New Biotechnology 35 (2017) 54–61

Contents lists available at ScienceDirect

New Biotechnology

journal homepage: www.elsevier.com/locate/nbt

Full length Article

Functional components of the bacterial CzcCBA efflux system reduce cadmium uptake and accumulation in transgenic tobacco plants

Andrea Nesler[a,b], Giovanni DalCorso[b], Elisa Fasani[b], Anna Manara[b], Gian Pietro Di Sansebastiano[c], Emanuele Argese[d], Antonella Furini[b,*]

[a] Actual address: Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy
[b] Department of Biotechnology, University of Study of Verona, St. Le Grazie 15, Verona, Italy
[c] Department of Biological and Environmental Sciences and Technologies, University of Salento, S.P. 6, Lecce, Italy
[d] Department of Molecular Sciences and Nanosystems, Ca' Foscari University of Venice, Via Torino 155, Venezia, Italy

ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form 11 November 2016
Accepted 25 November 2016
Available online 27 November 2016

Keywords:
CzcCBA
Cadmium
Phytoremediation
Food safety
Applied biotechnology

ABSTRACT

Cadmium (Cd) is a toxic trace element released into the environment by industrial and agricultural practices, threatening the health of plants and contaminating the food/feed chain. Biotechnology can be used to develop plant varieties with a higher capacity for Cd accumulation (for use in phytoremediation programs) or a lower capacity for Cd accumulation (to reduce Cd levels in food and feed). Here we generated transgenic tobacco plants expressing components of the *Pseudomonas putida* CzcCBA efflux system. Plants were transformed with combinations of the *CzcC*, *CzcB* and *CzcA* genes, and the impact on Cd mobilization was analysed. Plants expressing *PpCzcC* showed no differences in Cd accumulation, whereas those expressing *PpCzcB* or *PpCzcA* accumulated less Cd in the shoots, but more Cd in the roots. Plants expressing both *PpCzcB* and *PpCzcA* accumulated less Cd in the shoots and roots compared to controls, whereas plants expressing all three genes showed a significant reduction in Cd levels only in shoots. These results show that components of the CzcCBA system can be expressed in plants and may be useful for developing plants with a reduced capacity to accumulate Cd in the shoots, potentially reducing the toxicity of food/feed crops cultivated in Cd-contaminated soils.

Introduction

Human industrial activity has resulted in the pollution of the environment with metals and metalloids [1]. This widespread soil contamination is harmful to plants by interfering with physiological and metabolic processes [2]. Cadmium (Cd) is a toxic trace element released into the environment not only by industry but also by agricultural practices, especially the use of phosphate fertilizers contaminated with Cd. The environmental release of Cd has been in decline since the 1960s as production and disposal methods have improved, but the industrial consumption of Cd has risen steadily and the cumulative environmental concentration has therefore increased [3]. Cd is not required as a plant micronutrient but it is co-transported by proteins that mobilize essential minerals such as iron (Fe) and zinc (Zn), and it therefore accumulates in plants used for food, feed and smoking [4]. This is hazardous because Cd is toxic to humans, with severe adverse effects on kidney function [5] and an exposure-dependent increase in the risk of cancer [6,7].

The restoration of sites polluted with Cd and other heavy metals can be achieved adopting conventional physical or chemical treatments, or remediation strategies using plants and their microbiota to mobilize the metals for storage in plant organs (*phytoextraction*), or to immobilize the metals to make them inaccessible (*phytostabilization*) [8,9]. However, it is impossible to completely restore all soil types even with mild contamination, and it is not technically feasible to remove soils naturally containing heavy metals that are toxic to most plant species. One way to address this challenge is to develop plants with a lower capacity for the uptake of heavy metals even when growing in

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; MVBs, multivesicular bodies; RFP, red fluorescent protein; RND, resistance-nodulation-cell division; (RT)-PCR, reverse transcription PCR; TGN, trans-Golgi network; YFP, yellow fluorescent protein.

* Corresponding author at: Department of Biotecnology, University of Study of Verona, St. Le Grazie 15, 37134, Verona, Italy.
E-mail address: antonella.furini@univr.it (A. Furini).

http://dx.doi.org/10.1016/j.nbt.2016.11.006
1871-6784/

mildly contaminated soils, thus reducing the input of toxic elements in the food/feed chain [10].

Toxic heavy metals and essential micronutrients often share the same mobilization pathways, and plants have evolved mechanisms to maintain the concentration of essential metals within physiological limits. A complex homeostatic network controls the uptake, chelation, transport, accumulation and detoxification of metals [11]. Similarly, bacteria have evolved several mechanisms to tolerate the uptake of heavy metal ions, such as efflux pumps that selectively remove toxic metals, the complexation and accumulation of metal ions inside the cell, and the reduction of metal ions to a less toxic state [12]. Genes from heterologous sources have been expressed in plants to increase their capacity for metal accumulation, allowing the phytoremediation of contaminated soils, or to reduce their capacity for metal accumulation, to prevent toxic metals accumulating in edible crops and tobacco [10,13,14,15].

In Gram-negative bacteria, the CBA transporter (consisting of subunits C, B and A) is a member of the resistance-nodulation-cell division (RND) system that exports metals from the cytoplasm or the periplasm across the outer membrane [16]. The first characterized member of the RND family was the CzcA protein from *Ralstonia metallidurans* (formerly *Alcaligenes eutrophus* CH34; [17]). The presence of the pMOL30 plasmid in this species increased the minimal inhibitory concentrations of cobalt (Co), Zn and Cd (hence Czc) by several fold [18] and the corresponding genetic resistance determinants were amply characterized [19,20]. The *CzcC*, *CzcB* and *CzcA* genes encode a membrane-bound protein complex that achieves heavy metal resistance by active cation efflux driven by a cation-proton antiporter [21,22]. CzcC is the outer membrane factor, CzcB is a membrane fusion protein, and CzcA is the RND counterpart, the only subunit of the CzcCBA efflux protein complex with several transmembrane α-helicases [23]. The loss of CzcA and CzcB increases sensitivity to Co, Zn and Cd, whereas the loss of CzcC has no further impact [24,16]. Proteomic analysis of the *Pseudomonas putida* strain *Cd-001* isolated from a site contaminated with the heavy metals Zn, lead (Pb) and Cd showed that several proteins were modulated in response to Cd treatment, including members of the CzcCBA efflux system [25]. Here we investigated whether the *P. putida* CzcCBA complex affects Cd accumulation when constitutively overexpressed in tobacco plants exposed to excess Cd in their hydroponic growth medium.

