Cadmium-inducible expression of the ABC-type transporter *AtABCC3* increases phytochelatin-mediated cadmium tolerance in Arabidopsis

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Date of submission: 16/03/2015
Figures: 8
Colour figures in print: 7
Number of total words: 9588
Supplementary figures: 4
Running head: AtABCC3 is involved in cadmium tolerance

AtABCC3 detoxifies cadmium by transporting phytochelatin-cadmium complexes into the vacuoles, and it can functionally complement *abcc1 abcc2* mutants.

Abstract

The heavy metal cadmium (Cd) is a widespread environmental contaminant with harmful effects on living cells. In plants, phytochelatin (PC)-dependent Cd detoxification requires that PC-Cd complexes are transported into vacuoles. Here, we show that *Arabidopsis thaliana* seedlings defective in the ABCC transporter AtABCC3 (*abcc3*) have an increased sensitivity to different Cd concentrations, and that seedlings overexpressing *AtABCC3* (AtABCC3ox) have an increased Cd tolerance. Cellular distribution of Cd was analysed in protoplasts from *abcc3* mutants and *AtABCC3* overexpressors grown in the presence of Cd, by means of the Cd-specific fluorochromes BTC-5N and Leadmium™ Green AM dye. This analysis revealed that Cd is mostly localized in the cytosol of *abcc3* mutant protoplasts whereas there is an increase in vacuolar Cd in protoplasts from AtABCC3ox plants. Overexpression of *AtABCC3* in *cad1-3* mutant seedlings defective in PC production and in plants treated with L-buthionine sulfoximine (BSO), an inhibitor of PC biosynthesis, had no effect on Cd tolerance, suggesting that *AtABCC3* acts via PCs. In addition, overexpression of *AtABCC3* in *atabcc1 atabcc2* mutant seedlings defective in the Cd transporters AtABCC1 and AtABCC2 complements Cd sensitivity of double mutants, but not in the presence of BSO. Accordingly, the level of *AtABCC3* transcript in wild type seedlings was lower than that of *AtABCC1* and *AtABCC2* in the absence of Cd but higher after Cd exposure, and even higher in *atabcc1 atabcc2* mutants.

Our results point to AtABCC3 as a transporter of PC-Cd complexes, and suggest that its activity is regulated by Cd and is coordinated with the activity of AtABCC1/AtABCC2.

Key words: Cadmium stress, Arabidopsis, ABC-type transporters, Cadmium

tolerance, Phytochelatins, Vacuolar compartmentalization.

Introduction

Cadmium is a heavy metal that exerts a detrimental effect on plants and on human health by interfering with biochemical functions of essential metals. Higher plants respond to Cd exposure by producing phytochelatins (PCs), cysteine-rich peptides with the general structure (Glu-Cys) *n*-Gly where *n* is in the range of 2-11 (Grill *et al*., 1985; Rauser, 1990). Phytochelatins also protect plants from the toxic effects of other heavy metals/metalloids such as Pb, Hg and As., and have also been identified in the majority of algae, in fungi, including *Schizosaccharomyces pombe*, and in the worm *Caenorhabditis elegans* (Ha *et al*., 1999). Phytochelatins are synthesized by phytochelatin synthase (PCS) from the substrate glutathione (GSH) (Grill *et al*., 1989; Thangavel *et al*., 2007), and *PCS* genes were first isolated from *Arabidopsis thaliana, S. pombe, Triticum aestivum* and *C. elegans* (Ha *et al*., 1999; Vatamiunik *et al*., 1999; Cobbett CS, 2000*a, b*; Clemens *et al*., 1999, 2001). Subsequently *PCS* genes have been isolated from different plants such as *Brassica juncea* (Heiss *et al*., 2003) and invertebrate species such as the slime mould *Dictyostelium discoideum* (Cobbett CS, 2000*a*).

Phytochelatins are able to bind cytoplasmic Cd forming stable PC-Cd complexes, playing a major role in cadmium detoxification: PC-deficient mutants of *S. pombe* and Arabidopsis *-cad1,* mutated in *AtPCS1-* are hypersensitive to Cd (Ha *et al.*, 1999); accordingly, in most species *PCS* overexpression leads to increased Cd tolerance (Vatamaniuk *et al*., 1999; Gisbert *et al.*, 2003; Sauge-Merle *et al.*, 2003; Martinez *et al.*, 2006; Pomponi *et al.*, 2006; Gasic and Korban, 2007; Guo *et al*., 2008; Wojas *et al.*, 2010; Brunetti *et al.*, 2011). The mechanism of detoxification mediated by PCs requires that PC-Cd complexes are transported by specific proteins into the vacuoles where they form more stable high molecular weight complexes by sulphide bonds. In addition, Cd that can be transported directly into vacuoles by vacuolar Ca^{2+}/H^{+} antiporters (Salt and Wagner, 1993; Clemens *et al.*, 2001). Early experiments on isolated vacuoles from *Avena sativa* roots suggested that PC-Cd complexes transport is mediated

by ABC-type transporters (ATP-binding cassette; Salt and Rauser, 1995), ubiquitous transmembrane proteins that utilize ATP to translocate various substrates across membranes. ABC proteins have a characteristic modular structure consisting of a double set of two basic structural elements, a hydrophobic transmembrane domain (TMD) usually made of six membrane-spanning α-helices, and a cytosolic domain containing a nucleotide-binding domain (NBD) involved in ATP binding (Wanke and Kolukisaoglu, 2010); the two TMDs dimerize to form the substrate binding cavity (Procko *et al.*, 2009). The first protein that has been assigned a role as PC-Cd vacuolar transporter has been the half ABC transporter molecule HMT1 in *S. pombe* (HEAVY METAL TOLERANCE-FACTOR1); this transporter, which has only one NBD and one TMD domain, needs to homo- or heterodimerize to become functional (Ortiz *et al.*, 1995). Subsequently, *HMT1*-homologs have been identified in *C. elegans* (Vatamaniuk *et al.*, 2005) and in *Drosophila melanogaster* (Sooksa-Nguan *et al.*, 2009) but not in higher plants. More recently an ABCC-type transporter Abc2, (belonging to the ABCC/MRP subfamily of ABC transporters), has been identified as the main PC-Cd transporter in *S. pombe* (Mendoza-Cózatl *et al.*, 2010). On the other hand, it has been shown that in *Saccharomyces cerevisiae*, which lacks PCS and does not produce PCs, the ABCC-type transporter YCF1 is able to transport GSH-Cd complexes into the vacuole (Li *et al.*, 1997), and overexpression of *ScYCF1* increases Cd tolerance in Arabidopsis seedlings (Song *et al.*, 2003).

In Arabidopsis, the ABCC family consists of 15 ABC proteins, characterized by the presence of an additional N-terminal transmembrane domain (TMD0) of unknown function (Klein *et al.*, 2006), although it has been shown that in some human and yeast ABCCs TMD0 is involved in protein targeting. Most ABCC proteins are localized in the vacuolar membrane and have been considered good candidates as transporters of PC-heavy metal complexes. In particular, AtABCC3, AtABCC4, and AtABCC7 when expressed individually in *S. cerevisiae* are able to complement the loss of YCF1, partially restoring Cd tolerance (Klein *et al.*, 2006). Very recently, it has been shown that AtABCC1 and AtABCC2 - first identified as transporters of PC-Arsenic complexes play a role in Cd (and Hg) tolerance

(Park *et al.*, 2012). However, it has not yet been established whether AtABCC3, which is also upregulated by Cd treatment together with AtABCC6 and AtABCC7 (Gaillard *et al.*, 2008), plays also a role in PC-mediated Cd detoxification. Here, by analysis of Cd tolerance of *abcc3* knockout mutants defective in *AtABCC3*, and by *AtABCC3* overexpression in wild type, PC-deficient lines, and *atabcc1 atabcc2* double mutants, combined with analysis of cellular Cd localization, and comparative analysis of Cd tolerance between *abcc3* and *atabcc1 atabcc2* double mutants, we show that AtABCC3 is involved in the vacuolar transport of PC-Cd complexes.

Material and Methods

Plant growth conditions and metal treatments

Wild type, mutant lines *abcc3* -kindly provided by Markus Klein (Philip Morris International, Switzerland)- *abcc1 abcc2* (Song et al., 2010) -kindly provided by Enrico Martinoia (University of Zurich, Switzerland)- *cad1-3* (Cobbett CS, 2000*a*) -kindly provided by Chris Cobbett (University of Melbourne, Australia)- AtPCSox-21, AtPCSox-20, AtPCSox-26, AtABCC3ox-*cad1*-53 AtABCC3ox-*cad1*-59, AtABCC3ox-*abcc1abcc2*-1, AtABCC3ox-*abcc1abcc2*-3 and AtABCC3ox-*abcc1abcc2*-5 seedlings were germinated on half strength MS basal agar medium (pH 5.8) (Murashige and Skoog, 1962) in a growth chamber in a 16/8 h light/dark cycle at 22 °C. After 7 days, 10 seedlings were transferred to a half-strength MS basal medium with 0.5% sucrose, at different concentrations of $CdSO_4$ (0, 15, 30, 60, 90 μM) in the presence of 10 μM β-estradiol when indicated. Seedlings fresh weight and root length were measured after 5 or 9 days of further growth.

To assess the effect of BSO on Cd sensitivity, 7-d-oldseedlings were transferred to medium containing 60 μM $CdSO_4$ with or without 0.5 mM BSO. Seedlings fresh weight and root length were measured after 9 days of further growth.

The experiments were performed in triplicate.

To analyse Cd content two experiments were performed as follows:

a) Seven days after germination approximately 50 seedlings for each plant were placed into holes of a plastic septum in phytatray (SIGMA), so that only roots were immersed in liquid medium. A half-strength MS (0.5% sucrose) was supplemented with 10 μM β-estradiol and 60 μM $CdSO_4$ was added. Seedlings, shaken occasionally, were harvested after 9 days.

b) Seven days after germination approximately 130 seedlings for each line were transferred to a half-strength MS basal medium with 0.5% sucrose, at 30 or 60 μM $CdSO_4$ in the presence of 10 μM β-estradiol. Seedlings were harvested after two weeks. The experiments were performed in triplicate.

