Chapter 1

Chloride Channels: A General Introduction

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INTRODUCTION

The physiology of plants strongly depends on solute and water fluxes across the cell plasma membrane, the tonoplast and other endomembranes. Among the different transporter systems involved in transport, ion channels represent a large class with important roles. These proteins facilitate passive fluxes of ions down their respective electrochemical gradients. The CIC proteins constitute a family of transmembrane transporters that either function as anion channel or as H⁺/anion exchanger. Part of the CIC family members are anion selective ion channels, which provide passive pores which allow anions to move according to their electrochemical gradient. The others are anion/proton antitransporters, which couple the transport of two anions with one proton. The first member of the CIC family, called ClC-0 was isolated from the Torpedo marmorata electric organ by expression cloning in Xenopus oocytes (Jentsch et al., 1990). Figure 1 shows the phylogenetic relationship between different members of CIC family prokaryotes and eukaryotes. The CIC family has been best characterized in mammals, in which at least nine members have been identified that can be grouped in three branches (Jentsch et al., 2002). Several studies have demonstrated the importance of CIC family in human diseases, such as kidney stones, muscle disorder myotonia, cystic fibrosis, deafness, and the bone disease osteoporosis (Jentsch et al., 2005). The first plant member of the CIC family was ClC-Nt1 from Nicotiana tabacum (Lurin et al., 1996), which was cloned by a PCR-based cDNA library screening approach (Lurin et al., 1996). CIC proteins in the two model plants, Arabidopsis thaliana and rice, have been shown to encode anion channels and transporters involved in nitrate homeostasis. CIC proteins in plants participate in various physiological processes, such as, osmoregulation, stomatal movement, cell signaling, nutrient uptake and metal tolerance (Barbier et al., 2000). However, detailed knowledge on the role of CIC proteins in plant cells is still lacking as a result of the absence of distinctive effects of knock-outs, the unknown intracellular localization and the co-operation between the different family members.
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Figure 3. Neighbor-Joining Consensus tree of ClC proteins of various kingdoms
The tree was calculated using the Geneious program. The branch containing the AtClCe and AtClCf proteins is indicated with “cyanobacteria, mitochondria, chloroplasts”. Arabidopsis thaliana: AtClCa: CAA96057.1; AtClCb: CAA96058.1; AtClCc: CAA96059.1; AtClCd: P92943.2; AtClCe: AAK53390.1; AtClCf: AAK53391.1; AtClCg: P60300.1; Escherichia coli: EcClC1: P37019.2; Homo sapiens: HsClC1: P35523.2; HsClC2: P51788.1; HsClC3: P51790.2; HsClC4: P51793.2; HsClC5: P51795.1; HsClC6: P51797.1; HsClC7: P51798.2; HsClCKa: P51800.1; Neurospora crassa: NcClCx1: EAA33130.2; NcClCx2: EAA28009.2; NcClCx3: EAA28099.2; Nostoc punctiforme: NpClCx1: YP_001868013.1; NpClCx2: BAB73778.1; NpClCx3: YP_001866371.1; NpClCx4: YP_001865245.1; NpClCx5: YP_001865422.1; Saccharomyces cerevisiae: ScClC (GEF1): P37020.1; Salmonella enterica: SeClC1: AAL19167.1; Synechococcus elongatus: SelClCx1: YP_170743.1; SelClCx2: YP_400274.1; SelClCx3: YP_400605.1; Torpedo marmorata: TmClC-0: CAA40078

Transport across cell membranes

The important function of the plasma membrane is to isolate the exterior from the interior of the cell in order to allow the biochemical processes and preservation of labile biological molecules. However, in order to facilitate the exchange of substrates, products and waste this isolation can not be absolute. Because only a very small number of small lipophilic molecules, like O₂ and CO₂, can traverse the membrane unmediated, biological membranes contain various systems that allow the controlled passage of molecules. These systems are hydrophobic proteins that are inserted in the
membrane and create pores or passageways for all kinds of molecules. These systems can be divided in two major groups: passive transporters and active transporters.

**Passive transport**

Transport is considered to be passive when the movement of the solute is solely driven by the concentration gradient that exists between the interior and the exterior of the cell. In this case there is always a net flux from the compartment with a high to the compartment with a low concentration. Subsequently, passive transport can be divided into simple diffusion and facilitated diffusion. Osmosis, in that sense, does not differ from solute transport. The only difference is that it concerns water transport. Osmotic water fluxes are also driven by the differences in concentration. The higher the concentration of dissolved solutes the lower the concentration of water and, thus, water moves to areas with a high solute concentration and, thus, a low water concentration (activity).

**Simple diffusion**

Simple diffusion through the membrane has only been demonstrated for a few uncharged lipophilic molecules, like for instance O₂, CO₂ and NH₃. However, transport can be slow and can not be controlled. Hence, most fluxes of molecules are facilitated by pores, formed by proteins.

**Facilitated diffusion**

Routes for facilitated diffusion are created by the insertion of hydrophobic proteins into the membrane. These proteins have a hydrophobic surface, which allow interaction with, and thus insertion into, the lipophilic membrane. Internally they either contain a hydrophilic pore or a hydrophilic pathway, which allows the passage of the solutes. These pores can be highly specific, in the sense that they allow only passage of one single type of molecule, for instance potassium channels that only allow the passage of K⁺ ions. Another group of ion-specific channels are the chloride channels, they are named chloride channels because this was the first activity of these channels detected. However, they are able to mediate fluxes of a few anions (Cl⁻, Br⁻, I⁻, NO₃⁻). Other pores are less specific and allow the passage of various types of molecules. Fluxes through the channels continue until equilibrium in concentration is reached (in the case of uncharged molecules) or if the Nernst potential for the ions
transported is established. The Nernst potential considers next to the difference in concentration also the fact that charges are transported. As soon as for instance $K^+$ flows through a potassium-specific channel it leaves a negative charge behind and thus a potential difference across the membrane is generated. At a certain moment the polarization is so large that $K^+$ ions cannot move anymore. The potential at which this happens is called the Nernst potential for that particular ion.