Materials and methods

Plant materials and growth conditions

Tobacco seeds (*Nicotiana tabacum* cv. Petit Havana SR1) were sown and cultivated *in vitro* on MS medium [26] at 22 °C/18 °C day/night temperature with a 16-h photoperiod. The plants were used for leaf disc genetic transformation as previously described [27]. The presence and expression of the transgenes were confirmed by PCR and reverse transcription (RT)-PCR. Transgenic T_1 plants were transferred to the greenhouse, tested for transgene expression by real-time RT-PCR, and three independent lines representing each genotype were selected based on the highest transgene expression levels. These plants were self-pollinated and the T_2 progeny were used for further analysis.

Cloning and generation of tobacco plants overexpressing PpCzcC, PpCzcB and PpCzcA

The three genes encoding the CzcCBA efflux system were separately amplified by PCR from *P. putida* strain *Cd-001* genomic DNA [25] using gene-specific primer pairs 1–3 (Supplementary Table 1) designed according to the *P. putida* KT2440 genome sequence (GenBank: AEO15451). The *PpCzcA* sequence was modified to add the Kozak consensus at the 5′ end, whereas native sites were already present in *PpCzcB* and *PpCzcC*. The three PCR products were placed in separate vectors downstream of the CaMV 35S promoter. *PpCzcA* was cloned in the Gateway pENTR/D-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to the expression vector pH2GW7 by LR recombination (The Gateway® LR Clonase™ enzyme mix kit, Thermo Fisher Scientific). The final pH2GW7-*PpCzcA* vector was introduced by electroporation into competent *Agrobacterium tumefaciens* cells, strain GV3101 pMP90RK [28]. The *PpCzcB* and *PpCzcC* sequences were cloned in vector pMD1 [29], and the constructs pMD1-*PpCzcB* and pMD1-*PpCzcC* were transferred to *A. tumefaciens* strain EHA105 [30]. The transformed *A. tumefaciens* strains were used for tobacco leaf disc transformation [31]. Tobacco plants transformed with the empty pMD1 vector were used as negative controls in all experiments.

Genomic DNA isolation, RNA extraction, and cDNA synthesis

Genomic DNA for PCR analysis was isolated from control plants and plants carrying the *PpCzcC*, *PpCzcB* and *PpCzcA* genes using the DNeasy Plant Mini Kit (Qiagen, Redwood City, CA, USA). Total RNA was extracted from fresh tissue using TRIzol reagent (Thermo Fisher Scientific). After DNase treatment, first-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific).

Transcript quantification by real-time RT-PCR

Real-time RT-PCR was used for the analysis of transgene expression in several independent transgenic lines. The first-strand cDNA prepared above was amplified in 40 cycles using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) using KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). Each reaction was performed in triplicate using primer pairs 4, 5, and 6 (Supplementary Table 1) and melting curves were analysed to confirm the amplification of a single product. Solanaceae actin was used as an endogenous reference gene and was amplified using primer pair 7 (Supplementary Table 1). The data were analysed using the $2^{-\Delta\Delta CT}$ method [32].

Crosses, selection and phenotypic analysis

T_2 transgenic tobacco plants expressing the individual *PpCzcC*, *PpCzcB* or *PpCzcA* genes at the highest levels were crossed to obtain plants carrying both *PpCzcB* and *PpCzcA* (named CzcBA) or the whole efflux system comprising *PpCzcC*, *PpCzcB* and *PpCzcA* (named CzcCBA). The presence of different transgenes was confirmed by genomic PCR as described above. Phenotypic analysis was carried out on plants germinated and maintained *in vitro*, with or without the addition of $CdSO_4$ for 2 weeks, as well as plants transferred to the greenhouse and cultivated in hydroponic solution in the presence or absence of $CdSO_4$, as described below.

Cd. treatment, tolerance and quantification of Cd content

After *in vitro* selection, T_2 transgenic plants expressing each transgene at the highest level were either transferred to Petri dishes containing different concentrations of $CdSO_4$ (0, 25, 50 and 75 μM) for another 2 weeks, or moved to the greenhouse and inserted into 1.5-cm holes in polyethylene discs used as floating supports. In the latter case, plants were grown in continuously-aerated hydroponic half-strength Hoagland's solution [33] with the pH adjusted to 5.7. Hydroponic culture was chosen since it provides good experimental reproducibility, due to the allowed

strict control on the metal concentration and bioavailability. After 2 weeks, the nutrient solution was supplemented with 0.7 μM $CdSO_4$ and the plants were harvested 22 days later. The nutrient solution was maintained by adding the same volume of half-strength Hoagland's solution to each pot to avoid dehydration. After harvesting, roots and shoots were washed with 5 mM $CaCl_2$ before oven-drying at 60 °C for 36 h, and the dry weight was determined. Dried samples were homogenized in a Wiley mill followed by microwave-assisted acid digestion (Method 3051A, 2007 – https://www.epa.gov/hw-sw846/sw-846-test-method-3051a-microwave-assisted-acid-digestion-sediments-sludges-soils-and-oils) and by quantification of Cd by means of inductively coupled plasma mass spectrometry (ICP-MS) (EPA 6010D, 2014 – https://www.epa.gov/hw-sw846/sw-846-test-method-6010d-inductively-coupled-plasma-optical-emission-spectrometry-icp-aes).

Protoplast transient expression and subcellular localization analysis

The preparation and transformation of tobacco protoplasts was carried out as previously described [34]. The subcellular localization of each protein under investigation was determined by generating fluorescent fusion constructs for transient expression. The fluorescent protein tags were prepared by amplifying the red fluorescent protein gene *dsRED* (RFP, primer pair 11) from vector pGJ1425 [35], the green fluorescent protein gene *eGFP* (GFP, primer pair 12) from vector pB7FWG2 [36], and the yellow fluorescent protein *eYFP* (YFP, primer pair 13) from vector pGreen [37]. These sequences were cloned downstream of the CaMV 35S promoter in vector pMD1. The *PpCzcA*, *PpCzcB* and *PpCzcC* genes without stop codons were amplified from *P. putida* strain *Cd-001* genomic DNA using primer pairs 8, 9 and 10 (Supplementary Table 1) and inserted between the CaMV 35S promoter and the corresponding fluorescent protein tag, resulting in the final constructs pMD1-*PpCzcA*:*GFP*, pMD1-*PpCzcB*:*RFP* and pMD1-*PpCzcC*:*YFP*. Protoplasts were transfected with 20 μg of each plasmid. Four markers were utilized to label the main cellular endomembranes: GFP-KDEL [38] and RFP-KDEL [39] for the endoplasmic reticulum (ER), Cherry: BP80 for the pre-vacuolar compartment (PVC) [40], and GFP:At51F for the trans-Golgi network (TGN) and tonoplast [40]. Protoplasts were examined 16 h after transfection under a LSM 710 Zeiss confocal laser microscope using ZEN software (Carl Zeiss AG, Jena, Germany). GFP and YFP were both detected within the short 505–530 nm wavelength range and RFP within the 560–615 nm range. Excitation wavelengths of 488 and 543 nm were used simultaneously.