Plant expression construct, transformation, and selection

An XbaI-XbaI fragment harbouring the coding region of *AtABCC3* was cloned into the SpeI site of the binary plasmid pER8, under the control of an estrogen-inducible promoter (Zuo *et al.*, 2000). *Agrobacterium tumefaciens* strain GV3101 carrying the construct *pER8::35S-ABCC3* was used to transform *Arabidopsis thaliana* wild type plants (ecotype Columbia) by standard dip floral transformation (Clough and Bent, 1998). Transformed plants were analysed by PCR with the following primers: LexA 4096 For 5'-GCCATGTAATATGCTCGACT-3', MRP3 Rev 4467 5'-GAGCTGACTTAAACCCAAAAT-3'and by real-time RT-PCR (see below). Homozygous T2 generations were obtained by self-fertilization of primary transformants and the seeds were grown as described below (Cecchetti *et al.*, 2008).

Quantitative RT-PCR analysis

RNA was extracted from 50 mg of seedlings grown at the indicated $CdSO_4$ concentration in the presence or absence of the inducer β-estradiol and reverse-transcribed as previously described. SYBR Green-based quantitative assays were performed using a Bio-Rad iCicler iQ as described in Cecchetti *et al.* (2013). The primers used to analyse *AtABCC3* transcript levels were:

RTmrp3 For 3835 5'-CTTCAGGTCCGATATGCTCCA-3', RTmrp3 Rev 3885 5'-TGTTATTCCTCGCAACACAAGAG-3'; ACTIN2 For 5'-

CCGATCCAGACACTGTACTTCCTT-3' ACTIN2 Rev 5'-CTTGCACCAAGCAGCATGAA-3', and were designed as previously described (Cecchetti *et al.*, 2004). The experiments were performed in triplicate.

Cross pollination

Homozygous *cad1-3* lines were used for crosses with homozygous AtABCC3ox-21 lines. F2 lines, homozygous for AtABCC3ox construct and for *cad1-3* mutation, were selected on hygromycin, and *cad1-3* mutation was verified by PCR with the following primers: *cad1-3* For 5'-TCAAGTATCCCCCTCACTGC-3'; *PCS1* For 5'-TCAAGTATCCCCCTCACTGG-3'; *PCS1* Rev 5'-CGGGTTCTCTGTGTGGTCTA-3'. Three independent homozygous lines named AtABCC3ox-20, AtABCC3ox-21 and AtABCC3ox-26 were used for subsequent Cd tolerance analysis.

Statistical analysis

Two-tailed and one-tailed Student's t tests were used to evaluate statistical significance. All the statistical analyses were performed using Graph Pad Prism 5 (Graph Pad Software Inc.).

Intracellular Cd localization through Cd-sensing fluorescent dyes

Wild type, *abcc3* and AtABCC3ox seedlings were grown onto half-strength MS agarized medium in the absence or presence of 60 μM $CdSO_4$. 10 μM β-estradiol was added in experiments conducted with AtABCC3ox lines when indicated. Leaf protoplasts were prepared from wild type and *abcc3* plants after 9 or 22 days of treatment, whereas from AtABCC3ox lines after 5 and 9 days of treatment. The enzymatic digestion was carried according to Lindberg *et al.* (2004). The same number of isolated protoplasts from wild type, *abcc3* and AtABCC3ox were loaded either with 0.5% of 5-nitrobenzothiazole coumarin (BTC-5N) (Lindberg *et al*., 2004) in DMSO/ Pluronic aqueous solution (Molecular Probes, Leiden, the Netherlands) or 0.5% Leadmium™ Green AM dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA) in DMSO, and treated as described for BTC-5N. The

fluorescence signal was observed using a DMRB microscope equipped with a specific filter sets (excitation at 415 nm and emission at 500-530 nm for BTC-5N, and excitation at 484/15 nm and emission at 517/30 nm for Leadmium™ Green AM dye). Images were acquired with a LEICA DC500 digital camera and analysed with the IM1000 image-analysis software (Leica). Regions inside the vacuole and within the cytosol were selected from 30 single protoplast images per genotype and the mean intensity value of the epifluorescence was quantified using the ImageJ 1.36 b analysis software (National Institute of Health, Bellevue, WA, USA) and expressed in arbitrary units (AUs, from 0 to 255). The experiment was repeated three times; data from one experiment was reported.

Cadmium accumulation through ICP analysis

Wild type, AtABCC3ox-20 and AtABCC3ox-21 seedlings, cultured as described above, were washed with distilled water- shoots and roots separated when necessary- and dried at 80°C overnight. Dried tissues were weighed and then ground in a mortar. Homogenized material was mineralized in a microwave oven (Milestone Ethos 1600) with HNO_3 and H_2O_2 (3:1) under high temperature and pressure. Mineralized samples were analysed for total Cd detection, using ICP-MS (ThermoFisher X Serie II). All analyses were performed in three replicates. The amounts of acids used were the same as the amounts of additives to the digested samples in the digestion batch. Analytical accuracy was determined using certified reference material of the Community Bureau of Reference.

Results

Cd tolerance is decreased in *abcc3* mutants and enhanced in *AtABCC3* overexpressors

To assess whether *AtABCC3* contributes to Cd tolerance, the growth of wild type and *abcc3* seedlings was analysed at different Cd concentrations. In a previous paper, we showed that growth of Arabidopsis seedlings is not affected at Cd concentrations up to 15 µM, while is slightly reduced at 30 and 60 µM $CdSO_4$, and severely inhibited at 90 µM (Brunetti *et al.*, 2011).

Here, 7 days after germination, wild type and *abcc3* seedlings were grown in the presence of 0, 15, 30, 60 and 90 µM $CdSO_4$, and fresh weight and root length were analysed after 9 days. As shown in Fig. 1, in the absence of Cd and at 15 µM $CdSO_4$, the growth of *abcc3* seedlings and that of wild type ones were comparable, whereas in the presence of all Cd concentrations from 30 µM onwards the former was slightly but significantly more inhibited than the latter (Fig. 1A-C). In terms of fresh weight, the growth of *abcc3* seedlings was inhibited from 30 µM $CdSO_4$ concentration onwards (Fig. 1A-C), whereas roots were significantly shorter only at 30 and 60 µM $CdSO_4$ (Fig. 1B and C).

These results suggest an involvement of *AtABCC3* in Cd tolerance, and to confirm this notion we produced Arabidopsis lines overexpressing *AtABCC3* (AtABCC3ox) under the control of a β-estradiol-inducible promoter (Zuo *et al.*, 2000). Overexpression of *AtABCC3* was analysed by means of real-time RT-PCR (qRT-PCR) in three independent homozygous lines named AtABCC3ox-20, AtABCC3ox-21 and AtABCC3ox-26. Seedlings from wild type and these AtABCC3ox lines were grown in the presence of 60 µM $CdSO_4$ with or without the inducer β-estradiol, and *AtABCC3* transcript levels were analysed after 9 days of growth. As shown in Fig. 2A, the *AtABCC3* mRNA level increased about 15-, 17- and 13-fold compared to wild type in AtABCC3ox-20, AtABCC3ox-21 and AtABCC3ox-26 seedlings, respectively.

We ruled out an effect of β-estradiol on seedling growth, as no significant differences in fresh weight and root length were observed between wild type and AtABCC3ox seedlings after 9 days of growth in the presence or absence of β-estradiol, without Cd (Fig. S1).

To assess Cd tolerance, AtABCC3ox-20, AtABCC3ox-21 and AtABCC3ox-26 seedlings were grown in the presence of 0, 30, 60 and 90 µM $CdSO_4$ with or without β-estradiol, and fresh weight and root length analysed after 9 days. No significant differences in either growth indicators were observed at 30 µM $CdSO_4$ (Fig. 2B and C) in any of the AtABCC3ox seedlings grown in presence or absence of the inducer. At 60 µM $CdSO_4$, all three AtABCC3ox lines showed a significant increase in root length when grown in the presence of the inducer (Fig. 2C and D), whereas fresh weight

was comparable in seedlings grown in the presence or absence of β-estradiol (Fig. 2B).

At 90 μM $CdSO_4$, all three AtABCC3ox lines showed a significant increase in root length (Fig. 2C), but not in fresh weight when grown in the presence of the inducer (Fig. 2B).

These results confirm an involvement of *AtABCC3* in Cd tolerance.

AtABCC3 is involved in vacuolar Cd^{2+} sequestration

To determine whether AtABCC3 plays a role in Cd transport into the vacuole, the cellular distribution of Cd was compared in wild type and *abcc3* mutants by means of selective Cd-sensing fluorochromes: BTC-5N (Lindberg *et al.*, 2004, 2007) and Leadmium™ Green AM dye (Lu *et al.*, 2008), specific for cytosolic and vacuolar Cd accumulation, respectively. Wild type protoplasts have been preliminary used to define the cytosolic and vacuolar regions independently of the fluorescence, as shown in Fig. S2. Leaf protoplasts were isolated from wild type and *abcc3* plants grown in the absence or presence of 60 μM $CdSO_4$ for 9 and 22 days, and loaded with either one of the two fluorochromes.

As shown in Fig. 3, BTC-5N-loaded protoplasts isolated from wild type and *abcc3* plants grown in the absence of $CdSO_4$ exhibited an orange-green signal due to red chlorophyll autofluorescence, and a green signal due to complexes between the fluorochrome and cytosolic divalent ions other than Cd (Fig. 3A, C, I, K and Q left panel). When wild type and *abcc3* plants were cultured in the presence of Cd, after 9 days BTC-5N-loaded protoplasts showed a comparable Cd-specific cytosolic fluorescence signal (Fig. 3B, D and Q left panel), whereas after 22 days the Cd-cytosolic signal decreased in wild type protoplasts but significantly increased in *abcc3* protoplasts ($P<0.01$) (Fig. 3J, L and Q left panel).

Leadmium green-loaded protoplasts isolated from wild type and *abcc3* plants grown in the absence of Cd had a very low fluorescence signal that could be detected in the vacuole by quantitative analysis (see Material and Methods) (Figs 3Q right panel and 4M) but was not detectable in fluorescence images (Fig. 3E, G, M and O). This is possibly due to interactions between the fluorochrome and Ca^{2+} that occur in the absence of

Cd. When wild type and *abcc3* protoplasts from plants cultured for 9 days in the presence of Cd were analysed, a slightly but significantly higher ($P<0.05$) fluorescence signal was detectable in the vacuoles of the former (Fig. 3F) than in those of the latter (Fig. 3H and Q right panel). After 22 days in the presence of Cd the vacuolar signal was almost unchanged in wild type vacuoles (Fig. 3N), whereas in vacuoles of *abcc3* protoplasts became significantly lower ($P<0.01$) than in wild type (Fig. 3P and Q right panel).

