Running head: CAX1 involvement in Ca-dependent Cd tolerance of A. halleri

*Corresponding author:
Nathalie Verbruggen
Laboratory of Plant Physiology and Molecular Genetics
Université Libre de Bruxelles
Bd du Triomphe, 1050 Brussels
Belgium

Telephone: +32-2-6502128
Fax: +32-2-6505421
e-mail: nvebru@ulb.ac.be

Research area: Responses of plant to trace metallic excess
CAX1 co-segregates with Cd tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in *Arabidopsis*.

Cecilia Baliardini¹, Claire-Lise Meyer¹, Pietrino Salis¹, Pierre Saumitou-Laprade² and Nathalie Verbruggen¹.

¹ Laboratory of Plant Physiology and Molecular Genetics, Université Libre de Bruxelles, Bd du Triomphe, 1050 Brussels, Belgium; ² Laboratoire Evolution, Ecologie, Paléontologie, UMR CNRS 8016, Université de Lille 1, F-59655 Villeneuve d’Ascq Cedex, France

Summary:
A gene encoding a calcium/proton antiporter is involved in cadmium tolerance of the Cd-hyperaccumulator *Arabidopsis halleri* and of the Cd-sensitive species *Arabidopsis thaliana*, and acts to limit oxidative stress.
Financial sources: The authors thank the Fonds national de la Recherche scientifique for financial support (PDR T.0206.13 and fellowships of C. B. and C-L. M.).

Corresponding author:
Nathalie Verbruggen (e-mail: nvebru@ulb.ac.be)
Abstract:

*Arabidopsis halleri* is a model species for the study of plant adaptation to extreme metallic conditions. In this species, cadmium (Cd) tolerance seems to be constitutive and the mechanisms underlying the trait are still poorly understood. A previous QTL analysis, performed on a *A. halleri* x *A. lyrata* back-cross population (BC1), identified the metal-pump gene *HMA4* as the major genetic determinant for Cd tolerance. However, although necessary, *HMA4* alone is not sufficient for determining this trait. After fine-mapping, a gene encoding a 
Ca$^{2+}$/H$^+$ antiporter, *CAX1*, was identified as a candidate gene for the second QTL of Cd tolerance in *A. halleri*. BC1 individuals displaying the *A. halleri* allele for the *CAX1* locus exhibited significantly higher *CAX1* expression levels compared to the ones with the *A. lyrata* allele, and a positive correlation between *CAX1* expression and Cd tolerance was observed. Here, we demonstrate that this QTL is conditional and it is only detectable at low external Ca concentration. *CAX1* expression in both roots and shoots was higher in *A. halleri* than in the close Cd sensitive relatives species *A. lyrata* and *A. thaliana*. Moreover, *CAX1* loss of function in *A. thaliana* led to higher Cd sensitivity at low concentration of Ca, higher sensitivity to methyl-viologen and stronger accumulation of reactive oxygen species (ROS) following Cd treatment. Overall the present study identifies a novel genetic determinant of Cd tolerance in the metal hyperaccumulator *A. halleri* and offers a new twist for the function of *CAX1* in plants.
INTRODUCTION

Pollution by excessive amounts of trace metal elements (TMEs) has become a serious and widespread problem at the global level (Koptsik, 2014). In particular, mining and industrial activities have released large amounts of zinc (Zn), cadmium (Cd) and lead (Pb) into the air, water and soil. In addition, the application of phosphate fertilizers and sewage sludge contributed to the accumulation of TMEs in agricultural soils and aquatic environments. Cd represents one of the most toxic pollutants released into the environment and it is recognized as a main threat to human health (Clemens et al., 2013).

Moreover, compared to other trace metals, Cd is rapidly absorbed and accumulated in plant tissues, and its transfer to the food chain is a severe consequence of widespread low-level contamination of soil (Clemens et al., 2013). Although plant responses to Cd toxicity are well-reported and described in the literature (Lux et al., 2011), the mechanisms underlying Cd toxicity are not completely understood. Cd has been reported to affect the uptake of other minerals from the soil (e.g. Fe, Mg, K and Ca). At the cellular level, Cd ions lock on many different targets. Cd ions, indeed, can induce mineral deficiencies by competing with the uptake of essential metallic elements, displace other metals from protein active sites (e.g. Fe, Zn, Ca) and bind sulfhydryl groups of structural proteins and enzymes leading to misfolding and inhibition of activity (Dal Corso et al., 2008; Gallego et al., 2012). Furthermore, Cd can also impair photosynthetic and respiration activities, and induce oxidative stress (Heyno et al., 2008). Cd presence inside the cell leads to the arousal of ROS (Reactive Oxygen Species), which can react with lipids, proteins, nucleic acids and pigments causing toxic effects and oxidative burst (Sharma and Dietz, 2009; Cuypers et al., 2010). Despite Cd toxicity, a class of plants, referred to as hyperaccumulators, have evolved a combination of mechanisms to reach high level of tolerance to and accumulation of this element. In these species, some individuals display an extraordinary capacity to accumulate more than 100 µg of Cd·g⁻¹ dry weight (DW) in their shoots in their natural habitats (Baker, 1981; Verbruggen et al., 2009; Krämer, 2010).

In the last decade, great attention has been given to two Zn and Cd hyperaccumulators, the Brassicaceae Arabidopsis halleri and Noccaea caerulescens. The former is a clonal and self-incompatible plant, able to grow on both metal contaminated and non-contaminated soils. In Europe, A. halleri occurs at low altitudes in industrial sites polluted by Zn, Cd and Pb (e.g. those in northern France, Poland, Germany and northern Italy, among others) and at moderate and high altitudes in soils containing low levels of TMEs. A. halleri exhibits constitutive Zn and Cd tolerance, as well as Zn hyperaccumulation, while
also maintaining a high natural variability in all of these traits (Bert et al., 2002; Pauwels et al., 2006). Currently, *A. halleri* is considered a model species for metal accumulation and tolerance studies, but also for metal homeostasis, since it is a close relative of *Arabidopsis thaliana* (Al-Shehbaz and O’Kane, 2002). Therefore, the molecular and genetic tools that have been developed for Arabidopsis can also be used for *A. halleri*. Moreover, since *A. halleri* is inter-fertile with *Arabidopsis lyrata petraea*, a close phylogenetic relative sensitive to and non-accumulator of heavy metals, metal tolerance and accumulation can be analyzed in segregating populations.

The genetic basis of Cd tolerance and accumulation have been investigated in a backcross (BC1) population that was obtained from crosses between *A. halleri* (metallicolous accession Auby, France) and *A. lyrata petraea* (Courbot et al., 2007; Willems et al., 2010). Three chromosomal regions associated with Cd tolerance trait were identified (Courbot et al., 2007). These quantitative trait loci (QTL), named CdTol1, CdTol2 and CdTol3, explained together 83% of the phenotypic variance (43%, 24% and 16% respectively). Another QTL, that was found to be responsible for 21% of the phenotypic variance for Cd accumulation, was identified and it co-localized with CdTol1 and with the *Heavy Metal ATPase 4* gene (*HMA4*). *HMA4* encodes a Zn/Cd pump that mediates root-to-shoot metal translocation, and it has been validated as a fundamental genetic determinant for Zn/Cd tolerance and accumulation in *A. halleri* (Hanikenne et al., 2008). Nonetheless, although necessary to modify metal allocation and accumulation, *HMA4* alone is not sufficient to explain the high level of Zn and Cd tolerance exhibited by *A. halleri*. Thus, additional genetic determinants are required for an efficient detoxification mechanism in shoots, considering the massive metal flux generated by HMA4 activity (Hanikenne et al., 2008). Results from transcriptomic analyses performed on *A. halleri* and *N. caerulescens*, suggest that one of the main mechanism underlying metal tolerance is the constitutive high expression of genes involved in metal transport and detoxification processes (Weber et al., 2006; Van De Mortel et al., 2008). Among the genes found to be over-expressed in these tolerant species, those involved in metal uptake (e.g. ZIP members), xylem loading (in particular *HMA4* and *HMA2*), synthesis of ligands (e.g. NAS, YSL and MTs members) and vacuolar sequestration and detoxification (e.g. *CAX2*, *MTP1* and *HMA3*) are of particular interest (Verbruggen et al., 2013). In *N. caerulescens*, several lines of evidence support the key role of elevated transcript levels of the tonoplast Cd transporter HMA3 for the extraordinary vacuolar sequestration capacity of some populations (Ueno et al., 2011). Taken together, these studies suggest that the genes that
contribute to hyperaccumulation and hypertolerance are not species-specific or novel, but rather differently regulated between tolerant and sensitive species. Nevertheless, apart from \textit{HMA4} and \textit{HMA3}, the molecular mechanisms responsible for Cd tolerance and accumulation in hyperaccumulators still remain elusive. Therefore, the aim of the present study was to further analyze CdTol2 QTL in order to identify new genetic determinants involved in Cd tolerance. Marker densification in the QTL CdTol2 genomic region resulted in the identification of \textit{CAX1} as possible candidate gene. In \textit{Arabidopsis}, \textit{CAX1} encodes a tonoplast Ca$^{2+}$/H$^+$ exchanger that plays a key-role in cellular Ca homeostasis (Cheng et al., 2003; Mei et al., 2007; Conn et al., 2011). By using multiple approaches, we demonstrated that \textit{CAX1} is involved in the maintenance of Ca homeostasis limiting the accumulation of ROS species upon Cd stress.
RESULTS

