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

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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, it is shown 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. The cellular distribution of Cd was analysed in protoplasts from abcc3 mutants and AtABCC3ox plants grown in the presence of Cd, by means of the Cd-specific fluorochromes 5-nitrobenzothiazole coumarin (BTC-5N) and Leadmum™ 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 sulphoximine (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 the 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. The results point to AtABCC3 as a transporter of PC–Cd complexes, and suggest that its activity is regulated by Cd and is co-ordinated with the activity of AtABCC1/AtABCC2.

Key words: ABC-type transporters, Arabidopsis, cadmium stress, cadmium tolerance, phytochelatins, vacuolar compartmentalization.

Introduction

Cadmium (Cd) 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,
PCs also protect plants from the toxic effects of other heavy metals/metalloids such as lead (Pb), mercury (Hg), and arsenic (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). PCs are synthesized by phytochelatin synthase (PCS) from the sub- strate glutathione (GSH) (Grill et al., 1989; Thangavel et al., 2007), and PCS genes were first isolated from Arabidopsis thaliana, S. pombe, Trichum aestivum, and C. elegans (Ha et al., 1999; Clemens et al., 1999, 2001; Vatamaniuk et al., 1999; Cobbett, 2000a, b). 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, 2000a).

PCs are able to bind cytoplasmic Cd, forming stable PC–Cd complexes, playing a major role in Cd 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; Wojis 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 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 transport of PC–Cd complexes is mediated by ATP-binding cassette (ABC)-type transporters (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 up 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 a PC–Cd vacuolar transporter is the half ABC transporter molecule HMT1 (HEAVY METAL TOLERANCE-FACTOR1) in S. pombe; this transporter, which has only one NBD and one TMD, needs homo- or heterodimerize to become functional (Ortiz et al., 1995). Subsequently, HMT1 homologues have been identified in C. elegans (Vatamaniuk et al., 2005) and in Drosophila melanogaster (Sooksa-Ngwan et al., 2009), but not in higher plants. More recently, an ABC-type transporter Abc2 (belonging to the ABC/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 ABC-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 TMD (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–As 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 up-regulated by Cd treatment together with AtABCC6 and AtABCC7 (Gaillard et al., 2008), also plays 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 abcc1 abcc2 double mutants, combined with analysis of cellular Cd localization, and comparative analysis of Cd tolerance between abcc3 and abcc1 abcc2 double mutants, it is shown that AtABCC3 is involved in the vacuolar transport of PC–Cd complexes.

**Plant and methods**

**Plant growth conditions and metal treatments**

Wild type, mutant lines abcc3 (kindly provided by Markus Klein of Philip Morris International, Switzerland), abcc1 abcc2 (Song et al., 2010), kindly provided by Enrico Martinoia (University of Zurich, Switzerland), and cad1-3 (Cobbett, 2000a; kindly provided by Chris Cobbett of University of Melbourne, Australia) AtPCSox-21, AtPCSox-26, AtABCC3ox-cad1-53, AtABCC3ox-cad1-59, AtABCC3ox-abcc1abcc2-1, AtABCC3ox-abcc1abcc2-3, and AtABCC3ox-abcc1abcc2-5 seedlings were germinated on half-strength Murashige and Skoog (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 d, 10 seedlings were transferred to a half-strength MS basal medium with 0.5% sucrose, at different concentrations of CdSO₄ (0, 15, 30, 60, and 90 μM) in the presence of 10 μM β-oestradiol when indicated. Seedling fresh weight and root length were measured after 5 d or 9 d of further growth.

To assess the effect of l-buthionine sulfoximine (BSO) on Cd sensitivity, 7-day-old seedlings were transferred to medium containing 60 μM CdSO₄ with or without 0.5 mM BSO. Seedling fresh weight and root length were measured after 9 d of further growth. The experiments were performed in triplicate.