These results indicate a decrease in vacuolar Cd and a concomitant increase in cytosolic Cd in *abcc3* mutant protoplasts compared to wild type ones, suggesting a role of ABCC3 in Cd transport into the vacuole.

To confirm the involvement of ABCC3 in Cd compartmentalization, the vacuolar Cd signal was analysed in two different AtABCC3ox lines. To detect a possible increase in vacuolar Cd, AtABCC3ox-21 and AtABCC3ox-26 plants were grown in the presence of 0 and 60 μM $CdSO_4$, with or without β-estradiol; leaf protoplasts isolated after 5 or 9 days were loaded with Leadmium™ Green AM dye. After 5 days of treatment with Cd, protoplasts from AtABCC3ox-21 and AtABCC3ox-26 plants grown in the presence of β-estradiol showed a significant increase ($P<0.05$ and $P<0.01$, respectively) in the vacuolar signal (Fig. 4C, F and M left panel) compared to protoplasts grown without β-estradiol (Fig. 4B and E). Analogously, after 9 days of treatment with $CdSO_4$ in the presence of β-estradiol, both AtABCC3ox-21 and AtABCC3ox-26 protoplasts exhibited a vacuolar signal (Fig. 4I, L and M right panel) significantly higher ($P<0.01$) than that of protoplasts from plants grown without β-estradiol (Fig. 4H and K).

We also analysed the Cd cytosolic signal in protoplasts from AtABCC3ox-21 and AtABCC3ox-26 plants. After 9 days of treatment with Cd in the presence of β-estradiol, AtABCC3ox-21 and AtABCC3ox-26 protoplasts exhibited a cytosolic signal (Fig. 5C, F and G) significantly lower ($P<0.01$) than that of protoplasts grown in the absence of β-estradiol (Fig. 5B and E). These results indicate a lower cytosolic Cd accumulation and a corresponding increase in vacuolar Cd in AtABCC3ox protoplasts.

All in all, these data on the cellular distribution of Cd in *abcc3* and in AtABCC3ox leaf protoplasts indicate that AtABCC3 plays an essential role in vacuolar cadmium sequestration.

To determine whether in ABCC3ox lines the increase in vacuolar Cd corresponds to an increase in total Cd accumulation, we analysed Cd content in wild type, AtABCC3ox-21 and AtABCC3ox-26 seedlings by means of Inductively Coupled Plasma Mass Spectrometry (ICP). After 9 days of treatment with 60 µM $CdSO_4$ in the presence of β-estradiol, wild type, AtABCC3ox-21 and AtABCC3ox-26 seedlings showed comparable content of total Cd (624 ± 51.6, 696 ± 12.22 and 698 ± 40.01 µg g^{-1} FW, respectively).

To confirm this data we analysed Cd content, separately in shoots and roots from wild type, AtABCC3ox-21 and AtABCC3ox-26 seedlings exposed for two weeks at 30 or 60 µM $CdSO_4$. As shown in Figure 5, no significant difference in Cd content was observed at these Cd concentrations, in roots (Figure 5H) or shoots (Figure 5I) -as well as in seedlings (Figure 5J)- of the overexpressing lines compared to the wild type. Altogether this data rules out an effect of *AtABCC3* overexpression on Cd accumulation.

Overexpression of *AtABCC3* has no effect on Cd tolerance of seedlings lacking or with reduced PC synthesis

To assess whether vacuolar sequestration of Cd by AtABCC3 is mediated by PCs, we overexpressed *AtABCC3* in a *cad1-3* mutant line defective in PCS and, consequently, in PC production (Howden *et al.*, 1995). We generated AtABCC3ox-*cad1* plants by crossing AtABCCox-21 with *cad1-3* lines and we analysed *AtABCC3* overexpression in different lines homozygous for the *cad1* mutation and the AtABCC3ox construct. Two lines AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 lines overexpressing *AtABCC3* in the presence of β-estradiol (Fig. S3A) were used for subsequent analysis. To assess Cd tolerance, AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 seedlings together with seedlings of the two parental lines *cad1-3* and AtABCC3ox-21, were grown in the presence of 0, 30 and 60 µM $CdSO_4$ with or without β-estradiol. After 9 days root length and fresh weight were analysed. As shown in Fig. 6A and Fig. S3B, in the

absence of β-estradiol at 30 and 60 µM $CdSO_4$, *cad1-3*, AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 seedling growth was completely inhibited, whereas root length and fresh weight of AtABCC3ox-21 seedlings are comparable to that of wild type seedlings (Fig. 2B and C). In the presence of β-estradiol, (Figs 6A and S3B) both concentrations of Cd resulted toxic to AtABCC3ox-*cad1*-53, AtABCC3ox-*cad1*-59 pointing to a lack of effect of *AtABCC3* overexpression in growth rescue in the absence of PCs, and suggesting that AtABCC3 acts in concert with PCs to control Cd tolerance.

Interestingly, at 60 µM $CdSO_4$ while AtABCC3ox-21 seedlings showed, as described above, a significant increase in root length upon addition of β-estradiol (Figs 6A and 2C), the growth of *cad1-3*, AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 seedlings was unaffected by addition of the inducer (Figs 6A and S3B). To determine whether *ABCC3* overexpression enhances Cd tolerance at lower Cd concentrations that only slightly affect *cad1-3* seedling growth, AtABCC3ox-*cad1*-53, AtABCC3ox-*cad1*-59, *cad1-3* and AtABCC3ox-21 seedlings were grown in the presence of 15 µM $CdSO_4$ with or without the inducer β-estradiol. As shown in Fig. 6A and B, after 9 days in the absence of β-estradiol AtABCC3ox-21 seedlings show a growth comparable to that without Cd, whereas *cad1-3*, AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 seedling growth was slightly but significantly inhibited in terms of root length (Fig. 6A). In the presence of the inducer, the growth of AtBCC3ox-21 seedlings is comparable to that of seedlings grown without the inducer or without Cd (Fig. 6A and B). More interestingly, when grown in the presence of β-estradiol AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 are as much inhibited in growth as the parent line *cad1-3* in terms of root length (Fig. 6A and B) but not of fresh weight (Fig. S3B).

To provide further evidence that the effect of AtABCC3 is mediated by PCs, we assessed Cd tolerance of AtABCC3ox-21 and AtABCC3ox-26 seedlings in the presence of BSO, an inhibitor of γ -glutamylcysteine synthetase (-GCS), an enzyme that modulates GSH and PC synthesis (Howden and Cobbett 1992). AtABCC3ox-21 and AtABCC3ox-26 seedlings were grown at 60 µM $CdSO_4$ with or without β-estradiol, in the presence or absence of 0.5 mM BSO, and root length was measured after 9

days. As shown in Fig. 6C and D, the increase in Cd tolerance observed in AtABCC3ox-21 and AtABCC3ox-26 seedlings when exposed to Cd in the presence of β-estradiol, was not observed when BSO was added to the medium.

These results indicate that when PC biosynthesis is abated or reduced, Cd severely affects Arabidopsis growth even when *AtABCC3* is overexpressed.

AtABCC3 contributes to Cd tolerance and its expression is regulated by Cd

It has been reported that *AtABCC1* and, to a lesser extent, *AtABCC2* have a key role in Cd tolerance (Park *et al.*, 2012). To determine the contribution of *AtABCC3* on Cd tolerance relative to *AtABCC1* and *AtABCC2*, the growth of wild type, *abcc3* and *atabcc1 atabcc2* double mutant seedlings was comparatively analysed at high Cd concentration (60 µM) where *AtABCC3* was shown to have an effect (see above). After 9 days in the absence of Cd the growth of wild type, *abcc3* and *atabcc1 atabcc2* seedlings was comparable, whereas in the presence of 60 µM $CdSO_4$ the growth of all seedlings was inhibited and, interestingly, *atabcc1 atabcc2* seedling growth was only slightly more inhibited than that of *abcc3* in terms of root length and fresh weight. This suggests a substantial contribution of AtABCC3 to Cd tolerance (Fig. 7A-C).

We have shown above that ABCC3 acts in the transport of PC-Cd complexes as do ABCC1 and ABCC2: we then asked whether ABCC3 could complement the *abcc1 abcc2* double mutation. To perform a complementation assay we produced Arabidopsis *abcc1 abcc2* lines overexpressing *AtABCC3* by transforming *abcc1 abcc2* double mutant plants with the construct *pER8::35S-ABCC3* (see Material and Methods). Overexpression of *AtABCC3* was measured by means qRT-PCR in three independent homozygous lines denominated AtABCC3ox-*abcc1abcc2*-1, AtABCC3ox-*abcc1abcc2*-3 and AtABCC3ox-*abcc1abcc2*-5 (see Fig. S3C). We assessed Cd tolerance of AtABCC3ox-*abcc1abcc2*-1 and AtABCC3ox-*abcc1abcc2*-3 seedlings at 0 and 60 µM $CdSO_4$ with or without β-estradiol, in the presence or absence of 0.5 mM BSO. After 9 days, seedling fresh weight and root length were analysed. As shown in Fig. 7D-F, a significant

increase in both fresh weight and root length was observed in AtABCC3ox-*abcc1abcc2*-1 and AtABCC3ox-*abcc1abcc2-3* seedlings grown with β-estradiol compared to uninduced seedling.

The increase in root length of AtABCC3ox-*abcc1abcc2*-1 was not observed in the presence of 0.5 mM BSO (Fig. 7D-F), indicating that the observed BSO effect is specific for the transporter ABCC3.

To determine whether the relative transcript levels of *AtABCC1*, *AtABCC2* and *AtABCC3* are consistent with the above reported Cd tolerance of *abcc3* and *atabcc1 atabcc2* seedlings, a qRT-PCR analysis of mRNA extracted from wild type, *abcc3* and *atabcc1 atabcc2* seedlings grown for 9 days at 0 or 60 µM $CdSO_4$ was performed. As shown in Fig. 8A, in the absence of Cd the transcript levels of *AtABCC1* and *AtABCC2* are, respectively, 4- and 2-fold higher than that of *AtABCC3*. In contrast, at 60 µM $CdSO_4$ the transcript levels of *AtABCC1* and *AtABCC2* do not increase, whereas the transcript relative to *AtABCC3* increases by 6.9-fold, resulting 1.7- and 3.4-fold higher than, respectively, that of *AtABCC1* and *AtABCC2*. Interestingly, in *abcc3* mutants at 60 µM $CdSO_4$ the transcript levels of *AtABCC1* and *AtABCC2* are comparable to those of wild type seedlings, whereas in *atabcc1 atabcc2* seedlings the level of *AtABCC3* transcript further increases, compared to that of wild type seedlings, resulting 3.2- and 6.8-fold higher than that of, respectively, *AtABCC1* and *AtABCC2*. The Cd-induced high level of *AtABCC3* transcript accounts for the slight differences in Cd sensitivity between *abcc3* and *atabcc1 atabcc2* seedlings at 60 µM $CdSO_4$ concentration (Fig. 7A-C).

