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Ectopic expression of *SaNRAMP3* from *Sedum alfredii* enhanced cadmium root-to-shoot transport in *Brassica juncea*

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ABSTRACT

SaNRAMP3 gene cloned from a Zn/Cd hyperaccumulator *Sedum alfredii* was ectopicly expressed in *Brassica juncea*, a fast-growing and high-biomass crop plant. In a tissue culture experiment, transgenic plants were grown on MS medium with 0, 25, 50, 100, 200 μM Cd. It was shown that, at the same Cd treatment, the Cd tolerance of transgenic plants had no significant difference with those of wild-type plants (WT). However, the shoot Cd content and accumulation were improved significantly while the root Cd content and accumulation were descended significantly by *SaNRAMP3* gene expression, which obviously enhanced the Cd root-to-shoot translocation factor (TF). In the hydroponic experiment, plants were cultured in nutrition solution with 0, 2.5, 25 μM Cd. Data showed that the Cd tolerance of transgenic plants had no significant difference with that of WT under the same Cd exposure. Whereas, the shoot Cd content and accumulation was increased 1.43–1.81 times and the TF was enhanced 3.09–3.51 times by *SaNRAMP3* gene expression. Those results indicated that ectopic expression of *SaNRAMP3* in *B. juncea* didn't lead to Cd sensitivity, but enhanced Cd root-to-shoot transport, so that increased shoot Cd accumulation. This study provided a possibility to improve phytoextraction efficiency of heavy metal through gene engineering.

1. Introduction

Soil heavy metal pollution pervaded many parts of the world, especially developing countries such as China (M. Chen et al., 2017; Yan et al., 2016; Li et al., 2014). Among these heavy metals, cadmium (Cd) pollution was increasingly serious in soils, occurring both naturally and artificially (Li et al., 2017; Yuan et al., 2017; Wen et al., 2015). Although it was a non-essential element, Cd was taken up by plants through the essential element transporters and entered the food chain, posing a threat to human health (Luo et al., 2017; Dou et al., 2017; Clemens et al., 2013). Furthermore, Cd was classified as a human carcinogen that affected many cellular processes including the cell cycle, cell proliferation and DNA repair (Nersesyan et al., 2016; P. Chen et al., 2016; Bertin and Averbeck, 2006).

Plants which were able to accumulate high concentrations of heavy metals were known as hyperaccumulators (Brooks et al., 1977; Krämer, 2010; Wei and Twardowska, 2013). To reduce Cd content in contaminated soils, several Cd hyperaccumulators were characterized. Among them, *Noccaea caerulescens, Arabidopsis halleri, Sedum alfredii* and *Solanum nigrum* were well studied (Baker et al., 1994; Bert et al., 2002; Yang et al., 2004; Wei et al., 2006, 2016). However, the usefulness of natural hyperaccumulators appeared to be limited since they were small plants with slow growth rates, consequently, cleaning up a heavy-metal contaminated site could take years (J. Zhang et al., 2016; Liu et al., 2015; Robinson et al., 2015). The ideal hyperaccumulators were supposed to be fast-growing, have high biomass as well as deep roots, be easy to harvest, tolerate and accumulate a range of heavy metals in their aerial and harvestable part (Liang et al., 2016; Xu et al., 2017; Clemens et al., 2002). The lack of an ideal hyperaccumulators pushed researchers to explore the possibility of increasing metal tolerance and accumulation in fast-growing and high-biomass crop plants through expressing exogenous genes involved in metal uptake, translocation or detoxification (Hilgert et al., 2017; Zhang et al., 2014; Antosiewicz et al., 2014).

Brassica juncea, a crop of *Brassicaceae* family, was frequently used as a suitable host plant to express Cd-related genes because it was a fast-growing, high-biomass and Cd accumulating crop (Bhuiyan et al., 2011a, 2011b; Reisinger et al., 2008; Gasic and Korban, 2007; Lindblom et al., 2006). Meanwhile, previous studies showed that *B. juncea* could accumulate Cd in its vegetative organ rather than seed, which indicated that risk of food-chain contamination through the seeds was less and oil produced from the seeds would not pose high

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risks (Pan et al., 2017).