Marker densification in the CdTol2 region and identification of CAX1 as candidate gene

Five markers were added to the Ah x Alp genetic map published by Courbot et al. (2007) in order to confirm the localization of QTL CdTol2 and to narrow down its size. BC1 individuals from the study of Courbot et al. (2007) were genotyped for these markers and were used for QTL fine-mapping. As result, the CdTol2 locus was reduced to a 3 centiMorgan (cM) region between markers ACT3 and At2g40140 (Fig. 1A). In A. thaliana, the region between these markers is approximately 600 kb and it contains about 150 genes. Among them, four genes showed gene ontology annotation associated with Cd response or metal ion transport (Table S1). CAX1 was selected for further studies based on: (i) current literature, suggesting a role for CAXs in metal transport in the vacuole (Hirschi et al., 2000; Shigaki et al., 2005; Koren’kov et al., 2007; Mei et al., 2007; Wu et al., 2011) and (ii) expression analysis of BC1 Ah x Alp individuals displaying different alleles at the CdTol2 locus. Indeed, BC1 individuals with the Ah allele at the CdTol2 locus showed significantly higher CAX1 expression levels in shoots than the ones displaying the Alp allele (Fig. 1B). Furthermore, a second expression analysis identified a positive correlation between CAX1 expression levels in shoots and Cd tolerance in BC1 individuals (n=20) (data not shown).

Cloning of A. halleri CAX1 and in silico analyses

In A. thaliana, three CAX1 transcripts are annotated. The At2g38170.3 sequence has been used as model in all previous studies of AtCAX1, whereas the other two transcripts (At2g38170.1 and At2g38170.2) are predicted as alternative splicing products (TAIR10). The isolated AhCAX1 transcript exhibited a high level of sequence identity with At2g38170.1 and A. lyrata CAX1 (AICAX1) (95% and 96%, respectively), and a lower identity with the two other A. thaliana transcripts At2g38170.2 and At2g38170.3 (90% and 85% respectively). The percent identity of AhCAX1 at the amino acid level with AICAX1 and At2g38170.1 is 97% and 95%, respectively. The few differences that exist between the deduced sequence of AhCAX1 and the sequences of AtCAX1 and AICAX1 all occur outside the annotated functional domains that are the cation-selection, exchange and auto-inhibitory regions (Fig. S1). These results suggest that the three proteins share a similar function and that substrate specificity of CAX1 transporter is conserved in the three species.
Given the high expression of CAX1 in the BC1 individuals displaying the Ah allele (Fig. 1b) and the several reports of gene duplication linked to adaptation to extreme environments (Hanikenne et al., 2008; Ueno et al., 2011; Kondrashov, 2012), the copy number of CAX1 in A. halleri as investigated. In quantitative real-time PCR essays performed with genomic DNA from the Auby population (the grand-parent of the BC1 individuals), and with the A. thaliana single-copy gene SHR (short root) used as internal control, it is estimated that there is only one copy of CAX1 in the A. halleri genome (mean = 1.08 ± 0.15) (Fig. S2).

Study of CAX1 expression following Cd treatment at different Ca concentrations

To investigate the impact of Cd treatment on the regulation of CAX1 expression and compare transcript levels in the different species, quantitative real-time RT-PCR assays were performed. Since CAX1 is known to have a significant role in Ca homeostasis, levels of CAX1 expression were assayed at two different Ca concentrations. Shoot and root samples were harvested from A. halleri, A. lyrata and A. thaliana plants grown in a hydroponic system at 0.5 mM Ca (low Ca concentration, used in the QTL analysis of Courbot et al., 2007) and 2mM Ca (moderate Ca concentration). Half set of samples was further treated with 10 µM CdSO₄ for 72 h (Fig. 2A and 2B).

Under non-contaminated condition at low Ca, at least 3-fold higher levels of CAX1 were detected in the shoots and roots of A. halleri compared with A. lyrata (Fig. 2A). Between A. halleri and A. thaliana, expression of CAX1 in the shoots was similar. However, in the roots, expression of CAX1 was 5.6-fold higher in A. halleri. Following Cd treatment, CAX1 expression increased 5.5-fold and 2-fold in the roots of the Cd-sensitive species A. lyrata and A. thaliana, respectively. In contrast, CAX1 expression did not increase in the shoots of these two species. An increase in CAX1 transcript levels after Cd treatment was also observed in A. halleri roots, although this difference was not statistically significant (Fig. 2A).

At 2 mM Ca, for all of the conditions tested and for all three species, the expression of CAX1 was at least 2-fold higher than the levels detected at low Ca (Fig. 2B). Under non-contaminated condition, CAX1 expression in the shoots was similar in all three species. For the root samples, similar transcript levels were detected in A. halleri and A. lyrata, whereas A. thaliana exhibited 12-fold lower levels of CAX1 transcript than the other two species. In addition, Cd exposure did not modify CAX1 expression in any of the three species at 2 mM Ca (Fig. 2B). The increased expression of CAX1 transcript in roots upon...
Cd treatment at low Ca concentration was then checked at the protein level by western blot. An increase in levels of CAX1 protein in all three species were confirmed (Fig. 2C).

**CdTol2 is a Ca-conditional QTL**

At moderate Ca concentration, no difference in CAX1 expression was detected between the parental species of the BC1 (Fig. 2B). Therefore, to verify the validity of the candidate gene, individuals from the BC1 progeny were phenotyped for Cd tolerance through a sequential growth test at moderate Ca concentration (2 mM). The broad-sense heritability value ($H^2$), that was estimated using at least three clones of each BC1 genotype, was relatively high (0.51). In addition, segregation of Cd tolerance for the new BC1 individuals was bimodal and similar to that observed by Courbot et al. (2007) (data not shown). As result of the QTL analysis, no QTL was detected on the Linkage Group 4 (Fig. 3, red line), whereas, with the same set of markers and the phenotyping of Courbot et al. 2007 (performed at 0.5 mM Ca), the CdTol2 QTL was detectable (Fig. 3, green line).

**Loss of function in A. thaliana impairs Cd tolerance at low Ca**

The A. thaliana CAX1 knockout (k.o.) mutant, cax1-1, has been well-studied and already characterized for metal tolerance by Cheng et al., (2003). However, no Cd tolerance phenotype has been described for this mutant. Therefore, Cd tolerance of A. thaliana WT (Col-0 background) and cax1-1 was reassessed at different Ca concentrations using the primary root length and the shoot surface as growth parameters (Fig. 4A, B). Tests were performed *in vitro* at low and moderate Ca (0.5mM and 2mM) in the presence or absence of 100µM Cd for 7 days.

Similar root lengths and shoot areas were observed under the control conditions at the two tested Ca concentrations (Fig. 4). In contrast, Cd treatment induced a significant decrease in both growth traits at both concentrations of Ca. Yet, a more significant decrease was observed in the cax1-1 mutant compared with the WT at 0.5 mM Ca, with decreases of 19% and 24% observed, respectively, for primary root length and shoot area (Fig. 4A, B).

In the presence of 2 mM Ca, no significant differences were detected between the two genotypes (Fig. 4). An *in vitro* Zn tolerance test was also performed with A. thaliana WT and cax1-1 plantlets, grown at 0.5 mM or 2 mM Ca. No difference in either growth parameter was detected between the two genotypes following exposure to 150 µM ZnSO4 (data not shown), thereby excluding a role for CAX1 in Zn tolerance. Shoot tissues were pooled and full mineral profiles were assayed. No difference in Cd accumulation was
observed between WT and cax1-1 plants at both Ca concentrations (data not shown).