To determine whether the relative slight differences in growth in the presence of Cd between *abcc3* and *atabcc1 atabcc2* mutants would be shown when Cd was added during the germination phase - see Park *et al.*, (2012)- the same Cd tolerance assay was performed by incubating wild type, *abcc3* and *atabcc1 atabcc2* seeds on a medium containing 60 µM $CdSO_4$. As shown in Fig. S4A, after 14 days in the absence of Cd the growth of *abcc3* seedlings and that of wild type and *atabcc1 atabcc2* ones was comparable. In contrast, in the presence of 60 µM $CdSO_4$, the growth of *abcc3* seedlings was similar to that observed when seeds were germinated without Cd, whereas that of *atabcc1 atabcc2* double mutants was severely

inhibited in terms of root length (Figs S4A and B). This data suggest that, contrary to *AtABCC1* and *AtABCC2*, *AtABCC3* does not play a role in Cd tolerance during seed germination.

The relative transcript levels of *AtABCC1*, *AtABCC2* and *AtABCC3* under these experimental conditions were evaluated by means of a qRT-PCR analysis of mRNA extracted from wild type seedlings 5 days after germination at 60 µM $CdSO_4$. As shown in Fig. S4C in the absence of Cd the levels of *AtABCC1* and *AtABCC2* transcripts were 4- and 2-fold higher than that of *AtABCC3* respectively, similar to that described in the previous experiment (Fig. 8A), whereas in the presence of Cd the transcript levels of *AtABCC3* did not increase. The lack of Cd-induced *AtABCC3* expression during germination accounts for the dramatic differences in Cd sensitivity of *abcc3* and *atabcc1 atabcc2* seedlings under these experimental conditions.

As we know that *abcc3* seedlings are not sensitive to low Cd concentrations - 15 µM - and only slight sensitive to 30 µM $CdSO_4$ (Fig. 1), to determine whether *AtABCC3* expression was induced at low Cd concentrations, we analysed the transcript level of *AtABCC3* at different Cd concentrations in comparison to that of *AtABCC1* and *AtABCC2*. A qRT-PCR analysis of mRNA extracted from wild type seedlings grown for 9 days at 0, 15, 30 or 60 µM $CdSO_4$ was performed. As shown in Fig. 8B, the level of *AtABCC1* and *AtABCC2* transcripts in seedlings grown in the presence of all Cd concentrations was comparable to that in the absence of Cd. In contrast, while at 15 µM $CdSO_4$ *AtABCC3* transcript level was comparable to that in the absence of Cd, at 30µM $CdSO_4$ a slight but significant increase (about 1.5-fold) was observed.

This data indicate that little expression of *AtABCC3* occurs at low Cd concentrations.

Discussion

The ABC transporter AtABCC3 has been considered for a long time a good candidate for Cd transport into the vacuole as it partially complements the loss of the ABC protein YCF1 involved in Cd detoxification in *S. cerevisiae* (Tommasini *et al.*, 1998). Furthermore, *AtABCC3* expression is induced by

Cd (Bovet *et al.*, 2003), and the AtABCC3 protein is localized in the vacuolar membrane (Dunkley *et al.*, 2006). However, the role of AtABCC3 in Cd tolerance and the substrates transported by AtABCC3 remained to be examined (Kanga *et al*., 2011).

Here, utilizing Arabidopsis mutant deficient in AtABCC3 (*abcc3*), and plants overexpressing an inducible form of AtABCC3 in a wild type and in a PC-deficient mutant background, we provide strong evidence that AtABCC3 confers Cd tolerance by sequestering PC-Cd complexes in vacuoles.

In our overexpressor lines, the *AtABCC3* gene is under the control on a β-estradiol-inducible promoter, allowing us to induce *AtABCC3* overexpression only when Cd was present in the medium. Seedling growth was evaluated by using two different parameters, fresh weight and root growth, as in Brunetti *et al.* (2011).

We show here that growth of *abcc3* mutant seedlings is hampered at any tested Cd concentration, except at very low concentrations which are not inhibitory for wild type seedlings. In agreement, *AtABCC3* overexpressing plants show a slight but significant higher root growth rate compared to wild type at relatively high Cd concentrations. By contrast, no effects were observed at a lower Cd concentration, that causes just a slight reduction in wild type seedling growth. A possible explanation is that the Cd transport activity exerted by AtABCC3 is low at low Cd concentrations, as suggested by qRT-PCR analysis that shows a low level of *ABCC3* transcript at 30 µM $CdSO_4$, and as previously shown for arsenic transport by the ABCC transporters AtABCC1 and AtABCC2 when expressed in yeast (Song *et al.*, 2010). Our results on Cd tolerance of *abcc3* mutant seedlings are not in contrast with those presented by Park *et al.* (2012) where *abcc3* mutant seedling growth was shown to be comparable to that of wild type seedlings in the presence of Cd. The experimental conditions utilized by Park *et al.* (2012) were different from ours, as we exposed seedlings to Cd after germination. When we germinated seeds in the presence of Cd, we obtained results similar to Park *et al.* (2012), as *abcc3* seedlings under those conditions show only a very slight Cd sensitivity.

By analysing the cytosolic and vacuolar Cd distribution in *abcc3* mutant and

in AtABCC3-overexpressing protoplasts, we show here that the effects of AtABCC3 on Arabidopsis Cd tolerance are due to its capacity to transport Cd into the vacuole. To distinguish between vacuolar and cytosolic Cd in protoplasts of the same lines, we performed an innovative single-cell analysis based on two different fluorochromes, BTC-5N and Leadmium™ Green AM dye. BTC-5N has been previously used to detect Cd in the cytosol of wheat root and shoot protoplasts (Lindberg *et al.*, 2004, 2007), while Leadmium™ Green AM dye has been used to detect Cd in the vacuole of Arabidopsis plant protoplasts (Park *et al.*, 2012) or to determine Cd distribution in entire organs, such as roots of two different *Sedum alfredii* ecotypes (Lu *et al.*, 2008). We show here that in protoplasts isolated from *abcc3* mutant lines there is a decrease in vacuolar Cd and a concomitant increase in cytosolic Cd compared to wild type ones, whereas in AtABCC3ox protoplasts there is an increase in vacuolar Cd and a decrease in cytosolic Cd.

We also show that the total amount of Cd is not altered in all AtABCC3ox seedlings grown in the presence of Cd, under different experimental conditions. Similarly roots and shoots from the overexpressing lines have Cd content similar to the wild type, suggesting that the transport of cytosolic Cd into the vacuole has no effect on total Cd accumulation in the cell.

Three lines of evidence based on the effects of *AtABCC3* overexpression indicate that this ABCC protein acts by transporting PC-Cd complexes into the vacuole. Firstly, overexpressing *AtABCC3* in *cad1-3* mutant lines defective in PC production (Howden *et al.*, 1995) no enhanced Cd tolerance was induced even when lines were exposed to low Cd concentrations. Secondly, overexpressing *AtABCC3* in the presence of BSO, which prevents the accumulation of PCs by reversibly inhibiting the key enzyme in GSH biosynthesis, -no enhanced Cd tolerance was induced by *AtABCC3* overexpression-. Lastly, *AtABCC3* overexpression in *atabcc1 atabcc2* double mutant background defective in the PC-Cd transporters *AtABCC1* and *AtABCC2* (Park *et al.*, 2012) restores Cd sensitivity of *atabcc1 atabcc2* double mutant seedlings, but not in the presence of BSO, indicating that BSO effects are specifically on *AtABCC3*.

By analysing the relative abundance of *AtABCC1*, *AtABCC2* and *AtABCC3*

612 transcripts at different Cd concentrations, we showed that *AtABCC3*
613 expression is regulated by Cd and that its activity is coordinated with the
614 activity of AtABCC1 and AtABCC2. The constitutive level of *AtABCC3* is
615 lower than that of *AtABCC1* and *AtABCC2* at low Cd concentrations (15
616 µM) and during seed germination, but its transcript level increases at high
617 Cd concentration, (60 µM), being higher than that of *AtABCC1* and
618 *AtABCC2*. In addition a further increase of *AtABCC3* mRNA is observed in
619 *atabcc1 atabcc2* double mutant seedlings exposed to high Cd concentrations
620 suggesting a compensative regulation of this Cd-inducible gene, in the
621 absence of *AtABCC1* and *AtABCC2*.
622 Our results are in accord with those of Park *et al.*, (2012), which showed
623 that the Cd-sensitive phenotype of the *atabcc1 atabcc2* double mutant
624 defective in *AtABCC1* and *AtABCC2* PC-Cd transporters is not as severe as
625 that of *cad1-3* (lacking PCs) suggesting that other transporter(s) may be able
626 to compartmentalize PC-Cd complexes. Taken all together, these results
627 indicate that in Arabidopsis several different ABCC Cd-PC transporters act
628 in compartmentalizing Cd into the vacuole. This redundancy may be due to
629 a lack of transporter specificity since all three proteins are involved in the
630 transport of other xenobiotics/metabolites: AtABCC1 is involved in the
631 transport of glutathione *S*-conjugates of xenobiotics and folate, while
632 AtABCC2 and AtABCC3 are able to transport glutathione *S*-conjugates of
633 xenobiotics and chlorophyll catabolites (Lu *et al.*, 1997; Frelet-Barrand *et*
634 *al.*, 2008). Interestingly, while *AtABCC3* expression is induced by Cd
635 (Bovet *et al.*, 2003, this paper), thus ensuring a response related to Cd
636 concentration or to PC-Cd complexes in the cell, *AtABCC1* and *AtABCC2*
637 are constitutively expressed at a higher level and do not respond to Cd
638 exposure. Furthermore, *AtABCC3* is part of a cluster - possibly due to gene
639 duplication (Kolukisaoglu *et al.*, 2002) - of three Cd-regulated *ABCC/MRP*
640 genes (*AtABCC6*, *AtABCC3* and *AtABCC7*) localized in chromosome 3. A
641 slight sensitivity to Cd has been described for *atabcc6* mutant seedlings
642 (Gaillard *et al.*, 2008), while Park *et al.* (2012) report that root length was
643 not altered in *atabcc6* seedlings at different Cd concentrations. On the other
644 hand, an increase in Cd tolerance was observed by overexpressing
645 *AtABCC7* in tobacco lines, while no Cd sensitivity was exhibited by *atabcc7*

seedlings after exposure to Cd (Park *et al.,* 2012). Further work is therefore necessary to assess whether *AtABCC6* and *AtABCC7* are also involved in Cd tolerance as members of a Cd-inducible transport system.

