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PHYSIOLOGICAL CHARACTERIZATION OF ANION CHANNEL MUTANTS OF *ARABIDOPSIS THALIANA*

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Summary: To study the role of CLC proteins in Arabidopsis we obtained three T-DNA insertion lines for AtCLCa, b, d and generated the three double and the single triple mutants. Seed germination on media supplemented with different concentrations of NaCl, choline chloride, KCl and KNO_3 did not show differences between the different genotypes. The micro-electrode ion flux estimation technique was used to monitor and characterize H^+ and Cl^- fluxes from intact leaf tissue and the changes in these fluxes induced by changes in the external NaCl concentration. Although NaCl induced a transient change in the fluxes of chloride and protons, the responses of the mutant plants were not distinguishable from the wild type. Also, morphologically the mutant plants were similar to the wild type. However, when pH of the growth medium was increased from 5.8 to 6.2, root growth in mutant lines AtCLCa and AtCLCd, the double mutant AtCLCad and the triple mutant AtCLCabd was reduced.

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Key words: *Arabidopsis*; chloride channel mutants; ion fluxes; root growth.

Abbreviations: CLC proteins – members of a large family of chloride transport proteins; AtCLC – *Arabidopsis thaliana* CLC proteins; T-DNA – transfer DNA; MIFE – micro-electrode ion flux estimation; NASC – Nottingham Arabidopsis Stock Centre; BMS - Basic measuring solution; PMF - Proton-motive force.

INTRODUCTION

Chloride is essential for the optimal activity of several enzymes. In photosynthesis chloride plays a role in the water splitting complex. Chloride also plays an important role in osmoregulation and growth (Barbier-Brygoo et al., 2000). Cell expansion is driven by turgor pressure which results from the osmotically driven uptake of water into the cell. Water enters the cells due to the high concentrations of potassium and anions, like chloride and malate. During rapid growth, chloride accumulates in the vacuole of plants cells, where concentrations up to 40 mM

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can be measured (Barbier-Brygoo et al., 2000). A specialized, but closely related role is the involvement of chloride in the opening and closing of stomata. Stomata open as a result of an increase in turgor due to the uptake of water into the guard cells. The continuous redistribution of chloride between the guard cells and the surrounding tissue upon changing conditions is one of the mechanisms controlling the opening and closing of stomata (Pandey et al., 2007). Another role of chloride is associated with the negative charge of the ion. Transport of anions across a membrane can compensate for the currents, which result from transport of positive ions (like protons), thereby allowing a steeper concentration gradient of these cations across the membrane. In this context a role of chloride has been demonstrated in proton transport by the plasma membrane H^+-ATPase and the vacuolar V-type ATPase (Felle, 1994). Other transport processes in which chloride is either directly of indirectly involved are, for instance, Na^+/H^+ and HCO_3^-/Cl^- exchange (Jentsch et al., 2002).

At low external concentrations, Cl^- can be taken up via a H^+/Cl^- co-transporter, while at high Cl^- concentrations the anion might enter passively through anion channels. Active chloride uptake and transport by roots has been demonstrated (reviewed by White and Broadley, 2001) and also Cl^- transport to the shoot is controlled by the roots (Sauer, 1968; Downton, 1977; Storey et al., 2003). It is still unknown which systems actually mediate Cl^- uptake into the symplast of the root. Considering the concentration gradient, which exists between the apoplast and the symplast, the systems involved should be active transporters. Only under high saline conditions the concentration gradient might be in favor of passive uptake of Cl^- mediated by chloride-specific channels or more non-specific anion channels.

The chloride channel (CLC) proteins represent an important group of anion transporting proteins. Seven CLC genes have been identified in the *Arabidopsis* genome. CLC genes are present in most organisms: their presence has been shown in fungi, bacteria, animals, and plants. The CLC protein family has been best characterized in mammals, in which nine different CLC members are present. These proteins can be divided into three groups based on their localization in the plasma membrane or intracellular membranes (Jentsch et al., 2002). The 3D structure of these proteins has been solved (Dutzler et al., 2002; Dutzler et al., 2003) which allows a detailed study of the structure-function relationship in these proteins. In *Arabidopsis* the seven members can be divided into two groups (Lv et al., 2009). This division is based on their molecular structure and seems to be related to their mode of action. The seven proteins have been demonstrated as a group to be present in cell membranes where the different members have their own specific localization (De Angeli et al., 2007; Marmagne et al., 2007; Lv et al., 2009). They are involved in turgor and osmoregulation, membrane potential control, vesicle trafficking, nutrient transport, stomatal movement, and resistance to heavy metals and salt stress (Barbier-Brygoo et al., 2000; Tyerman et al., 1997). Despite the key role of chloride in various processes, little is known about the role of the CLC proteins in Cl^- homeostasis and, for instance,

