Chapter 5

The role of AtClCa and AtClCd in heavy metal tolerance in

*Arabidopsis thaliana*

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ABSTRACT

In higher plant cells, anion channels play a role in acclimation of plant cells to abiotic and biotic environmental stresses, in the control of metabolism and in the maintenance of electrochemical gradient. A number of studies have demonstrated that anion channels are present in various cell types of plants. Seven genes encoding chloride channel (ClC) proteins have been identified in the Arabidopsis genome. To assess the function of ClCs in Arabidopsis, we obtained three single mutant plants (Atclca, Atclcb, and Atclcd) with an T-DNA in the respective genes and we made the three double and the triple mutant plants. One of the aims of our study was to elucidate the role of anion channels in heavy metal (i.e. cadmium) resistance and specifically their involvement in the alleviation of cadmium effects by calcium.

The primary root growth and the morphology of cells between the meristematic and elongation zone, were significantly affected by exposure to 60 μM Cd$^{2+}$ in all genotypes. Adding Ca$^{2+}$ to Cd$^{2+}$-exposed plants, restored primary root growth and the normal shape of cells, except in the double mutant Atclcad and the triple mutant Atclcabd. We propose that both AtClCa and AtClCd proteins play a role in the detoxification of cadmium by allowing efficient sequestration of the metal in the vacuole or in acidic intracellular vesicles. Plants that lack both transporter proteins, therefore have decreased cadmium resistance.
INTRODUCTION

Heavy metals are elements that have an atomic weight between approximately 63-200 Daltons. Over 50 elements have been classified as heavy metals, 17 of which are very toxic and relatively accessible. They are naturally occurring minerals that are found throughout our natural environment. Contamination of soil with heavy metal is a serious worldwide problem both for human health and agriculture (Gairola et al., 1992; Mazess and Barden, 1991 and Ryan et al., 1982). Metal toxicity interferes with cellular activity by several mechanisms: displacement of essential cations, induction of oxidative stress, and direct interaction with proteins. Cadmium is a toxic heavy metal that enters the environment, and also the food chain, through industrial processes and phosphate fertilizers (Pinto et al., 2004). In plants, cadmium is taken up easily by the roots of many plants species, where it can be loaded into the xylem and transported to the leaves. Cadmium has a 2-20 times higher toxicity than most of the other heavy metals (Jagodin et al., 1995). Cadmium toxicity is associated with growth inhibition and imbalances in many macro and micronutrient levels. Cadmium toxicity symptoms are more apparent in the root than the shoot, as the accumulation of Cd$^{2+}$ in the root is significantly higher than in the shoot (Breckle, 1991). In plants a low concentration (5-10 $\mu$M) of Cd$^{2+}$ reduces chlorophyll content and the photosynthetic yield in Brassica napus (Baryla et al., 2001; Larsson et al., 1998), displaces Ca$^{2+}$ in the photosystem II (Faller et al., 2005), is negatively affecting in respiration (Greger and Ogren, 1991; Reese and Roberts, 1985) and inhibits water transport (Barcelo and Poschenrieder, 1990). Cadmium inhibits almost all enzymes of the Calvin cycle in pigeon pea and wheat plants (Sheoran et al., 1990; Malik et al., 1992). Cadmium also induces the generation of reactive oxygen species (ROS), resulting in the unspecific oxidation of proteins and membrane lipids and DNA damage (Dean et al., 1993; Ames et al., 1993), inhibits germination (Sarath et al., 2007) and suppresses root cell elongation (Stohs and Bagchi, 1995; Schutzendubel et al., 2001). Uptake studies suggest that transport of Cd$^{2+}$ into the cytoplasm and vacuole might depend on both active and passive transport systems (Costa and Morel, 1993; Costa and Morel, 1994; Hall, 2002; Hart et al., 1998; Salt and Wagner, 1993).