**CAX3 does not appear to have a role in Cd tolerance**

Among the CAX transporters, CAX3 is the closest homolog to CAX1 (77% sequence identity). CAX3 has been shown to have a similar role in Ca homeostasis and it is able to partially compensate for the loss of CAX1 in the cax1-1 mutant (Cheng et al., 2005; Zhao et al., 2008; Zhao et al., 2009; Conn et al., 2011). To investigate whether CAX3 is also involved in the Cd stress response, an *in vitro* tolerance test was performed for WT *A. thaliana* and *A. thaliana* CAX3 k.o. mutant (cax3-1). The test was performed at low and moderate Ca concentrations (0.5 mM and 2 mM) in control condition or after 100 µM CdCl₂ treatment for 7d. For all the tested conditions, cax3-1 exhibited similar growth to *A. thaliana* WT. However, following growth of the plants at 0.5 mM Ca and CdCl₂ treatment, only cax1-1 mutant exhibited a significant decrease in the primary root length compared to the WT (Fig. 5).

**CAX1 limits ROS accumulation following Cd treatment**

The link between ROS production and stress-induced spikes of cytosolic Ca is well-established (Pei et al., 2000; Demidchik, 2003; Takeda et al., 2008; Hua et al., 2012). To investigate a potential role for CAX1 in managing ROS accumulation following Cd treatment, levels of H₂O₂ and O₂⁻ were measured in *A. thaliana* (WT and cax1-1), *A. lyrata*, and *A. halleri* plants grown in a hydroponic system and treated with 10 µM CdSO₄ for 72 h. Only results for the root samples are presented, because ROS accumulation was not detected in the shoots after 72 h Cd treatment. At 0.5 mM Ca, all of the root samples presented pale staining. In contrast, *A. lyrata* and both *A. thaliana* genotypes, WT and cax1-1, exhibited stronger staining after Cd treatment, corresponding to an accumulation of H₂O₂ (Fig. 6A). Remarkably, cax1-1 roots appeared to accumulate a higher level of ROS than the WT *A. thaliana* and *A. lyrata* (Fig. 6A, panels E, F, H). Accumulation of H₂O₂ in *A. halleri* following Cd treatment was not detected (Fig. 6A, panels C, G). At 2 mM Ca, there was no difference in the staining patterns between the control and the Cd-treated root samples for all of the species and genotypes assayed (data not shown). Quantification of H₂O₂ accumulation is shown in Fig. 6B. Following growth at low Ca and Cd treatment, the cax1-1 mutant showed a 4-fold greater accumulation of H₂O₂ than *A. thaliana* WT. In comparison, H₂O₂ accumulation following Cd treatment was slightly lower for *A. lyrata* than for *A. thaliana* WT. Quantification of H₂O₂ levels allowed differences to be detected
following Cd treatment, even when plants were grown with 2 mM Ca. Levels of H$_2$O$_2$ were the highest for the cax1-1 mutant (818.97 µg·mg$^{-1}$), followed by A. thaliana WT (350.12 µg·mg$^{-1}$), A. lyrata (91.57 µg·mg$^{-1}$) and A. halleri (26.57 µg·mg$^{-1}$). However, the induction of ROS was at least 2-fold lower at moderate Ca concentrations than at low Ca. For all four genotypes, we observed similar accumulation patterns of O$_2^\cdot$ (Fig. S3).

**CAX1 involvement in oxidative stress**

To investigate whether the role for CAX1 in ROS accumulation was Cd-specific or general for response to oxidative stress, we performed in vitro tolerance test using methyl-viologen (MV). This herbicide, that affects electron-transducing reactions in chloroplast and mitochondria, has been widely used to study ROS-mediated damage in plants. For the tolerance test, A. thaliana WT and cax1-1 plantlets were grown in the presence of 0.5 mM and 2 mM Ca as control conditions, and then treated for 7 d with 0.1 µM MV. The cax1-1 plants exhibited higher sensitivity to MV than the WT at the two Ca concentrations. Indeed, cax1-1 plants exhibited approximately 20% and 17% reduction in primary root growth and shoot surface area compared with the A. thaliana WT plants, respectively (Fig. 7).
DISCUSSION

To identify new genetic determinants of Cd tolerance in the metal hyperaccumulator *A. halleri*, a fine-mapping analysis was performed on the *A. halleri x A. lyrata petraea* BC1 progeny previously studied by Courbot et al. 2007. This analysis allowed the identification of CAX1 as a candidate gene at the locus of the QTL CdTol2, the second major QTL for Cd tolerance, explaining 23.7% of the phenotypic variance observed. In first support of CAX1 as candidate gene for CdTol2 QTL, a positive correlation between CAX1 expression and the tolerance level of individuals of the BC1 progeny was observed. In addition, individuals with the highest levels of CAX1 expression displayed the *A. halleri* allele at the CdTol2 locus. In *A. thaliana*, CAX1 encodes a Ca²⁺/H⁺ exchanger that localizes to the vacuolar membrane (Cheng et al., 2003; Conn et al., 2011). Plant cation/H⁺ exchangers (CAXs) are members of a multigene family that have the capacity to transport a broad range of cations (Manohar et al., 2011). In Arabidopsis, there are six CAX genes, and CAX1 is a major tonoplast Ca²⁺ transporter (Cheng et al., 2003; Shigaki & Hirschi, 2006).

Other members of the CAX family display the capability to transport Cd into the vacuole, besides Ca (e.g. CAX2 and CAX4; Hirschi et al., 2000; Mei et al., 2009). Furthermore a mutant of an activated N-terminal form of AtCAX1, that differs by one amino acid in the selectivity domain, named CAXcd, showed high apparent Cd transport (Shigaki et al., 2005) and Petunia plants expressing this CAXcd exhibited greater Cd accumulation than control plants, along with fewer toxicity symptoms (Wu et al., 2011).

In the present study the expression of CAX1 was found to be more than 3-fold higher in the shoots and roots of *A. halleri* compared with the Cd-sensitive species *A. lyrata*, and 5-fold higher in the *A. halleri* roots compared with the *A. thaliana* ones. One possible explanation for the high levels of CAX1 expression observed in *A. halleri* is copy number expansion, a common strategy that has evolved in plants in order to survive and adapt to unfavorable environments (Flagel and Wendel, 2009; Kondrashov, 2012). Indeed, some of the genes involved in metal tolerance (e.g., HMA4, MTP1) have multiple copies in the *A. halleri* genome (Talke et al., 2006; Hanikenne et al., 2008; Shazad et al., 2013). Our data suggest that AhCAX1 is a single copy gene in *A. halleri* and that differential expression between species can be attributed to cis- or trans-regulation. High levels of AhCAX1 expression could have evolved in ancestral non-metallicolous populations from which metallicolous populations derived (Pauwels et al., 2005). Indeed non-metallicolous populations in several parts of Europe are found in calcareous areas (Saumitou-Laprade, personal communication) and levels of CAX1 expression was found to be dependent on
the external Ca concentration (Hirschi, 1999; this study). Besides a link between Ca concentration in the soil and level of CAX1 expression was previously suggested for soils with low level of Ca. The loss of function of CAX1 in Arabidopsis conferred better growth in culture conditions similar to serpentine soils, characterized by low Ca/Mg ratio (Bradshaw, 2005). CAX1 transcript levels were also found to vary according to the exposure of Cd. At low Ca concentration, CAX1 was induced by Cd treatment in the roots of all three species. To further investigate the possible role of CAX1 in Cd tolerance, a knock-out mutant of Arabidopsis was studied. In the in-vitro tolerance tests that were performed, loss of CAX1 in A. thaliana significantly affects the Cd-tolerance phenotype at low Ca but not at moderate concentrations. As a result, significant reductions in primary root length and shoot surface area were observed compared to A. thaliana WT. The metal sensitivity of the cax1-1 mutant has been studied, but no particular phenotype linked to Cd was identified before, most probably due to the Ca level used during the phenotype assays performed (Cheng et al., 2003).