salinity stress. Moreover, interaction and coordination of the activities of the seven ClC proteins can be expected. In order to address these issues we set out to isolate mutants of CLC genes. We have identified three *Arabidopsis* mutants lines which carry a T-DNA insert in the At CLC-a, At CLC-b, and At CLC-d genes, respectively. We showed the absence of transcripts of these genes in the mutant plants and made the three double mutants and the triple mutants and performed a preliminary evaluation of these six mutant lines. Under the conditions we used, no obvious phenotypes were observed for the single mutants. We could only observe a phenotype in the double and triple mutants containing insertions in both the At CLC-a, and At CLC-d genes when the plants were grown at pH higher than 5.8.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Arabidopsis* T-DNA insertion lines were obtained from NASC (*At* CLC-*a*: *WiscDsLox477-480I4; At* CLC-*b*: SALK_ 27349; *At* CLC-*d*: SALK_42895). For *in vitro* growth experiments, seeds were surface sterilized with gaseous chlorine (derived from acidified sodium hypochlorite) and sown on a half-strength Murashige and Skoog medium (MS/2), supplemented with 1% (w/v) sucrose and buffered with 10 mM MES-Tris, pH 5.8 and 0.8% (w/v) of agar. For the selection of the mutants 50 μg/ml kanamycin was added. The dishes were sealed with surgery tape (3M). For the growth analysis the proper salts and additives were added or pH was adjusted accordingly. For growth on soil, seeds were surface sterilized and sown in pots containing organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands). All dishes and pots were incubated in the dark at 4°C for 3 days. Subsequently, the dishes were transferred to a growth chamber, set at a 16h/8h light light/dark cycle, 20±2°C temperature and 72% relative humidity while the pots were transferred to the greenhouse at 20±1°C during the 16h day period and 18±1°C during the night period at 72% relative humidity and with supplementary light when necessary, or to the growth chamber under the conditions described above.

Screening for T-DNA insertion mutants

The T-DNA insertion disrupting AtCLC-b and AtCLC-d were identified in the database in the SALK Institute Genome Analysis Laboratory (Salk-027349 for AtClCb, Salk-042895 for AtCLC-d) and Wiscd Siox 477-480i4 was identified in the WiscDsLox T-DNA collection for AtCLC-a. To obtain homozygous mutants 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). To generate double mutants, single mutant plants were crossed with each other. The genotypes of F_1 plants were checked using a PCR-based screen with the respective primers for each gene (Table 1). All F1 double mutants (*Atclcab*, *Atclcad*, and *Atclcbd*) were allowed to self-pollinate. Seeds were sown on plates. Plants were subsequently checked again with the primer sets of the respective T-DNAs (Table 1) and segregated in the F_2 population progeny in a 1:15 ratio. A

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			
At CLC-a	5'-CCAGATAAATCTTCACTTTCTGATGG	5'-TGTCAATGCCATTAAGGTAAGC	5'-GCGTGGACCGCTTGCTGCAACT
At CLC-b	5'-GGAGTTCTGTAGCCCCAGTTG	5'-GTAATCGGTGGAATTCTTGGG	5'-TGGTTCACGTAGTGGGCCATCG
At CLC-d	5'-GGAACTGGATTAGCTGCTGTG	5'-CCTACCATGATGTGACCTCCTC	5'-TGGTTCACGTAGTGGGCCATCG
Gene expression analysis			
At CLC-a	5'-ACTGCATTTTGGGTCTTA	5'- GAAATGCTTGTAGAATGTTA	
At CLC-b	5'-ACTGCATCTTGGGGCTTT	5'-AGGCTTGTAGAATGTTGTAT	
At CLC-d	5'-TTTACACATTAGCTGTAG	5'-TTGACTGTTGGAGTTCCACT	
β-Tubelin	5'-GAGCCTTACAACGCTACTCTGTCTGTC	5'- ACACCAGACATAGAGCAGAAATCAAG	

triple mutant was created by crossing between two double mutants *Atclcad* and *Atclcbd*. F_1 progeny from this cross was allowed to self-pollinate. Seeds were sown and the progeny was checked and analyzed for its genotypes (Fig. 1).

