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Functional characterization of *BjCET3* and *BjCET4*, two new cation-efflux transporters from *Brassica juncea* L.

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Abstract

Brassica juncea is promising for metal phytoremediation, but little is known about the functional role of most metal transporters in this plant. The functional characterization of two B. juncea cation-efflux family proteins BjCET3 and BjCET4 is reported here. The two proteins are closely related to each other in amino acid sequence, and are members of Group III of the cation-efflux transporters. Heterologous expression of BjCET3 and BjCET4 in yeast confirmed their functions in exporting Zn, and possibly Cd, Co, and Ni. Yeast transformed with BjCET4 showed higher metal resistance than did BjCET3 transformed. The two BjCET–GFP fusion proteins were localized to the plasma membrane in the roots when expressed in tobacco, and significantly enhanced the plants' Cd tolerance ability. Under Cd stress, tobacco plants transformed with BjCET3 accumulated significant amounts of Cd in shoots, while maintaining similar shoot biomass production with vector-control subjects. Transformed BjCET4 tobacco plants showed significantly enhanced shoot biomass production with markedly decreased shoot Cd content. The two transporter genes have a lower basal transcript expression in B. juncea seedling tissues when grown in normal conditions than under metal-stress, however, their transcripts levels could be substantially increased by Zn, Cd, NaCl or PEG, suggesting that BjCET3 and BjCET4 may play roles in several stress conditions, roles which appear to be different from those of previous characterized cation-efflux transporters, for example, AtMTP1, BjCET2, and BjMTP1.

Key words: *B. juncea* L., cation-efflux transporter, heavy metal stress, heavy metal tolerance, osmotic stress, transgenic plant.

Introduction

Transition metals such as Zn and Cu are essential for many processes in plants, but are toxic when they accumulate to high levels (Godbold and Hüttermann, 1985; Dietz *et al.*, 1999; Nies, 1999). However, a few plants have special mechanisms for metal-ion homeostasis and detoxification, and allow normally toxic levels of metals accumulate in their tissues. For example, two members of the Brassicaceae family, *Thlaspi caerulescens* and *Thlaspi goesingense*, are able to grow in soil containing Cd^{2+}, Ni^{2+}, Zn^{2+}, and accumulate 100–1000 times higher concentrations of such heavy metals in the shoots than typical species, without any symptoms of toxicity (Persans *et al.*, 2001; Rigola *et al.*, 2006). In recent years, it has been learned that metal membrane transport systems probably play a critical role in this metal hyperaccumlation (Hall and Williams, 2003; Krämer *et al.*, 2007; Milner and Kochian, 2008; Kim *et al.*, 2009)

With the application of molecular and genetic techniques, a number of gene families involved in metal transport and homeostasis have been identified. These include: the natural resistance-associated macrophage protein (NRAMP) family with members encoding multispecific metal transporters, described in Fe and Mn homeostasis (Curie *et al.*, 2000; Thomine *et al.*, 2000); the cation exchanger (CAX)

family involved in Ca^{2+}/H^+, Na^+/H^+, or Mn^{2+}/H^+ antiport (Hirschi *et al.*, 2000); the Zrt, Irt-like protein (ZIP) family involved in transporting a variety of divalent cations including Zn^{2+}, Fe^{2+}, Mn^{2+}, and Cd^{2+} (Guerino, 2000); and the cation efflux (CE) family including members from *Arabidopsis thaliana* involved in Zn homeostasis (van der Zaal *et al.*, 1999). Proteins belonging to the cation-efflux family (also known as the cation diffusion facilitator family) were first described by Nies and Silver in 1995 (Nies and Silver, 1995), and have been implicated in conferring Zn^{2+}, Cd^{2+}, Co^{2+} or Ni^{2+} tolerance in a range of organisms. Recently a distinct phylogenetic group of cation-efflux proteins (ShMTP8 and AtMTP11) were shown to be specific for Mn export from the cytoplasm (Delhaize *et al.*, 2003, 2007; Peiter *et al.*, 2007). Most cation-efflux proteins are transporters that either sequester metal ions within cells or export metal ions out of cells (Paulsen and Saier, 1997). Interest in cation-efflux proteins has greatly increased because of evidence that cation-efflux proteins may be useful in phytoremediation, food fortification, and treating human disease (Ford, 2004; Krämer *et al.*, 2007). Although there is evidence that overexpression of a cation-efflux member in plants can decrease metal toxicity and enhance metal accumulation, up to now little is known about the function and mechanisms of cation-efflux transporters.

The *A. thaliana* genome encodes 12 putative cation-efflux proteins (AtMTP1 to AtMTP12), which can be divided into three distinct groups (Blaudez *et al.*, 2003). Group I (AtMTP8 to AtMTP11 and AtMTP6, AtMTP7) and Group II (AtMTP5 and AtMTP12) proteins contain only a subset of cation-efflux features, described as having 15 (AtMTP12) or four to five predicted transmembrane domains, only part of the N-terminal signal sequence, or lacking the His-rich domain (Paulsen and Saier, 1997). Group III (AtMTP1 to AtMTP4) contains all the basic cation-efflux features (Paulsen and Saier, 1997). Most of the cation-efflux proteins are involved in metal tolerance. For example, the *ZAT* gene of *Arabidopsis* (also known as *AtMTP1*) confers Zn^{2+} tolerance when over-expressed in plants (van der Zaal *et al.*, 1999). The increased tolerance was thought to be attributable to internal sequestration of Zn^{2+}, which was confirmed later by the subcellular location of the AtMTP1–GFP fusion protein to the vacuolar membrane in root and leaf of *Arabidopsis* cells (Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005). Homologues of *AtMTP1* have been found in metal hyperaccumulators including *TgMTP1* in *Thlaspi goesingense* (Persans *et al.*, 2001), *TcMTP1* in *Thlaspi caerulescens* (Assucao *et al.*, 2001), and *AhMTP1* in *Arabidopsis halleri* (Dräger *et al.*, 2004). All these MTP1 proteins showed a constitutively higher shoot expression in the hyperaccumulators than in non-accumulators, a feature that is therefore supposed to be a key contribution to metal hyperaccumulation, a hypothesis supported from recent molecular genetic evidence (Filatov *et al.*, 2006; Milner and Kochian, 2008). Based on the localization of MTP1–GFP fusion proteins in yeast or plant cells, AhMTP1 was found in the vacuolar membrane (Dräger *et al.*, 2004), whereas TgMTP1 was localized to both the cytoplasmic membrane

and vacuolar membrane (Kim *et al.*, 2004; Gustin *et al.*, 2009). MTP1 proteins have been shown to confer zinc tolerance to *Saccharomyces cerevisiae* mutants defective in one or both of the cation-efflux transporter COT1 and ZRC1 that transport zinc ions into the vacuole (Persans *et al.*, 2001; Bloss *et al.*, 2002; Blaudez *et al.*, 2003; Dräger *et al.*, 2004; Kim *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005; Arrivault *et al.*, 2006).

Phytoremediation is a major concern (Chaney, 1983). Because of its multi-metal (such as Zn, Cr, Cu, Cd, Se, and Pb) accumulating ability and relatively high biomass production with fast growth rate and susceptibility to genetic manipulation, *B. juncea* has been taken as a potential metal hyperaccumlator for practical use in phytoremediation (Salt *et al.*, 1995, 1997, 1998). A molecular understanding of *B. juncea* cation-efflux transporters, and their role in metal homeostasis, has important biotechnological applications, such as the design of metal accumulating plants to help combat metal-deficient and metal-contaminated soil conditions, as well increasing the metal ion content of plant foods. Four novel cation-efflux family transporters (*BjCET1–BjCET4*) have previously been cloned and recombinantly expressed from Cd treated seedlings of *B. juncea* (Lang *et al.*, 2005a), and some functional properties of *BjCET2* have been reported (Xu *et al.*, 2009). As both BjCET3 and BjCET4 show high sequence similarity with each other and are in Group III of the CE family, it is hypothesized that BjCET3 and BjCET4 may perform similar functions of metal ion homeostasis in *B. juncea*, especially for Zn^{2+}. The molecular and functional characteriztion of *BjCET3* and *BjCET4*, which have some different functional properties from *BjCET2*, are described here. The proteins were expressed in mutant yeast and tobacco in medium oversupplied with metal to investigate their role in metal homeostasis and metal tolerance and accumulation. CET–GFP fusion proteins were used to determine their subcellular location in plant cells and semi-quantitative RT-PCR was used to check their mRNA levels in *B. juncea* under normal and adverse stress conditions.