Previous studies on cax1-1 mutant have reported very subtle phenotypes after Ca²⁺ stress due to functional compensation by CAX3 (Cheng et al., 2005; Conn et al., 2011). Therefore in the present study Cd sensitivity of the A. thaliana cax3-1 ko mutant was evaluated. Knockout of CAX3 did not affect Cd sensitivity compared with the WT phenotype. These results suggest a specific role of CAX1 in response to Cd stress.

After cloning AhCAX1, the deduced amino acid sequences were analyzed considering the different variants issued from alternative splicing and compared with the proteins of A. thaliana and A. lyrata. AhCAX1 shares 97% and 95% identity at the amino acid level with AlCAX1 and AtCAX1, respectively. Moreover the annotated functional domains are all entirely conserved among A. lyrata, A. halleri, and A. thaliana, including the selectivity domain. Therefore it could be hypothesized that AlCAX1 and AhCAX1 have the same substrate specificity as AtCAX1 and that the role of CAX1 in Cd tolerance is probably not linked to Cd transport. Furthermore, given that Cd accumulation is similar between the cax1-1 mutant and A. thaliana WT, it is unlikely that CAX1 participates in the sequestration of Cd in the vacuole. It was further investigated how CAX1 could impact Cd sensitivity. Cd treatment is known to induce oxidative stress (Sandaliu et al., 2001; Cuypers et al., 2010; Gallego et al., 2012). It has been reported that an increase of cytosolic ROS level is responsible for the activation of Ca channels in pollen, guard cells and root cells (Pei et al., 2000; Demidchik, 2003; Hua et al., 2012; Wudick and Feijó, 2014) and that Ca accumulation in the cytosol appears to enhance the cellular accumulation of ROS.
(Potocký et al., 2007; Takeda et al., 2008). A positive feedback loop was accordingly showed between ROS production and stress-induced cytosolic Ca spikes (Pei et al., 2000; Demidchik, 2003; Takeda et al., 2008; Hua et al., 2012). Given that CAX1 has a major role in Ca homeostasis, a possible role for CAX1 in limiting ROS production and oxidative burst in response to Cd stress by sequestration of Ca in the vacuole was investigated in the present study. Loss of CAX1 function in A. thaliana led to an enhanced accumulation of ROS following Cd treatment. The patterns of ROS accumulation in roots also correlated with the expression profile of CAX1 in the different species examined. Indeed, the genotypes that resulted in null or low levels of CAX1 expression showed a higher augmentation of ROS after Cd treatment. The strongest accumulation was observed in the cax1-1 mutant (ROS accumulation was 4-fold higher than in A. thaliana WT). The only genotype that did not accumulate ROS species after exposure to Cd was A. halleri, which well fits the exceptional Cd tolerance of that species. The absence of H2O2 accumulation in the A. halleri root samples could be due to: (i) the efficient transfer of Cd to the upper part of the plant as a result of high levels of HMA4 expression; (ii) the constitutive high levels of CAX1 expression that lead to a stable maintenance of Ca homeostasis in the cytosolic compartment; (iii) a synergistic action of these mechanisms.

On the whole, the evidence collected suggests that CAX1 expression may play an important role in maintaining cytosolic Ca levels in order to avoid uncontrolled ROS accumulation. Furthermore, the high sensitivity to ROS-generating reagent of the cax1-1 mutant suggests that CAX1 plays a role not limited to Cd tolerance but also in the general response to oxidative stress. In addition, we cannot exclude that changes of CAX1 expression may have an impact on downstream targets responsive to Cd stress. For example, Catala et al. (2003) showed that CAX1 negatively regulates the expression of the CBF/DREB1 transcription factor gene, which plays a role in adaptation to freezing after cold acclimation.

In the present study, the impact of CAX1 on Cd tolerance could only be observed at low Ca supply in the external medium, in the CAX1 loss of function in Arabidopsis as well as in the genetic analysis of Cd tolerance architecture in A. halleri. The apparent discrepancy in the observations at low and moderate Ca can be explained by two different sides of plant response to Cd and Ca supplies. First the sensitivity of the cax1-1 mutant to Cd was only detected at low level of Ca in the medium, while its sensitivity to MV-induced oxidative stress was independent of Ca concentration in the medium. These contrasting results may be due to stronger Ca/Cd competition when Ca levels in the medium are increased.
Correspondingly, it has previously been reported in the literature that high external Ca mediates a “protection effect” against Cd stress due to competition at ca-binding motifs of structural proteins and enzymes (Rivetta et al., 1997; Perfus-Barbeoch et al., 2002; Heyno et al., 2008; Rodriguez-Serrano et al., 2009; Lu et al., 2010; Tian et al., 2011; Zorrig et al., 2012). Second, the CdTol2 QTL was only detectable at low Ca concentration (Courbot et al., 2007). The absence of the CdTol2 detection at moderate Ca concentration is probably due to the absence of differences in CAX1 expression between Cd-sensitive species and the Cd-tolerant A. halleri under those conditions. The fact that CdTol2 is Ca-conditional further strongly supports CAX1 as the candidate gene for this QTL in A. halleri.

It will be interesting to further investigate the role of CAX1 in the Cd hypertolerance of A. halleri in its natural environment. This will contribute to a better understanding of the evolution of naturally-selected tolerance to extreme conditions in plants. Overall the present study identifies a novel genetic determinant of Cd tolerance in the metal hyperaccumulator A. halleri and offers a new twist for the function of CAX1 in plants.

MATERIAL AND METHODS

Plant materials and growth conditions

For expression analysis and ROS detection experiments, seeds of Arabidopsis halleri ecotype Auby (Bert et al., 2000), Arabidopsis lyrata ssp petraea (Unhost, Central Bohemia, Czech Republic; Macnair et al., 1999), Arabidopsis thaliana ecotype Col-0 and Arabidopsis thaliana cax1 ko mutant (cax1-1; Cheng et al., 2003) were used. Seeds were sown on humid vermiculite and four weeks after germination seedlings were transferred to hydroponic culture. The hydroponic solution was a modified Murashige and Skoog culture (Courbot et al., 2007): K₂SO₄ (0.88 mM), KH₂PO₄ (0.25 mM), NaCl (10 µM), CaCl₂ (0.5 or 2 mM), MgNO₄ (1 mM), FeEDDHA (20 µM), H₂BO₃ (10 µM), ZnSO₄ (1 µM), MnSO₄ (0.6 µM), CuSO₄ (0.1 µM), and (NH₄)₆Mo₇O₂₄ (0.01 µM), adjusted to pH 5.7 with KOH and buffered with 0.25 mM MES (2-morpholino-ethane-sulphonic acid). Plants were cultivated in a climate-controlled growth chamber (20°C day/17°C night; 8h light, 100 µmol photons m⁻² s⁻¹/16 h darkness, 60% humidity), and the hydroponic solution was changed every week. After four weeks in non-contaminated solution, 10 µM CdSO₄ was supplied to half of the plants for 72h.

QTL mapping at 2mM Ca was performed using [Ah x Alp] x Alp BC1 individuals generated from a batch of BC1 seeds from the same original cross described in Courbot et al. (2007).
This new set of individuals was produced with the same parental genotypes because BC1 individuals used for the previous QTL mapping (Courbot et al., 2007) died before starting our experiment. QTL analysis was performed using three cuttings from 125 genotypes of the new BC1 progeny. Cuttings were grown on sand for four weeks in glasshouses and rooted ones were then moved to hydroponic culture. Tolerance was assessed through a sequential growth test (Courbot et al., 2007). The range of CdSO₄ concentrations tested was 10, 25, 50, 75, 100, 150 and 250 µM. The composition of the hydroponic solution was the same as reported in Courbot et al. (2007), except 2 mM CaCl₂ was used instead of 0.5 mM.

In-vitro Cd tolerance test was performed by using 7-days old plantlets previously grown on half-strength MS medium (Murashige and Skoog, 1962) containing 1% sucrose and 0.8% agar. Cd concentration was chosen after preliminary assays performed with half-strength MS plates supplemented with 75, 100 and 150 µM CdCl₂. The concentration (100 µM Cd) inhibiting root length of wild-type by 50% 7 days after the transfer was selected. This concentration is higher than concentration used in hydropony due to the presence of agar which is known to bind cations.

The same transfer procedure was used to test the tolerance of Col-0 and cax1-1 to the ROS-inducing reagent methyl-viologen (MV) (0.1µM) (Sigma-Aldrich Co., St. Louis, MO, USA). All plates were incubated in a growth chamber (22°C under 8 h light/16 h dark illumination cycle and 100 µmol photons m⁻² s⁻¹ irradiance).