Reverse Transcript PCR

Total RNA from shoots and roots was isolated according to Chomczynski et al. (1997) using TRIZOL reagent (Invitrogen). First-strand cDNA synthesis on 3µg of total RNA was done using reverse transcriptase (Fermentas) and a Oligo(dT) primer according to the suppliers manual. Two µL from total 20 µl volume of cDNA was used for PCR amplification using polymerase in a 50 µL reaction volume according to the following program: 94°C for 2 min, then 32 (*AtCLC-a* and *At CLC-d*) or 35 (*At CLC-b*) cycles consisting of 94°C for 15 s; 54°C for 30 s (*At CLC-a* and *At CLC-d*), 45°C for 30 s (*At CLC-b*); 72°C for 1 min and 72°C for 5 min. Table 1 shows the RNA-specific primers for the *CLC genes* and the control gene, tubelin.

Ion flux measurement using MIFE technique

Net fluxes of H^+ and Cl^- from leaves were measured using H^+- and Cl^--selective microelectrodes with the MIFE technique (Shabala et al., 1997; Newman, 2001; Lanfermeijer et al., 2008). Microelectrodes were pulled from borosilicate glass capillaries (GC150-10; Harvard Apparatus) and silanized with tributylchlorosilane (Fluka 90974). The H^+- selective electrodes were back filled with 15 mM NaCl plus 40 mM KH_2PO_4 and front filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297). The Cl^--selective electrodes were back filled with 500 mM KCl adjusted to pH 6 with NaOH and front filled with Cl^- (chloride ionophore I, cocktail A, Fluka 24902). The response of the electrode was typically 48 mV/decade. This somewhat low response voltage was always observed and was considered to be the result of a small leakage of ions in the tip of the electrode. Apparently, the sealing between the silanized glass

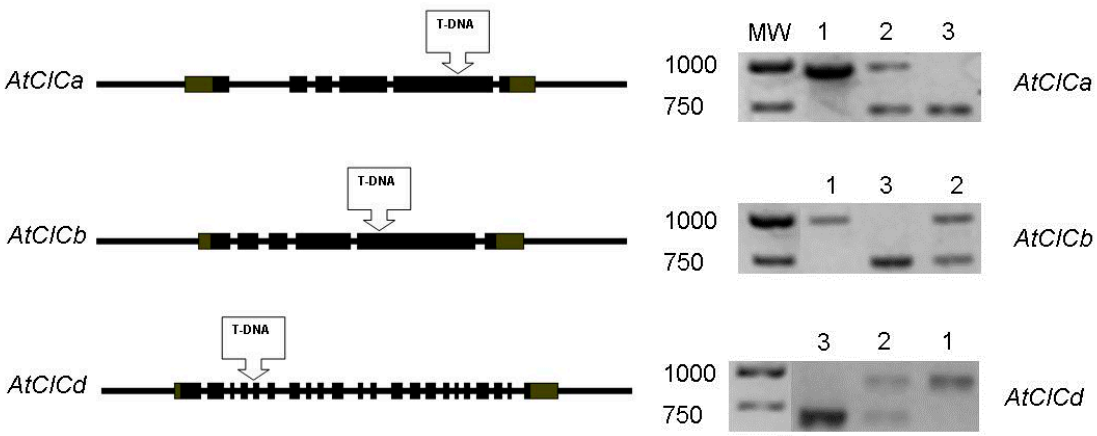

Figure 1. The T-DNA insertion in the three single mutant parental lines Left: The position of the T-DNA insertions in *AtCLC-a, AtCLC-b* and *AtCLC-d* genes. Right: PCR-based markers for the presence and absence of the T-DNA insertions. Lane 1: Homozygous wild type genotype, 2: Heterozygous genotype 3: Homozygous T-DNA insertion genotype. The MW markers are indicated on the left and the gene studied is indicated on the right.

and the ionophore mix was not optimal. To avoid potassium ion leakage from the reference electrode, the reference electrode was placed in a compartment different from the measuring chamber. The two compartments were electrically connected via a salt bridge, which consisted of 300 mM $(NH_4)_2SO_4$ in 2% (w/v) agar.