Materials and methods

Plant materials and growth conditions

Seeds of *B. juncea* were a gift of the North Central Regional Plant Introduction Station (NCRPIS) of the United States National Plant Germplasm System (NPGS). *Nicotiana tabacum* cv. W38 was grown routinely in a greenhouse and *B. juncea* was grown as previous described by Lang *et al.* (2005b). All plants were grown under a 16 h light and 8 h dark cycle at 25 °C.

Cloning of BjCET genes

Using a PCR-enriched cDNA pooling method, four cation-efflux transporter genes were cloned from Cd-treated seedlings of *B. juncea*, which include *BjCET3* and *BjCET4* (Lang *et al.*, 2005a).

Stress and semi-quantitative RT-PCR (sqRT-PCR)

B. juncea seedlings were hydroponically grown in 4.0 l of aerated 0.5× Hoagland's medium, changed every third day. Up to the 5–6

leaf stage, the seedlings were subjected to stress for 4, 8, 12, and 24 h or only for 12 h, by transferring to the aerated liquid medium with or without salt (250 mM NaCl), PEG (20% w/v PEG6000) or heavy metals (200 μM $CdCl_2$ or 1000 μM $ZnCl_2$). After each treatment, seedlings were washed with distilled water and the roots, stems, and leaves were separated and snap frozen in liquid nitrogen immediately. Total RNA was extracted using the RNAiso Plus reagent (TaKaRa), following the manufacturer's protocol and using the DNase digestion step to reduce DNA contamination. cDNA was reverse transcribed using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) by using 1 μg total RNA as the template. The *B. juncea* actin gene was used as an internal control for the relative quantification of transcript levels. The primer sequences were as follows: 5′-CTGTTACCACTCGTCACCATC-3′ and 5′-ACCTTGTTGAGCACCATATCTG-3′ for *BjCET3* to amplify a 499 bp product, 5′-TGATGAAGAACAGAGAACAACAAC-3′ and 5′-CGCAGCAACAAGGAACATAAG-3′ for *BjCET4* to amplify a 445 bp product, and 5′-AAGATCTGGCATC ACACTTTC-3′ and 5′-TAGTCAACAGCAACAAAGGAG-3′ for *BjACTIN* to amplify a 529 bp product, respectively. Three biological replicate samples were used for all analyses, and various cycle numbers were applied to ensure that, under the chosen PCR conditions, the products (both actin and the specific BjCET) were in the linear amplification range (data not shown). PCR band intensities were measured using Carestream MI software.

Yeast experiments for the metal tolerance assay

For yeast expression, the *BjCET* cDNAs were cloned into the pYES2 vector (Invitrogen), and transformed into three *S. cerevisiae* mutant strains (a kind gift from Professor DH Nies) by means of a chemical method (Gietz and Woods, 1994): YK40 (*ura3-52 his3-200, Δcot1, MATα*), YK41(*ura3-52 his3-200, Δzrc1, MATα*), and YK44 (*ura3-52 his3-200, Δzrc1, Δcot1, MATα*). The transformed yeast cells were selected on SMM minimal medium lacking uracil [SMM-uracil, 6.7 g yeast nitrogen base without amino acids, 20 mg histidine, 2% (w/v) glucose, 2% (w/v) agar 1^{-1}] and confirmed by PCR amplification of *BjCET* inserts. The metal tolerance assay was performed according to Persans *et al.* (2001). Briefly, yeast strains were grown to 1.5 OD_{600} in 10 ml of SMM–uracil liquid medium. Using 1 ml of yeast culture to inoculate 50 ml of SMM–uracil medium [replacing glucose with 4% (w/v) galactose and adding 1.5% (w/v) top agar 1^{-1}], the solution was mixed well and then poured into 90×15 mm plastic sterile dishes and allowed to cool. Five 4 mm sterile paper filters were placed at regular intervals on the surface of the top agar for each dish, and solutions containing various metal ions were spotted on to the discs. The plates were cultured in a 30 °C incubator for 3–4 d.

Yeast experiments for the metal accumulation assay

To determine the efflux transport activity of BjCET proteins, Zn accumulation in yeast cells was tested after an oversupply of Zn. Overnight cultures of single *zrc1* yeast colonies transformed with BjCET3, BjCET4, and a empty vector were added to 200 ml SMM–uracil liquid medium to OD_{600}=0.2. The cultures were then incubated to reach OD_{600}=0.4. $ZnCl_2$ (125 μM final concentration) was added, and the culture was incubated for another 4 h. Yeast cells were collected by centrifugation and first washed with 20 mM EDTA and then washed with sterilized deionized water 3–4 times. The samples were dried in an oven and then weighed. The Zn accumulation in samples was determined by the inductively coupled plasma-atomic emission spectrophotometry (ICP-MS) method.

Generation of transgenic tobacco expressing BjCET:GFP fusion proteins and confocal microscopy

The pBI121-GFP vector (a kind gift from Dr Jim Haseloff, Cambridge, England) contains the cauliflower mosaic virus 35S promoter and an NOS (nopaline synthase) terminator, and an *npt*II cassette was used for selection in plants under the control of the nos promoter and terminator. The *BjCET*s cDNAs were inserted into the *Xba*I and *Bam*HI sites of the pBI121–GFP vector, and fused with GFP under the control of 35S promoter. The expression vector pBI121–BjCET–GFP and the empty vector pBI121–GFP were introduced into *Agrobacterium tumefaciens* (*EHA105* strain) by the freeze–thaw method (Walkerpeach and Velten, 1994). About 1 cm^2 regions of tobacco leaves were excised and co-cultured with *Agrobacterium* by the leaf disc-infection method (Horsch *et al.*, 1985). Transformants were selected on MS medium supplemented with 200 mg 1^{-1} of kanamycin and 250 mg 1^{-1} of Cef. Putative transgenic plantlets were screened under a fluorescence microscope (485 nm) to assess green fluorescence and checked with PCR using the forward primer 5′-CCCGGATCCAAGGAGATATAAC-3′ and the reverse primer 5′-CCCGAGCTCTTATTTGTATAGTTCATCC-3′ to detect about 800 bp GFP fragments by using the transgenic plants' genomic DNA as a template. Positive plantlets were then transferred to soil and allowed to flower and set seed in a greenhouse. The T_0 transgenic plantlets were amplifed by using a tissue culture method. The differentiation and regeneration medium MT_0 is MS supplemented with 1 mg 1^{-1} 6-BA, 0.1 mg 1^{-1} NAA , 200 mg 1^{-1} kanamycin, 250 mg 1^{-1} Cef ,and 8 g 1^{-1} agar, pH 5.8. The rooting medium MT_1 is 1/2 MS supplemented with 0.1 mg 1^{-1} NAA, 200 mg 1^{-1} kanamycin, 250 mg 1^{-1} Cef, and 8 g 1^{-1} agar, pH 5.8. The tissue culture amplified T_0 transgenic plants were used for the following heavy metal tolerance and accumulation assay.