Statistical analysis

For the analysis of Cd accumulation, three lines (three biological replicates) were considered for each genotype, with the exception of CzcCBA plants, for which only two lines were available. The values reported in the histograms are percentages compared to control plants (lines transformed with the empty pMD1 vector). Data are presented as means ± SD. Differences between means were tested by the two-way analysis of variance (ANOVA) F-test and statistical significance was assumed at $p < 0.05$.

Results and discussion

Transgene expression

Stable transformation was confirmed by PCR for all transgenic plants grown *in vitro* on selective medium. Transgenic tobacco lines (T_1) containing the *PpCzcC*, *PpCzcB* and *PpCzcA* genes were transferred to the greenhouse for self-pollination. T_2 seeds were sown *in vitro* on selective medium, to identify homozygous T_2 candidates that were subsequently used for all further analysis and crossing. Transgene expression levels were determined by real-time RT-PCR in six independent T_2 lines representing each genotype, revealing the different expression levels shown in Supplementary Fig. 1. The presence of heterologous mRNA representing each transgene indicated that the bacterial genes can be functionally transcribed in plant cells. For each construct, the three T_2 lines with the highest expression levels (p35S:*PpCzcC*#4, #5 and #6; p35S:*PpCzcB*#2, #4 and #5; and p35S:*PpCzcA*#3, #4 and #6) were used for phenotypic analysis and the measurement of Cd tolerance and accumulation. Lines p35S:*PpCzcC*#4, p35S:*PpCzcB*#4 and p35S:*PpCzcA*#6 were subsequently used for crossing.

Phenotypic analysis

The *Escherichia coli* membrane ZntA transporter conferred resistance to Cd, Pb and Zn and reduced the heavy metal content of transgenic *Arabidopsis thaliana* plants [10]. Furthermore, tomato plants expressing bacterial ACC deaminase accumulated more metal than untransformed plants and showed enhanced tolerance in the presence of a number of metals [41]. Similarly, transgenic petunia plants co-expressing bacterial ACC deaminase and the *A. tumefaciens iaa*M gene showed increased tolerance to metals and grew larger that control plants in metal-contaminated soils [42]. These reports indicate that a variety of bacterial genes coding for proteins involved in bacterial metal homeostasis or coding for enzymes that synthesise compounds involved in plant physiology, when expressed in plants can confer resistance to heavy metals, so we tested the Cd tolerance in our transgenic lines p35S:*PpCzcC*, p35S:*PpCzcB* and p35S:*PpCzcA* selected as described above and compared to the transgenic control carrying the empty pMD1 vector. T_2 seeds from each line were sown *in vitro* on selective medium, and one-week-old plantlets were transferred to half-strength solid MS medium supplemented with different concentrations of $CdSO_4$ (0, 25, 50 and 75 μM) for a further 2 weeks before phenotypic analysis (Fig. 1). No differences were observed between the Czc and control transgenic lines under standard growth conditions (Fig. 1a). Overall, all the plant lines were comparable in terms of shoot expansion and leaf chlorosis (Fig. 1b) as well as the progressive reduction of root length in the presence of increasing concentrations of Cd (Fig. 1c). These data suggest that the bacterial genes considered for plant transformation in this work, when expressed in the entire plant body, including shoots and roots (Sup. Figs. 1 and 2) do not substantially interfere with normal physiological processes or have any effect on Cd tolerance when expressed individually.

Phenotypic analysis was repeated on plants expressing *PpCzcA* and *PpCzcB* together, obtained by crossing parents carrying the corresponding individual transgenes. These double transgenic CzcBA plants were further crossed with the p35S:*PpCzcC* line to obtain CzcCBA progeny simultaneously expressing all three transgenes. The expression of *PpCzcC* alone and in the CzcCBA plants had a minimal impact on Cd accumulation, so the remaining double transformants (CzcCA and CzcCB) were not considered for further experiments. Following PCR analysis to verify the double and triple transgenic lines, and after confirmation of transgene expression in both roots and shoots by Real Time RT-PCR (Sup. Figs. 1 and 2), T_2 plantlets were grown *in vitro* in the presence of $CdSO_4$ (25, 50 and 75 μM) as described above for the single transgenic lines. Again, there were no substantial differences in phenotype between the double or triple transgenic lines and the controls (Fig. 2a). A growing inhibition of root elongation was observed in all genotypes with increasing concentrations of Cd in the medium (Fig. 2b).