**Marker densification of the QTL CdTol2**

In order to confirm localization of the QTL CdTol2 on linkage group 4 and decrease its size, five markers (3 microsatellite markers and 2 single nucleotide polymorphism (SNP) markers, see Table S1) were added to the genetic map of Courbot et al. (2007). These markers were selected to cover the CdTol2 region according to their position in the *A. thaliana* genome. The new microsatellite marker was developed as described in Frérot et al., (2010). Re-sequencing data were used to design the two SNP markers. Illumina paired-end sequencing reads from six *A. halleri* populations (P Saumitou-Laprade, unpublished data) were aligned to the Araly1 assembly of the *A. lyrata lyrata* genome. Within regions of interest, the criteria used for the selection of SNPs were: (1) frequency of the polymorphism in *A. halleri* and *A. lyrata* equal to 1, (2) 50 base flanking regions with low polymorphism within *A. halleri* and between *A. halleri* and *A. lyrata*, (3) read depth >
20. BC1 individuals from the study of Courbot et al. (2007) were genotyped for the microsatellite according to Frérot et al. (2010) and for the SNPs using competitive allele-specific polymerase chain reaction KASP chemistry (LGC genomics, Teddington, UK). KASP assays were performed in a final reaction volume of 8 μL containing 4 μL KASP master mix V2 low ROX (LGC genomics), 0.125 μL of KASP mix assay (LGC genomics) and ~100 ng of genomic DNA. The PikoReal real-time PCR system (Thermo Scientific, Loughborough, UK) was used with the following cycling conditions: 15 min at 94°C; 10 touchdown cycles of 20 s at 94°C and 60 s at 61–55°C (the annealing temperature for each cycle being reduced by 0.6°C per cycle); 26 cycles of 20 s at 94°C and 60 s at 55°C. Fluorescence detection was performed at the end of each cycle and the data were analyzed using the allelic discrimination RFU-based method of the PikoReal software 2.1. The new Ah x Alp linkage map was constructed with Joinmap 3.0 program (Van Ooijen et al., 2002). Markers along each linkage group were ordered using the sequential method implemented in Joinmap 3.0. Kosambi’s mapping function was used to translate recombination frequencies into map distances (Kosambi, 1944). Detection of QTLs was performed using the MapQTL 4.0 software (Van Ooijen et al., 2002). A Multiple-QTL Model (MQM) analysis was performed every cM, in which markers close to detected QTLs (by IM mapping) were selected as cofactors to take over the role of the nearby QTLs in the approximate multiple-QTL models used in the subsequent MQM analysis. The calculated LOD scores were compared with a LOD score threshold obtained by a permutation test (1000 permutations), which corresponds to a linkage group-wide empirical significance threshold at the 5% level (Churchill and Doerge, 1994).

QTL CdTol2 detection at 2mM Ca

The new BC1 progeny (n=125) was genotyped for 8 markers covering half of the linkage group 4 of the Ah x Alp map. Four of these markers are described in the previous paragraph. The 4 other microsatellite markers (see Table S1) were developed and genotyped according to Frérot et al. (2010). For map construction and QTL mapping, we used the same procedure as above.

Cloning of the Arabidopsis halleri CAX1 sequence

To clone the coding sequence of AhCAX1, A. halleri tissues were homogenized in liquid nitrogen and RNA was extracted with Aurum™ Total RNA Mini Kit (BIO-RAD Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. cDNA was then
The primers used for the cloning were designed on conserved regions of AtCAX1 (At2g38170) and AlCAX1 (fgenesh2_kg.4__1885__AT2G38170.1 on Araly assembly) and are reported in Table S2. In order to obtain the full length AhCAX1 transcript sequence, 5' and 3'RACE-PCR were performed (Scotto-Lavino et al., 2006a; Scotto-Lavino et al., 2006b). The deduced aminoacid sequence of AhCAX1 (GeneBank accession number KT156755) was obtained using the cDNA translation tool on Expasy (http://web.expasy.org/translate/).

Real-time RT–PCR analysis

For CAX1 transcript quantification by qRT-PCR, leaf and root tissues of A. halleri, A. thaliana and A. lyrata were separately homogenized in liquid nitrogen and RNA was extracted as described above. Extraction quality and quantity was checked through nanodrop detector. cDNA was then synthesized and Real-time PCR was performed in 96-well plates with the PikoReal real time PCR system (Thermo Scientific, Loughborough, UK). In each 10 uL reaction, there were 2.5 µL cDNA, 5 µL SYBR mastermix (VeriQuest Fast SYBR Green qPCR Master Mix, Affymetrix, Santa Clara, CA, USA), 2 µL H2O, and 0.5 µM of each primer. A total of three technical repeats were run per cDNA and primer pair combination. qPCR reaction thermal profile was: pre-incubation at 95°C for 3 min, 40 cycles at 95°C for 30 s, 60°C for 1 min. Relative transcript levels were calculated by normalization to EF1α as a constitutively expressed reference gene (Talke et al., 2006; Deinlein et al., 2012).

For gene copy number analysis, genomic DNA of A. halleri and A. thaliana species was extracted using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Loughborough, UK). qPCR was performed using the single-copy gene SHR (At4g37650) as internal control (Ueno et al., 2011; Craciun et al., 2012). Reactions were carried out as described above.

All primers were designed in conserved genomic and transcript regions of A. thaliana and A. lyrata, using Primer 3 (http://primer3.sourceforge.net/) and checked on Beacon-free software (Premier Biosoft) and mfold (Zuker, 2003). Amplicons were sequenced and reaction efficiencies were determined for each primer pair (Table S2).

Protein extraction and immunoblotting

Proteins were extracted from plants grown in hydroponic culture for four weeks (under the
same conditions used for the plants analyzed by real-time RT-PCR analysis). Root samples were homogenized in liquid nitrogen and then resuspended in 0.15 M Tris, pH 7.5. After centrifugation, the supernatants were recovered and the total protein content of each sample was estimated using the Bradford protein assay (Quick Start™ Bradford protein assay, Bio-Rad Laboratories, Hercules, CA, USA). As a negative control, the total protein fraction extracted from cax1-1 mutant was used. Proteins were separated at 4 °C by SDS-PAGE (12% poly-acrylamide gel) in SDS-Tris-glycine buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS). Western blotting was then performed by transferring proteins to a nitrocellulose membrane. CAX1 protein was detected using a CAX1-specific antibody (sc-27240, Santa Cruz Biotechnology, Dallas, TX, USA). Bound antibodies were visualized using Lumi-Phos WB Chemiluminescent Substrate for AP (Thermo Scientific, Loughborough, UK).

Analysis of in-vitro tolerance and mineral analysis

After one week of growth in half-MS media supplied with different Ca concentrations (under control or Cd treatment conditions, as described above), plates were scanned and images were analyzed using the RootNav platform (Pound et al., 2013) to calculate primary root length. At the same time, shoot surface area was measured using ImageJ software (Abràmoff et al., 2004) and was used as an important parameter of plant growth in response to stress conditions (Claeys et al., 2014). Shoot tissues were then harvested, dried at 60°C, pooled to have sufficient weight and full mineral profiles of the bulks were assayed by ICP-OES. Roots were not analyzed because of their insufficient weight. Tolerance level of plants grown on MV containing plates, was assessed by measuring primary root length and shoot surface.

ROS detection and quantification

In situ detection of hydrogen peroxide \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) was carried out for leaves and roots of Arabidopsis plants (A. halleri, A. lyrata, A. thaliana WT and A. thaliana cax1-1 mutant) according to protocols reported in Daudi et al., (2012) and Ramel et al., (2009). To quantify the \( \text{H}_2\text{O}_2 \) content after DAB (3,3'-diaminobenzidine) staining, samples were frozen in liquid nitrogen, grounded to a powder, resuspended in 0.2 M HClO₄, and centrifuged for 10 min at 12,000 × g. \( A_{450} \) values were immediately measured and compared with a standard curve obtained from known amounts of DAB resuspended in HClO₄ (Ramel et al., 2009). For \( \text{O}_2^- \) quantification after NBT (nitroblue tetrazolium) staining, samples were
homogenized in liquid nitrogen and resuspended in 2M KOH-DMSO (1:1.16 v/v). After centrifugation (10 min at 12,000g), $A_{530}$ was measured and plotted on a standard curve.