Heavy metal accumulation and tolerance assay

About 1 cm^2 regions of leaves were excised from WT and T_0 transgenic tobaccos and placed in MT_0 medium in which kanamycin had been replaced with 200 μM $CdCl_2$. Each bottle contained 10 leaf explants and was cultured in an incubator at 25 °C with 16 h d^{-1} of light. After 35 d, the differentiated seedlings were counted. In the meantime, the 2–3 cm regenerated seedlings of WT and transgenic plants from regular medium were planted onto MT_1 rooting medium containing 200 μM $CdCl_2$. After 15 d, the rooting results were observed and, after 30 d, the plants were harvested and the plant growth index was measured. For determining Cd content, plant samples were firstly washed 5–7 times with distilled water, then dried overnight at 70 °C, weighed, and finely ground. Samples (0.1 g) were digested with 2 ml nitric acid by heating discontinuously in microwave for 2 min. After the solution was cooled, it was transferred to a 50 ml flask and made up to volume with deionized water before being analysed by the ICP-MS method.

Statistical analysis

Data were analysed using a one-way ANOVA test to our endpoints, followed by a Tukey test when data were statistically different (P <0.05) (Zar, 1996). Values are hte average number of each genotype (arithmetic mean ±SD of n=3 independent experiments).

Results

Cloning and sequence analysis of B. juncea BjCET3 and BjCET4 cDNAs

Lang *et al.* (2005a) isolated four previous unidentified full-length cation-efflux family cDNA sequences from Cd stressed *B. juncea* and named them *BjCET1–BjCET4* (Fig. 1A). Muthukumar *et al.* (2007) cloned BjMTP1, another cation-efflux family member of *B. juncea*. cDNAs of

A

B

Fig. 1. BjCET sequence analysis. (A) Alignments of predicted amino acid sequences for BjCET1, BjCET2, BjCET3, BjCET4, and BjMTP1 from *B. juncea*, TgMTP1 from *T. goesingense* and AtMTP1 from *A. thaliana*. Sequence alignment was performed by CLUSTALW. The following protein features have been highlighted: the six transmembrane domains (TMs) identified by TMHMM (version 2.0) (underlined in dark green); the CDF signature sequences (yellow); the histine-rich loop region (dashed dark green line); the highly conserved aspartate (filled inverted triangles)in domain II and V (Bloss *et al.*, 2002); the C-terminal LZ motif (red) (Blaudez *et al.*, 2003); and the C-terminal putative Zn binding site HD(E)XHXWXL(I)TX$_8$H (dark green) (Dräger *et al.*, 2004). (B) Phylogenetic analysis of the identified CE proetins from *B. juncea*. The Neighbor–Joining phylogenetic tree was constructed using MEGA4.1 after CLUSTALW alignment of the full-length amino acid sequences.

BjCET3 and *BjCET4* contained putative ORFs of 1155 and 1128 bp encoding 385 and 376 amino acid residues with predicted molecular masses of 42.4 and 41.4 kDa, respectively (see Supplementary Table S1 at *JXB* online). The deduced amino acid sequences showed strong similarities (pairwise identity of 81–89%) among the five cation-efflux proteins from *B. juncea*, and to the previously characterized CE proteins ZAT, AhCDF1-3, and TgMTP1 (76–82%)

(Fig. 1B). *BjCET3* and *BjCET4*, and *BjCET2* and *BjMTP1* are the most similar pairs in a phylogenetic tree analysis (Figs 1B, 2). *BjCET3* and *BjCET4* contain features of Group III cation-efflux proteins (Fig. 1A).

The predicted amino acid sequences of 32 cation-efflux genes from different species were analysed using Clustal X (see Supplementary Fig. S1 at *JXB* online). Cation-efflux members with common features from the same source mostly group together. Analysis through a phylogenetic tree (Figs 1B, 2), showed that the plant Group III cation-efflux proteins are more closely grouped than others (Blaudez *et al.*, 2003). The phylogenetic tree also showed that all the cloned cation-efflux members with common features could be grouped based on species, for example, *ZiTB* and *czcD*, bacteria source; *COT1* and *ZRC1*, yeast source. Almost all plant source cation-efflux members with common features are situated on one clade, and cation-efflux members from the same plant species are usually situated on one subclade, for example, *Thlaspi* source of *TgMTP1* and *TcMTP1*, *Arabidopsis* source of *AhMTP1-1*, *AhMTP1-2*, and *AhCDF1-3*. The newly identified *Brassica* cation-efflux members *BjCET1-4* and *BjMTP1* are also situated on one

subbranch, and *BjCET3* and *BjCET4* are grouped together in the third subclade, suggesting that the two cDNAs are more evolutionarily and functionally related (Figs 1B, 2). However, the Group III proteins AtMTP1–4 from *A. thaliana* showed more divergence compared with BjCETs. Montanini *et al.* (2007) classified the majority of CDF family members into three groups (Zn, Fe/Zn, and Mn) based on phylogenetic reconstruction, and each group prefered a special metal selectivity. Our new BjCET members fall into Zn-CDF group, which suggests they may transport zinc. Their other possible substrates are Co^{2+}, Cd^{2+}, and Ni^{2+}.

BjCET3 and BjCET4 response to multiple abiotic stress in addition to Cd and Zn

The expression patterns of BjCET3 and BjCET4 genes in different tissues (roots, stems, and leaves) and under different growth conditions were examined by sqRT-PCR. Specific primers for each *BjCET* gene were designed and tested for amplifying only the target BjCET (data not shown). As *B. juncea* is a Cd accumulator and tolerant to

Fig. 2. Phylogenetic analysis of the CE proteins from *B. juncea* and other genera. The Neighbor–Joining phylogenetic tree based on the protein alignment was created with MEGA 4.1. The sequences were aligned with Clustal X software, and the scale bar indicates an evolutionary distance of 0.05 amino acid substitution per site. The number at the nodes show bootstrap values (>800) after 1000 replicates. Accession numbers and species names of each gene are shown in Supplementary Table S2 at *JXB* online.

high Cd concentration than normal plants (Salt *et al.*, 1995), a little higher Cd concentration (200 μM CdCl$_2$) was used to stress the *B. juncea* plantlets, but that concentration was not lethal to the plant during the 24 h and even longer times of treatment (see Supplementary Fig. S2 at *JXB* online).

The basal mRNA levels of *BjCET3* and *BjCET4* in the roots, stems, and leaves of 5–6 leaf stage *B. juncea* seedlings grown in normal conditions were checked. The sqRT-PCR could hardly detect the *BjCET4* transcript in the leaf and stem, and was only detected a little more in the root. The method showed a higher *BjCET3* transcript level in all three tissues than for *BjCET4* (Fig. 3A).

A 12 h stress treatment was then made for the 5–6 leaf stage of *B. juncea* seedlings with 200 μM CdCl$_2$, 1000 μM ZnCl$_2$, 250 mM NaCl, and 20% PEG 6000 (w/v), respectively, and the various stress effects on *BjCET3* and *BjCET4* transcript levels in different tissues was checked (Fig. 3B). In the root, it was found that the *BjCET4* transcript level was increased 6.8-fold upon PEG treatment, and it showed a slight increase upon Zn and NaCl stress, but vitually no change upon Cd stress. The *BjCET3* transcript level did not change much in response to Zn, NaCl, and PEG treatment, but showed some decrease in response to Cd treatment. In the stem, the *BjCET4* transcript level showed 1.9-, 1.7-, and 2.1-fold increase in response to Cd, Zn, and NaCl stress, respectively, while nearly no change was seen in response to PEG treatment. However, in addition to the up-regulation upon Cd (3.9-fold) and NaCl (3.7-fold) stress, the *BjCET3* transcript level increased 4.0-fold after 12 h of PEG treatment, and it also showed a minor increase with Zn stress. In the leaf, both *BjCET3* and *BjCET4* transcript levels were markedly increased with Cd (6.4-fold for *BjCET3*, 4.9-fold for *BjCET4*), NaCl (5.7-fold for *BjCET3*, 5.5-fold for *BjCET4*), and PEG (3.6-fold for *BjCET3*, 4.4-fold for *BjCET4*) treatments, while in response to the Zn treatment, the *BjCET3* transcript level still showed a small (1.5-fold) increase, but *BjCET4* showed a stronger (3.5-fold) increase.