To analyse Cd content, two experiments were performed as follows. (i) Seven days after germination, ~50 seedlings for each plant were placed into holes of a plastic septum in a phytatray (Sigma), so that only roots were immersed in liquid medium. A half-strength MS medium (0.5% sucrose) was supplemented with 10 μM β-oestradiol, and 60 μM CdSO₄ was added. Seedlings, shaken occasionally, were harvested after 9 d. (ii) Seven days after germination, ~130 seedlings for each line were transferred to a half-strength MS basal medium with 0.5% sucrose, at 30 μM or 60 μM CdSO₄ in the presence of 10 μM β-oestradiol. Seedlings were harvested after 2 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 oestrogen-inducible promoter (Zuo et al., 2000). Agrobacterium tumefaciens strain GV3101 carrying the construct pER8:35S-ABCC3 was used to transform A. thaliana wild type plants (ecotype Columbia) by standard floral dip 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′-GAGCTGACCTTTAAACCCTAAAT-3′; and by real-time reverse transcription–PCR (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., 2013).

Quantitative RT–PCR analysis

RNA was extracted from 50 mg of seedlings grown at the indicated CdSO4 concentration in the presence or absence of the inducer β-oestradiol and reverse-transcribed as previously described. SYBR Green-based quantitative assays were performed using a Bio-Rad iCycler iQ as described in Cecchetti et al. (2013). The primers used to analyse AtABCC3 transcript levels were: RTmrp3 For 3835 5′-CTTCAAGTGTCGATATGCTCCA-3′, RTmrp3 Rev 3885 5′-TGTATTCTCAGCAACACAAGAG-3′; ACTIN2 For 5′-CTTCAGGTCCGATATGCTCCA-3′, ACTIN2 Rev 5′-CTTTGCCCAAGACGACATGAA-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 the AtABCC3ox construct and for the cad1-3 mutation, were selected on hygromycin, and the cad1-3 mutation was verified by PCR with the following primers: cad1-3 For 5′-TCAAATGTCGATCCCTCAGTCG-3′; PCS1 For 5′-TCAAATGTCGATCCCTCAGTCG-3′; and PCS1 Rev 5′-CGGTTCTCAGTGATGGTCTA-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 on half-strength MS agarized medium in the absence or presence of 60 μM CdSO4, β-Oestradiol (10 μM) was added in experiments conducted with AtABCC3ox lines when indicated. Leaf protoplasts were prepared from wild type and abcc3 plants after 9 d or 22 d of treatment, whereas they were prepared from AtABCC3ox lines after 5 d and 9 d 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% 5-nitrobenzoxadiazole coumarin (BTC-5N) (Lindberg et al., 2004) in dimethylsulphoxide (DMSO)/pluronic aqueous solution (Molecular Probes, Leiden, The Netherlands) or with 0.5% Leadchrom™ Green AM dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA) in DMSO, and treated as described. 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 Leadchrom™ 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 (AU); from 0 to 255. The experiment was repeated three times; data from one experiment were reported.

Cadmium accumulation through ICP-MS analysis

Wild type, AtABCC3ox-26, and AtABCC3ox-21 seedlings, cultured as described above, were washed with distilled water—with 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 HNO3 and H2O2 (3:1) under high temperature and pressure. Mineralized samples were analysed for total Cd detection, using inductively coupled plasma-mass spectrometry (ICP-MS; ThermoFisher Série II). All analyses were performed in three replicates. The amounts of acids used were the same as the amounts of additives in 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 study, it was shown that growth of Arabidopsis seedlings is not affected at Cd concentrations up to 15 μM, while in the presence of 30 μM Cd and 60 μM CdSO4, and severely inhibited at 90 μM (Brunetti et al., 2011). Here, 7 d after germination, wild type and abcc3 seedlings were grown in the presence of 0, 15, 30, 60, and 90 μM CdSO4, and the fresh weight and root length were analysed after 9 d. As shown in Fig. 1, in the absence of Cd and at 15 μM CdSO4, the growth of abcc3 and wild type seedlings was 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 a concentration of 30 μM CdSO4 onwards (Fig. 1A–C), whereas roots were significantly shorter only at 30 μM and 60 μM CdSO4 (Fig. 1B, C).

These results suggest an involvement of AtABCC3 in Cd tolerance, and, to confirm this notion, Arabidopsis lines over-expressing AtABCC3 (AtABCC3ox) under the control of a β-oestradiol-inducible promoter were produced (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 the wild type and these AtABCC3ox lines were grown in the presence of 60 μM CdSO4 with or without the inducer β-oestradiol, and AtABCC3 transcript levels were analysed after 9 d of growth. As shown in Fig. 2A, the AtABCC3 mRNA level increased ~15-, 17-, and 13-fold compared with the wild type in AtABCC3ox-20, AtABCC3ox-21, and AtABCC3ox-26 seedlings, respectively.