An important aspect of channel-proteins is that they can be controlled. The channels can be opened or closed according to the needs of the cell. This phenomenon is called “gating”. Gating can be controlled by ligands, by membrane potential (voltage-gated), by post-translational modifications or mechanically. Worth mentioning in this context is the presence of two CBS domains in the ClC-proteins. It is suggested that the CBS domains form a sensor that switches transporters between an inactive and an active state (channels: gating) by interaction of the CBS domains with the negatively charged membrane surface in response to the ionic strength. This switching mechanism is an effective means for cells to respond to osmotic shifts, because an increase in medium osmolality will result in a decrease in cell volume, and the accompanying increase in cytoplasmic ionic strength will activate the transporter (Poolman et al., 2006). The presence of these sensors in ClC proteins can be related with the role of these channels in osmo-regulation, turgor-homeostasis and cell growth.

**Active transport**

Cells need to accumulate compounds for different reasons and, thus need to transport solutes against their concentration gradient. This can be achieved in three ways. Firstly, the uphill transport of a solute is driven by the release of chemical energy from the hydrolyzation of ATP or pyrophosphate (PPi), by redox reactions (respiratory chain) or by light energy (photosynthetic apparatus). Secondly, the uphill transport of a solute is coupled to the down hill transport of an other solute, and finally, charged molecules move as a result of membrane potential against their concentration gradient. The first type of transport is called primary active transport, the second is a form of secondary active transport and the third is passive transport down the electro-chemical gradient (but up the chemical gradient).
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Primary active transport

While the respiratory chain and the photosynthetic apparatus are special cases, solute transport is usually energized by the hydrolysis of ATP. Four transport systems exist which mediate primary active transport of solutes by ATP hydrolysis: P-type ATPases, V-type ATPases, \( F_0F_1 \)-ATPase and ABC-transporters. Important in plant cell growth are the three primary transporters located in the plasma membrane and tonoplast. These are the plasma membrane (PM) \( \text{H}^+\)-ATPase, the tonoplast V-type \( \text{H}^+\)-ATPase and the tonoplast \( \text{H}^+\)-pyrophosphatase (PPase). Because these primary transporters generate the proton-motive force across the plasma membrane and tonoplast, they play an important role in the growth of cells. In that context their activity is regulated by for instance growth controlling plant hormones like auxins (Kitamura et al., 1997).

The PM \( \text{H}^+\)-ATPase

The PM \( \text{H}^+\)-ATPase has two roles in the plant cell: firstly it plays a role in maintenance of the cytosolic pH and secondly it generates the proton-motive force across the PM which is used for the uptake of other solutes. The PM \( \text{H}^+\)-ATPase is a single-subunit protein and belongs to the P-type ATPases that extrudes \( \text{H}^+ \) from the cell. This proton pump is able to generate membrane potentials ranging from -120 to -160 mV (negative inside) and a pH gradient of 1.5 to 3 units (acid outside). The membrane potential and the pH gradient form the proton-motive force which enables the uptake of other solutes (Sze et al., 1999).

The Tonoplast \( \text{H}^+\)-ATPase and Tonoplast \( \text{H}^+\)-pyrophosphatase

The tonoplast \( \text{H}^+\)-ATPase is a V-type ATPase and, as such, a multimeric complex encoded by at least 26 genes (Strompen et al., 2004). The tonoplast \( \text{H}^+\)-pumping pyrophosphatase (\( \text{H}^+\)-PPase) is single subunit proteins. Both these primary transporters pump \( \text{H}^+ \) into the vacuole. This action results in a pH gradient and a membrane potential across the tonoplast. The pH of the vacuole is usually in the range 3-6 while membrane potentials up to 60 mV have been measured (positive in the vacuole). These two primary pumps generated a proton motive force across the tonoplast, which is used for the accumulation of solutes into the vacuole. These solutes can be waste or toxic compounds (\( \text{Na}^+ \)), however, the majority of these solutes are accumulated in order to generate a low water potential necessary for water uptake,
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turgor and growth. V-type ATPases have also been found in the endoplasmic reticulum and trans-Golgi network. (Chanson and Taiz., 1985; Strompen et al., 2005 and Dettmer et al., 2005 and 2006), where they play a role in directing the transport vesicles to their destination.

*Secondary active transport*

Secondary active transporters or co-transporters couple the uphill transport of solutes to a downhill transport of another solute. In plants energy is stored in the proton motive force (PMF) generated by the three major primary proton pumps and most secondary active transporters use this PMF by coupling the transport of their solute to the downhill transport of H⁺. Two distinct types can be distinguished. First of all there are the symporters, where the transport of the solute is in the same direction as the co-transported H⁺. The second type are antiporters in which the direction of the substrate is opposite to the transport of the H⁺.

In the large family of CIC membrane proteins, transmembrane movement of Cl⁻ and NO₃⁻ is facilitated by an antiporter mechanism in which a H⁺ is transported in the opposite direction (Accardi and Miller 2004; Scheel et al. 2005). A recent electrophysiological and molecular study demonstrated that CIC homologues are antiporters in the vacuole of *Arabidopsis* that, through NO₃⁻/H⁺ exchange, concentrate NO₃⁻ in a plant vacuole (De Angeli et al 2006).

