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Effect of Long-Term Stress of High Pb/Zn Levels on Genomic Variation of *Sedum alfredii* Hance

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Abstract In this study, the heavy metal contents were detected in plants of *Sedum alfredii* and soils from Pb/Zn mined area and non-mined area, and a dendrogram was generated by using RAPD methods based on the hyperaccumulating ecotype (HE), the non-hyperaccumulating ecotype (NHE) and other species of *Sedum*. The results showed that the available Pb of the Pb/Zn mined soil was 77-fold higher, and available Zn and Cd were 10-fold and 16-fold higher in the mined soil than in the non-mined soil, respectively. The dendrogram showed that the HE *S. alfredii* was the nearest relative to NHE *S. alfredii*. However, genomic variation of two ecotypes was still notable, indicating that heavy metal stress had great impacts on the genetic diversity and plant evolution, and HE may be a mutant from the NHE. Ten RAPD bands were observed only in the HE as compared with other species of *Sedum*. The character of Zn/Cd hyperaccumulation in HE appeared to be related to SH-containing compounds and resist osmotic stress, and also many unknown genes.

Keywords Genomic variation · Heavy metal pollution · Dendrogram · *Sedum alfredii*

Heavy metal pollution influences plant evolution and induces natural genetic variations among species or populations. Previous studies showed that heavy metal stresses, such as high levels of lead, copper, manganese, and cadmium, could induce the genomic instability in kidney-beans (*Phaseolus vulgaris*) or barley (*Hordeum vulgare* L.) (Liu et al. 2005; Enan 2006). A new Zn/Cd co-hyperaccumulating ecotype of *S. alfredii* Hance (HE) was found in an old Pb/Zn mined area of Southern China (Yang et al. 2002, 2004), which was probably a mutant from the non-hyperaccumulating ecotype of *S. alfredii* (NHE) after a long period of inhabitation (more than 1,000 years) in a highly heavy metal polluted environment. The HE is a perennial herb with clumped population and has characteristics of large biomass, fast growth and easy propagation, and has obvious morphological differences from the NHE which was collected from the agricultural area of Hangzhou suburb, Zhejiang Province of China (Long et al. 2002). Previously reports confirm that the HE exhibits much greater ability to tolerate heavy metal toxicity and accumulate Zn, Cd, and Pb in shoots than other species of *Sedum*. However, the global genomic differences among different species of *Sedum*, especially between the two ecotypes of *S. alfredii* H., are not clear.

The random amplified polymorphic DNA (RAPD) is one of the commonly used DNA-based markers, developed by Williams et al. (1990), with traits of being simple, fast, sensitive and relatively cheap. Previous studies indicated that it has the potential to detect a wide range of DNA damage (e.g., DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements) and therefore can be applied to genotoxicity studies (Conte et al. 1998; Liu et al. 2005; Enan 2006). In the present study, we focus on generating a dendrogram by using RAPD methods, and the analysis of the band sequences that are only observed in HE as compared with other species of *Sedum*, including *S. Sarmentosum* Bunge, *S. Emarginatum* Migo, *S. Bulbiferum* Makino, *S. Makinoi* Maxim, and NHE of *S. alfredii* Hance.

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Materials and Methods

Six species of *Sedum* were collected from Zhejiang Province of China. The HE *Sedum alfredii* and *Sedum Bulbiferum* Makino (SBM) were collected from an old Pb/Zn mined area of Quzhou city, whereas the NHE *S. alfredii*, *Sedum Emarginatum* Migo (SEM), and *Sedum Sarmentosum* Bunge (SSB) were collected from Hangzhou suburb, *Sedum Makinoi* Maxim (SMM) was collected from Lin'an suburb. The shoots of HE and NHE were rinsed with bi-distilled water, then dried and ground with a stainless steel mill for elemental analysis. A given amount of dry sample were digested with HNO_3–$HClO_4$ (v/v = 4/1) at 180°C. The digest solution was transferred to a 1,000 mL volumetric flask, made up to volume and filtered. Contents of heavy metals in the filtrate were analyzed using ICP-MS (Agilent 7500a, America).