Changes in the cytoplasmic calcium concentration are used by the cell as an almost universal second messenger system for many signals. Disturbance of calcium
homeostasis and displacement of calcium have been suggested as possible mechanisms of Cd$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, or Al$^{3+}$ toxicity (Kinraide et al., 2004). For instance, the vacuolar Ca$^{2+}$/H$^+$ antiporter CAX2 in Arabidopsis is able to transport Ca$^{2+}$, Cd$^{2+}$ and Mn$^{2+}$ (Hirschi et al., 2000). Cadmium competes with Ca$^{2+}$ at both the Ca$^{2+}$-channel (Nelson, 1986) and at intracellular Ca$^{2+}$ binding proteins (Rivetta et al., 1997). Exposure to cadmium resulted in a decrease of the calcium content in different plant species (Gussarson et al., 1996; Sandalio et al., 2001). This competition between calcium and cadmium seems to work both ways: increasing the external calcium concentration alleviates the effects of cadmium, an effect that is assumed to result from the competition for transporters between the two ions (Suzuki, 2005).

Anion channels are well documented in various tissues, cell types and membranes of animals, protists and plants and current evidence supports a central role in cell signaling, osmo-regulation, nutrient uptake and metal tolerance (Barbier-Bryggo et al., 2000). Seven ClC genes have been identified in the Arabidopsis genome. Subcellular localization is still largely putative, but AtClCa-b-c and g are assumed to function in the tonoplast, AtClCd and AtClCf are localized to the Golgi membrane and AtClCe is assumed to be targeted to the tylakoide membrane (De Angeli et al. 2007; Marmagne et al., 2007; Lv et al., 2009). Recently, the localization in the tonoplast of AtClCa and its role as a NO$_3^-$/H$^+$ antiporter was demonstrated (De Angeli et al., 2006).

The aim of this study was to determine the link between Cd$^{2+}$ toxicity on the one hand and Ca$^{2+}$ and ClC transporter-related heavy metal resistance on the other hand. We show that Cd$^{2+}$ at concentrations of 60 to 90 $\mu$M causes serious damage in the primary roots of all genotypes. Ca$^{2+}$ was able to alleviated Cd$^{2+}$ reduction of root growth in most genotypes, except in plants that were defective in both the AtClCa and AtClCd transporter.
MATERIALS AND METHODS

Plant material and culture conditions

Plant material and standard culturing conditions used are the similar to those described in Chapter 2. To determine Cd\(^{2+}\) toxicity, the role of Ca\(^{2+}\) and pH in this, plants were transferred to new plates with the same solid medium but supplemented with the appropriate concentrations of CdCl\(_2\), ZnCl\(_2\), PbCl\(_2\), BaCl\(_2\), MgCl\(_2\) or CaCl\(_2\). Also the pH of the Tris/Mes-buffered medium was adjusted when needed.

Screening for T-DNA insertion mutants

Selection for single, double and triple mutants has been described in Chapter 2.

Cell imaging in primary root

Imaging was performed using a Nikon Coolpix 990 digital camera mounted on an inverted optical microscope (CX41, Olympus, Tokyo, Japan) equipped with objectives of 20\(\times\) and 40\(\times\) magnification. After 15 days of growth (after sowing) on the vertically placed agar plate the primary root length and the distance between root tip and first epidermal cell with visible root hair bulge (DFEH) were measured using the microscope. For every experiment the average of the root length of at least 8 plants was calculated and every experiment was repeated twice.
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RESULTS

Reverse transcription PCR analyses

RT-PCR for *Atclc* single, double and triple mutant plants was carried out with gene specific primers as described in chapter 2 and the bands were compared with the tubulin product. RT-PCR confirmed the expression of *AtClCa*, *AtClCb* and *AtClCd* in root tissue. In all mutant genotypes the absence of transcripts confirmed that the T-DNA insertions all resulted in null alleles (Figure 1).

**Figure 1.** The absence of expression of the *AtClCa*, *AtClCb* and *AtClCd* genes in the double and triple mutant lines. The five genotypes were analysed using the primers shown in table 1 of chapter 2. The *Tubulin* transcript levels of the five genotypes are shown as a loading control.