*Anion transporters*

*Chloride channel proteins*

An important group of anion transporters in plants is the chloride channel (CIC) family. Since the cloning of first member of the CIC family from the *Torpedo* electric organ (CIC-0), these transporter-proteins have been identified in almost all organisms (Gurnett et al., 1995; Klock et al., 1994). In mammals, CIC proteins form a family of at least 9 different genes, which can be classified in three subfamilies (Jentsch et al 2005). While more and more individual *CIC* genes have been identified recently, a nice synopsis of the presence of this gene family in plants can be obtained from the complete genome sequencing projects. In the *Arabidopsis* genome 7 *CIC* genes are present (Hechenberger et al 1996). In plants, CIC proteins participate in various physiological functions, such as, osmoregulation, stomatal movement, cell signaling,
nutritent uptake and metal tolerance (Barbier-Brygoo et al., 2000). Like for all other organisms, the discussion concerning the real substrates of the ClC proteins in plants is still continuing. Proteins are designated a chloride channel (CIC) based on the fact that the cDNA from the prime example, CIC-0, isolated from Torpedo, gave currents typical for the Torpedo electric organ chloride channel in Xenopus oocytes (Hirono 1987; Gundersen 1984). However, during the last years a dualistic character of these proteins has surfaced. Some members of this family are indeed functional Cl⁻ channels, but recently evidence has come forward showing that other members of this family mediate fluxes of NO₃⁻ and, even more surprising, in some cases the transport the anions is coupled to a proton counterflux, which changes the nature of the channel into that of an antiporter.

**Structural organisation of ClC transporters**

The *Escherichia coli* EcClC and *Salmonella typhimurium* StClC proteins were the first CIC proteins to be crystallized and provided the second structure of a transmembrane channel protein (Dutzler et al., 2002; Dutzler et al., 2003). These studies revealed that the members of the CIC family share a conserved structural organization, consisting of a transmembrane channel domain and in many cases of cytoplasmic regulatory domains, like the two cystathionine-β-synthetase domains (CBS1 and CBS2) at the carboxyl end (see above). EcClC crystallizes, and probably functions, as a homodimer with each subunit containing an independent ion translocation pore. The subunits exhibit an ‘antiparallel architecture’: one subunit contains two structurally related halves spanning the membrane with opposite orientations (Dutzler 2006; Dutzler et al., 2002, 2003). This topology shows similarity to other transporter proteins, namely the presence of broken α-helices and partly inserted α-helices and the anti-parallel architecture. A common topology of CIC proteins has been presented in Dutzler (2006), in which 18 α-helices are recognized,
<table>
<thead>
<tr>
<th>Protein</th>
<th>TIAR code</th>
<th>Presence of structural element</th>
<th>Predicted Function</th>
<th>Confirmed function by</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtClCa</td>
<td>AT5G40890.2</td>
<td>P + + + +</td>
<td>H⁺/NO₃⁻</td>
<td>De Angeli et al., 2006</td>
</tr>
<tr>
<td>AtClCb</td>
<td>AT3G27170.1</td>
<td>P + + + +</td>
<td>H⁺/NO₃⁻</td>
<td>Gaxiola et al., 1998; Lv et al., 2009</td>
</tr>
<tr>
<td>AtClCc</td>
<td>AT5G49890.1</td>
<td>S + + + +</td>
<td>H⁺/Cl⁻</td>
<td>Gaxiola et al., 1998; Hechenberger et al., 1996; Lv et al., 2009</td>
</tr>
<tr>
<td>AtClCd</td>
<td>AT5G26240.1</td>
<td>S + + + +</td>
<td>H⁺/Cl⁻</td>
<td>Gaxiola et al., 1998; Hechenberger et al., 1996; Lv et al., 2009</td>
</tr>
<tr>
<td>AtClCe</td>
<td>AT4G35440.1</td>
<td>-</td>
<td>K to P</td>
<td>A⁻</td>
</tr>
<tr>
<td>AtClCf</td>
<td>AT1G55620.1</td>
<td>-</td>
<td>K to P</td>
<td>A⁻</td>
</tr>
<tr>
<td>AtClCg</td>
<td>AT5G33280.1</td>
<td>S</td>
<td>E to A</td>
<td>A⁻, no pH gating</td>
</tr>
</tbody>
</table>

Table 1: Structural characteristics of the *Arabidopsis thaliana* ClC proteins and their predicted function, based upon these characteristics. a: The presence of a proline or a serine at position 2 of the motif is indicated by a “P” or an “S”, respectively. The absence of the motif in the protein is indicated by “-“. b: The presence of the exact motif is indicated by “+”, if the motif is present in a modified form the change is indicated by the one letter amino acid code. c: The presence or the absence of the tyrosine is indicated by “+” or “-“, respectively.
of which 17 are fully or partly inserted into the membrane. If this structure can be also applied to the plant CIC proteins, remains to be seen. At least AtClCa,b,c,d, and g, which show a high homology with EcCIC and contain clearly the conserved functional domains (GP/SGIP and GK/REPG), might show this topology (Table 1). AtClCe and f have a lesser homology with the archetype and for instance lack the GP/SGIP motif (Table 1) and might therefore differ structurally. If plant CIC proteins function as homodimers, like their bacterial counterparts, also remains to be determined. Most bacteria contain only one CIC gene, whereas for instance Arabidopsis contains seven. If some of these plant CIC proteins are targeted to the same membrane the formation of heterodimers is a possibility.