ACKNOWLEDGMENTS

The authors thank Simon J. Conn for cax1-1 and cax3-1 seeds, Susanne Reiner and Nicolaus Von Wirén for mineral analysis and Eugeniusz Małkowski and the GDRI Locomet network for interesting discussions. We are also grateful to Cecile Godé and Sophie Galina for technical assistance on design and genotyping of molecular markers.

Fig. 1: Identification of CAX1 as candidate gene for the QTL CdTol2. A. Densification of the QTL CdTol2. Five additional markers (in red) were used to confirm the localization and to reduce the CdTol2 locus to a 3 cM region. The vertical dashed lines represent the LOD score threshold (1.5) for QTL detection at an error level of $\alpha=0.05$. Bars indicate the one-LOD (10-fold) support interval and whiskers (lines extending beyond bars) indicate the two-LOD (100-fold) support intervals. BC1 individuals were phenotyped at 0.5 mM CaNO$_3$ in the study of Courbot et al. (2007). B. CAX1 transcript levels in BC1 individuals displaying the Ah/Alp (h) or Alp/Alp (a) allelic combinations at the CdTol2 locus. At least three cuttings for each BC1 genotype were transferred in hydroponic solution according to Courbot et al. (2007) for 4 weeks. Clones were pooled together and CAX1 expression levels were assessed through qPCR analysis. Data are means ± SD ($n=9$). $^*P < 0.05$ (t-test).

Fig. 2: Expression analysis of CAX1. CAX1 transcript levels at (A) low Ca and (B) moderate Ca concentration in control condition (grey bars) and upon 72h 10µM CdSO$_4$ treatment (white bars) of A. halleri, A. lyrata petraea and A. thaliana. Data are means ± SD, $n=8-12$. Lower case letters for comparison between conditions in the same species; capital letters for comparison between species in the same condition (Kruskall Wallis test). (C). Western blot showing relative levels of CAX1 protein in root from A. halleri, A. lyrata and A. thaliana. Samples have been collected from plants grown at low Ca concentration in control condition (lanes 1, 3 and 5) or upon 72h 10µM CdSO$_4$ treatment (lanes 2, 4 and 6). As negative control, we loaded total protein fraction extracted from A. thaliana cax1-1 mutant (lane 7). Equal amounts of total protein (35ng) were loaded onto
the gel.

**Fig. 3:** QTL mapping of Cd tolerance in the CdTol2 region at two different Ca concentrations. Red line, LOD score obtained with 125 BC1 individuals phenotyped for Cd tolerance at 2 mM CaNO₃ and genotyped for six markers in the CdTol2 region. Green line, LOD score obtained with the phenotyping at 0.5 mM CaNO₃ (Courbot et al. 2007) and the 6 markers. The vertical dashed lines represent the LOD score threshold for QTL detection at an error level of α=0.05.

**Fig. 4:** Analysis of Cd tolerance in *A. thaliana* WT and *cax1-1* mutant. Comparison of Cd tolerance between *A. thaliana* WT (Col-0, grey bars) and *cax1-1* mutant (white bars) was based on (A) primary root length and (C) total shoot surface. Plants were sown *in vitro* on half-strength MS medium containing 1% sucrose solidified with agar. After one week, plantlets were transferred to half-strength MS agar plates containing low (0.5 mM) or moderate (2mM) Ca concentrations and 0 or 100 µM CdCl₂ for one week. Data are means ± SD, n=58-92 for primary root length measurements, n=25-47 for shoot surface analysis. *P < 0.05* (t-test). The representative phenotypes for root (B) and shoot (D) at Low Ca are report. Scale bars represent respectively 2cm for roots and 1cm for shoots.

**Fig. 5:** Cd tolerance assessed in *A. thaliana* K.O. mutant *cax3-1* at low Ca concentration. Comparison of Cd tolerance was performed between *A. thaliana* WT (grey bars), *cax1-1* (white bars) and *cax3-1* (striped bars). Tolerance was assessed *in vitro* by measuring primary root length as a parameter for growth. One week after germination, plantlets were transferred at low (0.5 mM) Ca concentration in control condition or with 100 µM CdSO₄ for another week. Data are means ± SD, n= 15-27. *P < 0.05* (t-test).

**Fig. 6:** Hydrogen peroxide (H₂O₂) detection and quantification in DAB-stained samples. *A. thaliana* (WT and *cax1-1*), *A. halleri* and *A. lyrata* roots were stained with DAB reagent in control condition (panels A, B, C and D) or after 72h at 10µM CdSO₄ treatment (E, F, G and H) at low Ca. Polymerization of DAB is visible as a brown precipitate in the presence of H₂O₂. Scale bars represent 50µm. **B.** The amount of H₂O₂ was measured in roots of *A. thaliana* WT (black bars), *cax1-1* (striped bars), *A. halleri* (white bars) and *A. lyrata* (checked bars). H₂O₂-DAB content in the samples was determined using a standard curve prepared with known amounts of DAB. Plants were grown in hydroponic solution with low or moderate Ca contaminated for 72h with 0µM CdSO₄ or 10µM CdSO₄. In order to obtain sufficient material for H₂O₂ quantification, roots (n=3) were pooled before measurements.

**Fig. 7:** *In vitro* tolerance to ROS-inducing reagent methyl-viologen (MV). Comparison between *A.
*thaliana* WT (Col-0, grey bars) and *cax1-1* mutant (white bars) was based on primary root length (A) and total shoot surface (B). Plants were grown at low (0.5 mM) and moderate (2mM) Ca concentrations, in control condition or with 0.1µM methyl-viologen. Data are means ± SD; n=20-30. * for *P* < 0.05 (t-test). Pictures of *A. thaliana* WT and *cax1-1* roots and shoots are reported in (C) and (D) respectively. Scale bars represent 2cm length.
REFERENCES


Clemens S, Aarts MGM, Thomine S, Verbruggen N (2013) Plant science: The key to


uptake and translocation by the hyperaccumulator Sedum alfredii. J Hazard Mater 183: 22–28


soil. New Phytol 187: 368–379


Wudick MM, Feijó J a (2014) At the Intersection: Merging Ca2+ and ROS Signaling Pathways in Pollen. Mol Plant 7: 1595–7


**Fig. 1:** Identification of *CAX1* as candidate gene for the QTL CdTol2. 

**A.** Densification of the QTL CdTol2. Five additional markers (in red) were used to confirm the localization and to reduce the CdTol2 locus to a 3 cM region. The vertical dashed lines represent the LOD score threshold (1.5) for QTL detection at an error level of \( \alpha = 0.05 \). Bars indicate the one-LOD (10-fold) support interval and whiskers (lines extending beyond bars) indicate the two-LOD (100-fold) support intervals. BC1 individuals were phenotyped at 0.5 mM CaNO\(_3\) in the study of Courbot et al. (2007).

