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SaZIP4, an uptake transporter of Zn/Cd hyperaccumulator *Sedum alfredii* Hance

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ABSTRACT

Sedum alfredii Hance is a zinc (Zn)/cadmium (Cd) hyperaccumulator plant. However, the molecular mechanism of the Zn/Cd uptake of roots and shoots has not been thoroughly elucidated to date. In this work, two isoforms of the putative Zn/Cd transporter of *S. alfredii* Hance, SaZIP4, were investigated. *SaZIP4h* and *SaZIP4n* were cloned from the hyperaccumulating and non-hyperaccumulating ecotypes of *S. alfredii*, respectively. Transcriptional analysis, subcellular localization analysis, yeast functional complementation analysis and transgenic plants were used to characterize *SaZIP4*. The transcription levels of *SaZIP4h* are significantly and constitutively higher than those of *SaZIP4n*, and the expression levels of *SaZIP4h* and *SaZIP4n* in the roots were highly induced by Zn deficiency. A subcellular localization analysis indicated that both SaZIP4h and SaZIP4n are localized to the plasma membrane. In addition, expressing *SaZIP4h* and *SaZIP4n* in the yeast mutant ZHY3 can reverse the Zn uptake deficiency. However, expressing *SaZIP4h* alone in the yeast mutant *Δzrc1* increased sensitivity to Cd. Transgenic *Arabidopsis thaliana* mutant *zip4-2* expressing *SaZIP4h* reversed the Zn/Cd uptake defect, and wild-type *A. thaliana* ectopically overexpressing *SaZIP4h* displayed increased Zn accumulation both in roots and shoots. Together, these results suggest that SaZIP4 is an important Zn uptake transporter that takes up Zn in the roots and shoots of *S. alfredii*. These findings help to elucidate the molecular mechanisms of heavy metal and micronutrient uptake and accumulation in hyperaccumulator plants.

1. Introduction

The heavy metals zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and molybdenum (Mo) are essential plant nutrients that are required for physiological processes, while being toxic at supra-optimal concentrations (White and Pongrac, 2017). Cadmium (Cd), on the other hand, is a nonessential element that can be taken up by plants through Fe and Zn transporters and can be bound by sulfhydryl groups and other amino acid side chains, leading to extreme toxicity (Gallego et al., 2012). Large quantities of heavy metals, such as Zn, Cu, Fe and Cd, are often released into the environment as a result of soil acidity, flooding, mining, industrial activities or other pollution (White and Pongrac, 2017). Plants have developed many strategies to cope with the excess of heavy metals. One of these strategies, exhibited by "hyper-accumulator" plants, is the ability to accumulate heavy metals in the above-ground tissues at much higher concentration levels than normal plants without exhibiting toxicity symptoms (Baker and Brooks, 1989). The use of the hyperaccumulator plants has been proposed for the remediation of heavy metal-contaminated soils (Salt et al., 1998).

Sedum alfredii Hance of the hyperaccumulating ecotype (HE) is a Zn/Cd hyperaccumulator belonging to the *Crassulaceae* family of plants native to China, and was discovered in an old mining region in Quzhou, Zhejiang Province, China (Yang et al., 2002, 2004a). Laboratory analysis showed that HE *S. alfredii* was able to accumulate up to 29,000 mg kg^{-1} dry weight (DW) of Zn (Yang et al., 2002) and 9000 mg kg^{-1} DW of Cd (Yang et al., 2004a) in the shoots without any symptoms of toxicity. While other Zn/Cd hyperaccumulator plants, such as the *Brassicaceae* family members *Arabidopsis halleri* and *Noccaea caerulescens* with a relatively low biomass, have been studied as model plants for harnessing the useful features of the hyperaccumulation processes, the development of other hyperaccumulators such as *S. alfredii* is necessary because of its higher biomass and therefore higher potential for the efficient bioremediation of contaminated soils. Another non-hyperaccumulating ecotype of *S. alfredii* discovered by Yang et al. (2004b) in Hangzhou Jiuxi Tea Garden (120°09′42″E, 30°15′12″N), Zhejiang Province, is usually used to compare the

Abbreviations: HE, hyperaccumulating ecotype of *Sedum alfredii*; NHE, non-hyperaccumulating ecotype of *Sedum alfredii*

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differences of physiological (Lu et al., 2008) and molecular function (Yang et al., 2017; Zhang et al., 2016) from HE *S. alfredii* due to their homology (Chao et al., 2008) but contrasting Zn/Cd accumulating abilities (Yang et al., 2004b).