Leaf material was immobilized on a glass capillary using grease (consisting of 49% petroleum jelly, 34% bee wax, and 17% lanoline) with the abaxial epidermisless side exposed to the solution and placed in a measuring chamber with a transparent bottom. The chamber was filled with 1 ml of basic measuring solution (BMS; 1 mM KCl plus 0.5 mM $CaCl_2$, pH 5.8), submerging the leaf material. The whole chamber was placed on a Nikon TMS inverted microscope. The ion-selective microelectrodes were mounted at an angle between 30° and 40° with the horizontal in a holder (MMT-5; Narishige) on a three-way piezo-controlled micromanipulator (PCT; Luigs and Neumann) driven by a computer-controlled motor (MO61-CE08; Superior Electric). The electrodes were positioned 10 μm from the surface of the tissue. During measurements, the distances between the tissue and the electrodes were changed from 10 to 50 μm at a frequency of 0.1 Hz. The chemical activities of H^+ and Cl^- in solution were continuously recorded at the two distances from the tissue, and from these data, net H^+ and Cl^- fluxes were calculated according to Newman (2001) whereas positioning the material in the MIFE apparatus was performed under light conditions (150 μmol m^{-2} s^{-1}) and the measurements were performed in the dark at ambient room temperature.

RESULTS

Isolation of homozygous knockout lines with PCR-based screen

The obtained three single homozygous T-DNA insertion lines in *Arabidopsis* ecotype Columbia in *At CLC-a*, *At CLC-d* and *At CLC-b* were used to generate the three double and the one triple mutant combinations. The exact locations of the T-DNA insertions in the loci At5g40890, At3g27170 and At5g26240 were obtained from the TAIR database (http://www.Arabidopsis.org) (Fig. 1a). The correct genetic conformation of the three parental single mutant lines was confirmed by PCR (Fig. 1b).

Tissue specific expression of the *AtClCa*, *b*, and *d* genes

Transcripts of *At CLC-a* and *d* were detected in both shoot and root. Transcripts of *AtClCb* were detected only in the root (Fig. 2).

In the T-DNA insertion lines only transcripts of the *CLC* genes, which were not disrupted, were detected (Figs. 3-1, 3-2).

Ion fluxes

The MIFE technique allows monitoring of ion fluxes in and out of almost intact tissues. We explored the potential of this technique to study NaCl-induced changes in ion fluxes in leaf tissue. We focused on H^+ and Cl^- fluxes because of the potential role of the so-called CLC proteins in Cl^- fluxes and the potential linkage between this flux and the H^+ flux (Fig. 4). By convention a positive flux means an influx of the ions, both in the case of cations and anions (Newman 2001). To relate this to the currents measured in electrophysiology an

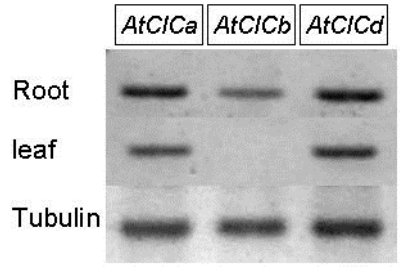

Figure 2. Semi-quantative expression of the *At CLC-a*, *At* CLC-*b* and *At CLC-d* genes in different plant tissues. The amounts of PCR products resulting from 32 (At CLC-a and At CLC-d) or 35 (At CLC-b) PCR cycles are shown. The *Tubulin* transcript levels are shown as a loading control. The used primers for the respective genes are shown in Table 1.

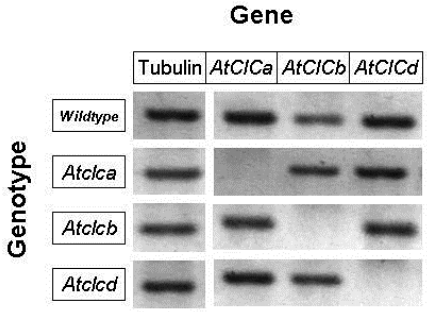

Figure 3-1. The lack of expression of the At CLC-*a*, At CLC-*b* and At CLC-*d* genes in their respective T-DNA insertion lines. The three genotypes were analysed using the primers shown in Table 1. The *Tubulin* transcript levels are shown as a loading control.