Natural resistance-associated macrophage protein (*NRAMP*) families were membrane spanning proteins and recognized as a ubiquitous family of metal transporters with several homologues in fungi, animals, plants and bacteria (S. Chen et al., 2017; Z. Chen et al., 2017; Qin et al., 2017; Bozzi et al., 2016; Wu et al., 2016; Nevo and Nelson, 2006). The first gene was *NRAMP1* cloned from rat as a locus involved in intracellular bacterial pathogen sensitivity (Vidal et al., 1993; Ge et al., 1996). Then, *NRAMP2* cloned from mouse was reported to function as iron (Fe) transporter (Fleming et al., 1998). When it came to plants, six *NRAMP* genes were identified in *Arabidopsis thaliana* while eight in *Oryza sativa* (Mäser et al., 2001; Gross et al., 2003). *AtNRAMP1, 3, 4* could transport Fe, manganese (Mn) and Cd (Curie et al., 2000; Thomine et al., 2000; Lanquaret al, 2004, 2005, 2010). *AtNRAMP4* could also transport zinc (Zn) (Oomen et al., 2009; Pottier et al., 2015). *AtNRAMP6* was a Cd transporter with functions inside the cell either by mobilizing Cd from its storage compartment or by moving Cd into a cellular compartment (Cailliatte et al., 2009). *OsNRAMP1* could transport Fe and Cd and it was involved in tolerance and accumulation of Cd (Takahashi et al., 2011). *OsNRAMP3* was a vascular bundles-specific Mn transporter that was responsible for Mn distribution in rice (Yamaji et al., 2013; Yang et al., 2013). *OsNRAMP5* was demonstrated to have transport ability to Fe, Mn and Cd (Ishimaru et al., 2012; Sasaki et al., 2012).

Sedum alfredii, a Zn/Cd hyperaccumulator of the *Crassulaceae* family, was found at an old Pb/Zn mining area in Quzhou, Zhejiang Province, China (Yang et al., 2004, 2005). The function of some genes cloned from *S. alfredii* was demonstrated to be involved in Cd uptake, translocation or detoxification (J. Zhang et al., 2014, 2016; M. Zhang et al., 2011, 2016; M. Zhang and Yang, 2014). Though *NRAMP* genes were cloned and analyzed in many plant species, the function of *NRAMPs* in *S. alfredii* is still unclear. Transcriptomic analysis of *S. alfredii* showed that the expression level of metal transporter *SaNRAMP3* was up-regulated by Cd exposure (Gao et al., 2013). In this study, we cloned *SaNRAMP3* from *S. alfredii* and addressed the question of whether the use of *SaNRAMP3* for ectopic expression under the constitutive CaMV35S promoter could alter Cd tolerance and accumulation, as well as modify Cd distribution in *B. juncea*.

2. Materials and methods

2.1. Materials

2.1.1. Gene material

The 3′ and 5′ RACE primers of *SaNRAMP3* was designed from the hyperaccumulating ecotype of *S. alfredii,* gained the full length cDNA by PCR amplification and named it as *SaNRAMP3h* (GenBank: KP684936. 1). To be easily stated, *SaNRAMP3h* was called as *SaNRAMP3* in this study. The 3′ RACE primer was TCA GGA GGT TAT TGG TAG TGC TAT TGC and the 5′ RACE primer was TCA GGA GGT TAT TGG TAG TGC TAT TGC. The amplification system was performed using the Advantage 2 Polymerase Mix Kit (Clontech, Takara, Japan) according to the manufacturer's instructions, as follows (totally 50 μL): 34.5 μL PCR-Grade Water, 5.0 μL 10 × Advantage 2 PCR Buffer, 1.0 μL 10 mM dNTP Mix, 1.0 μL 50 × Advantage 2 Polymerase Mix, 5.0 μL 10 × UPM, 1.0 μL Gene specific Primer (shown above), 2.5 μL 5′-RACE Ready cDNA / 3′-RACE Ready cDNA. The amplification was performed with the following conditions: 94 °C for 5 min, 5 cycles of (94 °C for 30 s, 72 °C for 3 min), 5 cycles of (94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min), 20 cycles of (94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min).