In conclusion our data indicates ~~a substantial role of AtABCC3 in Cd detoxification~~ that AtABCC3 detoxifies Cd by transporting PC-Cd complexes into the vacuoles, and that it can functionally complement *abcc1 abcc2* mutants. Further studies are needed to define whether AtABCC3 is also involved in tolerance to As and to other metals.

Supplementary material

Supplementary data are available at *JXB* online

Figure S1

Effects of β-estradiol on wild type and AtABCC3ox seedling growth.

Figure S2

Cytosolic and vacuolar regions in wild type, *abcc3* and AtABCC3ox protoplasts.

A) A protoplast from wild type leaves stained with neutral red. The vacuole is in red colour. (B,C) Leaf protoplasts from *abcc3* leaves, loaded with BTC-5N. White-light image (B), and fluorescent image with the bright green signal in the cytosol (C).

(D,E) Protoplasts from AtABCC3ox-26 leaves loaded with leadmium green. White-light image (D), and fluorescent image with the fluorescent green signal in the vacuole (E). The arrows show the tonoplast. Bars = 10 μm.

Figure S3

Quantitative analysis of *AtBCC3* in *cad1-3,* and in wild type, *cad1-3* and *abcc1 abcc2* lines overexpressing *AtABCC3*.

A) *AtBCC3* transcript levels in *cad1-3*, AtABCC3ox-21, AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 seedlings grown for 9 days in the absence or presence of β-estradiol. Error bars indicate SE. B) Fresh weight of AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 seedlings grown for 9 days at 15, 30, and 60 μM $CdSO_4$, in the absence or presence of β-estradiol.

Values correspond to means (*n*=3). C) *AtABCC3* transcript levels in AtABCC3ox-*abcc1abcc2-1,* AtABCC3ox-*abcc1abcc2-3* and AtABCC3ox-*abcc1abcc2-5* seedlings grown for 9 days in the absence or presence of β-estradiol. Error bars indicate SE. Asterisks indicate a significant difference from seedlings grown in the absence of β-estradiol (***P<0.001).

3ox, AtABCC3ox; est, β-estradiol.

Figure S4

Cd tolerance of *abcc3* and *atabcc1 atabcc2* mutant seedlings exposed to Cd during the germination phase (A,B) and quantitative analysis of *AtABCC3*, *AtABCC2* and *AtABCC1* transcripts in wild type seedlings exposed to Cd during the germination phase (C). Values correspond to means (*n*=3). Error bars indicate SE. Asterisks indicate a significant difference from wild type grown in the presence of 60 □M $CdSO_4$ (**P<0.01, ***P<0.001). A single dot indicates a significant difference from *abcc3* roots grown at 60 □M $CdSO_4$ (•P <0.05). wt, wild type.

Funding

This work was supported by Progetti di Ricerca di Interesse Nazionale (PRIN) to MC, to GF and to PC, and grants from Università La Sapienza to PC and to MMA.

Acknowledgements

We are grateful to Enrico Martinoia and Markus Klein (University of Zurich, Switzerland) for kindly providing *abcc3* mutant seeds, Chris Cobbett (University of Melbourne, Australia) for *cad1-3* mutant seeds and Sylvia Lindberg (Stockholm University, Sweden) for providing the Cd fluorochrome.

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Figure Legends

Figure 1

Cd tolerance of wild type and *abcc3* seedlings.

(A, B) Wild type and *abcc3* were incubated on medium containing 0, 15, 30, 60, 90 μM $CdSO_4$. (A) Fresh weight and root length (B) were measured after 9 days. (C) Wild type and *abcc3* seedlings at 0 μM $CdSO_4$ and 30 μM $CdSO_4$.

Values correspond to means (*n*=3). Error bars indicate SE. Asterisks indicate a significant difference from wild type (*P <0.05, **P<0.01). wt, wild type.

Figure 2

Quantitative analysis of *AtABCC3* transcript and Cd tolerance of wild type and AtABCC3ox seedlings.

(A) Real-time RT-PCR of mRNA extracted from wild type, AtABCC3ox-20, AtABCC3ox-21 and AtABCC3ox-26 seedlings grown for 9 days at 60 μM $CdSO_4$, in the absence or presence of β-estradiol. Data are expressed as a mean value (*n*=3) of *AtABCC3* cDNA levels relative to actin cDNA. Error

bars indicate SE. (B, C) Wild type, AtABCC3ox-20, AtABCC3ox-21 and AtABCC3ox-26 seedlings were incubated on medium containing 0, 30, 60, 90 μM $CdSO_4$ in the absence or presence of β-estradiol. (B) Fresh weight and root length (C) were measured after 9 days. (D) AtABCC3ox-21 seedlings compared to wild type seedlings after 9 days, at 60 μM $CdSO_4$, in the presence of β-estradiol.

Values correspond to means (*n*=3). Error bars indicate SE. Asterisks indicate a significant difference from seedlings grown in the absence of β-estradiol (A) or a significant difference from wild type roots (C) (*P<0.05, ***P<0.001). est, β-estradiol; wt, wild type.

Figure 3

Analyses of cytosolic and vacuolar Cd in wild type and *abcc3* leaf protoplasts. Protoplasts from wild type and *abcc3* mutant plants grown in the absence or presence of 60 μM $CdSO_4$, were loaded with the cytosolic Cd-sensing fluorochrome BTC-5N and the vacuolar Cd-sensitive probe, Leadmium™ Green AM dye.

(A-D, I-L) Fluorescent images of protoplasts loaded with BTC-5N at 9 and 22 days respectively. Wild type (A, I) and *abcc3* (C, K) protoplasts from plants grown in the absence of Cd for 9 or 22 days. Wild type (B, J) and *abcc3* (D, L) protoplasts from plants grown in the presence of 60 μM $CdSO_4$ for 9 or 22 days. Cd-specific cytosolic fluorescence signal is indicated by arrows (B, D, L). (E-H, M-P) Fluorescent images of protoplasts loaded with Leadmium™ Green AM dye at 9 and 22 days respectively. Wild type (E, M) and *abcc3* (G, O) protoplasts from plants grown in the absence of Cd for 9 or 22 days. Wild type (F, N) and *abcc3* (H, P) protoplasts from plants grown in the presence of 60 μM $CdSO_4$ for 9 and 22 days. (Q, R) Fluorescence signal intensity in the cytosol and in the vacuole of wild type and *abcc3* protoplasts from plants grown in the absence or presence of 60 μM $CdSO_4$ for 9 days and 22 days.

Values are means (*n*=30). Error bars indicate SE. Asterisks indicate a significant difference from wild type protoplasts (*P <0.05, **P<0.01). Dots indicate a significant difference between Cd treatment and control within

the same genotype ($^{\bullet}P<0.05$,$^{\bullet\bullet}P<0.01$). d, days; V, vacuole; wt, wild type. Scale bars= 10 μm.

Figure 4

Analyses of vacuolar Cd in AtABCC3ox leaf protoplasts.

Protoplasts from AtABCC3ox-21 and AtABCC3ox-26 overexpressing plants grown in the absence or presence of 60 μM $CdSO_4$ with or without the inducer β-estradiol were loaded with the vacuolar Cd-sensitive probe, Leadmium™ Green AM dye. (A-L) Fluorescent images of protoplasts loaded with Leadmium™ Green AM dye. AtABCC3ox-21 (A, G) and AtABCC3ox-26 (D, J) protoplasts from plants grown in the absence of Cd for 5 or 9 days. AtABCC3ox-21 (B, C, H, I) and AtABCC3ox-26 (E, F, K, L) protoplasts from plants grown in the presence of 60 μM $CdSO_4$ and β-estradiol for 5 and 9 days. (M) Fluorescence signal intensity in the vacuole of AtABCC3ox-21 and AtABCC3ox-26 protoplasts from plants grown in the absence or presence of 60 μM $CdSO_4$ with or without β̃estradiol for 5 and 9 days.

Values are means (*n*=30). Error bars indicate SE. Asterisks indicate a significant difference from the absence of β-estradiol, within the same genotype (*P <0.05, **P<0.01). d, days; est, β-estradiol; V, vacuole.

Scale bars= 10 μm.

Figure 5

Analyses of cytosolic Cd in AtABCC3ox leaf protoplasts and of Cd content in AtABCC3ox shoots, roots and seedlings.

Protoplasts from AtABCC3ox-21 and AtABCC3ox-26 overexpressing plants grown in the absence or presence of 60 μM $CdSO_4$ with or without the inducer β-estradiol, were loaded with the cytosolic Cd-sensing fluorochrome BTC-5N. (A-F) Fluorescent images of protoplasts loaded with BTC-5N. AtABCC3ox-21 (A) and AtABCC3ox-26 (D) protoplasts from plants grown in the absence of Cd for 9 days. AtABCC3ox-21 (B, C) and AtABCC3ox-26 (E, F) protoplasts from plants grown in the presence of 60 μM $CdSO_4$ with (C, F) or without (B, E) β-estradiol for 9 days compared to those from plants grown without β-estradiol. Cytosolic signal is indicated

by arrows. (G) Fluorescence signal intensity in the cytosol of AtABCC3ox-21 and AtABCC3ox-26 protoplasts from plants grown in the absence or presence of Cd with or without β-estradiol.

Values are means (*n*=30). Error bars indicate SE. Asterisks indicate a significant difference from the absence of β-estradiol within the same genotype (**P<0.01).

(H-J) Cd content in roots (H), shoots (I) and seedlings (J) of AtABCC3ox-21 and AtABCC3ox-26 seedlings over-expressing *AtABCC3* compared to wild type seedlings. Error bars indicate SE (n = 3).

est, β-estradiol. Scale bars= 10 μm.

Figure 6

Cd tolerance of *cad1-3* seedlings overexpressing AtABCC3 and of AtABCC3ox seedlings in the presence of BSO.