Time-dependent changes in the expression of *BjCET3* and *BjCET4* in response to Cd and NaCl treatment were also examined (Fig. 3C). In the root, both *BjCET3* and *BjCET4* mRNA levels peaked at 4 h and then decreased during the following 8–24 h treatment. In the stem, the *BjCET4* mRNA level peaked at 12 h upon Cd and NaCl treatment, then dropped and fell below the limit of detection at 24 h. The *BjCET3* transcript level was also observed to peak at 12 h (3.2-fold increase) upon Cd treatment, then decreased. However, it showed a higher level at both 12 h (4.0-fold increase) and 24 h (4.5-fold increase) during the NaCl treatment. In the leaf, the *BjCET4* mRNA level had increased at 4 h and peaked at 12 h with Cd and NaCl treatments. However, at 24 h, the mRNA level of *BjCET4* was markedly reduced during the NaCl treatment, but continued to be higher in the Cd treatment. The *BjCET3* expression peaked at 8 h and 12 h upon Cd and NaCl treatment, respectively, and then dropped to normal levels.
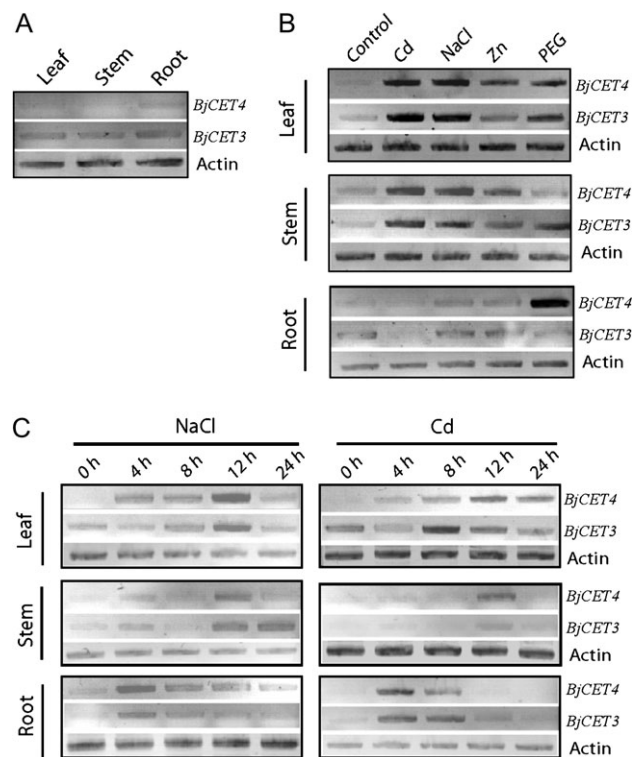

Fig. 3. sqRT-PCR analysis of steady-state levels of *BjCET3* and *BjCET4* transcripts and their regulation by Zn, Cd, NaCl, and PEG in root, stem, and shoot tissues of *B. juncea*. (A) *BjCET3* and *BjCET4* mRNA expression level in tissues of the 5–6 leaf stage of *B. juncea* seedlings growing in normal conditions; (B) *BjCET3* and *BjCET4* expression level in tissues of the 5–6 leaf stage of *B. juncea* seedlings after 12 h treatment with 200 μM CdCl$_2$, 1000 μM ZnCl$_2$, 250 mM NaCl, and 20% w/v PEG6000, respectively; (C) *BjCET3* and *BjCET4* expression level in tissues of the 5–6 leaf stage of *B. juncea* seedlings after 4, 8, 12 , and 24 h treatment with 200 μM CdCl$_2$ and 250 mM NaCl, respectively. 25 cycles were used for all PCR reactions, which were tested in the linear amplification range for each target gene.

BjCET3 and BjCET4 confer a broad range of metal tolerance in yeast complementation assay

To investigate the substrate specificity of BjCET3 and BjCET4 and their functional properties, they were expressed in yeast mutants deficient in their orthologues COT1 and ZRC1. These mutants are hypersensitive to Co and Ni, or Cd and Zn.

Supplementary Fig. S3 at *JXB* online shows representative zinc-tolerance assay plates, with zones of yeast growth inhibition being clearly visible and significantly different around the zinc ion-soaked discs between the vector control and BjCET4 expressed *cot1&zrc1* (YK44) strains. Expression of BjCET3 and BjCET4 in *cot1&zrc1* double mutant yeast complemented the Zn, Cd, and Co sensitivity of these strains (Fig. 4A–C), but not Ni sensitivity (data not shown). However, when expressed in the *cot1* yeast strain, Ni sensitivity was rescued by the two BjCETs (Fig. 4D). The two BjCETs also complemented *zrc1* yeast's sensitivity to Cd and Zn (Fig. 4E, F). In all of the yeast strains tested, the
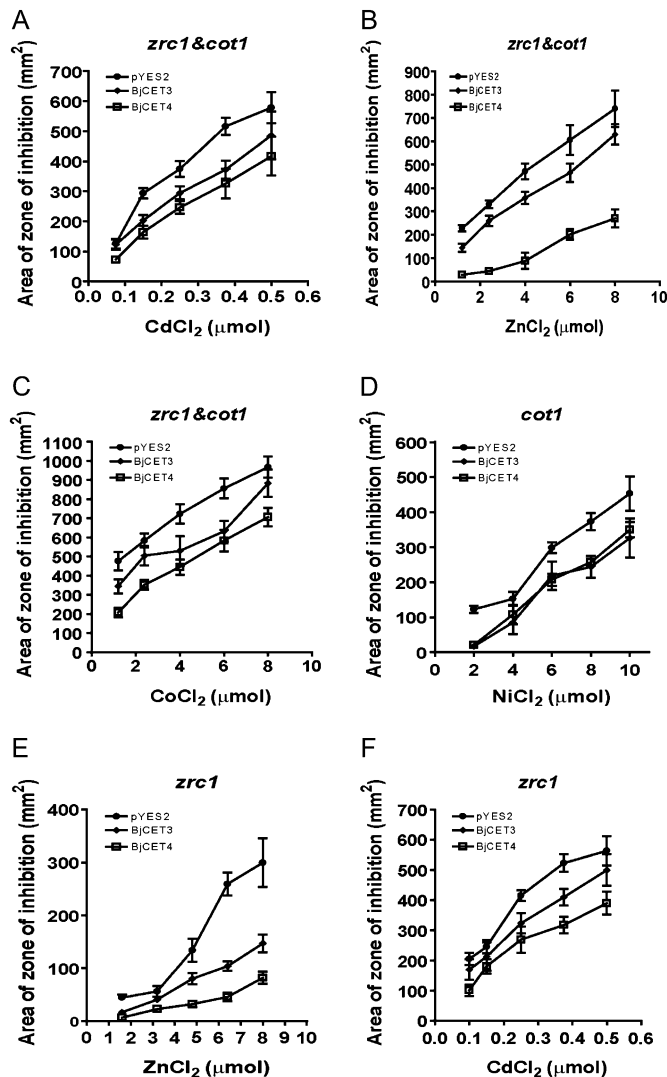
Fig. 4. Quantification of metal tolerance of recombinant yeast. (A–C) Quantification of Cd, Zn and Co tolerance of *zrc1&cot1* mutant strains (YK44) expressing *BjCET3* and *BjCET4*, respectively. (D) Quantification of Ni tolerance of *cot1* mutant strains (YK40) expressing *BjCET3* and *BjCET4*. (E, F) Quantification of Zn, Cd tolerance of *zrc1* mutant strains (YK41) expressing *BjCET3* and *BjCET4*. Metal tolerance was quantified by using the assay system described in Persans *et al.* (2001). Data are means ±SD of three biological repeats.

two BjCET proteins show more pronounced tolerance to Zn (Fig. 4B, E) than to Cd, Co, and Ni, and BjCET4 showed consistently higher tolerance to these metals than BjCET3. The same experiments were done with medium containing 2% glucose. No complement to the metal sensitivity of mutant yeast was observed for all the recombinant yeast strains (data not shown). In other experiments, it was also demonstrated that expression of BjCET4 and BjCET3 could restore the growth of vector control *cot1* (YK40) yeast when cultured on medium containing different concentration of Zn, Ni, Co, and Cd (see Supplementary Fig. S4 at *JXB* online). Taken together, the results suggest that the two BjCET transporters could

play a similar role to COT1 and ZRC1 in yeast, and they may either be targeted to the vacuolar membrane to sequester metal ions or targeted to the plasma membrane to export metal ions for rescuing the mutant yeast.