The hyperaccumulation mechanism of Zn/Cd involves a highly efficient uptake of heavy metals into the roots from the soil and translocation of the elements to the shoots of plants, coupled with a subsequent effective detoxification process. HE *S. alfredii* roots showed an initial rapid linear Zn absorption followed by a slower linear phase. Compared to the non-hyperaccumulating ecotype of *S. alfredii* (NHE), obtained from a tea plantation in Hangzhou, Zhejiang Province, China, the V_{max} of Zn influx was three-fold higher in HE (Li et al., 2005). On the other hand, the Zn affinity of transporters was lower in HE than in NHE (K_m value of 34.83 μM HE and 20.43 μM NHE) (Li et al., 2005), indicating that more transporters located in the membrane play important roles in Zn uptake for HE (Li et al., 2005). The K_m of ^{109}Cd influx into the roots of both ecotypes was similar, but the V_{max} was two-fold higher in the HE compared to NHE *S. alfredii* (Lu et al., 2008). The measured Cd accumulation in HE *S. alfredii* was three to four times higher than the Cd accumulation calculated from the transpiration rate (Lu et al., 2009), which implies that symplastic pathway contributes greater than apoplastic pathway to this ecotype for Cd uptake (Lu et al., 2009).

Members of the ZIP family, which is named after the Zinc-regulated transporter/Iron-regulated transporter-like Protein (Grotz et al., 1998), have been identified as important uptake transporters of heavy metals in *A. halleri*, *N. caerulescens*, *A. thaliana*, *Oryza sativa*, *Hordeum vulgare* and other plant species (Assuncao et al., 2001; Chiang et al., 2006; Ishimaru et al., 2005; Pence et al., 2000; Tiong et al., 2014). In previous studies, *AhZIP3*, *AhZIP6*, *AhZIP9*, *AhZIP12* and *AhIRT3* were proposed to be responsible for the Zn/Cd uptake activity of *A. halleri* based on the genomic expression analysis (Becher et al., 2004; Chiang et al., 2006; Talke et al., 2006; Weber et al., 2004). The ZNT1 transporter, which was identified from *N. caerulescens* (Nc), is a member of the ZIP family that also mediates Zn uptake (Pence et al., 2000) as well as Zn long-distance transport (Milner et al., 2012). This NcZNT1 protein has been shown to be highly homologous with AtZIP4 (Grotz et al., 1998). *AtZIP4* was induced in both shoots and roots under Zn deficient conditions and its expression is associated with vasculature, thus might transport Zn intracellularly or between plant tissues (Grotz et al., 1998; Lin et al., 2016; Wintz et al., 2003). Although many studies have been conducted on the Zn/Cd uptake function of the ZIP family in other species, there is no report on the detailed function of a member of the ZIP family in *S. alfredii*.

To elucidate whether the ZIP family also plays a role in Zn/Cd uptake by *S. alfredii* Hance and is involved in the hyperaccumulation process, we cloned some members of the *ZIP* gene family from the HE and NHE plants of *S. alfredii* Hance. We then characterized the expression patterns of one of the *ZIP* genes, *SaZIP4*. The subcellular localization of the corresponding protein, SaZIP4, was then studied, followed by functional characterization through yeast functional complementation analysis and by using transgenic *A. thaliana* plants expressing *SaZIP4h*. The results of gene function analysis of *SaZIP4* provide the theoretical basis of phytoremediation of Zn/Cd contaminated soil, and also provide insights into biofortification Zn in food crops.

2. Materials and methods

2.1. Plant materials and growth conditions

HE *S. alfredii* was collected from an old Zn/Pb mining region in Quzhou City in Zhejiang province in China (29°17′ N, 118°56′ E) and NHE *S. alfredii* was collected from a tea plantation in Hangzhou in Zhejiang province in China (30°15′ N, 120°09′ E). To eliminate the internal heavy metal concentration, the HE plants were grown in non-polluted soils for several generations. The shoots were cut and subsequently several leaves were removed. The HE and NHE plants were hydroponically cultured in a basal nutrient solution to grow new roots (Yang et al., 2017). The basal nutrient solution contained 5 μM $ZnSO_4$. To evaluate the *ZIP4* expression levels in HE and NHE, HE plants were supplied with either control (basal nutrient solution), 5 μM $CdCl_2$ added, 100 μM $CdCl_2$ added, Zn deficiency (without Zn, -Zn), 50 μM $ZnSO_4$ added or 500 μM $ZnSO_4$ added for 24 h or 8 d, whereas the NHE plants were supplied with either control (basal nutrient solution), 5 μM $CdCl_2$ added, -Zn or 50 μM $ZnSO_4$ added for 24 h and 8 d, because the treatments of 100 μM $CdCl_2$ and 500 μM $ZnSO_4$ are too toxic for NHE plants to grow. The pH of the nutrient solution was adjusted to 5.8 every other day. The nutrient solution was renewed every three days with a continuous aeration. The plants were grown in a growth chamber with a day/night temperatures of 26/20 °C, with a 16/8 h photoperiod, under a light intensity of 400 μmol m^{-2} s^{-1}, and humidity of 70/85%. Three biological replicates were conducted for each treatment.