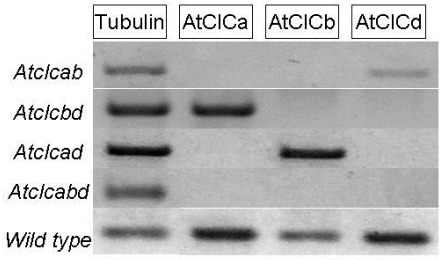

Figure 3-2. The lack of expression of the *AtCLC-a*, *AtCLC-b* and *AtCLC-d* genes in the double and triple mutant lines. The *Tubulin* transcript levels of the five genotypes are shown as a loading control.

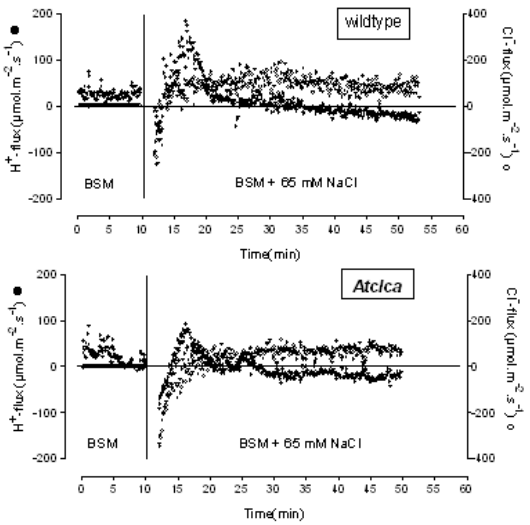

Figure 4. Typical changes in proton and chloride fluxes from wildtype *Arabidopsis thaliana* (upper panel) and the *Atclca* genotype (lower panel) leaf tissue induced by 65 mM of NaCl. The vertical line indicates the time point of the addition of 65 mM NaCl. When the flux becomes more positive, this means either an increase of the influx or a reduction of the efflux. Typical results of at least three experiments are shown.

influx of cations causes an inward current and a depolarization while an influx of anions causes an outward current and a hyperpolarization.

Suddenly challenging the leaf tissue with 65/75 mM of NaCl caused the influx of protons to increase transiently from around 25 µmol m^{-2} s^{-1} to 150 µmol m^{-2} s^{-1}. After 20 min the proton influx returned to its pre-salinity values and started slowly to change into a small efflux. Chloride fluxes were zero before NaCl was added but as soon as 65 mM of Cl^- was added this changed to an influx of chloride. However, no differences in the response to the sudden application of NaCl of the mutants mutually and wild type was observed.

Phenotypical characterization of mutant plants

For none of the mutant lines a difference in their growth and development could be observed when they were grown in soil (data not shown). The effects on germination were studied on MS/2 agar media supplemented with different concentrations (0-300 mM) of NaCl, KCl, KNO_3 and choline chloride (Fig. 5). No differences were observed between the six mutant lines mutually and in comparison with the wild type. However, germination was affected by the presence of different salts. Germination was least inhibited by KCl. A 50% reduction of germination was obtained at KCl concentrations above 275 mM. For both choline chloride and NaCl the concentration at which 50% reduction of germination was obtained was around 250 mM. The highest reduction in germination was observed when seeds