To determine the role of metal ion transport activity of BjCET3 and BjCET4, the ICP-MS method was used specifically to test for Zn accumulation in yeast cells. Yeast samples were treated with 125 μM $ZnCl_2$ for 4 h, and the cells collected and washed with EDTA to remove zinc ions that had adhered to the cell walls. Figure 5 showed that yeast cells expressing BjCET3 (P <0.001) and BjCET4 (P <0.01) significantly reduced Zn accumulation more than the vector controls under Zn stress conditions (Fig. 5). The result suggested a plasma membrane localization of BjCET3 and BjCET4 in yeast where they could export zinc ions out of cells.

Subcellular localization of the BjCET3 and BjCET4 proteins in transgenic tobacco

The pBI121-BjCET-GFP expression construct is designed for the constitutive expression of the *BjCET* genes by the enhancer-equipped CaMV 35S promoter, which is active in most cells of the plant. The kanamycin resistance gene *Npt*II was used to screen the transformants (Fig. 6A). Using transgenic tobacco genome DNA (data not shown) and cDNA as template, PCR amplification of *GFP* DNA fragments confirmed that 35S–BjCET:GFP cDNA constructs were integrated into the genome of tobacco and constitutively transcribed (Fig. 6B). Using confocal microscopy, BjCET3 and BjCET4 expressed in the root of T_0 transgenic tobacco seedlings was detected and their proteins had localized to the plasma membrane (Fig. 6C). To test that the BjCET:GFP proteins were not located at the cell wall, the root tissues were treated with saturated sucrose solution in order to make cell membrane plasmolyse before analysis. BjCET:GFP signals were detected which withdrew from the cell wall after 5–10 min of treatment (Fig. 6, C5 and C6). As plant root cells are usually highly vacuolated, to exclude the vacuole localization of the tested BjCET proteins, DAPI was used to stain the nucleus. Because nuclei are outside the vacuole but within the plasma membrane, if GFP-tagged proteins were localized to the vacuole, the DAPI signal should be outside the GFP signals. It was observed that the DAPI signals were surrounded by GFP signals (Fig. 6, C7 and C8) and, therefore, the BjCET:GFP proteins should not target to vacuoles. Taken together, our results indicate that BjCET3–GFP and BjCET4–GFP were located to the plasma membrane.

BjCET3 and BjCET4 over-expression increased Cd tolerance in transgeneic tobacco

B. juncea is a Cd accumulator (Salt *et al.*, 1995). Our yeast complementation results have shown that *BjCET3* and *BjCET4* expression enhanced Cd tolerance of a mutant yeast strain, which suggests the two proteins may have a role in Cd resistance and accumulation in *B. juncea*. As Cd is one of the most toxic heavy metals and a significant heavy
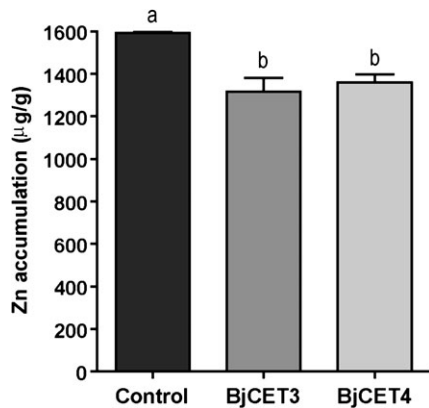
Fig. 5. ICP-MS assay of Zn content in *zrc1* yeast cells. Cultured yeast cells (OD_{600}=0.4) were incubated with 125 μM $ZnCl_2$ for 4 h before collection. Then yeast cells were washed with 20 mM EDTA and sterilized deionized water to remove the medium Zn and yeast cell wall unspecific bound Zn. The Zn content inside yeast cells was determined by ICP-MS. A significantly reduced Zn accumulation was detected in BjCET3 (P <0.001) and BjCET4 (P <0.01) expressing yeast compared to control (empty vector transformed) yeast. Comparison between different genotypes was made using a one-way ANOVA test to our endpoints data, followed by a Tukey test when data were statistically different (P <0.05) (Zar, 1996). Values are average of each genotype (arithmetic mean ±SD of n=3 independent experiments).

Fig. 6. Verifying *BjCET3* and *BjCET4* expression in transgenic tobaccos and their subcellular localization in roots. (A) Structures of plasmid pBI121-BjCET-GFP. nos-pro, Nopaline synthase promoter; *Npt II*, neomycin phosphotransferase; nos-ter, nopaline synthase terminator; CaMV 35S Pro, CaMV 35S promoter. (B) RT-PCR analysis of *BjCET3* and *BjCET4* transcript level in the leaves of transgenic plants. PCR products are their fused *GFP* cDNA fragments. *Actin* was used as the control. (C) Subcellular localization of BjCET proteins in root cells of transgenic tobaccos. C1–C3, images of GFP fluorescence in transgenic tobacco root cells in normal growing condition; C4–C6, images of GFP fluorescence in transgenic tobacco root cells treated with saturated sucrose solution for 5–10 min; C7, C8, images of GFP and DAPI (arrow) fluorescence in BjCET3 and BjCET4 transgenic tobacco root cells. Before analysis, root samples were treated with 2 μg ml^{-1} DAPI for 15 min. C1 and C4 represent pBI121-GFP transgenic tobacco tissues, C2, C5, and C7 represent pBI121-BjCET3-GFP transgenic tobacco tissues, and C3, C6, and C8 represent pBI121-BjCET4-GFP transgenic tobacco tissues. Fluorescence of GFP and DAPI was observed by using confocal laser scanning microscopy under 485 nm and 364 nm, respectively.

metal pollutant (Oberlunder and Roth, 1978; Staessen *et al.*, 1999), genetically engineering Cd-tolerant and -accumulating plants would have an application in phytoremediation.

The effect of over-expressing *BjCET3* and *BjCET4* on the ability of tobacco leaf explants to differentiate and regenerate under Cd exposure conditions was investigated. Under normal conditions, *BjCET3* and *BjCET4* over-expression had no significant effect on the number of regenerated shoots among *BjCET* transgenic plants and vector control subjects (Fig. 7B), however, the explants had significant differences in shoot regeneration when exposed to Cd (Fig. 7A, B). *BjCET4* explants regenerated the highest number of shoots, which was even more significant than *BjCET3* explants (P <0.001). These results showed that over-expression of *BjCET3* and *BjCET4* could significantly enhance the Cd tolerance of explants, and more so with *BjCET4* than with *BjCET3*.