**Cd**$^{2+}$, **Pb**$^{2+}$ and **Zn**$^{2+}$ inhibits primary root growth in *Atclc* mutant plants

Exposure of 7 days old seedling to 90 μM Cd$^{2+}$ resulted after 8 days of further growth in strongly reduced primary roots (Figure 2a). Exposure to 90 μM ZnCl$_2$ (Figure 3) or lower CdCl$_2$ (Figure 2a) concentrations only weakly inhibited primary and lateral root growth. In all treatments, root growth was not significantly different between single, double and triple mutant and wildtype plants.
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Figure 2. The inhibition of growth of the primary roots of the wildtype *Arabidopsis* and the 7 mutant genotypes by Cd²⁺ and the alleviation of the inhibition by Ca²⁺. a: Concentration dependence of Cd²⁺ inhibition in absence and the presence of 30 mM CaCl₂. b: Ca²⁺ concentration dependence of the alleviation of the inhibition of root growth by 90 μM Cd²⁺. Datapoints are the average of 3 experiments and the error bars indicate the standard deviation.

Alleviation of cadmium toxicity by Ca²⁺ is different between wildtype and Atclcad double mutants

Based on the results of Suzuki (2005), that increased external Ca²⁺ reduces the effects of Cd²⁺ we tested the effect of 30 mM CaCl₂ in plants exposed to toxic levels of cadmium. The application of CaCl₂ to Cd²⁺-stressed plants, restored the growth of the primary root in all genotypes, except in two. In the Atclcad double mutant and in the triple mutant Atclcadb the primary and lateral root was significantly shorter than in the other genotypes (Figure 2a). The effect of calcium is dose-dependent and with 90 μM Cd²⁺ the Kₘ for calcium is about 1 mM (Figure 2b). The restoration of root growth in plants exposed to cadmium seems to be a calcium-specific effect. Addition of BaCl₂, cholinchloride or MgCl₂ did not show any positive effect on the root growth of plants that were treated with 90 μM CdCl₂ (Figure 4).

Figure 3. Inhibition of root growth by Zinc and the absence of allevetation by Ca²⁺ in the wildtype *Arabidopsis* and the 4 multiple mutant genotypes. Datapoints are the average of 3 experiments and the error bars indicate the standard Deviation.
In animal cells a relation between Zn\(^{2+}\)-toxicity and anion channel function had been demonstrated (Duffield et al., 2005). To test whether a similar relation exists in plants, we studied the effects of Ca\(^{2+}\) on Zn\(^{2+}\)-treated wildtype and Atclc mutant plants. Figure 3 shows that, in contrast to Cd\(^{2+}\)-treated plants, calcium does not change the effect of zinc on root growth, nor does the genotype of the plant influence the extent of the inhibition.

**Figure 4.** Influence of different cations on the inhibition of root growth in the wildtype *Arabidopsis* and the 4 multiple mutant genotypes by Cd\(^{2+}\). The used concentrations of CaCl\(_2\), BaCl\(_2\), CholineCl and MgCl\(_2\) are 30 mM. Datapoints are the average of 3 experiments and the error bars indicate the standard deviation.

**Effects of Cd\(^{2+}\) on root morphology**

In plants exposed to cadmium the morphology and color of the root tip changes and the shape of the cells in the elongation zone is distorted (Suzuki, 2005). In our experiments, exposure to 90 μM Cd\(^{2+}\) also leads to deformation of the root tip and a change in morphology of the cells along the root rip. In cadmium-treated roots the diameter of the root tip is decreased and the color of the cells much darker (Figure 5). At lower concentrations of Cd\(^{2+}\) (30-60 μM) the diameter of the primary root also decreased and initiation of lateral root growth was still higher, but the shape and color of the cells in the root tip were not different from the controls. The experiment shown in figure 5 confirms that a high concentration of Cd\(^{2+}\) causes serious damage to the cells at the border between the elongation and meristematic zone.
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**Figure 5.** Phenotypes of the roots of the *Arabidopsis* wildtype and the four multiple mutants when grown on media with Cd$^{2+}$ or Cd$^{2+}$ and Ca$^{2+}$. The bar in the upper left photo indicates 0.5 millimeter.

These effects of cadmium poisoning can also be alleviated by increasing the external calcium concentration (Figure 5, right panels). In all genotypes the morphology of calcium-treated roots is not significantly different from the controls. For comparison we also exposed the plants to a high concentration of lead. 700 μM Pb$^{2+}$ does induce changes in the diameter and color of root, but does not affect the shape of the cells in the tip of the primary root cells in same way as 90 μM Cd$^{2+}$ does (data not shown).