**B.** *CAX1* transcript levels in BC1 individuals displaying the Ah/Alp (h) or Alp/Alp (a) allelic combinations at the CdTol2 locus. At least three cuttings for each BC1 genotype were transferred in hydroponic solution according to Courbot et al. (2007) for 4 weeks. Clones were pooled together and *CAX1* expression levels were assessed through qPCR analysis. Data are means ± SD \((n = 9)\). \(*P < 0.05\) (t-test).
Fig. 2: Expression analysis of CAX1. CAX1 transcript levels at (A) low Ca and (B) moderate Ca concentration in control condition (grey bars) and upon 72h 10µM CdSO₄ treatment (white bars) of *A. halleri*, *A. lyrata petraea* and *A. thaliana*. Data are means ± SD, n = 8-12. Lower case letters for comparison between conditions in the same species; capital letters for comparison between species in the same condition (Kruskall Wallis test). (C). Western blot showing relative levels of CAX1 protein in root from *A. halleri*, *A. lyrata* and *A. thaliana*. Samples have been collected from plants grown at low Ca concentration in control condition (lanes 1, 3 and 5) or upon 72h 10µM CdSO₄ treatment (lanes 2, 4 and 6). As negative control, we loaded total protein fraction extracted from *A. thaliana cax1-1* mutant (lane 7). Equal amounts of total protein (35ng) were loaded onto the gel.
**Fig. 3:** QTL mapping of Cd tolerance in the CdTol2 region at two different Ca concentrations. Red line, LOD score obtained with 125 BC1 individuals phenotyped for Cd tolerance at 2 mM CaNO₃ and genotyped for six markers in the CdTol2 region. Green line, LOD score obtained with the phenotyping at 0.5 mM CaNO₃ (Courbot et al. 2007) and the 6 markers. The vertical dashed lines represent the LOD score threshold for QTL detection at an error level of α=0.05.
**Fig. 4:** Analysis of Cd tolerance in *A. thaliana* WT and cax1-1 mutant. Comparison of Cd tolerance between *A. thaliana* WT (Col-0, grey bars) and cax1-1 mutant (white bars) was based on (A) primary root length and (C) total shoot surface. Plants were sown *in vitro* on half-strength MS medium containing 1% sucrose solidified with agar. After one week, plantlets were transferred to half-strength MS agar plates containing low (0.5 mM) or moderate (2 mM) Ca concentrations and 0 or 100 µM CdCl₂ for one week. Data are means ± SD, *n*=58-92 for primary root length measurements, *n*=25-47 for shoot surface analysis. *P* < 0.05 (t-test). The representative phenotypes for root (B) and shoot (D) at Low Ca are report. Scale bars represent respectively 2 cm for roots and 1 cm for shoots.
**Fig. 5:** Cd tolerance assessed in *A. thaliana* K.O. mutant *cax3-1* at low Ca concentration. Comparison of Cd tolerance was performed between *A. thaliana* WT (grey bars), *cax1-1* (white bars) and *cax3-1* (striped bars). Tolerance was assessed *in vitro* by measuring primary root length as a parameter for growth. One week after germination, plantlets were transferred at low (0.5 mM) Ca concentration in control condition or with 100 µM CdSO₄ for another week. Data are means ± SD, *n* = 15-27. *P* < 0.05 (t-test).
Fig. 6: Hydrogen peroxide (H$_2$O$_2$) detection and quantification in DAB-stained samples. A. A. thaliana (WT and cax1-1), A. halleri and A. lyrata roots were stained with DAB reagent in control condition (panels A, B, C and D) or after 72h at 10µM CdSO$_4$ treatment (E, F, G and H) at low Ca. Polymerization of DAB is visible as a brown precipitate in the presence of H$_2$O$_2$. Scale bars represent 50µm. B. The amount of H$_2$O$_2$ was measured in roots of A. thaliana WT (black bars), cax1-1 (striped bars), A. halleri (white bars) and A. lyrata (checked bars). H$_2$O$_2$-DAB content in the samples was determined using a standard curve prepared with known amounts of DAB. Plants were grown in hydroponic solution with low or moderate Ca contaminated for 72h with 0µM CdSO$_4$ or 10µM CdSO$_4$. In order to obtain sufficient material for H$_2$O$_2$ quantification, roots (n=3) were pooled before measurements.
Fig. 7: In vitro tolerance to ROS-inducing reagent methyl-viologen (MV). Comparison between A. thaliana WT (Col-0, grey bars) and cax1-1 mutant (white bars) was based on primary root length (A) and total shoot surface (B). Plants were grown at low (0.5 mM) and moderate (2mM) Ca concentrations, in control condition or with 0.1µM methyl-viologen. Data are means ± SD; n=20-30. * for P < 0.05 (t-test). Pictures of A. thaliana WT and cax1-1 roots and shoots are reported in (C) and (D) respectively. Scale bars represent 2cm length.
SUPPORTING INFORMATION
### Table S1: List of genes with gene ontology annotations for cadmium response or metal ion transport in the refined CdTol2 QTL

<table>
<thead>
<tr>
<th>Accession n.</th>
<th>Name</th>
<th>Description</th>
<th>A. thaliana</th>
<th>A. lyrata</th>
<th>Involved in</th>
<th>Located in</th>
</tr>
</thead>
<tbody>
<tr>
<td>At2g37760</td>
<td>AKR4C8</td>
<td>NADPH-dependent aldo-keto reductase</td>
<td>Chr2-15831</td>
<td>Scaffold 4: 18263</td>
<td>oxidation-reduction process, para-aminobenzoic acid metabolic process, response to cadmium ion, response to cold, response to salt stress, response to toxic substance, response to water deprivation</td>
<td>cytoplasm, cytosol</td>
</tr>
<tr>
<td>At2g38170</td>
<td>CAX1</td>
<td>high affinity vacuolar calcium antiporter</td>
<td>Chr2-15989</td>
<td>Scaffold 4: 18483</td>
<td>calcium ion transport, cellular cation homeostasis, cellular lipid catabolic process, cellular manganese ion homeostasis, cellular zinc ion homeostasis, cold acclimation, divalent metal ion transport, glucosinolate biosynthetic process, hydrogen ion transmembrane transport, phosphate ion homeostasis, regulation of protein localization, response to fructose, response to nematode, response to salt stress, water transport</td>
<td>cell, chloroplast, membrane, plant-type vacuolar membrane, vacuole</td>
</tr>
<tr>
<td>At2g39010</td>
<td>PIP2;6</td>
<td>Water channel activity</td>
<td>Chr2-16292</td>
<td>Scaffold 4: 18926</td>
<td>calcium ion transport, cellular cation homeostasis, cellular water homeostasis, defense response to bacterium, defense response, incompatible interaction, divalent metal ion transport, ion transmembrane transport, photosynthesis, light reaction, response to cold, response to fructose, response to nematode, response to salt stress, salicylic acid biosynthetic process, transport, water transport</td>
<td>plasma membrane, plasmodesma, vacuole</td>
</tr>
<tr>
<td>At2g39410</td>
<td>/</td>
<td>alpha/beta-Hydrolases superfamily protein</td>
<td>Chr2-16455</td>
<td>Scaffold 4: 19129</td>
<td>Transition metal ion transport</td>
<td>nucleus</td>
</tr>
</tbody>
</table>

Downloaded on February 12, 2021. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.
Table S2: Primer sequences.

Following primers have been used for AhCAX1 cloning, transcript analysis in the three species (A. halleri, A. thaliana and A. lyrata) and for gene copy number analysis. We designed these primers in conserved genomic and transcript regions of A. thaliana and A. lyrata.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cloning of CAX1 sequence</strong></td>
<td></td>
</tr>
<tr>
<td>cax1-seq_F1</td>
<td>GAACGGAAACCCAAGCATAA</td>
</tr>
<tr>
<td>cax1-seq_R1</td>
<td>TCAGCCAAAGGTGTCAGTCC</td>
</tr>
<tr>
<td>cax1-seq_F2</td>
<td>TGAGCGAGTCAGCTTTTTGA</td>
</tr>
<tr>
<td>cax1-seq_R2</td>
<td>TTGCTCCACATCGTCATCAT</td>
</tr>
<tr>
<td>cax1-seq_F3</td>
<td>ATGGTTGGTTGGAGGCACAC</td>
</tr>
<tr>
<td>cax1-seq_R3</td>
<td>TGATGAAGACATTTCCATTGGA</td>
</tr>
<tr>
<td>5'-RACE</td>
<td>AGCGATTTCCACACACAGA</td>
</tr>
<tr>
<td>3'-RACE</td>
<td>TCTTCTTGTTGGTGTTGGT</td>
</tr>
<tr>
<td>Ef1α_F</td>
<td>Talke et al. (2006)</td>
</tr>
<tr>
<td>Ef1α_R</td>
<td>Talke et al. (2006)</td>
</tr>
<tr>
<td>CAX1qPCR_F</td>
<td>GCGGGAATCGTGACAGAG</td>
</tr>
<tr>
<td>CAX1qPCR_R</td>
<td>GTGAGCGGTTCCTCCAAGTC</td>
</tr>
<tr>
<td>SHR_F</td>
<td>CCATGACTCGAAAGCAACCCTAAA</td>
</tr>
<tr>
<td>SHR_R</td>
<td>GTCGGAGAGAAGAGAAGGTGGCT</td>
</tr>
<tr>
<td>gCAX1_F</td>
<td>GAAGTCATTCTCGGAAACAAAGC</td>
</tr>
<tr>
<td>gCAX1_R</td>
<td>CTGAGCCACAAATAAGTGCAA</td>
</tr>
</tbody>
</table>

**Efficiency**

<table>
<thead>
<tr>
<th>A. thaliana</th>
<th>A. halleri</th>
<th>A. lyrata</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.95</td>
<td>1.94*</td>
<td>1.92</td>
</tr>
<tr>
<td>1.99</td>
<td>1.96</td>
<td>1.95</td>
</tr>
</tbody>
</table>

From Talke et al. (2006)
Table S3: List of markers used for the fine-mapping and the QTL mapping at 2mM CaCl₂.