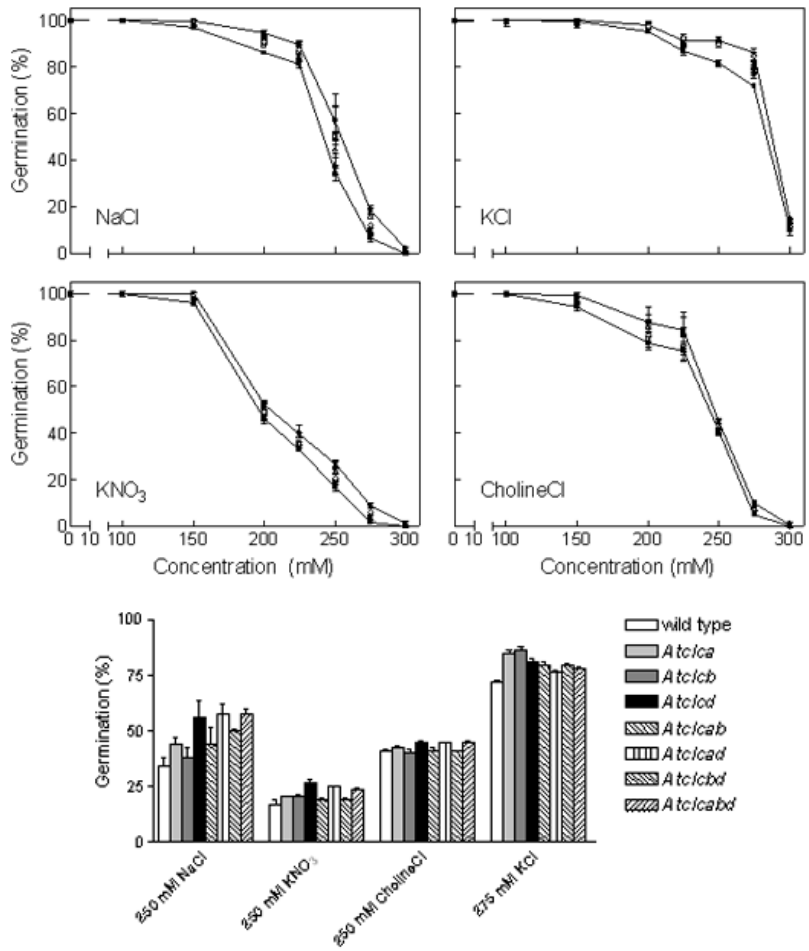

Figure 5. Germination of *Arabidopsis* seeds of 8 genotypes on a half strenght MS medium, supplemented with the indicated salts. Upper panel: Concentration dependent inhibiton of germination. Lines are only drawn based on the two outermost datasets. Datapoints are the average of 3 experiments and the error bars indicate the standard deviation. Lower pannel: Germination of the 8 genotypes at a single salt concentration. The data are the same as shown in Figure 5a , but for clarity shown separately. Datapoints are the average of 3 experiments and the error bars indicate the standard error.

were allowed to germinate on KNO_3, in this case 50% germination was already obtained at 200 mM.

In order to study the effect on growth and development plants were allowed to germinate and grow on vertical plates under a large number of different physiological conditions, like different salts, different concentrations of these salts and different pH-values. No differences were observed when plants were grown under standard conditions and various salt conditions (data not shown), whereas a difference in the growth of some plant lines could be observed when the plants were grown on MS/2 media with a higher pH (6.2) instead of 5.8 (Fig. 6). The single mutants *Atclca* and *Atclcd*