An effect of β-oestradiol on seedling growth was ruled out, as no significant differences in fresh weight and root length were observed between wild type and AtABCC3ox seedlings after 9 d of growth in the presence or absence of β-oestradiol, without Cd (Supplementary Fig. S1 available at JXB online).
To assess Cd tolerance, AtABCC3ox-20, AtABCC3ox-21, and AtABCC3ox-26 seedlings were grown in the presence of 0, 30, 60, and 90 μM CdSO₄ with or without β-oestradiol, and the fresh weight and root length were analysed after 9 d. No significant differences in either growth indicator were observed at 30 μM CdSO₄ (Fig. 2B, C) in any of the AtABCC3ox seedlings grown in the presence or absence of the inducer. At 60 μM CdSO₄, all three AtABCC3ox lines showed a significant increase in root length when grown in the presence of the inducer (Fig. 2C, D), whereas the fresh weight was comparable in seedlings grown in the presence or absence of β-oestradiol (Fig. 2B).

At 90 μM CdSO₄, 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.

To determine whether AtABCC3 plays a role in Cd transport into the vacuole, the cellular distribution of Cd was compared in the 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 preliminarily used to define the cytosolic and vacuolar regions independently of the fluorescence, as shown in Supplementary Fig. S2 at JXB online. Leaf protoplasts were isolated from wild type and abcc3 plants grown in the absence or presence of 60 μM CdSO₄ for 9 d and 22 d, 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 CdSO₄ 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, Q, left panel). When wild type and abcc3 plants were cultured in the presence of Cd, after 9 d BTC-5N-loaded protoplasts showed a comparable Cd-specific cytosolic fluorescence signal (Fig. 3B, D, Q, left panel), whereas after 22 d the Cd-specific cytosolic signal decreased in wild type protoplasts but significantly increased in abcc3 protoplasts (P<0.01) (Fig. 3J, L, 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 the Materials and methods) (Fig. 3Q, right panel, and Fig. 4M) but was not detectable in fluorescence images (Fig. 3E, G, M, O). This is possibly due to interactions between the fluorochrome and Ca²⁺ that occur in the absence of Cd. When wild type and abcc3 protoplasts from plants cultured for 9 d 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, Q, right panel). After 22 d in the presence of Cd, the vacuolar signal was almost unchanged in wild type vacuoles (Fig. 3N), whereas in vacuoles of abcc3 protoplasts it became significantly lower (P<0.01) than in the wild type (Fig. 3P, Q, right panel).
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These results indicate a decrease in vacuolar Cd and a concomitant increase in cytosolic Cd in abcc3 mutant protoplasts compared with those of the wild type, suggesting a role for 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 CdSO₄ with or without β-oestradiol; leaf protoplasts isolated after 5 d or 9 d were loaded with Leadmium™ Green AM dye. After 5 d of treatment with Cd, protoplasts from AtABCC3ox-21 and AtABCC3ox-26 plants grown in the presence of β-oestradiol showed a significant increase (P<0.05 and P<0.01, respectively).
respectively) in the vacuolar signal (Fig. 4C, F, M, left panel) compared with protoplasts grown without β-oestradiol (Fig. 4B, E). Analogously, after 9 d of treatment with CdSO₄ in the presence of β-oestradiol, both AtABCC3ox-21 and AtABCC3ox-26 protoplasts exhibited a vacuolar signal (Fig. 4I, L, M, right panel) significantly higher (P<0.01) than that of protoplasts from plants grown without β-oestradiol (Fig. 4H, K).