Previous studies have shown that Cd stress may significantly inhibit root growth and biomass production of plant (Balsberg Pahlsson, 1989). In our tests, under normal conditions, all the *BjCET* and vector control shoots grew normally and produced many roots 15 d after planting. However, when exposed to 200 μM $CdCl_2$, no roots were observed for the vector control subjects at that stage, whereas growing roots were observed in the *BjCET* transgenic plants, and the *BjCET4* plants grew longer roots than the *BjCET3* plants (Fig. 8), suggesting that over-expression of *BjCET4* makes plants more tolerant to Cd exposure than the others. After 50 d of Cd exposure, *BjCET3* and *BjCET4* plants survived better than the controls (Fig. 8). Under
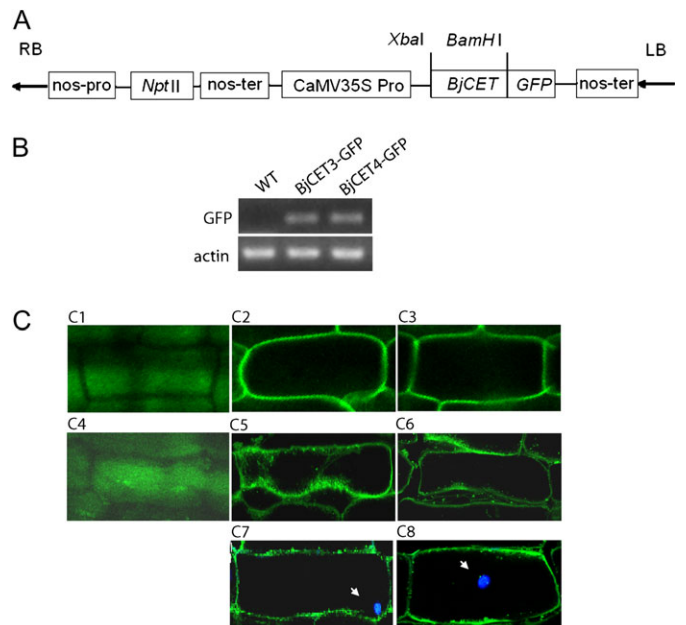
normal growing conditions, significant differences were not observed among the *BjCET* plants and vector control subjects at that stage (see Supplementary Fig. S5 at *JXB* online).

Figure 9 shows the growth index and Cd content of transgeic plants after 30 d of Cd exposure. *BjCET* plants have significantly longer root and higher root fresh weight in comparison with the vector control subjects (P <0.001 for *BjCET4* and P <0.01 for *BjCET3*), and *BjCET4* plants even showed a significantly higher root fresh weight than *BjCET3* plants (P <0.001) (Fig. 9A, B). The shoot fresh weight of *BjCET4* plants is also significantly higher than vector controls (P <0.001) and *BjCET3* plants (P <0.001), while that of *BjCET3* plants showed no significant difference from the vector controls (Fig. 9C).
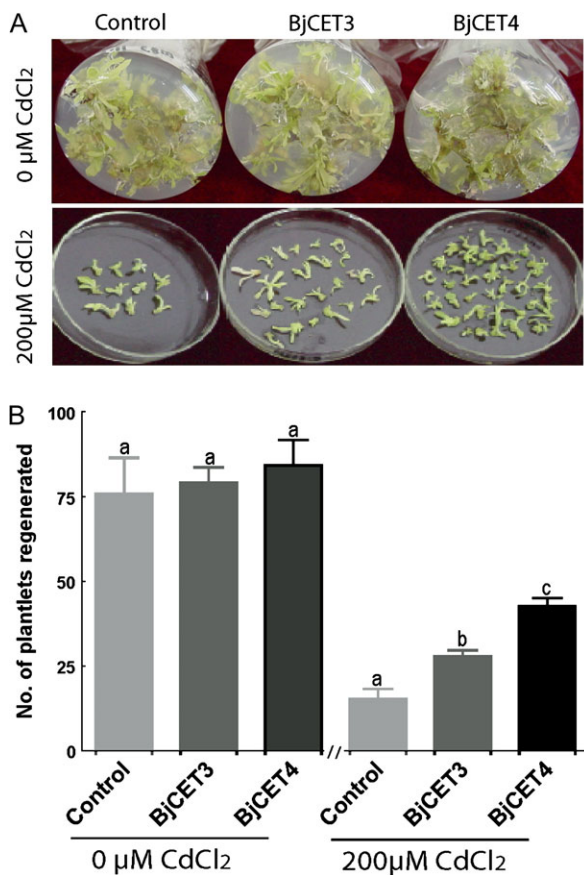
Fig. 7. Overexpression of BjCET protein confers Cd tolerance to tobacco leaf explants. (A) Showing an example of plantlets regenerated from 10 leaf explants of each genotype cultured in medium with or without 200 μM CdCl₂. (B) Quantitative analysis of numbers of regenerated seedlings from each genotype. Comparison between different genotypes upon Cd exposures was made using a one-way ANOVA test to our endpoints data, followed by a Tukey test when data were statistically different ($P < 0.05$) (Zar, 1996). Values are average number of each genotype (arithmetic mean $\pm$ SD of $n=3$ independent experiments). Different letters indicate significant differences between the groups. Control, pBI121-GFP vector transgenic tobacco; BjCET3, pBI121-BjCET3-GFP transgenic tobacco; BjCET4, pBI121-BjCET4-GFP transgenic tobacco.

Fig. 8. Over-expression of *BiCET3* and *BjCET4* enhanced Cd tolerance of transgenic tobaccos. The photographs are representative of the performance of *BiCET3* and *BjCET4* transgenic tobaccos on days 15 and 50 after planting on MT₁ medium with or without the addition of 200 μM CdCl₂. Control, pBI121-GFP vector transgenic tobacco; BjCET3, pBI121-BjCET3-GFP transgenic tobacco; BjCET4, pBI121-BjCET4-GFP transgenic tobacco.

In the analysis of the root Cd concentration (Fig. 9D), it was found that there was no difference between *BjCET3* plants and vector controls, while *BjCET4* plants showed about 160 μg Cd g^{-1} more than the vector controls ($P < 0.05$). For the shoot Cd accumulation (Fig. 9E), *BjCET3* plants showed, on average, about 90 μg Cd g^{-1} (dry weight) higher than the vector controls ($P < 0.05$), while *BjCET4* plants showed, on average, about 90 μg Cd g^{-1} (dry weight) lower than the vector controls ($P < 0.05$). This may be a result of the higher tolerance to Cd by over-expressing BjCET4 proteins, making the shoot grow faster than others thus diluting the shoot accumulated Cd, or hte BjCET4 proteins reduced Cd accumulation in the shoot thus reducing the damage caused by the Cd poison which led to a higher sho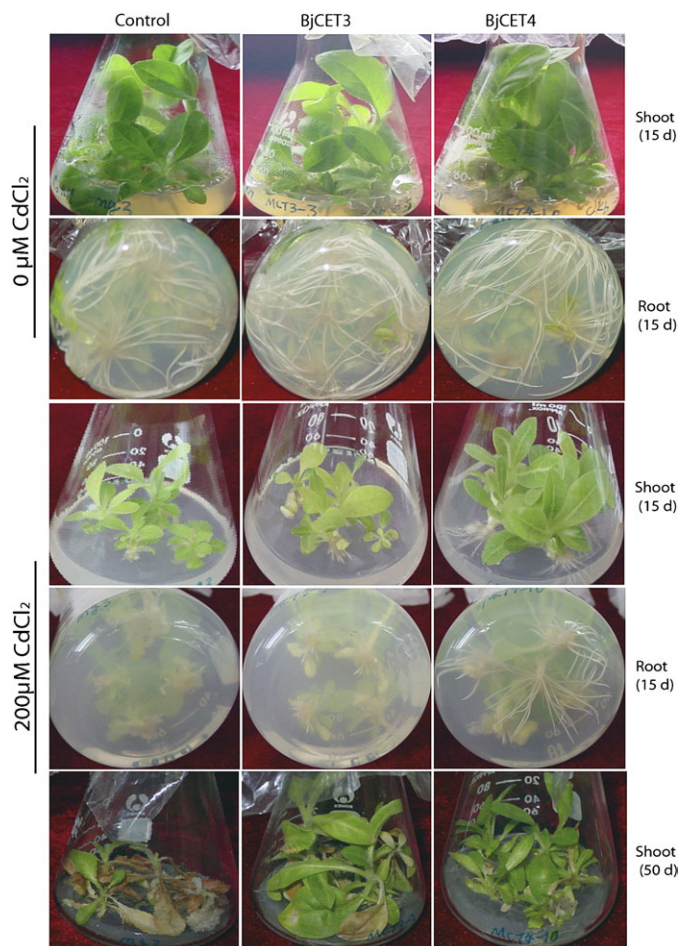ot biomass production. The shoot/root ratios for Cd content of *BjCET4*, *BjCET3* transgenic plants, and control subjects were 0.30 ± 0.01, 0.43 ± 0.02, and 0.38 ± 0.02, respectively, and these values were significantly different from each other ($P < 0.05$).