The CIC transporter family is an interesting group of transporters, as the overall structural organization of these proteins allow the members either to function as a channel or a transporter or even as both. This is not an oddity, but a universal property of possibly all eukaryotic CLC members (Dutzler, 2006, 2007). Therefore, the molecular architecture of the protein should be able to support both modes of transport. In the structure of EcCIC, but also present in the amino acid sequences of the Arabidopsis CIC proteins, several essential motives and amino acids have been recognized. In the crystal structures of the CIC proteins three Cl⁻ binding sites were recognized (Dutzler et al., 2002; 2003). The first one is, together with other elements, created by the 564Tyr residue (numbering for AtClCa) and the serine residue of the motif GSGIP (Figure 2). This site is referred to as the central binding site (Scen). The internal (close to the cytoplasm) binding site (Sint) is formed by main-chain amide nitrogen atoms of less conserved amino acid residues (Figure 2). The third binding site (Sext; Figure 2), which was only recognized after changing glutamate148 (counting in E.coli) to alanine is formed by residues from conserved motifs GK/REPG and GXFXP (Dutzler et al., 2000). Together these three sites in the channel protein form the path along which the Cl⁻ ions travel according their electrochemical potential.

Recently an important observation was made in relations to the NO₃⁻ versus Cl⁻ specificity of the transporters. The Arabidopsis AtClCa protein which is a NO₃⁻ transporter in which the transport of 2 nitrates into the vacuole is tightly coupled to movement of a proton in the opposite direction, contains, instead of the serine in the GSGIP motif, a proline. Mutating AtClCa (P to S) and the mammalian ClC-5 (S to P) at this position, showed the importance of these residues in substrate specificity. In
Figure 2a. The gating mechanism of CLC proteins which function as channels (see text)
The cartoon displays one monomer. A: Open conformation. B: Closed conformation. C− and CH: deprotonated and protonated carboxyl group, respectively, of the gating glutamate (see text). S_ext, S_int, and S_int indicate the three anion binding sites. The respective elements forming the binding sites are indicated in the open configuration. S_int is formed by main-chain amide nitrogen atoms.

Figure 2b. Model of the transport mechanism of CIC proteins which function as 2A+/H+ antiporters
The cartoon displays one monomer cycling through the different conformations. C− and CH: deprotonated and protonated carboxyl group, respectively, of the gating glutamate (see text). E− and EH: deprotonated and protonated gating glutamate (see text). The transport cycle: Step 1: All three binding site become occupied by an anion (in this case a Cl). The gating glutamate is protonated and in the open conformation. The proton-donating becomes protonated (can also take place at step 2). Step 2: The
gating glutamate deprotonates and “pushes” the anions through the channel. Two anions leave the channel. Step 3: The channel becomes blocked between $S_{\text{int}}$ and $S_{\text{cen}}$ (see figure 3) which prevents back flow of anions. A proton is transferred from the proton-donating glutamate to the gating glutamate and the gates opens and the system returns at step 1.

AtClCa, the P to S mutation resulted in a $\text{Cl}^-/\text{H}^+$ exchange comparable to $\text{NO}_3^-/\text{H}^+$ exchange, while in the wild type protein $\text{Cl}^-$ transport is negligible. The opposite change in the mammalian CIC-5 protein, which normally transports $\text{Cl}^-$ tightly coupled to $\text{H}^+$ and $\text{NO}_3^-$ almost uncoupled, resulted in a coupled $\text{NO}_3^-$ transport (Bergsdorf et al., 2009). Table 1 shows the distribution of the GS/PGIP variation over the Arabidopsis CLC proteins.

Two other important residues are the glutamates at positions 203 and 270 (numbering in AtClCa). Glutamate203 is part of the motif GK/REPG and is highly conserved in the CIC proteins. In \textit{Arabidopsis} only AtClCg has an alanine at this position (GKAPG), the other 6 contain this glutamate. In the first structures of the CIC proteins only two binding sites for chloride were recognized ($S_{\text{int}}$ and $S_{\text{cen}}$) because of their occupation by chloride ions. The third binding site ($S_{\text{ext}}$) was only recognized after the respective glutamate in EcCIC was mutated, resulting in an additional halogen anion in the crystal structure (Dutzler et al., 2003). As a consequence, the gating mechanism of the CIC channels is assumed to be mediated by this glutamate, which under the proper conditions (pH) mimics a chloride anion and binds in the $S_{\text{ext}}$ binding site and closes the channel. The change to an alanine results in a channel, which can not be closed (Dutzler et al. 2003; Dutzler, 2006; 2007; Jian et al., 2004). Recently, also a role of this glutamate in the functioning of the CIC transporters has been observed. In AtClCa, which in \textit{Xenopus} shows $\text{NO}_3^-/\text{H}^+$ exchange and to a lesser extend $\text{Cl}^-/\text{H}^+$ exchange, mutating $^{203}\text{Glu}$ results in uncoupled anion conductances, indicating a role of this glutamate in the coupling of the transport of protons to the anions (Bergsdorf et al., 2009). This effect of this amino acid change was also observed in other CIC transporters (Accardi and Miller., 2004; Zdebik et al., 2008).

