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Research article

Enhanced cadmium accumulation and tolerance in transgenic tobacco overexpressing rice metal tolerance protein gene *OsMTP1* is promising for phytoremediation

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ABSTRACT

One of the most grievous heavy metal pollutants in the environment is cadmium (Cd), which is not only responsible for the crop yield loss owing to its phytotoxicity, but also for the human health hazards as the toxic elements usually accumulate in the consumable parts of crop plants. In the present study, we aimed to isolate and functionally characterize the *OsMTP1* gene from *indica* rice (*Oryza sativa* L. cv. IR64) to study its potential application for efficient phytoremediation of Cd. The 1257 bp coding DNA sequence (CDS) of *OsMTP1* encodes a ~46 kDa protein belonging to the cation diffusion facilitator (CDF) or metal tolerance/transport protein (MTP) family. The *OsMTP1* transcript in rice plant was found to respond during external Cd stress. Heterologous expression of OsMTP1 in tobacco resulted in the reduction of Cd stress-induced phytotoxic effects, including growth inhibition, lipid peroxidation, and cell death. Compared to untransformed control, the transgenic tobacco plants showed enhanced vacuolar thiol content, indicating vacuolar localization of the sequestered Cd. The transgenic tobacco plants exhibited significantly higher biomass growth (2.2–2.8-folds) and hyperaccumulation of Cd (1.96–2.22-folds) compared to untransformed control under Cd exposure. The transgenic plants also showed moderate tolerance and accumulation of arsenic (As) upon exogenous As stress, signifying broad substrate specificity of OsMTP1. Together, findings of our research suggest that the transgenic tobacco plants overexpressing OsMTP1 with its hyperaccumulating activity and increased growth rate could be useful for future phytoremediation applications to clean up the Cd-contaminated soil.

1. Introduction

Metal pollutants have been an integral part of human civilization since its very inception. However, rapid industrial development has played a key role in intensifying the heavy metal toxicity in the biosphere, leading to severe environmental and health hazards. One of the most toxic heavy metals that possesses a significant health risk to the humans is cadmium (Cd), which ranks seventh among toxic substances for human health hazard by U.S. poison and disease registry (Priority List of Hazardous Substances, 2015, Agency for Toxic Substances and Disease Registry; http://www.atsdr.cdc.gov/spl). The contamination of Cd in agricultural soils occurs because of excessive fertilizer (phosphate) application in cultivated fields, use of sewage sludge as a soil amendment, or sometimes due to naturally high background levels (Dorlhac de Borne et al., 1998). The phytotoxicity caused due to this is an alarming concern in crop productivity nowadays. Cadmium has also been accounted to be accumulated in notable amount in the consumable parts of crop plants, and their subsequent internalizing in the food chain raises the question of potential health hazards (Verbruggen et al., 2009) as the cadmium (Cd) and arsenic (As), among the other heavy metals or metalloids known for the potential carcinogenic activity (Smith et al., 2002; Nawrot et al., 2006). The Cd element present in the food chain following ingestion is absorbed in the digestive tract in the consumer's body and is

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Abbreviations: As, Arsenic; AAS, Atomic absorption spectroscopy; Cd, Cadmium; CDF, Cation diffusion facilitator; CaMV, Cauliflower mosaic virus; CDS, Coding DNA sequence; *hptII*, Hygromycin phosphotransferase II; MDA, Malondialdehyde; MSI, Membrane stability index; MTPs, Metal tolerance or transporter proteins; MS, Murashige and Skoog medium; NOS, Nopaline synthase; RT-PCR, Reverse transcription polymerase chain reaction; TMDs, Transmembrane domains.

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accumulated in the kidney or liver. Accumulation in high levels causes stomach irritation leading to vomiting and diarrhea, and sometimes death. The high amount of Cd in the human body has also been associated with kidney damage, bone fragility, liver damage or even cancer (Toxicological Profile for Cadmium, 2012 ATSDR).

To overcome the problem of toxic substance contamination in soils, various methods have been used such as *ex-situ* or *in-situ* soil washing (Wood, 1997; GOC, 2003; Davies and Singh, 1995; Dikinya and Areola, 2010), chemical immobilization/stabilization of heavy metals in soil (Wang et al., 2009; Houben et al., 2012), electrokinetic remediation (Acar and Alshawabkeh, 1993; Reddy and Chinthamreddy, 1999), or land filling with fresh soil (Tokunaga, 1996) etc. However, most of these traditional approaches are very costly and is destructive to the soil (Evangelou et al., 2007). Therefore, in recent years, the low-cost plant-based phytoremediation of the toxic compounds from contaminated agricultural soil has been a subject of intense research (Suresh and Ravishankar, 2004; LeDuc and Terry, 2005; Chehregani and Malayeri, 2007; Lone et al., 2008; Singh and Prasad, 2011).

The fact that heavy metal pollutants cannot be chemically degraded makes their removal through absorption by plants as a very promising remediation technology (Pilon-Smits and Pilon, 2002). For possible application in phytoremediation, the critical factors of the selected plant species are their ability to heavy metal tolerance and accumulation turnout (Clemens, 2006; Kunze et al., 2002), thus plant species with enhanced metal tolerance and accumulation properties have been used for cleaning polluted soils and waters. However, the practical applicability of this emerging cheaper technology using natural metal hyperaccumulator plant species appears to be limited as they are mostly small plants with moderate growth rates, therefore cleaning up a heavy metal contaminated site could take several years (Pilon-Smits and Pilon, 2002). The absence of ideal natural plant species for phytoremediation applications, have made genetic engineering a suitable strategy to improve the phytoremediation efficiency of the plants (Verbruggen et al., 2009) by increasing the metal tolerance and accumulation patterns in high-biomass, naturally non-accumulating plant species through expressing genes involved in uptake and/or detoxification processes (Kunze et al., 2002; Kramer, 2005; Pilon-Smits and Pilon, 2002; Eapen and D'Souza, 2005).