The Cd cytosolic signal was also analysed in protoplasts from AtABCC3ox-21 and AtABCC3ox-26 plants. After 9 d of treatment with Cd in the presence of β-oestradiol, AtABCC3ox-21 and AtABCC3ox-26 protoplasts exhibited a cytosolic signal (Fig. 5C, F, G) significantly lower (P<0.01) than that of protoplasts grown in the absence of β-oestradiol (Fig. 5B, E). These results indicate a lower cytosolic Cd accumulation and a corresponding increase in vacuolar Cd in AtABCC3ox protoplasts.
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Taken together, 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, Cd content was analysed in wild type, AtABCC3ox-21, and AtABCC3ox-26 seedlings by means of ICP-MS. After 9 d of treatment with 60 μM CdSO₄ in the presence of β-oestradiol, wild type, AtABCC3ox-21, and AtABCC3ox-26 seedlings showed a comparable content of total Cd (624 ± 51.6, 696 ± 12.22, and 698 ± 40.01 μg g⁻¹ FW, respectively).
To confirm these data, Cd content was analysed separately in shoots and roots from wild type, AtABCC3ox-21 and AtABCC3ox-26 seedlings exposed for 2 weeks at 30 μM or 60 μM CdSO₄. As shown in Fig. 5, no significant difference in Cd content was observed at these Cd concentrations, in roots (Fig. 5H) or shoots (Fig. 5I), as well as in seedlings (Fig. 5J), of the overexpressing lines compared with the wild type. Together these data rule 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, AtABCC3 was overexpressed in a cad1-3 mutant line defective in PCS and, consequently, in PC production (Howden et al., 1995). AtABCC3ox-cad1 plants were generated by crossing AtABCCox-21 with cad1-3 lines, and AtABCC3 overexpression was analysed in different lines homozygous for the cad1 mutation and the AtABCC3ox construct. Two lines, AtABCC3ox-cad1-53 and AtABCC3ox-cad1-59, overexpressing AtABCC3 in the presence of β-oestradiol (Supplementary Fig. S3A at JXB online) 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 CdSO₄ with or without β-oestradiol. After 9 d, the root length and fresh weight were analysed. As shown in Fig. 6A and Supplementary Fig. S3B at JXB online, in the absence of β-oestradiol at 30 μM and 60 μM CdSO₄, cad1-3, AtABCC3ox-cad1-53, and AtABCC3ox-cad1-59 seedling growth was completely inhibited, whereas root length...
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and fresh weight of AtABCC3ox-21 seedlings were comparable with those of wild type seedlings (Fig. 2B, C). In the presence of β-oestradiol (Fig. 6A; Supplementary Fig. S3B at JXB online) both concentrations of Cd were toxic to AtABCC3ox-cad1-53 and 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 CdSO₄, while AtABCC3ox-21 seedlings showed, as described above, a significant increase in root length upon addition of β-oestradiol (Figs 2C, 6A), the growth of cad1-3, AtABCC3ox-cad1-53, and AtABCC3ox-cad1-59 seedlings was unaffected by addition of the inducer (Fig. 6A; Supplementary Fig. S3B at JXB online). To determine whether AtABCC3 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-oestradiol. As shown in Fig. 6A and B, after 9 d in the absence of β-oestradiol, AtABCC3ox-21 seedlings show a growth comparable with 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 AtABCC3ox-21 seedlings is comparable with that of seedlings grown without the inducer or without Cd (Fig. 6A, B). More interestingly, when grown in the presence of β-oestradiol, 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, B) but not of fresh weight (Supplementary Fig. S3B at JXB online).

To provide further evidence that the effect of AtABCC3 is mediated by PCs, Cd tolerance of AtABCC3ox-21 and AtABCC3ox-26 seedlings was assessed in the presence of BSO, an inhibitor of γ-glutamylcysteine synthetase (γ-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-oestradiol, in the presence or absence of 0.5 mM BSO, and root length was measured after 9 d. 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-oestradiol 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 to 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 d 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).

It was shown above that AtABCC3 acts in the transport of PC–Cd complexes as do ABCC1 and ABCC2: it was thus asked whether ABCC3 could complement the abcc1 abcc2 double mutation. To perform a complementation assay, Arabidopsis abcc1 abcc2 lines overexpressing AtABCC3 were produced by transforming abcc1 abcc2 double mutant plants with the construct pER8::35S-ABCC3 (see the Materials and methods). Overexpression of AtABCC3 was measured by means of qRT-PCR in three independent homozygous lines denoted AtABCC3ox-abcc1abcc2-1, AtABCC3ox-abcc1abcc2-2, and AtABCC3ox-abcc1abcc2-5 (see Supplementary Fig. S3C at JXB online). Cd tolerance of AtABCC3ox-abcc1abcc2-1 and AtABCC3ox-abcc1abcc2-3 seedlings was assessed at 0 and 60 μM CdSO$_4$ with or without β-oestradiol, in the presence or absence of 0.5 mM BSO. After 9 d, 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-abcc1abcc2-2 seedlings grown with β-oestradiol compared with uninduced seedlings.