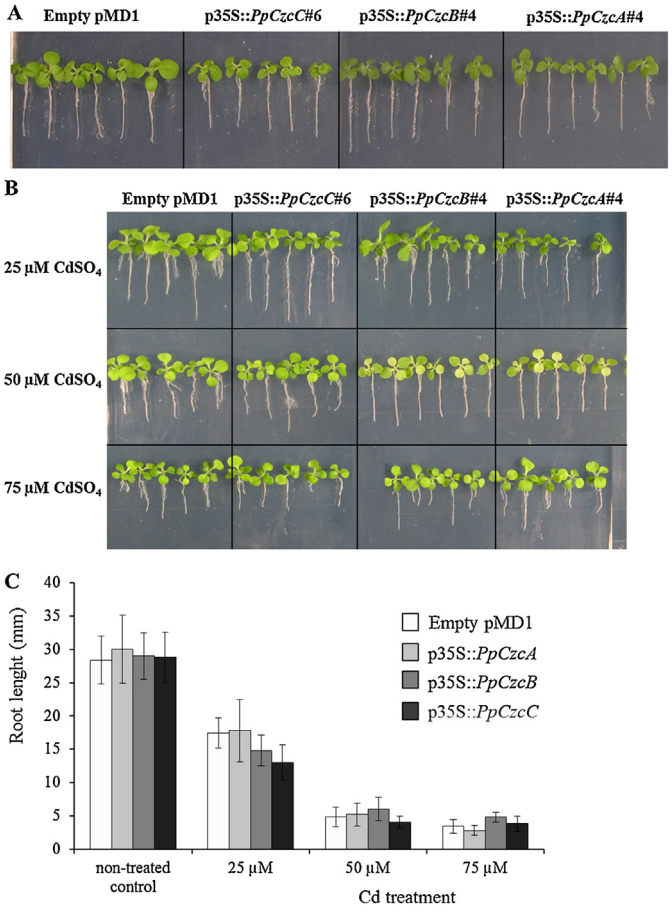

Fig. 1. Phenotypic analysis of single-transgenic lines carrying the individual *PpCzcC*, *PpCzcB* and *PpCzcA* transgenes compared to an empty-vector control. **(A)** Three-week-old plants grown on half-strength solid MS medium. **(B)** One-week-old plantlets grown on half-strength solid MS medium were transferred to medium supplemented with different concentrations of $CdSO_4$ (25, 50 and 75 µM) for a further 2 weeks. Only a single representative line has been shown for each construct. **(C)** Root length in transgenic lines exposed to different concentrations of $CdSO_4$ for 2 weeks. Three lines with the highest Czc gene expression were tested, and the values represented are means ± SD.

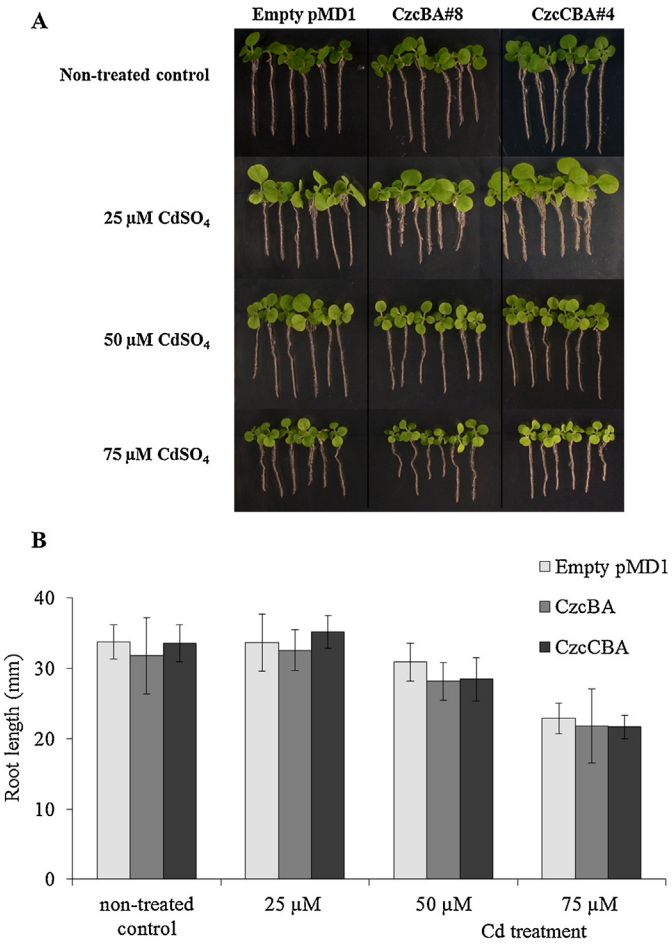

Fig. 2. Phenotypic analysis of transgenic lines expressing *PpCzcB* and *PpCzcA* simultaneously (line CzcBA#8), and *PpCzcC*, *PpCzcB* and *PpCzcA* simultaneously (line CzcCBA#4). **(A)** One-week-old plantlets grown on half-strength solid MS medium were transferred to medium supplemented with different concentrations of $CdSO_4$ (0, 25, 50 and 75 µM) for a further 2 weeks. Transgenic lines carrying the empty pMD1 vector were used as a control. **(B)** Root length in transgenic lines exposed to different concentrations of $CdSO_4$ for 2 weeks. Three lines with the highest Czc gene expression were considered, and the values represented are means ± SD.

Subcellular protein localization

To determine the subcellular localization of the three components, chimeric fusion genes were created by inserting the *PpCzcC*, *PpCzcB* and *PpCzcA* genes at the 5′ end of the *YFP*, *RFP* and *GFP* coding sequences, respectively, under the control of the 35SCaMV promoter. Each construct was transiently expressed in tobacco protoplasts and the fluorescence signal was compared to well-known markers of the endomembrane system co-expressed in the same cells. A diffuse PpCzcC:YFP signal was observed in the cytosol and nucleus, with no evidence of membrane localization or co-localization with the lytic sorting pathways represented by the PVC marker Cherry:BP80 (Fig. 3a) or the secretory pathway represented by the ER marker RFP-KDEL (Fig. 3b). PpCzcB:RFP was localized in discrete cytosolic aggregates, but these were not associated with the ER marker GFP-KDEL (Fig. 3c) or with ER-derived compartments such as the TGN and multivesicular bodies (MVBs) labelled using the vacuolar marker GFP:At51F (Fig. 3d). PpCzcA:GFP co-localized with RFP-KDEL in the ER (Fig. 4e), both in the tubular lumen and small ER-associated compartments (Fig. 3e, indicated by an arrow). PpCzcA:GFP was not exported from the ER to follow the vacuolar sorting pathway, and did not localize with the small pre-vacuolar compartments labelled using Cherry:BP80 (Fig. 3f indicated by an arrow). By analogy with GFPChi, an ER-to-Vacuole directed marker [34], PpCzcA:GFP may exit ER through still uncharacterized MVBs. To investigate putative interactions among PpCzcC, PpCzcB and PpCzcA, the constructs were transiently expressed in tobacco protoplasts simultaneously. Interestingly, the distribution of PpCzcC was modified by the presence of PpCzcB: the diffuse cytosolic localization of PpCzcC:YFP was no longer apparent and the fluorescence partially co-localized with PpCzcB:RFP-labelled structures (Fig. 3g). PpCzcC:YFP fluorescence appeared to engulf the PpCzcB:RFP aggregates, suggesting the inclusion of both proteins in MVBs. The ER localization of PpCzcA:GFP was not affected by the presence of either PpCzcB:RFP (Fig. 3h) or PpCzcB:RFP and untagged PpCzcC (Fig. 3i).