(A, B) *cad1-3*, AtABCC3ox-21, AtABCC3ox-*cad1*-53 and AtABCC3ox-*cad1*-59 were incubated on medium containing 0, 15, 30, 60 μM $CdSO_4$ in the absence or presence of β-estradiol. (A) Root length was measured after 9 days. (B) AtABCC3ox-*cad1*-59 and *cad1-3* seedlings after 9 days at 0 and 15μM $CdSO_4$, in the presence of β-estradiol. (C, D) AtABCC3ox-21 and AtABCC3ox-26 seedlings after 9 days at 60 μM $CdSO_4$ with β-estradiol in the presence and absence of BSO. (C) Root length was measured after 9 days. (D) AtABCC3ox-21 seedlings were incubated on medium containing 60 μM $CdSO_4$ with or without β-estradiol in the absence or presence of 0.5 mM BSO.

Values correspond to means (*n*=3). Error bars indicate SE. est, β-estradiol. Asterisks indicate a significant difference from roots grown in the absence of β-estradiol (*P<0.05, **P<0.01). A single dot indicates a significant difference from roots grown in the absence of Cd within genotypes (•P<0.05). 3ox-21, AtABCC3ox-21; 3ox-*cad1*-59, AtABCC3ox-*cad1*-59.

Figure 7

Comparative analysis of Cd tolerance of *abcc3* and *atabcc1 atabcc2* mutant seedlings and of *atabcc1 atabcc2* seedlings overexpressing AtABCC3 in the presence or absence of BSO.

(A, B) Wild type, *abcc3* and *atabcc1 atabcc2* seedlings were incubated on medium containing 0 and 60 µM $CdSO_4$. (A) Fresh weight and root length (B) were measured after 9 days. (C) Wild type, *atabcc1 atabcc2* and *abcc3* seedlings at 0 µM $CdSO_4$ and 60 µM $CdSO_4$. (D, E) Wild type, ABCC3ox-*atabcc1atabcc2-1* and ABCC3ox-*atabcc1atabcc2-3* seedlings were incubated on medium containing 60 µM $CdSO_4$ with and without β-estradiol, or with β-estradiol in presence of 0.5 mM BSO. (D) Fresh weight and root length (E) were measured after 9 days. (F) ABCC3ox-*atabcc1atabcc2-1* seedlings at 60 µM $CdSO_4$ with (middle) and without β-estradiol (left), or with β-estradiol in presence of 0.5 mM BSO (right).

Values correspond to means (*n*=3). Error bars indicate SE. est, β-estradiol. Asterisks indicate a significant difference from wild type grown in the presence of 60 µM $CdSO_4$ (*P<0.05, **P<0.01, ***P<0.001). A single circle indicates a significant difference from *abcc3* seedlings grown in the presence of 60 µM $CdSO_4$ (°P<0.05). Dots indicate a significant difference from seedlings grown in the presence of 60 µM $CdSO_4$ without β-estradiol and BSO within the same genotype (••P<0.01, •••P<0.001). wt, wild type; 3ox-*abcc1abcc2*-1, AtABCC3ox-*abcc1abcc2*-1; 3ox-*abcc1abcc2*-3, AtABCC3ox-*abcc1abcc2*-3.

Figure 8

Comparative analysis of *AtABCC3, AtABCC2* and *AtABCC1* transcript levels in wild type, *abcc3* and *atabcc1 atabcc2* seedlings exposed to 60 µM $CdSO_4$ and in wild type seedlings at different Cd concentrations. (A) Real-time RT-PCR of mRNA extracted from wild type, *abcc3* and *abcc1abcc2* seedlings grown for 9 days at 0 or 60 µM $CdSO_4$ (as indicated). Data are expressed as a mean value (*n*=3) of *AtABCC3, AtABCC1* and *AtABCC2* cDNA levels relative to actin cDNA. Error bars indicate SE.

(B) Real-time RT-PCR of mRNA extracted from wild type seedlings grown for 9 days at 0, 15, 30 and 60 µM $CdSO_4$. Data are expressed as a mean value (*n*=3) of *AtABCC3 AtABCC1* and *AtABCC2* cDNA levels relative to actin cDNA. Error bars indicate SE. Asterisks indicate a significant difference in *AtABCC3* transcript level from wild type seedlings at 0 µM

$CdSO_4$ (**P<0.01, ***P<0.001). Dots indicate a significant difference from *AtABCC3* transcript level in wild type seedlings at 60 μM $CdSO_4$ (••P <0.01). Circles indicate a significant difference in *AtABCC1* and *AtABCC2* transcripts from *AtABCC3* transcript level in wild type seedlings grown in the absence of Cd (°P< 0.05, °°°P< 0.001). wt, wild type.

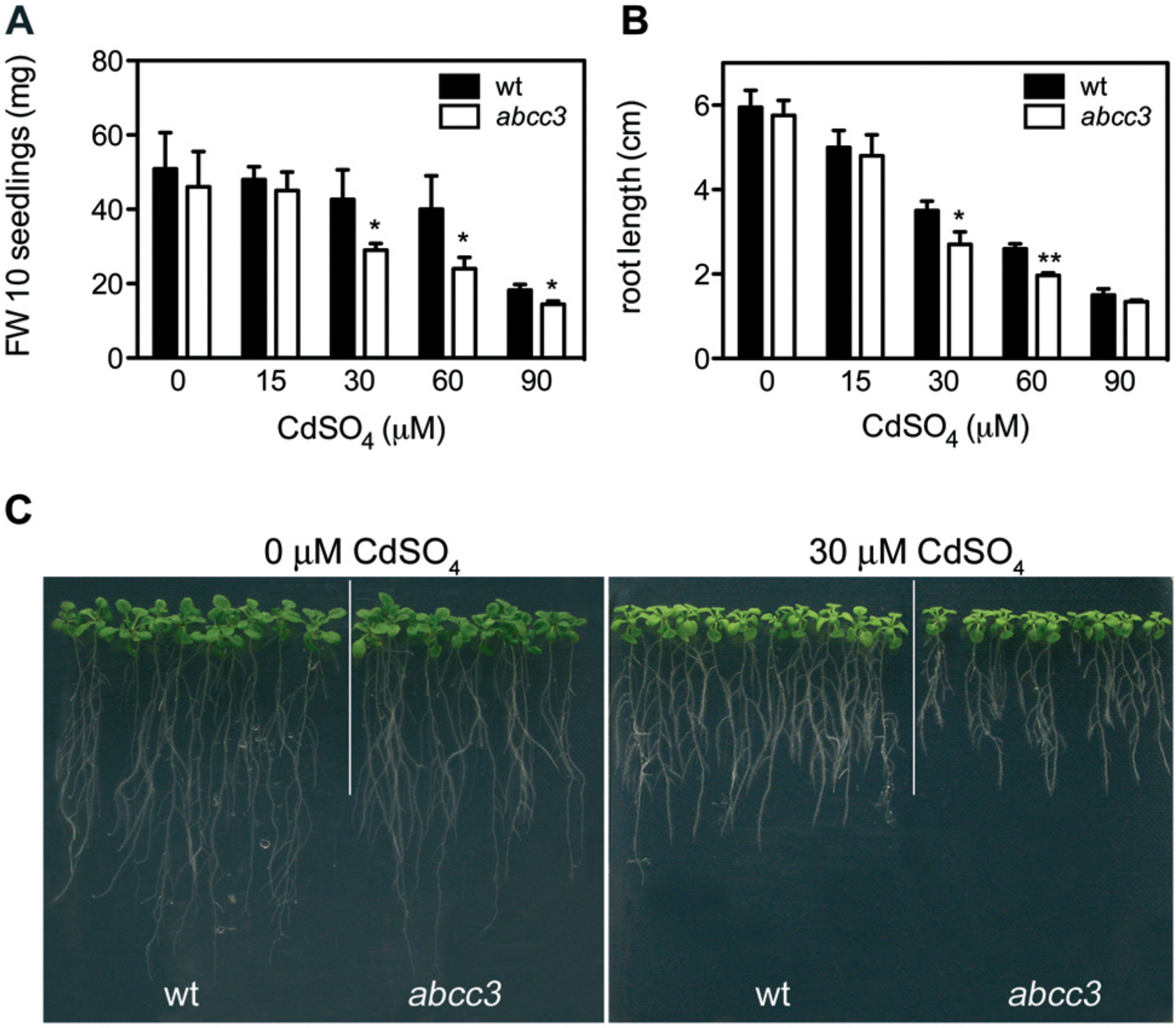

A
FW 10 seedlings (mg)
80
60
40
20
0
wt
abcc3
*
*
*
0
15
30
60
90
CdSO4 (μM)
B
root length (cm)
6
4
2
0
wt
abcc3
*
**
0
15
30
60
90
CdSO4 (μM)
C
0 μM CdSO4
30 μM CdSO4
wt
abcc3
wt
abcc3

Figure 1

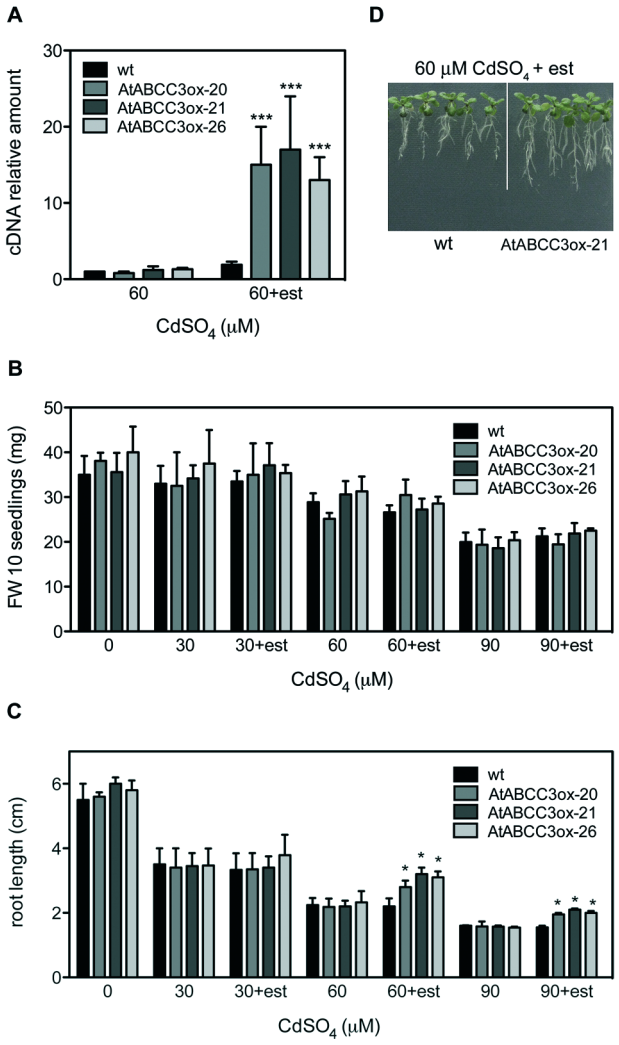

A
cDNA relative amount
wt
AtABCC3ox-20
AtABCC3ox-21
AtABCC3ox-26
***
***
***
60
60+est
CdSO4 (μM)
D
60 μM CdSO4 + est
wt
AtABCC3ox-21
B
FW 10 seedlings (mg)
wt
AtABCC3ox-20
AtABCC3ox-21
AtABCC3ox-26
0
30
30+est
60
60+est
90
90+est
CdSO4 (μM)
C
root length (cm)
wt
AtABCC3ox-20
AtABCC3ox-21
AtABCC3ox-26
*
*
*
*
*
*
0
30
30+est
60
60+est
90
90+est
CdSO4 (μM)