Changing the $^{270}\text{Glu}$ to an alanine completely abolished the anion currents mediated by AtClCa in \textit{Xenopus}-oocytes. However, currents could be restored by the uncoupling Glu203Ala mutation (Bergsdorf et al., 2009). The idea is that $^{270}\text{Glu}$, which is located at the cytoplasmatic site of the membrane, binds protons and hands
them over to the gating $^{203}$Glu, which results in the coupling of the anion flux to the proton flux (Accardi et al., 2005; Dutzler, 2007; Lim and Miller, 2009; Zdebik et al., 2008).

Based on the structural information, given above, predictions can be made about the function of the *Arabidopsis* CIC proteins (Table 1). The model described above can be applied to AtClCa, b, c, d and g resulting in AtClCa and b being $\text{NO}_3^-$ transporters and AtClCc and d being $\text{Cl}^-$ transporters. The absence of the equivalent glutamate residue of $^{203}$Glu in AtClCg suggests this might be a channel. However, its anion preference is difficult to deduce. AtClCa has been shown to function as a $\text{H}^+/\text{NO}_3^-$ (De Angeli et al., 2006). AtClCc and d are able to complement the chloride transporting CIC protein in yeast, GEF1. AtClCa was not able to do so. Those observations in yeast are in agreement with the role of these proteins as chloride transporter or nitrate transporter, respectively (Gaziola et al., 1998; Hechenberger et al., 1996). AtClCe and f, on the other hand, are more difficult to label. They show the lowest homology with EcCIC and the other *Arabidopsis* CIC proteins. They even lack some critical residues (for instance the $^564\text{Tyr}$) and motifs (GS/PGIP), hence the function and role of these two CIC proteins based on their sequence is difficult to predict.

Figure 1 shows the phylogenic tree containing a considerable set of CIC proteins from all the major kingdoms and the *Arabidopsis* proteins. As can be observed, 5 of the *Arabidopsis* proteins form their own branch. Only AtClCe and f mingle with CIC proteins from other kingdoms and more particularly with those from cyanobacteria. This suggests that these CIC proteins are more related to cyanobacterial proteins which could be explained by the cyanobacterial origine of the chloroplast and, this indicates that AtClCe and f are located in the chloroplast.

Calculating a phylogenic tree of a large set of plant CLC proteins resulted in another picture (Figure 3). In this situation the CIC proteins were organized according to their characteristics as also used in Table 1. First a large branch could be split off in which the GS/PGIP motif is absent and a modified GKEGP motif was present (the lysine was replace by a proline, hence: GPEGP). In this group the proton-donating glutamate is also absent. This branch contains both AtClCe and f and in combination with the location of these two *Arabidopsis* proteins in figure 1 this suggests that the other plant CIC proteins of this branch are also anion channels which function in chloroplasts or mitochondria. The absence of the GS/PGIP motif has until now not
Figure 3. Neighbor-Joining Consensus tree of plant ClC proteins

The tree was calculated using the Geneious program. Branches are grouped according the presence of elements in the sequence: GP/SGIP, GKEGP, GXFXP, “glu” indicates the proton-donating glutamate (see text). When the respective element has been struck through this element can not be detected in the protein sequences of the group. Bold and underlined residues indicate differences between this motif with the other groups or the consensus.

*Arabidopsis thaliana*: AtClCa: CAA96057.1; AtClCb: CAA96058.1; AtClCc: CAA96059.1; AtClCd: P92943.2; AtClCe: AAK53390.1; AtClCf: AAK53391.1; AtClCg: P60300.1; *Glycine max*: GmClC1: AY43007.1; *Medicago truncatula*: MtClCx1: ABE91957.1; *Nicotiana tabacum*: NtClC1: CAA64829.1; NtClC2: AAD29679.1; *Oryza sativa*: OsClC1: BAB97267.1; OsClC2: BAB97268.1; OsClCx3: NP_001047143.1; OsClCx4: NP_001047955.1; OsClCx5: NP_001066692.1; OsClCx7: NP_001054061.1; *Physcomitrella patens*: PpClCx1: EDQ80065.1; PpClCx2: EDQ78881.1; PpClCx3: EDQ52731.1; PpClCx4: EDQ64061.1; PpClCx5: EDQ63773.1; *Populus trichocarpa*: PtClCx1: EEE85399.1; PtClCx2: EEE77376.1; PtClCx3: EEF09978.1; PtClCx4: EEF01954.1; PtClCx5: EEF10085.1; PtClCx6: EEF96668.1; PtClCx7: EEE84906.1; *Ricinus communis*: RcClCx1: EEF34561.1; RcClCx2: EEF47977.1; RcClCx3: EEF31629.1; RcClCx4: EEF33157.1; RcClCx5: EEF50918.1; RcClCx6: EEF45376.1; *Solanium lycopersicum*: SlClCx1: CAC36403.1; *Solanium tuberosum*: StClCx1: CAA71369.1; *Vitis vinifera*: VvClCx1: CA047567.1; VvClCx2: CA071138.1; VvClCx3: CA067080.1; VvClCx4: CA066848.1; VvClCx5: CAO48998.1; VvClCx6: CAO69292.1; VvClCx7: CAO46902.1; *Zea mays*: ZmClCx1: ACN33881.1; ZmClCx2: AAP04392.2

been implicated with a characteristic of these ClC proteins. It is not known whether this affects ion-specificity or other transport characteristics. The effect of replacing the lysine next to the gating glutamate with a proline also is unknown, although an
effect on the pKa of the glutamate can be expected and, thus maybe on the
transporters pH-dependence. Hence, these proteins are probably pH-sensitive anion
channels.

A second group, which includes AtClCc, is characterized by the presence of the
two motifs GSGIP and GKEGP and the presence of the proton-donating glutamate.
These proteins are therefore probably H⁺/Cl⁻ exchangers.