Analysis of Cd accumulation in transgenic plants

More detailed analysis of the transgenic lines was carried out to investigate Cd accumulation in greenhouse plants cultivated in hydroponic solution containing 0.7 µM $CdSO_4$, a concentration that does not induce severe stress. After 22 days under this treatment regime, the Czc transgenic plants remained phenotypically indistinguishable from control plants (data not shown). Cd

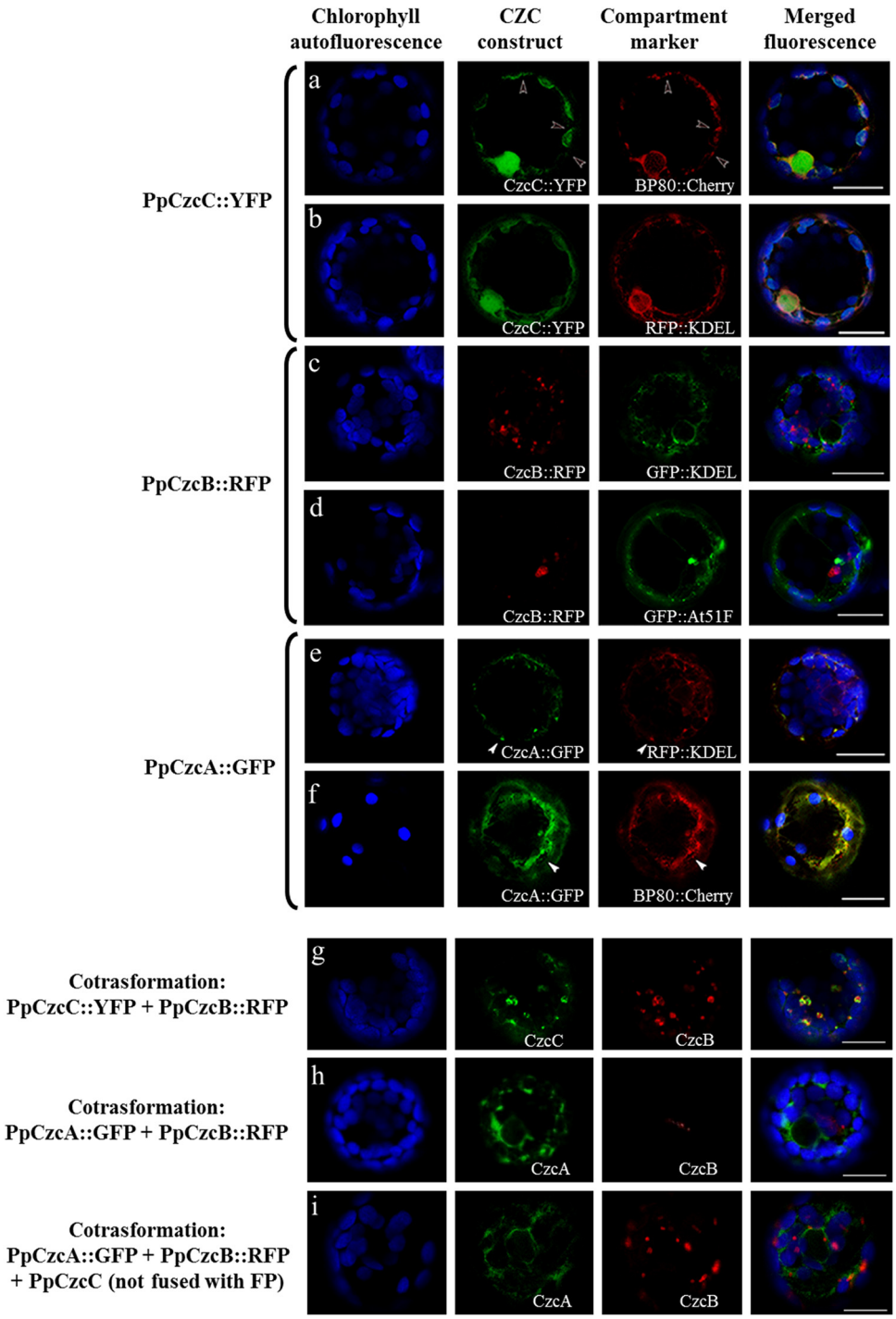

Fig. 3. Protein localization in tobacco protoplasts. PpCzcC:eYFP co-expressed with the PVC marker Cherry:BP80 (**A**) and the ER marker RFP-KDEL (**B**). PpCzcB:dsRED co-expressed with the ER marker GFP-KDEL (**C**) and the TGN and vacuolar marker GFP:At51F (**D**). PpCzcA:eGFP co-localized with the ER marker RFP-KDEL (**E**), ER-associated compartments (arrow) (**F**) and with the PVC marker Cherry:BP80 showing partial co-localization in the ER but not in the PVC (arrow). Co-expression of Czc constructs was also tested in tobacco protoplasts. PpCzcC:eYFP and PpCzcB:dsRED co-localize partially in aggregates (PpCzcC:eYFP is normally cytosolic) **(G)**. PpCzcA:GFP and PpCzcB:dsRED do not co-localize and maintain their normal distributions **(H)**. When PpCzcA:GFP is co-expressed with PpCzcB:RFP and PpCzcC (not fused with a fluorescent fusion partner (FP) due to the overlapping signals of GFP and YFP) there is no change from the normal distributions **(I)**. Fluorescent channels are presented separately in the first three columns and merged in the right-hand column. Scale bar = 20 μm.

levels were then determined by ICP-MS, based on three independent trials to control for variations caused by seasonal effects on growth conditions. The Cd levels in the Czc transgenic plants were expressed as relative values, *i.e.* a percentage compared to the control plants, which were set at 100%. There were no significant differences in Cd accumulation in the shoots and roots of the *PpCzcC* and control plants (Fig. 4a), but the *PpCzcA* and *PpCzcB* lines accumulated 30% and 23% less Cd in their shoots, respectively, compared to the controls (Fig. 4b and c). In contrast, the Cd content of the roots in the *PpCzcA* and *PpCzcB* transgenic lines was significantly greater than the controls (Fig. 4b and c). Three independent double-transgenic CzcBA lines and both of the

