wall acidification, the proton fluxes at the surface of the roots of 8-days-old plants were measured in wildtype and mutant plants in media with different pH’s. 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 between wildtype and mutants can be observed (Figures 3 and 4).

Figure 2. The effect of the pH of the growth medium on primary root length of wildtype Arabidopsis and the three single mutant lines. a: Phenotypes of roots of the wildtype and the three single mutants grown at different pH-values. The distance between two short lines on the reference is 1 mm. b: Quantification of primary root length of wildtype and the three single mutants grown at different pH-values. Datapoints are the average of 4 experiments and the error bars indicate the standard deviation.
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At higher pH’s (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 wildtype and \textit{Atclcb}. However, more significant was the almost complete disappearance of the H\(^+\) influx in the \textit{Atclea} and \textit{Atcleb} mutants.
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**Figure 3.** The proton flux profile along the roots of wildtype 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). Datapoints are the average of 4 experiments and the error bars indicate the standard deviation.

**Difference DFEH between wildtype and Atclca 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 pH’s 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 was decreased in all plants, but significantly more so 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, high enough. For both of these parameters a close interaction between transporter proteins is necessary. The apoplastic pH of root cells will reflect 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 stat. 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.

![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 roottip as shown in Figure 3. Lower panel: DFEH: the distance between root tip and first epidermal cell with visible root hair bulge. Datapoints are the average of 6 experiments and the error bars indicate the standard deviation.](image)

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
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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 CIC anion transporters and H⁺-pumping has been confirmed for the
mammalian CIC3, CIC5 and CIC7 transporters (Jentsch et al., 2002). The prokaryotic
CIC anion channel was shown to mediate a stoichiometrically fixed 2 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 we had selected for this study
were homozygous, and indeed lacked a full transcript (Figure 1) we used them to
eclucitate 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 Atcleca and
Atclecd on the one hand and wildtype and Atclecb on the other. In Atcleca and Atclecd
increasing the pH leads to 1) a more 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 Atcleca and Atclecb 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 wildtype and Atclcb (Figures 4 and 5).

2) The zone of highest expression of the CIC 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 zone, 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 mutants plant exhibit a reduction in root growth when compared to wildtype at elevated pH’s 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 hypocotyls 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 more strongly reduced H+ influx, are the most severely inhibited in root growth, is not immediately consistent with the accepted acid growth theory for expansion growth. Normally, reduction of H+ influx would result in
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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 (Taguchi et al., 1999; Vanderhoef and Dute, 1981; 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 lowest in the adjacent fast elongation zone (De Cnodder et al., 2006). Our results thus do not fit with this general model. We find that faster elongation correlates with higher influx, not with higher efflux. We hypothesize that for maintaining root growth at 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 wildtype 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 pH’s cell wall elasticity is lower and elongation will only be possible by higher turgor values.
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