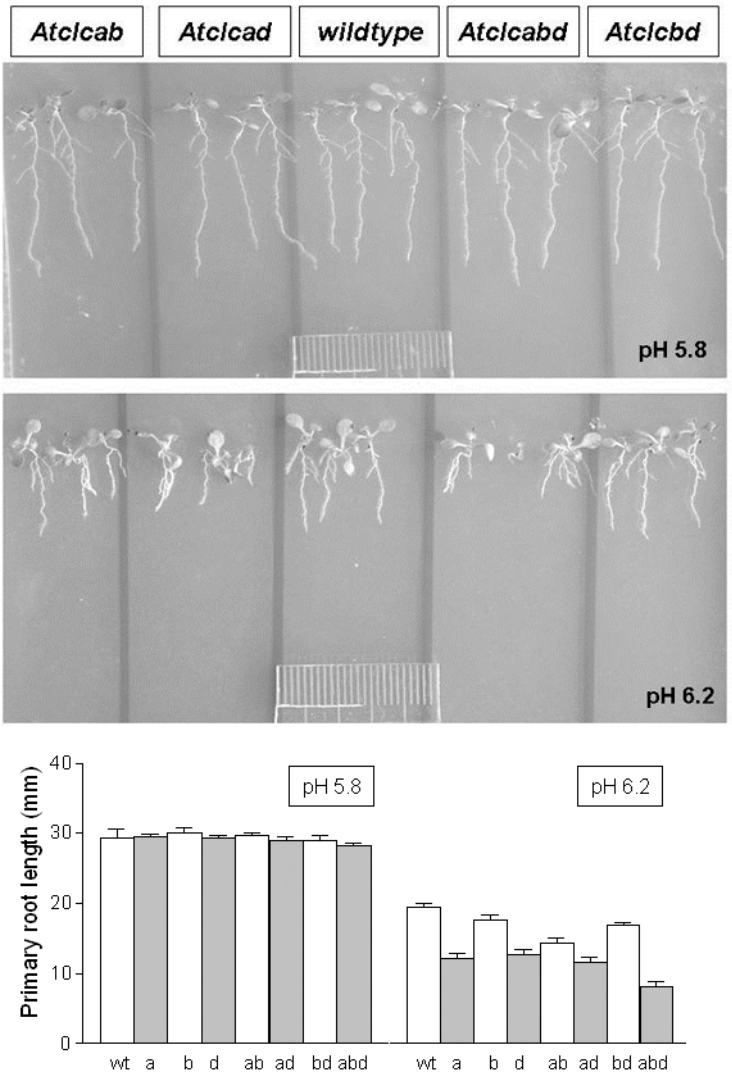

Figure 6. Upper panel: Primary root growth of the indicated *Arabidopsis* genotypes on a half strenght MS medium at two different pH values. Upper panel; pH 5.8; lower panel: pH 6.2. Length of the reference is 2.5 cm. Lower pannel: Length of the primary root of the indicated *Arabidopsis* genotypes on a half strenght MS medium at two different pH values. Datapoints are the average of 4 experiments and the error bars indicate the standard deviation. wt -wild type; a till adb - genotypes *Atclca* till *Atlcabd*.

had a significantly reduced primary root length compared with the wild type and the *Atclcb* mutant (Fig. 6). Combining the mutations showed that when *AtClCb* was also absent the effects of the absence of *At* CLC-*a* and *At* CLC-*d* were reduced. The double mutant *Atclcad* showed no additive effect due to the presence of both disrupted genes. However, the triple mutant *Atclcabd* showed the largest reduction in root elongation (Fig. 6).

DISCUSSION

We presently explored the potential of MIFE technique to study the physiological relevance of the Cl^- fluxes in plants. Using a double barreled configuration we assessed the changes of the H^+ and Cl^-fluxes when plants were suddenly exposed to a concentration of 65/75 mM NaCl. The addition of such a concentration had two effects. Firstly, the external concentrations of Na^+ and Cl^- changed drastically and secondly, the external water potential dropped. As a result of the latter water moved osmotically out of the cells resulting in a decrease of the turgor pressure in the cell. Turgor pressure has been shown to control ion fluxes, either via mechano-sensitive channels (Chang et al., 1998) or via the plasma membrane H^+-ATPase (Sabala and Lew, 2002). We observed a transient increase in the proton efflux upon NaCl exposure followed by a slow change into a small proton efflux. The time course of this phenomenon can be compared with the observations of Shabala et al. (2005) and Cuin et al. (2008), although those were made on roots. Their membrane potential measurements showed a transient depolarization and a partial recovery of the membrane potential 10 to 15 min after the addition of salt. The transient influx of protons observed in the present study fits well with the transient depolarization while the partial recovery could be due to the emerging efflux of protons. However, Shabala et al. (2005) did not observe this transient efflux of protons; they only observed the decreasing influx. The increase of chloride influx is caused first of all by the concentration gradient which favors an influx and, secondly, by the depolarization of the membrane potential. Our observations are in accordance with those of Shabala et al. (2005), who found an increase of the chloride flux from 40 to 100 nmol m^{-2} s^{-1} upon treatment of barley leaf tissue with 20 mM NaCl. The slow decrease of the chloride flux could be ascribed to either the slow recovery of the membrane potential or to the equilibration of the Cl^- ions across the membrane (Fig. 4).

We did not observe a difference between the response of the wild type and the mutants in the MIFE system. Single mutants reacted in an identical manner to a sudden exposure to 65 Mm NaCl (data not shown), which implied that disruption of the three CLC -proteins studied did not affect the observed fluxes.

