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

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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.

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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