Figure 2

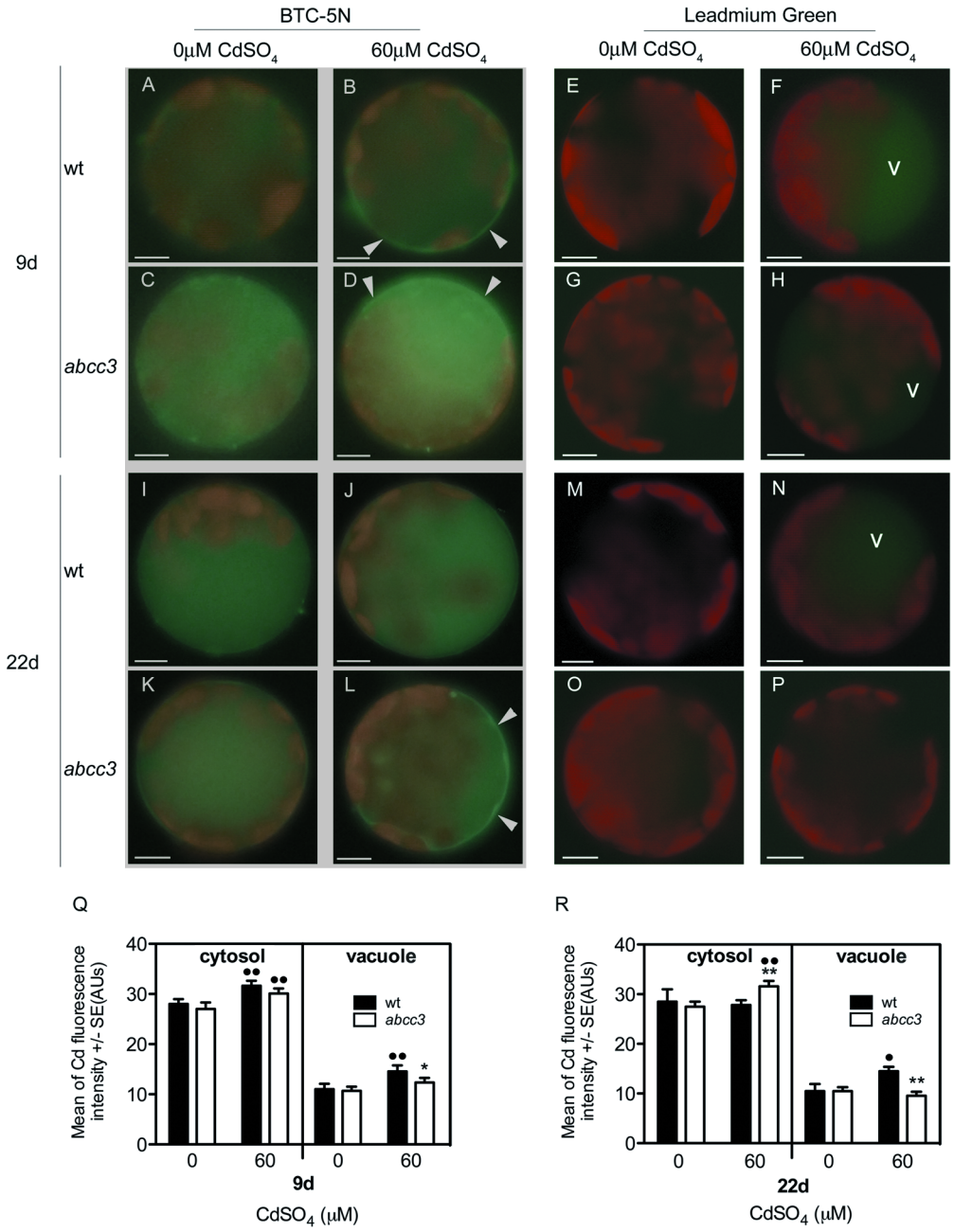
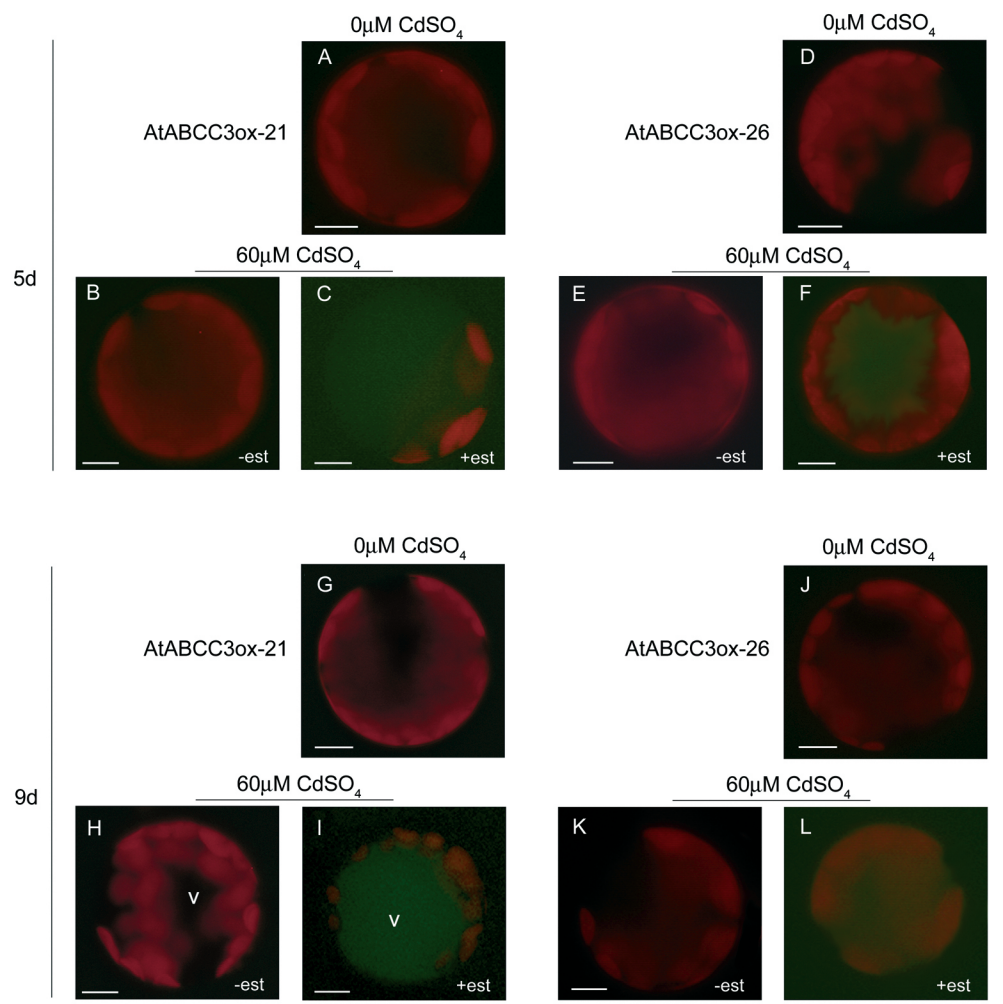

BTC-5N
Leadmium Green
0μM CdSO4
60μM CdSO4
0μM CdSO4
60μM CdSO4
9d
22d
wt
abcc3
wt
abcc3
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
V
V
V
Q
R
Mean of Cd fluorescence intensity +/- SE(AUs)
cytosol
vacuole
wt
abcc3
9d
22d
CdSO4 (μM)
0
60
0
60
0
10
20
30
40

Figure 3


0μM CdSO4
A
AtABCC3ox-21
0μM CdSO4
D
AtABCC3ox-26
5d
60μM CdSO4
B
-est
C
+est
60μM CdSO4
E
-est
F
+est
0μM CdSO4
G
AtABCC3ox-21
0μM CdSO4
J
AtABCC3ox-26
9d
60μM CdSO4
H
v
-est
I
v
+est
60μM CdSO4
K
-est
L
+est

M

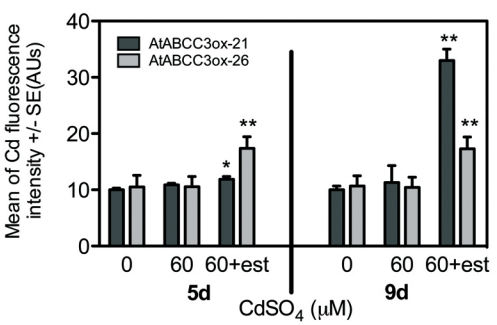

Mean of Cd fluorescence intensity +/- SE(AUs)
40
30
20
10
0
AtABCC3ox-21
AtABCC3ox-26
*
**
**
**
0
60
60+est
5d
0
60
60+est
9d
CdSO4 (μM)