2.1.2. Agrobacteria material

Promoted by CaMV35s, the recombinant plasmid vector pCAMBIA1300g containing *SaNRAMP3* full length cDNA sequence was introduced into *Agrobacteria tumefaciens* strain EHA101. The agrobacteria were cultured in yeast-extract-peptone (YEP) medium containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin with shaking at 200 rpm/min and 28 °C for 18–20 h. The agrobacteria were then collected by centrifugation at 3000 rpm/min for 20 min. The precipitate was gently suspended in agrobacterium infection medium (½MS salts and vitamins, 10 g L^{-1} sucrose and 20 g L^{-1} acetosyringone). This suspension (OD_{600} = 0.4) was used as the bacterial inoculum.

2.1.3. Explant material

Seeds of *B. juncea* cultivar Xikouhuazi (Ru et al., 2006) were sterilized by immersion in 75% ethanol (v/v) for 1 min, then in 0.1% $HgCl_2$ (v/v) for 10 min, followed by four-time rinses in sterilized distilled water for 10 min each time. The sterilized seeds were germinated on ½MS medium containing 30 g/L sucrose and 8 g/L agar (Murasnige and Skoog, 1962). The mediums were adjusted to pH 5.8 by 0.1 mol/L NaOH and autoclaved at 121 °C for 20 min. The culture condition was at 26 °C with a 16/8-h photoperiod. Hypocotyls about 0.5–1.0 cm were excised from 5-day old seedlings and taken as explants.

2.2. Genetic transformation protocol

The hypocotyls were planted on preculture medium containing ½MS salts and vitamins, 20 g/L sucrose, 8 g/L agar and 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Preculture condition was at 26 °C with a 16/8-h photoperiod. The induced callus were immersed in the bacterial inoculum for 3 min and then transferred to co-cultivation medium (½MS salts and vitamins, 20 g/L sucrose, 8 g/L agar, 2.0 mg/L 2,4-D, 20 mg/L acetosyringone). The culture condition was at 26 °C in the dark for 2 d. Following co-cultivation, the explant was transferred to shoot regeneration medium supplied with MS salts and vitamins, 3.0 mg/L 6-benzylaminopurine (6-BA), 0.1 mg/L 1-naphthylacetic acid (NAA), 30 g/L sucrose, 8 g/L agar, 2.5 mg/L $AgNO_3$, 5 mg/L hygromycin and 500 mg/L ampicillin. The culture condition was at 26 °C with a 16/8-h photoperiod. The shoot regeneration medium was updated per week. When the shoots were 2–3 cm high, the explants were moved into root induction medium (½MS salts and vitamins, 20 g/L sucrose, 8 g/L agar, 250 mg/L Ampicillin, 0.15 mg/L NAA). The culture condition was at 26 °C with a 16/8-h photoperiod. When the roots were 2–3 cm long, the rooted seedlings were transferred to organic nutrient soil after 3 d hardening. The transgenic plants were grown in a temperature-controlled growth chamber with a 16/8-h photoperiod, day/night temperatures of 26 °C/20 °C and day/night humidity of 70%/85%. The seeds of transgenic T_0 plants were collected after maturation of pods.

2.3. Molecular characterization of T_0 transgenic plants

2.3.1. Extraction of genomic DNA

Genomic DNA was extracted from plant leaves by the method of CTAB. The transgenic plants were confirmed by PCR analysis using the following primers: forward, 5′- AAG GTA CCA TGC CTC CTC CCT CT −3′, reverse, 5′- AAC TGC AGC TAT TTG GAA GGT CT −3′. The DNA amplification was performed with the following materials (totally 12.5 μL): 0.25 μL forward primer, 0.25 μL reverse primer, 0.125 μL Taq, 1 μL dNTP, 1 μL Template, 9.875 μL ddH_2O. The DNA amplification was performed with the following conditions: 94 °C for 5 min, 33 cycles of (94 °C for 45 s, 55 °C for 30 s), 72 °C for 50 s, and then 72 °C for 10 min. The PCR reaction was then analyzed on a 1.2% agarose gel and visualized by ethidium bromide staining.

2.3.2. Extraction of total RNA

Total RNA was extracted from plant leaves using the Plant RNA Preparation Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. RNA was purified and cDNA was compounded using the Primescript Reverse Transcription Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. The *SaNRAMP3* gene was amplified with the following