AtClCg is a member of a third group, which is typified by the presence of the
motifs GSGIP and GKAGP. Also the proton-donating glutamate is present in this
branch. As shown in Bergsdorf et al (2009) the engineered combination of the
presence of the proton-donating glutamate and the absence of the gating glutamate in
AtClCa resulted in an uncoupled Cl⁻ and NO₃⁻ conductance. Consequently, this branch
probably represents genuine anion channels. However, AtClCa is a H⁺/NO₃⁻
exchanger, based on the presence of the motif GPGIP which changes to an anion
channel with a higher conductance for Cl⁻ than for NO₃⁻ when the proline is changed
to a serine. How the serine in the GSGIP motif in the branch of AtClCg affects the
characteristics of these ClC proteins is unknown.

The final branch, which can be distinguished, is a branch representing H⁺/NO₃⁻
exchangers. The proteins in this branch contain the GPGIP and GKEGP motifs and
the proton-donating glutamate, which are all features in accordance with a H⁺/NO₃⁻
exchanger.

Moreover, if one considers the few plant species of which a (almost) complete
genome is available, Vitis vinifera, Oryza sativa, Arabidopsis thaliana and Populus,
all branches of the tree contain at least one protein of these species. This suggests an
early diversification of the CIC proteins in plants and a low redundancy of function
between the members of the different branches.

**Tissue and intracellular localization**

An important indication for the function of proteins is their functional localization.
In this respect both tissue and intracellular localization are important. Those two
levels of localization are mainly regulated in two different ways. Whereas tissue-
specific expression and developmental stage-specific expression are controlled at the
gene level, intracellular localization is controlled by sorting peptides present in the
protein. However, the nature of the translation product (different splice forms or
alternative translation initiation), controlled at gene or RNA level, can also affect
intracellular localization (Millar et al., 2009). Tissue expression is studied by gene-expression studies or by promoter fusions. Lv et al., (2009) made a thorough analysis of tissue-specific \textit{AtClC}-gene expression by RT-PCR and promoter-driven GUS expression. In their RT-PCR experiments ubiquitous expression of all \textit{CIC} genes throughout the plant was observed with only small variations in the level of expression amongst the tissues. Such an expression profile suggests that the various \textit{ClC} proteins have distinct individual functions and roles and have little or no redundancy. Interesting in this context, are the more or less inverse expression profiles of \textit{AtClCe} and \textit{f}. While \textit{AtClCe} is more expressed in leaf, flower and silique, \textit{AtClCf} is more expressed in root and stem. As suggested above these two proteins are probably functioning in either the chloroplast or mitochondrion.

The histochemical study of Lv et al. (2009) also demonstrated that \textit{CIC} members have individual functions. Their expression patterns overlapped, but they had also their differences. The largest differences were observed between \textit{AtClCa, b, c, d} and \textit{g}, on the one hand, and \textit{AtClCe} and \textit{f} on the other. The temporal and spatial distribution of \textit{AtClCe} and \textit{f} suggest a relation with the presence of functional chloroplasts. No evident expression was observed for these genes in the root. Moreover, it has been shown that photosynthesis is disturbed in mutants of \textit{AtClCe} (Marmagne et al., 2007).

Another important issue is the subcellular localization of the \textit{CIC} proteins. Predictions can be made using the Aramemnon web-based prediction tool (Table 2) but recently several studies using fusions of the \textit{AtClC} proteins with fluorescent passenger proteins (FP) like Green Fluorescent Protein derivatives or \textit{Discosoma sp. Red} (DsRed) has been used (De Angeli et al., 2006; Fecht-Bartenbach et al., 2007; Lv et al., 2009; Marmage et al., 2007) (Table 2). Also, a few \textit{ClC} proteins of \textit{Glycine max} and \textit{Oryza sativa} have been localized using fusions to fluorescent markers (Li et al., 2006; Nakamura et al., 2006). However, as Moore and Murphy (2009) state: “Determining protein localization inevitably is an exercise in imperfection.” They (Moore and Murphy, 2009) and Millar et al. (2009) discuss the state of the art of intracellular protein localization, summarize the strengths and weaknesses of the employed protocols and give guidelines for validation of the localization of proteins. Amongst the issues they raise are the use of strong, heterologous promoters to generate aesthetically pleasing images and the positioning of the fluorescent passenger proteins in an construct. Another important feature, which increases the risk
<table>
<thead>
<tr>
<th>Protein</th>
<th>Aramemnon prediction</th>
<th>Experimentally determined</th>
<th>Promoter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location FP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference</th>
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<tr>
<td>AtClCa</td>
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<td>tonoplast</td>
<td>35S</td>
<td>C</td>
<td>De Angeli et al., 2006</td>
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<td>AtClCb</td>
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<td>C</td>
<td>Lv et al., 2009</td>
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<tr>
<td>AtClCc</td>
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<td>C</td>
<td>Lv et al., 2009</td>
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<tr>
<td>AtClCd</td>
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<td>AtClCf</td>
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<td>AtClCg</td>
<td>weakly secretory pathway</td>
<td>tonoplast</td>
<td>35S</td>
<td>C</td>
<td>Lv et al., 2009</td>
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**Table 2**: Predicted and experimentally determined localization of the *Arabidopsis thaliana* ClC proteins

<sup>a</sup>: promoter of the fusion protein of the ClC and fluorescent protein, 35S: Cauliflower Mosaic Virus 35S promoter;  
<sup>b</sup>: location of the fluorescent protein, C: C-terminal.
of creating artifacts, is the routing of most proteins through various compartments before they reach their destination. This movement requires saturable transport and signaling systems, which can result in missorting (Moore and Murphy, 2009), especially in the case of over-expression. Moreover, during trafficking the various stations passed could have different amounts of the trafficking proteins. As a result higher protein amounts can be present at the intermediate stations and the fluorescence at these locations could outshine the fluorescence of the protein at the final destination. Even alternative locations, like the tonoplast or the plasma membrane could be reached due to congestion of the original route (Moore and Murphy, 2009). Hence, it can be asserted that in the case of membrane proteins, like CIC proteins, these artifacts are probable to occur. Membrane proteins have a lower degree of freedom and have to traffic via membranes.