**Relation between Cd$^{2+}$, Ca$^{2+}$ and pH condition in primary root growth**

In Chapter 4 we showed that root growth in the *Atleca* and *Atlecb* mutants is more sensitive to pH than in the wildtype plants. Since it is the combination of these same mutated genes in which Ca$^{2+}$ has a reduced capability to alleviate cadmium-toxicity we tested the effect of pH on cadmium toxicity in the different genotypes (Figures 6 and 7).
Figure 6. The pH dependence of Cd\(^{2+}\) inhibition of root growth and the pH dependence of Ca\(^{2+}\) alleviation of this inhibition in wildtype *Arabidopsis* and the four multiple mutants. a: pH of the growth medium was 5.8 b: pH of the growth medium was 6.2. The length of the reference in the pictures in 2.5 cm.

At pH 5.8 we obtained a result comparable with the data presented in figure 2. At the higher pH the mutations in both *AtClCa* and *AtClCd* genes resulted in a stronger pH-induced reduction of root growth. Curiously, at pH 6.2 the addition of 30 mM CaCl\(_2\) resulted in a complete reversal of the effect of Cd\(^{2+}\). At pH 6.2 no statistically significant difference was found between control, the addition of only Ca\(^{2+}\) and the
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addition of both Ca\(^{2+}\) and Cd\(^{2+}\). The difference between the genotypes was present also in the control situation, indicating that at pH 6.2 the effect of pH on root growth of the genotypes is dominating the response.

**Figure 7.** The pH dependence of Cd\(^{2+}\) inhibition of root growth and the pH dependence of Ca\(^{2+}\) alleviation of this inhibition in wildtype *Arabidopsis* and the four multiple mutants. Plants were grown in the presence of either 90 μM CdCl\(_2\), 30 mM CaCl\(_2\) or the combination of both salts. **a:** pH of the growth medium was 5.8 **b:** pH of the growth medium was 6.2
DISCUSSION

In order to understand the relationship between heavy metals and anion transporters in plant cells, we made single, double and triple anion channel mutants in *Arabidopsis thaliana* (Figure 1). With these genotypes we show, firstly, specific effects of cadmium on primary root growth and development, that cannot be mimicked by the other heavy metals tested (zinc and lead). Secondly, the effects of cadmium can be reversed by increasing the external calcium concentration. Thirdly, we show that this effect of calcium depends on the presence of AtClCa or AtClCd.

The effect of cadmium on roots is different from the effect of other heavy metals

In plants exposed to cadmium the accumulation in the roots will be higher than in the shoot and therefore phytotoxic effects will be more apparent in the root (Breckle, 1991). Although all three heavy metals tested resulted in inhibition of root growth, the mode of action of cadmium differs from that of zinc and lead. The effects of lead do resemble those of cadmium, as both result in a narrow root tip and a darkening of the cells. However, cell deformation of the cells in the root tip that are characteristic for exposure to sub-lethal concentrations of cadmium, were not observed after exposure to lead. In *Arabidopsis* root that cell death first appeared in around meristematic and then in elongation zone of root, where influx of Cd\(^{2+}\) caused cell death and inhibited primary root growth (Suzuki, 2005).

While no differences between the genotypes, concerning heavy metal sensitivity, were observed, a difference was observed when the capability of Ca\(^{2+}\) to alleviate the Cd\(^{2+}\) reduction of root growth was studied. Ca\(^{2+}\) was unable to alleviate the Cd\(^{2+}\) effects in the mutant plants lacking functional AtClCa and AtClCd. The reduction of root growth induced by Zn\(^{2+}\) is equal in all genotypes and is insensitive to calcium. We interpret this effect of calcium as an increased sensitivity of the genotypes *Atclcad* and *Atclcabd* for Cd\(^{2+}\).