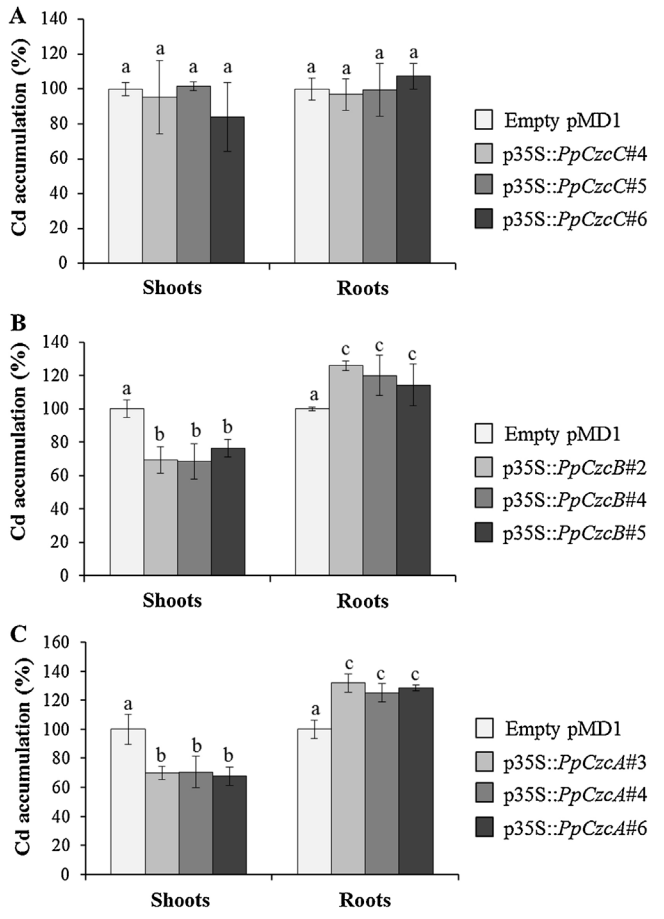

Fig. 4. Cd accumulation in shoots and roots of plants individually expressing *PpCzcC* **(A)**, *PpCzcB* **(B)** and *PpCzcA* **(C).** Cd values are presented for the three lines with the highest expression level of the Czc gene for each construct, and the values indicate percentages compared to control plants transformed with the empty pMD1 vector (set at 100%). These experiments were performed in triplicate on plants grown for 3 weeks in hydroponic culture supplemented with 0.7 μM $CdSO_4$, and the values reported are means ± SD. Different letters indicate significantly different values at $p \leq 0.05$.

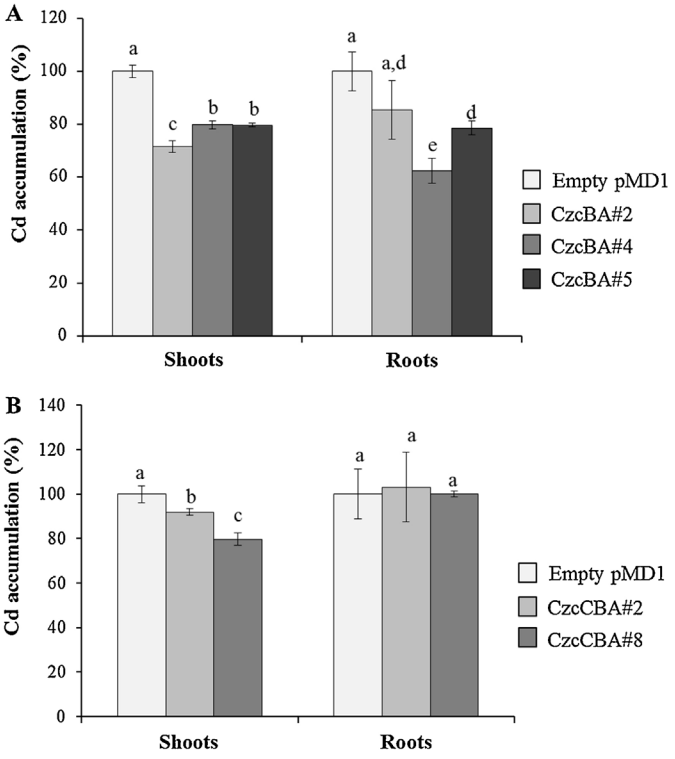

Fig. 5. Cd accumulation in shoots and roots of CzcBA plants expressing *PpCzcB* and *PpCzcA* simultaneously **(A)** and CzcCBA plants expressing *PpCzcC*, *PpCzcB* and *PpCzcA* simultaneously **(B)**. Cd values are presented for the three **(A)** and two **(B)** lines with the highest expression level of the Czc genes for each construct, and the values indicate percentages compared to control plants transformed with the empty pMD1 vector (set at 100%). These experiments were performed in triplicate on plants grown for 3 weeks in hydroponic culture supplemented with 0.7 μM $CdSO_4$, and the values reported are means ± SD. Different letters indicate significantly different values at $p \leq 0.05$.

available triple-transgenic CzcCBA lines were also cultivated under the same conditions. The double transgenic lines accumulated an average of ~23% less Cd in the shoots and 20% less Cd in the roots compared to the control plants (Fig. 5a). The Cd content of the roots in these lines was more variable, which may reflect the impact of position effects on transgene expression. It is notable that plants expressing *PpCzcB* or *PpCzcA* as individual transgenes accumulated lower levels of Cd in the shoots than controls but higher levels in the roots, whereas the combination of both transgenes reduced the level of Cd in both the shoots and the roots. The combination of all three transgenes resulted in a significant reduction in Cd levels in the shoots, but the amount of Cd in the roots was similar to the control lines (Fig. 5b).

These results suggest that the entire CzcCBA complex bacterial efflux system is not a prerequisite to reduce the Cd content of transgenic shoots. In bacteria, the loss of CzcA and CzcB functions reduced Co, Zn and Cd resistance, whereas mutated versions of CzcC did not further affect resistance towards these metals, indicating that CzcA and CzcB can function independently without the presence of CzcC [24,16]. In *R. metallidurans,* metal resistance is conferred by CzcA alone [24]. Moreover, an early sequencing project revealed the presence of two *CzcB* genes but no copy of *CzcC* in the *Helicobacter pylori* genome, supporting a model in which CzcB and CzcA can function as a metal efflux pump [43]. Interestingly, bacterial transformation with mutated versions of *CzcB* led to a slight decrease in metal resistance [24]. A recent study aiming to correlate bacterial resistance gene expression and metal bioavailability in contaminated sediments found the best correlation for *CzcA*, indicating that this gene is the most promising marker for the bioavailability of Cd/Zn/Co in aquatic sediments [44]. *CzcA* encodes the central antiporter subunit, and is promptly activated by an excess of these metals.