The references indicate the first use of corresponding markers in an A. halleri x A. lyrata petraea progeny. For KBD assay see http://www.lgcgroup.com/services/genotyping/#.VL4mTy6G9zM.

<table>
<thead>
<tr>
<th>Location in A. thaliana (Kb)</th>
<th>Marker Name</th>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr.5-06938</td>
<td>Ah-86</td>
<td>At5g20500</td>
<td>TTGTGCAAATCGAAGGATCA</td>
<td>TATCTCGATGGCTGTGTTGC</td>
</tr>
<tr>
<td>Chr.2-11702</td>
<td>Ah-52</td>
<td>IGR</td>
<td>GAGAGTGCAGAGGAGGTTG</td>
<td>CTGTGGCGGAGGAAGA</td>
</tr>
<tr>
<td>Chr2-12200</td>
<td>Ah-109B</td>
<td>IGR</td>
<td>CTATTTCTCCTCTCTCTCTC</td>
<td>GAAGAATAAATTACCTGTGGAAG</td>
</tr>
<tr>
<td>Chr2-13477</td>
<td>Ah-108</td>
<td>At2g31690</td>
<td>AAGCAACCAGGAGTAGTGA</td>
<td>TCATGTTCACTATAATACACAC</td>
</tr>
<tr>
<td>Chr2-14144</td>
<td>Ah-43</td>
<td>At2g33310</td>
<td>CTCACCTTCCCCATCTCAA</td>
<td>TCATCAGCTCTCCACCCACCT</td>
</tr>
<tr>
<td>Chr2-15990</td>
<td>CAX1-261CT</td>
<td>At2g38170</td>
<td>KDB assay</td>
<td></td>
</tr>
<tr>
<td>Chr2-16492</td>
<td>R67</td>
<td>At2g39510</td>
<td>KDB assay</td>
<td></td>
</tr>
<tr>
<td>Chr2-17890</td>
<td>Ah-27</td>
<td>IGR</td>
<td>Frérot et al. 2010</td>
<td></td>
</tr>
<tr>
<td>Chr2-18989</td>
<td>Ah-74</td>
<td>at2g46225</td>
<td>Frérot et al. 2010</td>
<td></td>
</tr>
</tbody>
</table>

IGR, inter genomic region
<table>
<thead>
<tr>
<th>AT2G38170.1</th>
<th>MAGIVTEPWSVAENGNSITAKGSSRELLGRTAEHMSSSSLRKKSDLRVIQKVFKGLK</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.lyrata</td>
<td>MAGIVTEPWSVAENGNSITAKGSSRELLGRTAEHMSSSSLRKKSDLRVIQKVFKGLK</td>
</tr>
<tr>
<td>A.halleri</td>
<td>MAGIVTEPWSVAENGNSITAKGSSRELLGRTAEHMSSSSLRKKSDLRVIQKVFKGLK</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>DFLSNLVQILVTKAIIIFQPAPAALICTYCGVQPSIIFGLSSLGLLPLAEVSPFLTEQL</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>DFLSNLVQILVTKAIIIFQPAPAALICTYCGVQPSIIFGLSSLGLLPLAEVSPFLTEQL</td>
</tr>
<tr>
<td>A.halleri</td>
<td>DFLSNLVQILVTKAIIIFQPAPAALICTYCGVQPSIIFGLSSLGLLPLAEVSPFLTEQL</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>AFYTGPTLGGLLNATCGNATELIIAILALTNNKVAVVKYSLLGSILSSLLVLGTSLFCG</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>AFYTGPTLGGLLNATCGNATELIIAILALTNNKVAVVKYSLLGSILSSLLVLGTSLFCG</td>
</tr>
<tr>
<td>A.halleri</td>
<td>AFYTGPTLGGLLNATCGNATELIIAILALTNNKVAVVKYSLLGSILSSLLVLGTSLFCG</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>GIANIRREQRFRDQFADLNFFLLLGLCHLLPLLVEYLANQKTSAAVSLDMLQLSISRGF</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>GIANIRREQRFRDQFADLNFFLLLGLCHLLPLLVEYLANQKTSAAVSLDMLQLSISRGF</td>
</tr>
<tr>
<td>A.halleri</td>
<td>GIANIRREQRFRDQFADLNFFLLLGLCHLLPLLVEYLANQKTSAAVSLDMLQLSISRGF</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>SIVMLIYAIVLYFQILWQHRQFDAGDQEDEYDQDVEQETAVISPWSGFAWLVGMTLVIA</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>SIVMLIYAIVLYFQILWQHRQFDAGDQEDEYDQDVEQETAVISPWSGFWLVGMTLVIA</td>
</tr>
<tr>
<td>A.halleri</td>
<td>SIVMLIYAIVLYFQILWQHRQFDAGDQEDEYDQDVEQETAVISPWSGFWLVGMTLVIA</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>LLSEYVATIEEASDKWNLSSVFISIIIFIPVNGEAAHAGAVIFAPKNKKLDISLGVALGS</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>LLSEYVATIEEASDKWNLSSVFISIIIFIPVNGEAAHAGAVIFAPKNKKLDISLGVALGS</td>
</tr>
<tr>
<td>A.halleri</td>
<td>LLSEYVATIEEASDKWNLSSVFISIIIFIPVNGEAAHAGAVIFAPKNKKLDISLGVALGS</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>ATQIGLFVPLTIVWAVILGIMNLNGFQGLETCLAVSIITAIQLDQDSHSHYMGKLVL</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>ATQIGLFVPLTIVWAVILGIMNLNGFQGLETCLAVSIITAIQLDQDSHSHYMGKLVL</td>
</tr>
<tr>
<td>A.halleri</td>
<td>ATQIGLFVPLTIVWAVILGIMNLNGFQGLETCLAVSIITAIQLDQDSHSHYMGKLVL</td>
</tr>
<tr>
<td>AT2G38170.1</td>
<td>LCYFIIAICFFVDKLPQKNAIHL HGQAMNNTVIATGCGGVESS*-----------------457</td>
</tr>
<tr>
<td>A.lyrata</td>
<td>LCYFIIAICFFVDKLPQKNAIHL HGQAMNNTVIATGCGGVESS*-----------------457</td>
</tr>
<tr>
<td>A.halleri</td>
<td>LCYFIIAICFFVDKLPQKNAIHL HGQAMNNTVIATGCGGVESS*-----------------439</td>
</tr>
</tbody>
</table>

**Fig. S1:** Alignment of the amino-acid sequences of AtCAX1 peptide (derived from AtCAX1 cDNA splicing variant AT2G38170.1), AICAX1 and the predicted sequence of AhCAX1. In grey, amino-acids that are conserved among AtCAX1 and AICAX1 but not in AhCAX1. Boxes represent the functional domains: auto-inhibitory regulation (red), interaction for the auto-inhibition (green), exchange activity (blue), cation selection (purple). *, stop codon. AhCAX1 deduced protein shares respectively 97% and 95% of sequence identity with AICAX1 and AT2G38170.1 proteins.
**Fig. S2:** Genomic copy number of CAX1 in *A. thaliana* and *A. halleri*. Data obtained from qPCR analysis for *A. thaliana* (grey bar) and *A. halleri* (white bar) were normalized with CT values for SHR gene. Data are means ± SD; *n*=3 and 5, respectively. *A. thaliana* genomic DNA served as calibrator.
**Fig. S3:** Detection and quantification of superoxide anion (O$_2^-$). Root samples of *A. thaliana* (WT and cax1-1), *A. halleri* and *A. lyrata* plants grown at low Ca concentration were stained with NBT agent and detection of O$_2^-$ was performed using an optical microscope. Panels from A to D show samples in control condition, panels from E to H samples after 72h of 10µM CdSO$_4$ treatment. NBT accumulation is visible as a blue precipitate when the staining agent is bounded to O$_2^-$. Scale bars represent 50µm. **B.** The amount of O$_2^-$-NBT complex in the samples was determined using a standard curve prepared with known amounts of NBT. In order to obtain sufficient material for O$_2^-$ quantification, roots (n=3) were pooled before measurements. Scale bars represent 50µm.


Wudick MM, Feijó J a (2014) At the Intersection: Merging Ca2+ and ROS Signaling Pathways in Pollen. Mol Plant 7: 1595-7