Plant cells have developed a variety of mechanisms to protect cells from heavy metals. One of them is the accumulation of soluble phenolic compounds in the cells, resulting in protection of tissues against oxidative stress. (Yamamoto *et al*., 1998; Schutzendubel *et al*., 2001; Suzuki, 2005), which is visible as the dark discoloration in Cd\(^{2+}\) or Cu\(^{2+}\) exposed root tips. Another is exclusion and/or sequestration.
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**In effects of calcium on cadmium toxicity: differential effect of Ca\(^{2+}\) on AtClcad double mutants**

Cadmium mainly enters the root in the first few 1-1.5 mm behind the root tip and the influx is significantly less, further up the root (Pineros *et al.*, 1998; Arduini *et al.*, 1996). This is also the normal pattern of uptake for plant nutrients and several studies indicate that it is likely that Cd\(^{2+}\) enters the plant, in a competitive way, through the same transporters that are involved in nutrient uptake. Exposure to cadmium can result in a decrease in the content of Ca, Zn, Cu, Mn and Fe in pea leaves (Rodriguez-Serrano *et al.*, 2009). Cadmium uptake could be competitively inhibited by other cations and by Ca\(^{2+}\)-channel blockers (Blazka and Shaikh, 1992; Clemens, 2006). Active and passive transport systems have been reported for Cd\(^{2+}\) in roots of several plant species (Cataldo *et al.*, 1981; Godbold, 1991; Gosta and Morel, 1993 and 1994; Hart *et al.*, 1998). Non-essential heavy metals might be transported via nutrient transporters or channels that are not completely selective (Clemens *et al.*, 1998). It has been observed that cadmium not only competes with calcium for calcium transporters, but also for intracellular Ca\(^{2+}\)-binding proteins (Rivetta *et al.*, 1997) and at plasma membrane (Kinraide, 1998). Alleviation by calcium of Cd\(^{2+}\) toxicity by reducing the Cd\(^{2+}\) uptake and accumulation, have been reported in radish (Rivetta *et al.*, 1997), tobacco (Choi *et al.*, 2001), rice roots (Kim *et al.*, 2002) and Arabidopsis seedlings (Suzuki, 2005). Also, in Arabidopsis thaliana AtHMA1 functions as a Ca\(^{2+}\)/ heavy metal pump (Moreno *et al.*, 2008). Analysis of the interaction between calcium and cadmium in wildtype and all mutant plants the extent of the effect of Ca\(^{2+}\) on Cd\(^{2+}\)- toxicity is related the concentrations of Cd\(^{2+}\) and Ca\(^{2+}\). Our results confirm this conclusion: 30 mM Ca\(^{2+}\) almost completely compensated the toxic effect of 30 and 60 \(\mu\)M Cd\(^{2+}\) (except for AtClCad and AtClCadbd in 60 \(\mu\)M Cd\(^{2+}\)). But at 90 \(\mu\)M Cd\(^{2+}\) the alleviation by 30 mM Ca\(^{2+}\) was not complete. Furthermore, in 90 \(\mu\)M Cd\(^{2+}\), 0.5 – 1 mM of Ca\(^{2+}\) reduces toxicity by 1/3 to 2/3 and 2.5-30 mM Ca\(^{2+}\) reduces the effect of cadmium by more than 2/3 (Figure 2b).

Our results showed that, of the different cations tested (BaCl\(_2\), MgCl\(_2\), CaCl\(_2\) and cholinchloride), only addition of CaCl\(_2\) alleviated cadmium-induced root growth inhibition. This rules out that the antagonism between cadmium and calcium depends on the occupation of extracellular binding sites, as for such a mechanism one would expect also a positive effect of the other divalent cations.
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The most intriguing result in our study is the differential effect of calcium on the cadmium toxicity in the \textit{Atclca} and \textit{Atclcd} mutants. Increasing the calcium concentration in the double mutant \textit{AtClCad} and triple mutant \textit{AtClCad} treated with Cd$^{2+}$, alleviated root growth significantly less than in other mutants and wildtype plants (Figure 2). Several reports have made a connection between anion transporters and heavy metal translocation. Heavy metals are transported across cell membranes by a number of complex mechanisms. In animals metal transport into cells is sensitive to the anion channel blocker DIDS (Simons, 1986; Lou \textit{et al.}, 1991). For animal cells it has been proposed that heavy metals can cross the membranes via the anion channel in the form of anionic complexes with carbonate, bicarbonate, hydroxyl, or chloride ions (Foulkes, 2000).