Figure 4

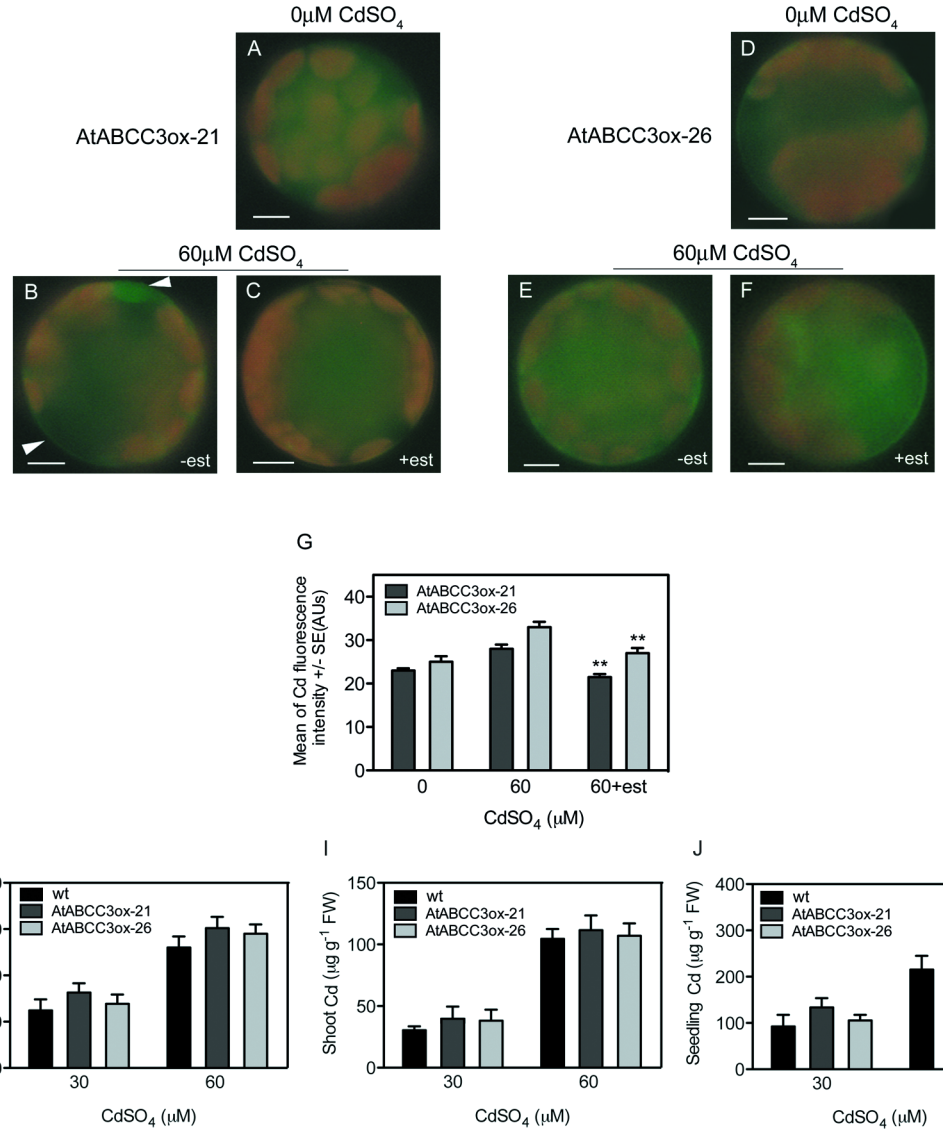

0μM CdSO4
A
AtABCC3ox-21
0μM CdSO4
D
AtABCC3ox-26
60μM CdSO4
B
C
-est
+est
60μM CdSO4
E
F
-est
+est
G
Mean of Cd fluorescence intensity +/- SE(AUs)
AtABCC3ox-21
AtABCC3ox-26
0
10
20
30
40
**
**
0
60
60+est
CdSO4 (μM)
H
Root Cd (μg g-1 FW)
wt
AtABCC3ox-21
AtABCC3ox-26
0
50
100
150
200
30
60
CdSO4 (μM)
I
Shoot Cd (μg g-1 FW)
wt
AtABCC3ox-21
AtABCC3ox-26
0
50
100
150
30
60
CdSO4 (μM)
J
Seedling Cd (μg g-1 FW)
wt
AtABCC3ox-21
AtABCC3ox-26
0
100
200
300
400
30
60
CdSO4 (μM)

Figure 5

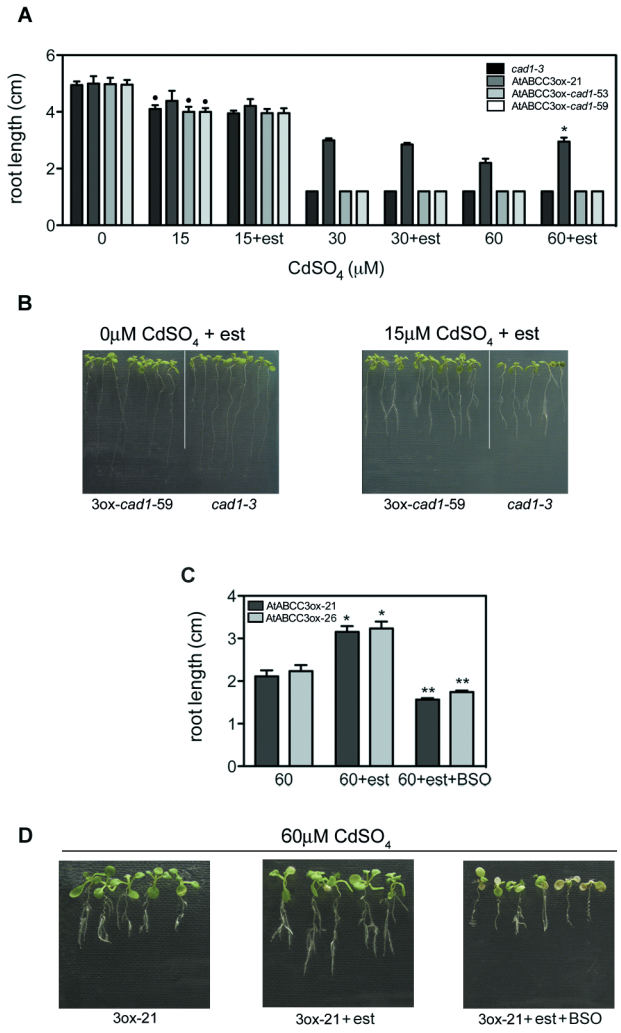

A
root length (cm)
cad1-3
AtABCC3ox-21
AtABCC3ox-cad1-53
AtABCC3ox-cad1-59
0
15
15+est
30
30+est
60
60+est
CdSO4 (μM)
B
0μM CdSO4 + est
15μM CdSO4 + est
3ox-cad1-59
cad1-3
3ox-cad1-59
cad1-3
C
root length (cm)
AtABCC3ox-21
AtABCC3ox-26
60
60+est
60+est+BSO
D
60μM CdSO4
3ox-21
3ox-21+est
3ox-21+est+BSO

Figure 6

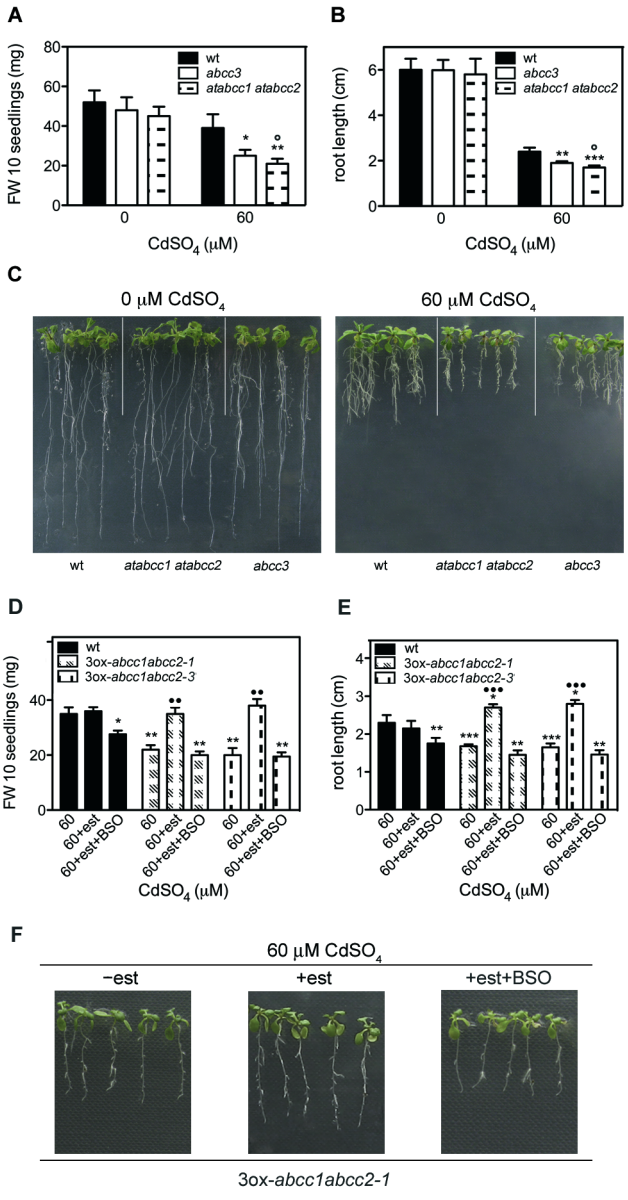

A
FW 10 seedlings (mg)
wt
abcc3
atabcc1 atabcc2
CdSO4 (µM)
B
root length (cm)
C
0 µM CdSO4
60 µM CdSO4
wt
atabcc1 atabcc2
abcc3
D
3ox-abcc1abcc2-1
3ox-abcc1abcc2-3
60
60+est
60+est+BSO
E
F
−est
+est
+est+BSO
3ox-abcc1abcc2-1

Figure 7

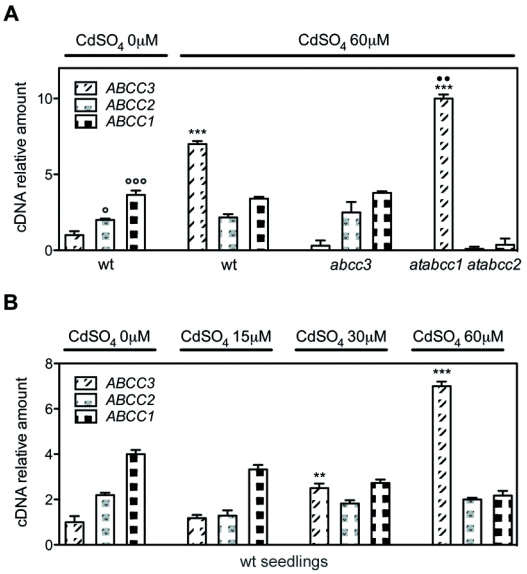

A
CdSO4 0µM
CdSO4 60µM
ABCC3
ABCC2
ABCC1
cDNA relative amount
10
5
0
wt
wt
abcc3
atabcc1
atabcc2
B
CdSO4 0µM
CdSO4 15µM
CdSO4 30µM
CdSO4 60µM
ABCC3
ABCC2
ABCC1
cDNA relative amount
8
6
4
2
0
wt seedlings

Figure 8