If we consider the guidelines for validation of the location of proteins as suggested by Millar et al. (2009), the experiments performed in order to determine the location of the CIC proteins are not optimal. Some of the major concerns are: 1) all studies use the Cauliflower Mosaic Virus 35S promoter, which results in an uncharacteristically high expression, presenting for membrane proteins an even larger ‘congestion’ problem, 2) in those studies the fluorescent passenger protein is attached to only one location in the protein. In the studies with the Arabidopsis, Glycine CIC proteins the FPs were all fused to the C-terminus of the proteins (De Angeli et al., 2006; Fecht-Bartenbach et al., 2007; Li et al., 2006; Lv et al., 2009; Marmage et al., 2007). In the studies with the Oryza proteins the FPs were fused to the N-terminus (Nakamura et al., 2006). Although the results are in agreement with the ideas of the function of the CIC protein, this means care must still be taken with the interpretation of the recent fluorescence data on the localization of the CIC proteins.

For example, the Glycine max CIC1 protein was placed in the tonoplast because of its co-localization with GmNHX1 (Li et al., 2006). NHX1 is an established tonoplast protein. However, in this study both proteins were visualized by the use of the strong Cauliflower Mosaic Virus 35S promoter. Although both proteins display a similar localization, we are doubtful about the result that shows a tonoplast localization. Apparently, both proteins accumulate in endomembrane vesicles which either outshine the proteins in the tonoplast, or are the result of congestion of the transport systems. Lurin et al. (2000) studied the localization of NtCIC1 using fractionation and Western-blotting and concluded that this protein localizes to mitochondria. The
closest homologue of NtClC1 in *Arabidopsis* is AtClCc, which is experimentally located in the tonoplast (Table 2). In spinach a CIC protein was found in the outer envelope of chloroplast by mass-spectrometry and membrane fractionation (Teardo et al., 2005). This spinach protein gave three peptides that had sequences identical to the partial sequences of the AtClCf protein. However, the AtClCf protein is experimentally located in the Golgi membrane, but predicted to be targeted to the mitochondria (Table 2).

**Differences between patch-clamp and molecular studies**

Electro-physiological studies have been performed on plants for at least 60 years. In the early years only membrane potentials could be measured by impalement of electrodes into cells and tissues. This is a technique that only allows a general study of the behavior of the membrane potential of plant cells upon varying conditions. Presently the high-resolution electro-physiological method, the patch-clamp technique, allows the study of single conductances in membranes. However, both patch clamp and the impalement of electrodes are invasive techniques, which require isolation of cells or protoplasts or wounding of the tissue. Especially, the patch clamp technique revealed an enormous number of ion-conductances present in plasma membrane and tonoplast. However, the matching of conductances with proteins and their corresponding genes is a laborious process. Forward genetics appears difficult, starting from a current and trying to find a protein, which is responsible for the current. However, reverse genetics has proved useful in identifying the transporter proteins, responsible for the conductances observed by patch clamp. A nice example of such a study is the characterization of the AtClCa protein in the tonoplast of *Arabidopsis thaliana* (De Angeli et al., 2006). In this study it is shown that in two independent knock-out plant lines, in which AtClCa is absent, a certain nitrate current could no longer be observed in the tonoplast, indisputably matching the nitrate conductance to the AtClCa protein. Recently, a new electrophysiological technique has been developed. The Micro-Electrode Ion Flux Estimation (MIFE) technique allows the noninvasive and simultaneous monitoring of different ion fluxes from intact tissues with a high spatial and temporal resolution (Shabala et al., 1997; Newman, 2001; Tegg et al., 2005; Vreeburg et al., 2005; Lanfermeijer et al., 2008). Without damaging the tissue this technique is able to detect changes in fluxes of
various ions by the use of ion-specific electrodes. However, no studies with this technique on CIC proteins are known to us.

**The physiology of anions**

The most abundant anions in plants are nitrate, chloride, sulfate, phosphate and malate. Carbonate, despite its low concentration, compared with other inorganic anions, occupies a particular status, as it plays a role in intracellular pH regulation and is the major carbon input for photosynthesis (Barbier et al., 2000). Most anions have important metabolic functions and most can be accumulated in the vacuole. In plant cells relative concentrations of anions vary, depending on the tissue and physiological and environmental parameters. In plant cells, the highest anion concentrations are found in the vacuole, while cytosolic levels are maintained in the millimolar range. Of these, the inorganic ions have to be taken up from the environment. In higher plants the root system is responsible for the uptake of nutrients and, thus, most inorganic anions. Subsequently, the anions (and their counter cations) are transported to the shoot by the transpiration stream. Although, most of the transport by the transpiration stream can be apoplastic and does not need passage of membranes, at least at the Casparian strips in the roots the ions have to enter the symplast. Hence, they have to pass the plasma membrane at least twice. Anion channels have been reported in the xylem parenchyma cells of barley roots (Wegner and Raschke., 1994; Kohler and Raschke. 2000; Kohler et al. 2002), root stellar cells of maize (Gilliham and Tester. 2005) and Arabidopsis root pericycle cells (Kiegle et al. 2000). Kohler and Paschke (2000) identified fast and slow activating anion channels in barley xylem parenchyma cells. After the anions have arrived at the sink tissue (growing leaves, fruits, etc) they have to enter cells. It is hypothesized that the influx of chloride occurs via H⁺/anion symporters or OH⁻/anion antiporters (Zeiger et al., 1978).