Several previous investigations have demonstrated the important role of Ca$^{2+}$ for root elongation, even in the absence of metal toxicity (Demidchik \textit{et al.}, 2002; Hanson, 1984; Kinraide, 1998). In plant guard cells (Schroeder and Hagiwara, 1989; Hedrich \textit{et al.}, 1990; Allen \textit{et al.}, 1999; Blatt, 1999 and Leonhardt \textit{et al.}, 2004) and in \textit{Arabidopsis thaliana} suspension cells (Trouverie \textit{et al.}, 2008) anion channels are Ca$^{2+}$-sensitive and activated by transient increases of [Ca$^{2+}$]. In \textit{Arabidopsis thaliana} hypocotyl protoplasts, activation of anion channels is directly dependent to the calcium concentration at the cytosolic site of the plasma membrane (Lewis \textit{et al.}, 1997).

Furthermore, reactive oxygen species (ROS), such as H$_2$O$_2$, are involved in signaling pathways through the activation of plasma membrane calcium channels (Pei \textit{et al.}, 2000; Murata \textit{et al.}, 2001, Kwak \textit{et al.}, 2003; Trouverie \textit{et al.}, 2008). One could hypothesize that activation of the anion current would result from the activation of calcium channels. Therefore, a simple explanation for the activation of anion channels by oxidative stress and calcium application would rely on the ability of heavy metal-induced increased ROS concentrations to promote Ca$^{2+}$ influx. However, the even simpler hypothesis that cadmium toxicity is prevented by external calcium through competition for transporters and thus reduction of the intracellular [Cd$^{2+}$], is a good possibility.

However, in both explanations the mechanistic role of \textit{AtClCa} and \textit{AtClCd} is not obvious. Here we present a model for the role of these anion transporter proteins in cadmium-tolerance (Figure 8). \textit{AtClCa} and \textit{AtClCd} are located on endomembranes, have H$^+$/anion antiporter activity and have the highest expression levels in the
expansion and transition zone of the root (Lv et al., 2009). In yeast, mutants of gefl, an anion transporter located in the trans-Golgi vesicles, fail to properly regulate pH and are more sensitive to heavy metals. Heterologous expression of AtClCa, AtClCc or AtClCd could at least partly complement gefl, and restore heavy metal resistance (Gaxiola et al. 1998).

Figure 8. Model for the role of ClC transporter proteins in the sequesteration of cadmium in intracellular compartments (e.g. the vacuole). A: Protons are actively pumped into the vacuole, resulting in a pH gradient and a tonoplast potential (positive inside), the activity of the ClC anion/proton antiporters proteins reduces the tonoplast potential and increases the concentration of anions in the tonoplast. This facilitates the accumulation of positively charged Cd\(^{2+}\) in the vacuole. B: When the ClC anion/proton antiporters are either inactive or absent this leads to an excess of positive charges and an acidic pH in the vacuole. This situation reduces sequestration of the Cd\(^{2+}\) in the vacuole.

We propose that in Arabidopsis thaliana AtClCa and AtClCd have a similar function as a H\(^+\) - and electrical shunt for the V-type ATPase and the heavy metal transporter, respectively. Functional AtClCa and AtClCd proteins facilitate a high accumulation of heavy metal in either the vacuole and/or acidic vesicles. The observation that only the double mutant is more sensitive implies that AtClCa and AtClCd have overlapping functionality. One possibility is that both are present in the membrane of the same compartment and thus are truly redundant. Another option is that they are localized in different compartments, but that heavy metal sequestration occurs in both compartments, viz. the acidic trans-Golgi vesicles and the vacuole. In this model the differential effect of Ca\(^{2+}\) on cadmium toxicity is based on two distinct mechanisms of heavy metal resistance: exclusion, which is aided by addition of calcium, and sequestration, which is facilitated by active AtClCa or AtClCd. Neither gives full protection when exposed to 90 \(\mu\)M Cd\(^{2+}\), but in combination they can restore full root growth and development.
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