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 AtABCC3.

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 d 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 were, respectively, 4- and 2-fold higher than that of AtABCC3. In contrast, at 60 μM CdSO$_4$, the transcript levels of AtABCC1 and AtABCC2 did not increase, whereas the transcript level relative to AtABCC3 increased by 6.9-fold, being 1.7- and 3.4-fold higher, respectively, than that of AtABCC1 and AtABCC2. Interestingly, in abcc3 mutants at 60 μM CdSO$_4$, the transcript levels of AtABCC1 and AtABCC2 were comparable with those of wild type seedlings, whereas in atabcc1 atabcc2 seedlings the level of AtABCC3 transcript further increased, compared with that of wild type seedlings, being 3.2- and 6.8-fold higher, respectively, than that of 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$_4$ (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 seen 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 Supplementary Fig. S4A at JXB online, after 14 d in the absence of Cd the growth of abcc3 seedlings and that of wild type and atabcc1 atabcc2 seedlings 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 (Supplementary Fig. S4A, B at JXB online). These data suggest that, in contrast 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 d after germination at 60 μM
AtABCC3 is involved in cadmium tolerance

Fig. 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 CdSO₄. (A) Fresh weight and root length (B) were measured after 9 d. (C) Wild type, atabcc1 atabcc2, and abcc3 seedlings at 0 and 60 μM CdSO₄. (D, E) Wild type, ABCC3ox-atabcc1atabcc2-1, and ABCC3ox-atabcc1atabcc2-3 seedlings were incubated on medium containing 60 μM CdSO₄ with and without β-oestradiol, or with β-oestradiol in the presence of 0.5 mM BSO. (D) Fresh weight and root length (E) were measured after 9 d. (F) ABCC3ox-atabcc1atabcc2-1 seedlings at 60 μM CdSO₄ with (middle) and without (left), or with β-oestradiol in the presence of 0.5 mM BSO (right). Values correspond to means (n=3). Error bars indicate the SE. est, β-oestradiol. Asterisks indicate a significant difference from the wild type grown in the presence of 60 μM CdSO₄ (*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 CdSO₄ (°P<0.05). Dots indicate a significant difference from seedlings grown in the presence of 60 μM CdSO₄ without β-oestradiol 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.
CdSO₄. As shown in Supplementary Fig. S4C at JXB online, in the absence of Cd the levels of AtABCC1 and AtABCC2 transcripts were 4- and 2-fold higher, respectively, than that of AtABCC3, similar to what was 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 it is known that abcc3 seedlings are not sensitive to low Cd concentrations (15 μM) and only slightly sensitive to 30 μM CdSO₄ (Fig. 1), to determine whether AtABCC3 expression was induced at low Cd concentrations, the transcript level of AtABCC3 was analysed at different Cd concentrations in comparison with that of AtABCC1 and AtABCC2. A qRT-PCR analysis of mRNA extracted from wild type seedlings grown for 9 d at 0, 15, 30, or 60 μM CdSO₄ 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 with that in the absence of Cd. In contrast, while at 15 μM CdSO₄ the AtABCC3 transcript level was comparable with that in the absence of Cd, at 30 μM CdSO₄ a slight but significant increase (∼1.5-fold) was observed.

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

**Discussion**

The ABC transporter AtABCC3 has for a long time been considered 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 (Kang et al., 2011).

Here, utilizing an 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, strong evidence is provided that AtABCC3 confers Cd tolerance by sequestering PC–Cd complexes in vacuoles.

In the overexpressor lines, the AtABCC3 gene is under the control on a β-oestradiol-inducible promoter, allowing AtABCC3 overexpression to be induced 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).

It is shown 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 with this, AtABCC3-overexpressing plants show a slight but significantly higher root growth rate compared with the wild type at relatively high Cd concentrations.