Also, no effects of the disruption of the CLC genes alone or in combination with the others were observed on germination. The effects of the different salts showed that KCl was the preferred salt for germination. This salt was probably accumulated rapidly and increased the ability of the seedling to take up water and grow. Sodium could not replace potassium in these experiments due to its toxicity and nitrate could not replace the chloride. Nitrate was probably

partly assimilated and turned into amino acids and proteins, while maybe at higher nitrate concentrations the nitrate was not fully assimilated and became nitrite. Nitrite is toxic and it inhibits growth. Hence, equivalent amounts of chloride and nitrate can not exert the same effect on water potential in growing cells.

Also other experiments failed to show differences between any of the mutants and the wild type suggesting either a delicate role of the chloride channels, which could not be observed by our crude observation methods. Moreover, it showed that the role of the CLC proteins was not obviously related with chloride and for instance, salinity stress.

The experiments on root growth showed an involvement of the CLC proteins with growth. Two explanations can be given. Firstly, the At CLC-a and At CLC-d proteins are involved in establishment of the proton-motive force (PMF). Root and cell growth, in general, is driven by pH gradient across the cell. The proton motive force resulting from this gradient across the membrane drives the uptake of solutes necessary for generation of low water potential, which subsequently is needed for the osmotically driven uptake of water and the generation of turgor pressure. When taking the external pH into account it is more difficult to establish the same PMF at higher pH. This is because at higher pH more protons are needed to achieve the same pH gradient and more protons transported across the membrane lead to a higher membrane potential. Hence, at higher external pH values it is advantageous to compensate for the positive charge moved across the membrane when a proton is extruded in order to limit the polarization of the membrane. Polarization of the membrane impedes the transport of the positively charged proton. Next to the coordinated influx of potassium ions the coordinated efflux of chloride ions can be used for this charge compensation. In this model removing AtCLC-a and AtCLC-d hampers this process, reduces the generation of the PMF and subsequently inhibits root growth.

Secondly, the At CLC-a and At CLC-d proteins are required for the accumulation of the osmotically active anions, Cl^- and NO_3^-, which is needed for cell expansion. The absence of the transporters limits the uptake of these osmolytes and, thus, reduces growth.

Recently a study appeared also describing a similar effect of the disruption of the *Atclcd* mutation of root growth in relation with pH of the external medium (Fecht-Bartenbach et al., 2007). These authors also suggested a role of the At CLC-d protein in charge compensation in relation with the establishment of a pH-gradient across the membrane of a Golgi-derived transport vesicle. However, the At CLC-d protein has all characteristics to be an $H^+/2A^-$ antiporter and this is difficult to match with a shunt function of this system. At CLC-a and At CLC-b showed also characteristics which suggest that these proteins are also $H^+/2A^-$ antiporters, however, these proteins are considered to be localized in the tonoplast membrane (Lv et al., 2009). The root-specific expression of the three CLC proteins is highly similar, with expression mostly restricted to maturation zone, and primarily in the vascular tissues (Lv et al., 2009) although At CLC-d seems to have considerable expression in the division

zone as well. In the study of Lv et al. (2009) the expression levels of At CLC-a and At CLC-d were comparable, while At CLC-b had a lower expression level. Although At CLC-a and At CLC-d are localized in two different membranes their effect on root growth could be similar. Cell growth needs an increase in volume but also an increase in surface or in other words of plasma membrane area. The increase of volume requires the generation of PMF or the uptake of osmotically active solutes. If this is disturbed by disruption of tonoplast-located At CLC-a cells will not grow. The increase of plasma membrane surface is accomplished by the continuous delivery of plasma membrane material (lipids and proteins) by golgi-derived vesicles. The trafficking of these vesicles is controlled by their internal pH. Hence, if the establishment of this pH is interrupted by for instance, the removal of At CLC-d, the plasma membrane can not increase its surface area, hence, cells and, thus, roots stop growing.

Our results point to a role of CLC proteins closely related to energizing the membrane. Apparantly CLC proteins are important electrical circuits in the membrane allowing the generation of steep ion gradients in combination with moderate membrane potentials. As such, these proteins play an important role in the general physiology of the cell. Further research will investigate the role and involvement of CLC proteins in growth.

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