In plants, tolerance to heavy metal is governed by the various interrelated physiological and molecular mechanisms. Usually the cellular detoxification process involves either chelation of heavy metals by low molecular weight compounds and their subsequent sequestration into organelles or expulsion of heavy metals to the extracellular space by specific transporters. Considering the importance of the transmembrane transport in metal sequestration within a cell, one possible approach to generate plants suitable for phytoremediation might consist of introducing transporter proteins involved in transporting heavy metals or their complexes to appropriate storage compartments like vacuole. In the case of Cd, one of the efficient detoxification mechanisms in the plant is associated with rapid binding of free ions in the cytoplasm and/or their sequestration into vacuoles, which are compartments of lower metabolic activity (Clemens, 2006). Genetic engineering approaches by expressing appropriate transporter molecules for increased resistance to environmental stresses, such as heavy metals (Hirschi et al., 2000; Pence et al., 2000; Song et al., 2003) have been used and termed as 'transport engineering' for enhanced phytoremediation.

The transporter proteins that have been found to play a critical role in maintaining cellular metal homeostasis by mediating heavy metal movement through membranes, have been grouped into channels, carriers, and pumps (Williams et al., 2000). Among these transporters, metal tolerance or transporter proteins (MTPs) belonging to the cation diffusion facilitator (CDF) family are one of the most important group of proteins that function in heavy metal homeostasis (Sharma et al., 2016). CDF transporters are phylogenetically ubiquitous being present in almost all kingdoms of life (Nies and Silver, 1995; Maser et al., 2001) and have been grouped into three sub-groups, i.e. Zn-CDF, Fe/Zn-CDF, and Mn-CDF (Montanini et al., 2007). In plants, the CDF or MTP family proteins are important metal transporter proteins having broad substrate specificity. These proteins are involved in compartmentalization or efflux of metal ions to reduce the toxicity, thereby maintaining the cellular homeostasis during metal stress. Till date, quite a few MTP family proteins have been characterized from both non-accumulator *Arabidopsis thaliana* (Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006) and hyper-accumulator plant species [*Arabidopsis halleri* (Drager et al., 2004), *Noccaea caerulescens*, *Thlaspi goesingense* (Kim et al., 2004) *Stylosanthes hamate* (Delhaize et al., 2003)]. All these MTPs have been found to respond to various metal ions including Zn, Cd, or other bivalent metal ions (Yuan et al., 2012).

The rice (*Oryza sativa*) orthologue OsMTP1 from *japonica* cultivar has been recently characterized by Yuan et al. (2012), Lan et al. (2012) and Menguer et al. (2013); and its expression was found to be significantly induced by exposure to metals such as Zn, Cd, Cu, and Fe (Lan et al., 2012). Moreover, both overexpression and silencing of OsMTP1 suggest its involvement in the transport of Zn, Cd, and Ni metal ions (Yuan et al., 2012; Lan et al., 2012; Menguer et al., 2013). Furthermore, the expression of OsMTP1 in yeast has been shown to increase tolerance to Zn, Cd, and Ni (Yuan et al., 2012). However, certain controversies regarding the subcellular localization and exact function of OsMTP1 during metal stress still exist. Hence, in this study, we aimed to functionally characterize the OsMTP1 from an *indica* rice cultivar and determine its suitability for phytoremediation of Cd using tobacco system.

2. Materials and methods

2.1. Plant materials

The *indica* cultivar (cv.) IR64 of rice (*O. sativa* L.) and tobacco (*Nicotiana tabacum*) plants were used in the present study. All the untransformed and transgenic lines were grown and maintained in contained growth chamber (Environmental system, Labtech, Daihan) under 16 h light/8 h dark period and natural field condition (custom made net-house) for different analyzes.

2.2. Isolation and cloning of OsMTP1

Total RNA was extracted from rice leaves using the RNeasy plant mini kit (Qiagen) and was treated with DNase I (Sigma). First-strand cDNA synthesis was carried out using 2 μg of RNA and oligo $d(T)_{23}VN$ primer (New England Biolabs) with MMuLV Reverse Transcriptase (New England Biolabs). The coding DNA sequence (CDS) of the *OsMTP1* gene was amplified from this cDNA sample by

Table 1
List of primers used in this study.

Serial no:	Primer name	Primer sequence
1	OsMTP1-FL5	5′-GGATCCATGGACAGCCATAACTCAGC-3′
2	OsMTP1-FL3	5′-GAGCTCCTACTCGCGCTCAATCTGAATG-3′
3	OSMTP1-RT5	5′-TCAAGATGCTGCGCAACATCC-3′
4	OsHis-RT5	5′-GGTCAACTTGTTGATTCCCCTCT-3′
5	OsHis-RT3	5′-AACCGCAAAATCCAAAGAACG-3′
6	NtACT-RT5	5′-GGHCGTACHACWGGTATTGTG-3′
7	NtACT-RT3	5′-CCTCCRATCCARACRCTRTACTT-3′