2.2. Cloning ZIP4 from S. alfredii

According to the transcriptome sequence of HE *S. alfredii* Hance (Gao et al., 2013), the sequences with relatively high expression levels and predicted to encode *ZIP* genes, Sa_Contig10290 (GenBank: HE727395.1), Sa_Contig10697 (GenBank: HE727802.1) and Sa_Contig22157 (GenBank: HE739262.1), were selected and extracted. These sequences were blasted in NCBI with *Arabidopsis thaliana* (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence Sa_Contig10290 was most similar with *AtZIP1* with 65% identity, thus termed as *SaZIP1h*. The sequence Sa_Contig10697 was similar with *AtZIP4* with 69% identity, thus termed as *SaZIP4h*. The sequence Sa_Contig22157 was most similar with *AtZIP11* with 72% identity, thus termed as *SaZIP11h*. The full lengths of *SaZIP1h*, *SaZIP4h* and *SaZIP11h* were isolated through 3′ and 5′ RACE as described by the protocol of the Smart RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). The primer used for *SaZIP1h* 5′-RACE is 5′-ACGTCGCAAAAGCGTCGATCA-3′ and the primer used for *SaZIP1h* 3′-RACE is 5′-TGCACGGACACTCGCACGGA-3′. The primer used for *SaZIP4h* 5′-RACE is 5′-GCTGCGATTAGTGGTCGGATGGTGC-3′ and the primer used for *SaZIP4h* 3′-RACE is 5′-TCGCCATCACAACACCAACAGG-3′. The primer used for *SaZIP11h* 5′-RACE is 5′-TGCGTGCCGAGGACAAGAAACCC-3′ and the primer used for *SaZIP11h* 3′-RACE is 5′-GTCTCTCCGTACTTCCTTAAATGG-3′.

The full lengths of *SaZIP4h* and *SaZIP11h* were used as references to design primers for amplification of *SaZIP4n* and *SaZIP11n* of NHE *S. alfredii*. The following primers: forward 5′-ATGGTGTTCTTCCAGGGTGT-3′ and reverse 5′-CTAAGACCAGAGAGCAAGTG-3′ were used to amplify the full length of *SaZIP4n*. The following primers, forward 5′-GTATCAACGCAGAGTAATGG-3′ and reverse 5′-CGAAACATCCGATGAAGAAG-3′, were used to amplify the full length of *SaZIP11n*. The total root RNA of NHE *S. alfredii* hydroponically cultured in a basal nutrient solution was extracted using the RNAout kit (Tiangen, China) and subsequently converted to cDNA using the Primescript™ RT reagent kit with a gDNA eraser (Takara, Japan). The cDNA of NHE *S. alfredii* roots was used as template to amplify *SaZIP4n* and *SaZIP11n*. The denaturation of PCR was 94 °C for 5 min, followed by 35 cycles of 20 s at 94 °C, 20 s at 55 °C, and 1 min at 72 °C and a final extension of 10 min at 72 °C. The FastPfu PCR SuperMix was used (TRANS BIOTECH, China). The PCR products were separated on a 1% agarose gel and purified with Gel DNA Purification Kit (Tiangen, China). The purified product was then cloned into the *pEASY*-Blunt Simple Cloning Vector (TRANS BIOTECH, China) and sequenced (Boshang, China). The sequence of *SaZIP4n* or *SaZIP11n* was blast in the NCBI with *S. alfredii* and *A. thaliana* to ensure that they are the different isoforms with *SaZIPh* or *SaZIP11h* between HE and NHE ecotypes.

The NCBI accession number of cDNA of *SaZIP1*, *SaZIP4h*, *SaZIP4n*,