We found that *PpCzcB* and *PpCzcA* had a profound impact on Cd accumulation in transgenic tobacco plants but that *PpCzcC* alone does not modulate Cd levels in either the shoots or roots. Our data confirm that these bacterial proteins are functional in plants and can reduce Cd levels in the shoots or the whole plant, presumably by conferring on cells the ability to partially exclude Cd from the cytosolic environment. It is notable that plants expressing *PpCzcB* or *PpCzcA* individually were able to exclude Cd from the shoots at the expense of higher Cd levels in the roots. This indicates that either of these proteins can counter the root-to-shoot translocation of Cd, perhaps by inducing some form of metal storage capacity in the roots. Based on our protein localization data, PpCzcA may facilitate the sequestration of Cd in the root ER, thus preventing vascular loading and translocation to the shoot. Similarly, the *Populus trichocarpa* cytochrome P450 monooxygenase PtCYP714A3 was localized to the ER in transgenic rice plants, allowing the regulation of Na^+/K^+ homeostasis and better salt tolerance, possibly by increasing the efficiency of Na^+ efflux [45]. In contrast, there was no definite localization for PpCzcB, and this protein may

therefore prevent root-to-shoot translocation by binding metal ions in solution and physically restricting their transport. In plants expressing both *PpCzcB* and *PpCzcA*, a functional efflux pump appears to assemble, resulting in the export of Cd into the apoplast, where Cd may deposit onto the negatively charged carboxyl groups of the cell wall, which provide sites for cation exchange [46], and thus reducing levels throughout the plant. However, since both proteins seem not to be clearly localized on the plasma membrane, an alternative explanation may be considered, which takes into account their localization in MVBs. These compartments can be indeed involved in secretion following unconventional mechanisms [47]. *PpCzcC* is unable to modulate the Cd content on its own, but the co-expression of *PpCzcC* with *PpCzcB* and *PpCzcA* reduces the level of Cd in the shoots and not the roots. Our localization experiments showed that PpCzcC:YFP is recruited from the cytosol by some sort of interaction with PpCzcB:RFP, so the modulation of Cd distribution in CzcCBA plants further supports the hypothesis that PpCzcB and PpCzcA directly affect Cd cell-to-cell transport, and that PpCzcB and PpCzcC physically interact. The presence of PpCzcC may sequester PpCzcB into a complex that limits its availability to interact directly with Cd, reducing its effect on Cd accumulation.

Conclusions

Our results confirm that it is possible to generate transgenic plants with a reduced capacity to accumulate Cd in the shoots by introducing PpCzcB, PpCzcA or both genes together. Such plants could be cultivated in mildly Cd-contaminated soils and will accumulate less Cd in their shoots without any other phenotypic effect, making this approach ideal for food and feed crops where the aerial organs are harvested. Indeed, Cd level in food is a current matter of concern. For example, the European Union Law regulates the maximum levels of Cd in foodstuffs with the Commission Regulation (EU) No 488/2014 of 12 May 2014, which is amending the Regulation (EC) No 1881/2006 [http://eur-lex.europa.eu/legal-ontent/EN/TXT/?uri=uriserv%3AOJ.L_.2014.138.01.0 075.01.ENG]. Ranging from vegetables and fruit, root and tuber vegetables to leaf vegetables and fresh herbs, the maximum allowed Cd content varies from 0.05 to 0.10 and 0.20 mg/kg ww respectively. Considering that Cd content in agricultural soils is largely increasing (for an updated review, refer to [48]), the moderate reduction of Cd absorption by plants expressing PpCzc genes may actually contribute to lower the Cd amounts in plant organs destined to food-feed production. A similar approach could be applied to high-biomass species with a deep root system. Because plants overexpressing *CzcA* and *CzcB* individually accumulate more Cd in their roots, this could be an interesting approach to achieve Cd phytostabilization in soils, thus preventing dangerous metals spreading in soil and water.

Funding information

AN received PhD funding from the Italian Ministry of University and Research (MIUR).

Authors' **contributions**

The author AN performed the most of the experiments, with the contributions of GDC, EF and AM. GPDS and AN performed the localization analysis in protoplast. EA contributed with ICP-mass analysis and result discussion. GDC and AF conceived the project and designed and supervised the experiments. AF, GDC and EF wrote the manuscript. All the authors listed approved the work for publication.

Acknowledgments

The authors acknowledge Prof. B Baldan (University of Padova, Italy) for kindly providing the vector pGreen and L. Gobbo (University Ca' Foscari of Venice, Italy) for the ICP-mass spectrometry analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2016.11.006.