**Anion fluxes in the plant cells**

The different cell compartments require all their specific concentrations of metabolites and minerals. These concentrations are all maintained by transport systems, which are energized by ion-gradients and potential differences, generated by the primary pumps. Anion fluxes play an important role in these processes. First of all
in the generation of the proton motive force. They relieve the membrane potential generated as a consequence of the transmembrane transport of protons. When for each proton moved an anion is transported in the same direction the membrane potential does not rise so drastically. This allows more protons to be transported and the proton gradient to become larger than in the absence of anion fluxes. Although the driving force on protons is thereby reduced, the power for transport of solutes coupled to protons is increased. This is the so called shunt-function of anion fluxes.

The second function of anion fluxes is related to the role of anions as osmotically active solutes. The accumulation of anions and their counter ions in cells drives the uptake of water into the cells and, subsequently, the generation of cell turgor. Cell turgor, in its turn drive cell expansion and cell growth.

Their role in water movement and turgor regulation makes anion fluxes also important in the opening and closing of stomata. Stomata are microscopic pores in the aerial parts of the plant, which provide a passageway for CO₂, that is needed for photosynthesis, to enter the leaf. Guard cells surround the pore and the swelling and shrinking of these cells modulate stomatal pore size by coordinating responses to environmental and physiological factors, including light, temperature, Ca²⁺, and the plant hormone abscisic acid. During stomatal opening and closing, chloride and malate are the major anionic species involved in turgor generation for opening. It has been known for several decades that guard cells can take up chloride ions during stomatal opening, but the molecular mechanism of that is still not fully understood.

**The role of pH in plant cell growth**

Many different physiological events in plant cells are regulated by changes in pH or depend on proton gradients. In plant cells pH is well characterized as a regulator of processes, such as modulation of Ca²⁺ signaling, protein synthesis, and enzyme activity. In plant cells, according to the plant species and the technique of measurement used, cytosolic pH values in resting conditions are between 6.8-7.9 (Guern, 1991). In response to osmotic stress and hormone treatment, the cytosolic and apoplastic pH both fluctuate (Guern., 1991; Tretyn et al., 1991; Nuhse et al., 2000). In plant cells, the PM H⁺-ATPase is the primary active transport system and mainly responsible for generating the membrane potential and the proton gradient and maintaining the cytosolic pH (Assman and Haubrick., 1996). It is also known that changes in cytosolic pH can act as a second messenger in plant cells (For review, see
Felle, 1989; Guern et al., 1992; Zimmermann et al., 1999). According to the acid growth theory (Rayle et al., 1970; Cleland, 1971; Hager et al., 1971), low pH induces rapid cell wall loosening and cell elongation. In pea leaves the increased extrusion of protons by the activated PM H^+-ATPase results in the enlargement of the pH gradient and the hyperpolarization of membrane potential across the plasma membrane, which result in an increase of the proton-motive force. This stimulates the uptake of nutrients and osmotically active solutes. Subsequently, water is also absorbed as a result of osmosis and cell turgor increases (Staal et al., 1994). The extracellularly located expansins react to the acidification of the cell wall by activation of their cellulose and hemicellulose degrading properties (Cosgrove, 1998), while Ca^{2+}-pectin cross-links are broken as a result of displacement of the Ca^{2+} by H^+ (Proseus & Boyer, 2006; den Os et al., 2007). Both these reactions increase the cell wall elasticity. The increases in turgor and cell wall elasticity result in cell expansion. But also in roots the elongation is regulated by acid growth phenomena (Edwards and Scott., 1974; Buntemeyer et al, 1998; Peters and felle, 1999). In Arabidopsis root, changes in root cap pH are required for the gravi tropism (Fasano et al 2001).

Anion and cation channels play an important role in this growth process. The movement of cations in the opposite direction or an anions in the same direction as the proton flux, can aid in the generation of the proton gradient (the shunt function; see above) and increase the extracellular acidification. Changes in the activity of ion channels can therefore result in changes in the membrane potential and in the pH gradient (Johannes et al., 1998) and therefore the ability of cells to grow. Secondly, anions are used as osmotics and a change in the transport capacity of these solutes can affect growth. The importance of anion fluxes is demonstrated by the fact that in AtClCd knock out mutants root growth is reduced compared to wildtype at a slightly alkaline pH of the growth medium (Fecht-Bartenbach et al).

**Perspectives**

Although their importance in processes like pH homeostasis, growth, abiotic and biotic stress resistance, osmotic acclimation, nutrient uptake and transport has been amply demonstrated, the physiological characterization and the knowledge of the position of CIC proteins in the complex network of membrane transport and solute fluxes is still incomplete. Mutant analysis, combined with detailed physiological
studies can provide us with much of the data necessary to fill these gaps in our understanding. In this study we used knock-out mutants to elucidate the role of members of the AtCIC transporter family with the use of the MIFE technique. In this thesis the role of AtClCa and AtClCd in pH homeostasis and metal-tolerance has been demonstrated