To compare total Cd accumulation in shoots, all the *BjCET* plants showed a significantly higher Cd extracting ability than the vector controls ($P < 0.01$ for *BjCET3*, $P < 0.001$ for *BjCET4*), and *BjCET4* plants nearly accumulated 2-fold more Cd compared with the vector controls (Fig. 9F). These results showed that biomass is an important factor for phytoextracting pollutants from the soil efficiently.

Discussion

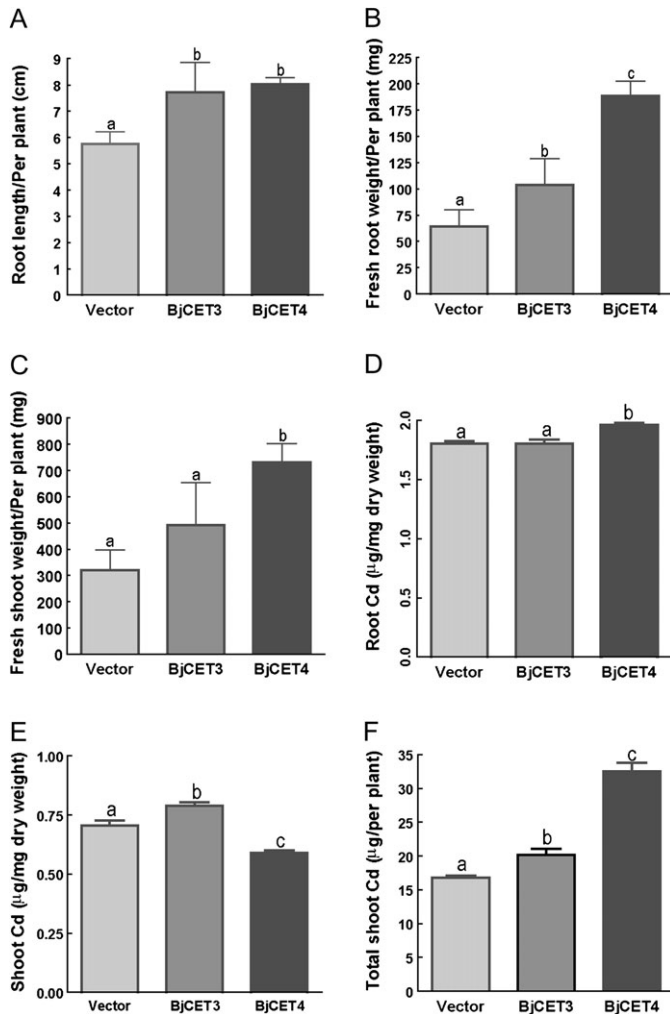*B. juncea* is characterized by rapid growth, high biomass, and an appreciable capacity to take up multi-toxic metals

Fig. 9. Cd tolerance and accumulation in roots and shoots of BjCET transgenic plants exposed to CdCl$_2$. (A–C) Quantitative analysis of root length (A), fresh root weight (B), and fresh shoot weight (C) of different genotype plantlets exposed to 200 μM CdCl$_2$ for 30 d. (D, E) Root and shoot Cd content (μg mg^{-1} dry weight) of different genotype plantlets exposed to 200 μM CdCl$_2$ for 30 d, respectively. (F) Total Cd accumulated in shoot of per plant for each genotype. The statistical analysis method used was the same as Fig. 7. Values are average number of each genotype (arithmetic mean ±SD of n=5–7). Different letters indicate significant differences between the groups.

including Cd (Rugh, 2004). The relatedness of *B. juncea* to numerous hyperaccumulators in the Brassicaceae family, and its ability to be self fertilized makes *B. juncea* a potential species for bioengineering of a practical hyperaccumulator for phytoremediation. This remains to be realized until more is known about the genes and mechanisms involved in metal uptake, detoxification, and deposition in plants. So far, five cation-efflux family members (BjCET1–4 and BjMTP1) have been identified and cloned from *B. juncea*, however, few have been well characterized. Sequence and phylogenetic tree analysis showed that *BjCET3* and *BjCET4* are more closely related than the other three cation-efflux members from *B. juncea*, suggesting that the two genes may

have similar functions in the plant. All the five cation-efflux transporters are in the Zn-CDF group of the plant cation-efflux family, exhibit the common structural features, including six predicted transmembrane domains (TMs) and two cytoplasmic loop regions (Gaither and Eide, 2001), in which the first region is localized between TM IV and V, and include a histidine rich motif (HX$_{n=3–6}$), and the second region includes the more tightly conserved motif H-D/E-X-HX-W-X-L-T-X$_8$-H (Fig. 1A). These structural similarities suggest the five cation-efflux transporters from *B. juncea*, including BjCET3 and BjCET4, may have a role in the homeostasis of Zn as do the other characterized CDF family members, for example, AtMTP1 and TgMTP1.

Over-expression of *BjCET3* and *BjCET4* in yeast mutants deficient in their evolutionary orthologues provided clear evidence that expression of *BjCET3* and *BjCET4* conferred Zn resistance in the Zn-sensitive yeast mutant strains *zrc1&cot1* or *zrc1* (Fig. 4B, F). To approve the Zn transport activity of BjCET proteins and their roles in rescuing the metal sensitivity of mutant yeast, the ICP-MS method was used directly to test the Zn content in yeast cell cultures over-supplied with Zn. The significantly reduced Zn accumulation in BjCET-expressing yeast cells (Fig. 5) suggested the plasma membrane targeting model of BjCET proteins for exporting metal ions out of yeast cells. In addition to rescuing the Zn-sensitivity of the *zrc1&cot1* and *zrc1* strains, expression of *BjCET3* and *BjCET4* also rescued or partially rescued the Cd, Co, and Ni- sensitivity of *zrc1*, *cot1*, and/or *zrc1* strains (Fig. 4A, F, C, D). Previous studies have characterized several plant Group III cation-efflux transporters in *zrc1 cot1* double or single mutant yeast strains. Most plant Group III cation-efflux transporters, for example, poplar *PtdMTP1* (Blaudez *et al.*, 2003), *Arabidopsis AtMTP1* (Bloss *et al.*, 2002; Desbrosses-Fonrouge *et al.*, 2005), *AtMTP3* and *AhMTP1-3* (Dräger *et al.*, 2004; Arrivault *et al.*, 2006), are likely to be Zn transporters capable of complementing the hypersensitivity of mutant strains to Zn but not to other metals, including Cd, Co, Mn, and Ni. Blaudez *et al.* (2003) identified a Leu zipper (LZ) motif in the C-terminal of PtdMTP1 sequence, which is highly conserved among plant CDFs. They found that the fourth and last Leu residue (Leu-314) of the LZ motif, together with the CDF signature sequence, are particularly critical for the function of PtdMTP1 for Zn detoxification (Blaudez *et al.*, 2003). Both BjCET3 and BjCET4 were found to contain the conserved LZ motif and CDF sequences (Fig. 1A). To our knowledge, only *T. goesingense* MTP1 protein has been reported to have a much broader substrate range than AtMTP1, AhMTP1-3, and AtMTP3, which were verified to mediate tolerance to Ni, Cd, Co, and Zn in yeast cells (Persans *et al.*, 2001). Although showing some differences in their ability to confer metal tolerance, BjCET3 and BjCET4 performed like TgMTP1- mediated tolerance to a range of metals in yeast cells. This may partially explain why *B. juncea* has the capability to tolerate and accumulate a range of toxic concentration of heavy metals including Zn, Cd, Co, Ni, while growing normally (Salt *et al.*, 1995, 1997).