References

[1] Anjum NA, Ahmad I, Mohmood I, Pacheco M, et al. Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids-a review. Environ Exp Bot 2012;75:307–24.
[2] Villiers F, Ducruix C, Hugouvieux V, Jarno N, et al. Investigating the plant response to cadmium exposure by proteomic and metabolomic approaches. Proteomics 2011;11:1650–63.
[3] Moulis JM, Thévenod F. New perspectives in cadmium toxicity: an introduction. Biometals 2010;23:763–76.
[4] Franz E, Römkens P, van Raamsdonk L, van der Fels-Klerx I. A chain modeling approach to estimate the impact of soil cadmium pollution on human dietary exposure. J Food Prot 2008;7112:2504–13.
[5] Satarug S, Moore MR. Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. Environ Health Perspect 2004;112:1099–103.
[6] Menke A, Muntner P, Silbergeld EK, Platz EA, et al. Cadmium levels in urine and mortality among U.S. adults. Environ Health Perspect 2009;117:190–6.
[7] Satarug S, Garrett SH, Sens MA, et al. Cadmium, environmental exposure, and health outcomes. Environ Health Perspect 2010;118:182–90.
[8] Farinati S, DalCorso G, Bona E, Corbella M, et al. Proteomic analysis of *Arabidopsis halleri* shoots in response to the heavy metals cadmium and zinc and rhizosphere microorganisms. Proteomics 2009;9:4837–50.
[9] Ma Y, Zhang C, Oliveira RS, Freitas H, et al. Bioaugmentation with endophytic bacterium E6S homologous to achromobacter piechaudii enhances metal rhizoaccumulation in host *Sedum plumbizincicola*. Front Plant Sci 2016;7:1–9, doi:http://dx.doi.org/10.3389/fpls.2016.00075.
[10] Lee J, Bae H, Jeong J, Lee JY, et al. Functional expression of a bacterial heavy metal transporter in *Arabidopsis* enhances resistance to and decreases uptake of heavy metals. Plant Physiol 2003;133:589–96.
[11] Clemens S. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 2001;212:475–86.
[12] Nies DH. Microbial heavy metal resistance. Appl Microbiol Biotechnol 1999;51:730–50.
[13] Sasaki Y, Hayakawa T, Inoue C, Miyazaki A, et al. Generation of mercury hyperaccumulating plants through transgenic expression of the bacterial mercury membrane transport protein MerC. Transgenic Res 2006;15:615–25.
[14] Koren'kov V, Park S, Cheng NH, Sreevidya C, et al. Enhanced Cd^{2+} −selective root-tonoplast transport in tobaccos expressing *Arabidopsis* cation exchangers. Planta 2007;225:403–11.
[15] He J, Hong Li H, Ma C, Zhang Y, et al. Overexpression of bacterial c-glutamylcysteine synthetase mediates changes in cadmium influx, allocation and detoxification in poplar. New Phytol 2015;205:240–54.
[16] Franke S, Grass G, Rensing C, Nies DH. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol 2003;185:3804–12.
[17] Saier MH, Tam R, Reizer A, Reizer J. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol 1994;11:841–7.
[18] Mergeay M, Nies D, Schlegel HG, Gerits J, et al. Alcaligenes eutrophus CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 1985;162:328–34.
[19] Kunito T, Kusano T, Oyaizu H, Senoo K, et al. Cloning and sequence analysis of czc genes in Alcaligenes sp strain CT14. Biosci Biotechnol Biochem 1996;60:699–704.
[20] Van der Lelie D, Schwuchow T, Schwidetzky U, Wuertz S, et al. Two component regulatory system involved in transcriptional control of heavy metal homoeostasis in *Alcaligenes eutrophus*. Mol Microbiol 1997;23:493–503.
[21] Nies DH. The cobalt, zinc, and cadmium efflux system CzcCBA from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. J Bacteriol 1995;177:2707–12.
[22] Kim EH, Nies DH, McEvoy MM, Rensing C. Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis. J Bacteriol 2011;193:2381–7.
[23] Nies DH. Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid 1992;27:17–28.
[24] Rensing C, Pribyl T, Nies DH. New functions for the three subunits of the CzcCBA cation-proton antiporter. J Bacteriol 1997;179:6871–9.

[25] Manara A, DalCorso G, Baliardini C, Farinati S, et al. *Pseudomonas putida* response to cadmium: changes in membrane and cytosolic proteomes. J Proteome Res 2012;11:4169–79.
[26] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473–97.
[27] Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, et al. A simple and general method for transferring genes into plants. Science 1985;227:1229–31.
[28] Koncz C, Schell J. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol Gen Genet 1986;204:383–96.
[29] Das M, Harvey I, Chu LL, Sinha M, et al. Full-length cDNAs: more than just reaching the ends. Physiol Genom 2001;6:57–80.
[30] Hellens R, Mullineaux P, Klee H. Technical focus guide to agrobacterium binary Ti vectrors. Trends Plant Sci 2000;5:446–51.
[31] Gallois P, Marinho P. Leaf disk transformation using Agrobacterium tumefaciens-expression of heterologous genes in tobacco. Methods Mol Biol 1995;49:39–48.
[32] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25:402–8.
[33] Hoagland DR, Arnon DI. The water culture method for growing plants without soil. Univ. Calif. Coll. Agric. Exp. Sta. Circ. Berkeley, 1938. CA 347-353.
[34] Stigliano E, Faraco M, Neuhaus JM, Montefusco A, et al. Two glycosylated vacuolar GFPs are new markers for ER-to-vacuole sorting. Plant Physiol Biochem 2013;73:337–43.
[35] Jach G, Binot E, Frings S, Luxa K, et al. Use of red fluorescent protein from *Discosoma* sp. (dsRED) as a reporter for plant gene expression. Plant J 2001;28:483–91.
[36] Karimi M, Inzé D, Depicker A. GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci 2002;7:193–5.
[37] Hellens R, Edwards A, Leyland N, Bean S, et al. pGreen:a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol Biol 2000;42:819–32.
[38] Di Sansebastiano GP, Paris N, Marc-Martin S, Neuhaus JM. Specific accumulation of GFP in a non-acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway. Plant J 1998;15:449–57.
[39] De Domenico S, Tsesmetzis N, Di Sansebastiano GP, Hughes RK, et al. Subcellular localisation of Medicago truncatula 9/13-hydroperoxide lyase reveals a new localisation pattern and activation mechanism for CYP74C enzymes. BMC Plant Biol 2007;7:58.
[40] De Benedictis M, Bleve G, Faraco M, Stigliano E, et al. AtSYP51/52 functions diverge in the post-Golgi traffic and differently affect vacuolar sorting. Mol Plant 2013;6:916–30.
[41] Grichko VP, Filby B, Glick BR. Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb and Zn. J Biotechnol 2000;81:45–53.
[42] Zhang Y, Zhao L, Wang Y, Yang B, et al. Enhancement of heavy metal accumulation by tissue specific co-expression of iaaM and ACC deaminase genes in plants. Chemosphere 2008;72:564–71.
[43] Tomb JF, White O, Kerlavage AR, Clayton RA, et al. The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 1997;388:539–47.
[44] Roosa S, Wattiez R, Prygiel E, Lesven L, et al. Bacterial metal resistance genes and metal bioavailability in contaminated sediments. Environ Pollut 2014;189:143–51.
[45] Wang C, Yang Y, Wang H, Ran X, et al. Ectopic expression of a cytochrome P450 monooxygenase gene PtCYP714A3 from *Populus trichocarpa* reduces shoot growth and improves tolerance to salt stress in transgenic rice. Plant Biotechnol J 2016;9:1838–51, doi:http://dx.doi.org/10.1111/pbi.12544.
[46] Van Belleghem F, Cuypers A, Semane B, Smeets K, et al. Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. New Phytol 2007;173:495–508.
[47] Ding Y, Robinson DG, Jiang L. Unconventional protein secretion (UPS) pathways in plants. Curr Opin Cell Biol 2014;29:107–15.
[48] Tóth G, Hermann T, Da Silva MR, Montanarella L. Heavy metals in agricultural soils of the European Union with implications for food safety. Environ Int 2016;88:299–309.