In 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 CdSO₄, and as previously shown for As transport by the ABC transporters AtABCC1 and AtABCC2 when expressed in yeast (Song et al., 2010). The present results on Cd tolerance of abcc3 mutant seedlings are not in contrast to those presented by Park et al. (2012) where abcc3 mutant seedling growth was shown to be comparable with that of wild type seedlings in the presence of Cd. The experimental conditions utilized by Park et al. (2012) were different from those used here, as seedlings were here exposed to Cd after germination. When seeds were germinated in the presence of Cd, results similar to those of Park et al. (2012) were obtained, as abcc3 seedlings under those conditions show only a very slight Cd sensitivity.
By analysing the cytosolic and vacuolar Cd distribution in the abc3 mutant and in AtABCC3-overexpressing protoplasts, it is shown 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, an innovative single-cell analysis was performed 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). It is shown here that in protoplasts isolated from abc3 mutant lines there is a decrease in vacuolar Cd and a concomitant increase in cytosolic Cd compared with those of the wild type, whereas in AtABCC3ox protoplasts there is an increase in vacuolar Cd and a decrease in cytosolic Cd.

It is also shown 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 a 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 ABC protein acts by transporting PC–Cd complexes into the vacuole. First, by 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, by overexpressing AtABCC3 in the presence of BSO, which prevents the accumulation of PC-s, by reversibly inhibiting the key enzyme in GSH biosynthesis, no enhanced Cd tolerance was induced. Lastly, AtABCC3 overexpression in the atabcc1 atabcc2 double mutant background defective in the PC–Cd transporters AtABCC1 and AtABCC2 (Park et al., 2012) restores the 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 transcripts at different Cd concentrations, it was shown that AtABCC3 expression is regulated by Cd and that its activity is co-ordinated with the activity of AtABCC1 and AtABCC2. The constitutive level of AtABCC3 is lower than that of AtABCC1 and AtABCC2 at low Cd concentrations (15 μM) and during seed germination, but its transcript level increases at high Cd concentration (60 μM), being higher than that of AtABCC1 and AtABCC2. In addition a further increase of AtABCC3 mRNA is observed in atabcc1 atabcc2 double mutant seedlings exposed to high Cd concentrations, suggesting a compensative regulation of this Cd-inducible gene in the absence of AtABCC1 and AtABCC2.

The results obtained are in accord with those of Park et al. (2012), who showed that the Cd-sensitive phenotype of the atabcc1 atabcc2 double mutant defective in AtABCC1 and AtABCC2 PC–Cd transporters is not as severe as that of cad1-3 (lacking PCs), suggesting that other transporter(s) may be able to compartmentalize PC–Cd complexes. Taken all together, these results indicate that in Arabidopsis several different ABC PC–Cd transporters act in compartmentalizing Cd into the vacuole. This redundancy may be due to a lack of transporter specificity since all three proteins are involved in the transport of other xenobiocumts/metabolites: AtABCC1 is involved in the transport of glutathione S-conjugates of xenobiocumts and folate, while AtABCC2 and AtABCC3 are able to transport glutathione S-conjugates of xenobiocumts and chlorophyll catabolites (Lu et al., 1997; Frelet-Barrand et al., 2008). Interestingly, while AtABCC3 expression is induced by Cd (Bovet et al., 2003; this study), thus ensuring a response related to Cd concentration or to PC–Cd complexes in the cell, AtABCC1 and AtABCC2 are constitutively expressed at a higher level and do not respond to Cd exposure. Furthermore, AtABCC3 is part of a cluster—possibly due to gene duplication (Kolukisaoglu et al., 2002)—of three Cd-regulated AtBCCMRP genes (AtABCC6, AtABCC3, and AtABCC7) localized in chromosome 3. A slight sensitivity to Cd has been described for atabcc6 mutant seedlings (Gaillard et al., 2008), while Park et al. (2012) report that root length was not altered in atabcc6 seedlings at different Cd concentrations. On the other hand, an increase in Cd tolerance was observed by overexpressing 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, the data indicate a substantial role for AtABCC3 in Cd detoxification whereby 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 data

Supplementary data are available at JXB online.

Figure S1. Effects of β-oestradiol on wild type and AtABCC3ox seedling growth.

Figure S2. Cytosolic and vacuolar regions in wild type, abcc3, and AtABCC3ox protoplasts.

Figure S3. Quantitative analysis of AtBCC3 in wild type, cad1-3 and abcc1 abcc2 lines overexpressing AtABCC3.

Figure S4. Cd tolerance of abcc3 and atabcc1 atabcc2 mutant seedlings exposed to Cd during the germination phase and quantitative analysis of AtABCC3, AtABCC2, and AtABCC1 transcripts in wild type seedlings exposed to Cd during the germination phase.

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