In *B. juncea*, the mRNA level of *BjCET3* and *BjCET4* were found to be transcriptionally regulated not only by heavy metal stresses like Cd^{2+} and Zn^{2+}, but also by osmotic stresses like salinity (NaCl) and PEG, a different expression pattern compared with currently characterized Group III cation-efflux transporters, for example, AtMTP1 from non-accumulator *A. thaliana* (Desbrosses-Fonrouge *et al.*, 2005), TgMTP1 from hyperaccumulator *T. goesingense* (Persans *et al.*, 2001; Kim *et al.*, 2004; Gustin *et al.*, 2009), and BjCET2 (Xu *et al.*, 2009) and BjMTP1 (Muthukumar *et al*, 2007) from *B. juncea*. PEG is an inert, non-ionic, and non-penetrating compound of high molecular weight, which is widely used to simulate water-deficit conditions in a similar manner to that observed in the cells of intact plants subjected to true drought conditions (Hohl and Schopfer, 1991; Attree *et al.*, 1991). In *B. juncea*, *BjMTP1* is strongly regulated by Cd^{2+} and Ni^{2+}, and weakly regulated by Zn^{2+}, but not the other forms of abiotic stress including cold, heat, and NaCl (Muthukumar *et al.*, 2007). Our previous work also demonstrated that the *BjCET2* mRNA level is regulated by Cd and Zn, but not cold, salt, and drought (Xu *et al.*, 2009), which showed that *BjCET2* and *BjMTP1* have similar functions, however, no available *in vivo* functional data for *BjMTP1* have been reported at present. As *B. juncea* is an amphidiploid plant resulting from the hybridization of *B. nigra* and *B. campestris* (syn. *rapa*), which contains the conserved genomes from both of its diploid parents (Muthukumar *et al.*, 2007), and our phylogenetic tree analysis also showed that *BjCET2* and *BjMTP1* are the most closely related (Fig. 1B), this suggested that the two genes may be the result of a relatively recent gene amplification or that the orthologues from the parent species. *BjCET3* and *BjCET4* may be a similar case due to their similar functions and relationship (Fig. 1B).

In *A. thaliana*, to date all studies have found that *AtMTP1* expression is not modulated by exposure to elevated Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+} or Mn^{2+} (van der Zaal *et al.*, 1999; Desbrosses-Fonrouge *et al.*, 2005), while *AtMTP3* expression is up-regulated under Fe deficiency, and under conditions of excess Zn^{2+} or Co^{2+}, which showed some similarity with *BjCET3* and *BjCET4*. In poplar, *PtdMTP1* mRNA was expressed constitutively throughout hybrid poplar and was not regulated by low or high concentrations of Zn, and also no response to Cd, Ni, and drought stress were found (Blaudez *et al.*, 2003). In hyperaccumulators, *AhMTP1* transcript levels did not show changes following exposure to Co, Cd, Cu or H_2O_2 in either the roots or shoots of *A. halleri*, but was up-regulated specifically in response to high Zn concentrations in the root (Dräger *et al.*, 2004). Expression of the Ni transporter *TgMTP1* from *T. goesingense* failed to increase during Ni exposure, although it is a MTP1-related Ni hyperaccumulator (Persans *et al.*, 2001). Similarly, *BjCET3* and *BjCET4*, like their homologues *AtMTP1*, *AtMTP3*, and *PtdMTP1* in non-accumulators, showed low transcript levels under normal conditions. Comparatively, *BjCET3* is more expressed than *BjCET4* in the root, stem, and leaf of *B. juncea*, and

both *BjCET3* and *BjCET4* accumulate relatively more in the root than the other tissues, which is similar to *AtMTP3* transcript abundance (Arrivault *et al.*, 2006). MTP1 is much more highly expressed in the hyperaccumulators (e.g. *A. halleri*, *T. caerulescens*, and *T. goesingense*) than in non-accumulator species (e.g. *A. thaliana*, poplar), presumably conferring enhanced ability to accumulate high concentration of metal ions, which exhibits a prominent character in the hyperaccumulator species (Kim *et al.*, 2004).

Through imaging BjCET–GFP fusions in transgenic tobacco root cells, BjCET3 and BjCET4 were localized to the plasma membrane, and our ICP-MS data also suggested the plasma membrane targeting model of the two BjCETs in yeast. To verify that the BjCET:GFP fused protein does not lose the metal transport function, the BjCET GFP fused protein was expressed in mutant yeast under Zn stress conditions, and similar rescue results were obtained as the non-GFP fused BjCET proteins (see Supplementary Fig. S6 at *JXB* online; Fig. 4E). Furthermore, it was shown that constitutive over-expression of *BjCET3* and *BjCET4* markedly enhanced the Cd tolerant ability of transgenic tobaccos. However, the two transporters may have different mechanisms to acquire the Cd-tolerant ability, compared with control subjects. *BjCET3* over-expressing plants accumulated significant amounts of Cd in shoots while producing similar amounts of biomass. However, *BjCET4* over-expressing plants markedly increased shoot biomass production with significantly reduced Cd content in shoot. The previous finding of the Zn-induced membrane relocalization of CDF protein ZNT6 from the *trans* Golgi network (TGN) to the plasma membrane may aid in this interpretation (Huang *et al.*, 2002). Several other reports also showed a variable location of CDF transporters. For example, the *S. hamata* CDF transporter ShMTP1 was found to be targeted to the vacuolar membrane *in planta* but localized to the endoplasmic reticulum membranes when expressed in yeast (Delhaize *et al.*, 2003), TgMTP1 from *T. goesingense* was localized to both the plasma membrane (in yeast cells and protoplasts of *A. thaliana* shoot tissues) and vacuolar membranes (in *A. thaliana* and *T. goesingense* shoot tissues) (Kim *et al.*, 2004; Gustin *et al.*, 2009). The LZ motifs presented in ZNT6 are suggested to be involved in membrane relocalization and because LZ motifs are very conserved in Group III cation-efflux members and critical to the function of Zn efflux (Blaudez *et al.*, 2003), the dynamic relocalization of CDF proteins between the plasma membrane and various endomembrane systems may play a critical role in Zn and/or other metal ion homeostasis and hyperaccumulation in metal hyperaccumulators (Huang *et al.*, 2002; Kim *et al.*, 2004).

In conclusion, our study of the *B. juncea* BjCET3 and *BjCET4* genes strongly indicates that they are functional and evolutionary orthologues with similar protein functions. In addition to Zn, the two proteins may also be involved in regulating Cd, Co or Ni metal ions homeostasis in *B. juncea*, which function in a manner similar to TgMTP1 from *T. goesingense*. Their strong up-regulation

in response to both Zn and Cd of heavy metal stresses and NaCl and PEG of osmotic stresses, suggests a novel role in balancing the ionic aspects of osmotic stress and transition metal homeostasis, which may not occur for other characterized cation-efflux members, although this role is not clear at present. Both BjCET3 and BjCET4 proteins were localized to the plasma membrane in root cells of transgenic tobaccos, and over-expression of *BjCET3* and *BjCET4* markedly enhanced the Cd-tolerant ability of transgenic plants, and greatly changed the Cd accumulation process in shoots. *BjCET4* over-expressing plants showed a reduced shoot Cd content under Cd excess conditions, however, because of its significantly enhanced shoot biomass production, the single *BjCET4* plant still accumulated much more Cd in the shoot than the others. Therefore, biomass is an important factor for phytoextracting pollutants from the soil efficiently. The two factors of metal concentration and shoot biomass should be balanced in order to achieve a high efficiency of phytoremediation.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Multiple alignment analysis of deduced amino acid sequences of the CE proteins.

Supplementary Fig. S2. Testing the effect of Cd stress on the growth of *B. juncea*.

Supplementary Fig. S3. A representative of the zinc-tolerance assay plates.

Supplementary Fig. S4. Analysis of the effect of expressing BjCET3 and BjCET4 in *cot1* mutant yeast with over-supplied metal ions.

Supplementary Fig. S5. The photographs show representative performance of BiCET3, BjCET4, and empty vector (control) transgenic tobaccos at day 50 of growing in normal conditions.

Supplementary Fig. S6. Testing the exporting activity of BjCET:GFP proteins.

Supplementary Table S1. Topology and hydrophile analysis of putative proteins of CE members from *B. juncea* and other species.

Supplementary Table S2. Gene name, GenBank accession number and species.

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