Soil samples were collected from the old Pb/Zn mined area of Quzhou (named as mined soil) and from the agricultural area of Hangzhou suburb (named as non-mined soil). A given amount of each soil sample was digested with aqua regia for the determination of total Pb, Zn, Cd, and Cu in soils. Available soil metals were extracted by 0.005 mol L^{-1} DTPA (pH 7.3, soil:extractant of 1:20). The total and extractable metal concentrations were analyzed using ICP-MS (Agilent 7500a, America).

In this study, all reagent and primers used for the experiment were bought from Sangon Company, China. The genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol by Murray and Thompson (1980). Both the concentration and purity of the extracted DNA were monitored using UV absorbance spectrophotometer (Biophotometer, Eppendorf, USA). Experiments were carried out with 25 μL reaction mixtures and amplification reactions were performed in a Programmable Thermal cycler (Mastercycler gradient, Eppendorf, USA). One hundred random primers (S201–S300) were screened in order to test amplification profiles for polymorphism, readability and reproducibility. A negative control reaction without DNA template was included in each amplification. The amplification products were separated on 1.5% (w/v) agarose gels at 100 V for about 2 h. The gels were stained with 5 μg mL^{-1} ethidium bromide and photographed under UV light.

The RAPD bands were analyzed by eyeballing, and the analysis of these bands was conducted using the computer program NTSYS V2.0 to determine the similar coefficient values between them. The figure of similar coefficient values were then used as input data for cluster analysis to generate dendrograms using the UPGMA method (Saitou and Nei 1987).

The RAPD bands shown only in HE compare with other species of *Sedum* were selected and excised from the gels, DNA extraction from excised gels and the product was cloned in pGEM-T Easy vectors (Promega USA) and after checking the cloned fragments on size, the clones per fragment were sequenced. Database searches were performed using the BLAST Network service [NCBI (National Center for Biotechnology Information); http://www.ncbi.nlm.nih. gov/BLAST]. The sequence of each genomic fragment was searched against all sequences in the databases using the BLASTN and BLASTX programs (Altschul et al. 1997).

Results and Discussion

The metal contents in the mined soil and the non-mined soil were shown in Table 1. Much higher total and extractable concentrations of Pb, Zn, Cd, and Cu were observed in the Pb/Zn mined soil than in the non-mined soil. For instance, the available Pb of the Pb/Zn mined soil was 77-fold higher, and available Zn and Cd were also reached 10-fold and 16-fold higher in the mined soil than in the non-mined soil, respectively. It is indicated that the heavy metal pollution in the mined soil should have been produced great stress on the plants grown on Pb/Zn mined soil.

The metal contents in shoots of HE and NHE were shown in Table 2. The Zn and Cd contents in shoots of HE collected from the Pb/Zn mined soil reached 12,521 and 1,023 mg/kg, respectively, and was 23- or 100-fold higher than those in NHE collected from the non-mined soil. It was recorded that this mined soil was derived from 1,000 years ago. The results demonstrated that *S. alfredii* had been inhabitant on high Zn/Pb pollution soil for long time, and was evolved be a Zn/Cd co-hyperaccumulator.

At the beginning, 100 random primers were used to detect the genomic difference of the six populations of *Sedum*, there were 14 primers could show reliable and replicable amplified bands and then were used to construct a dendrogram (Fig. 1). The dendrogram suggested that the

Table 1 Metal contents in two types of soil

	Type of metal	Mined soil	Non-mined soil
Total content (mg/kg dried soil)	Pb	12825.25	265.01
	Zn	3108.63	415.00
	Cd	34.63	2.75
	Cu	277.43	65.00
DTPA-extractable content (mg/kg dried soil)	Pb	1780.95	23.02
	Zn	127.96	12.96
	Cd	11.64	0.69
	Cu	16.34	2.95

Table 2 Heavy metal contents in shoots of two ecotypes of *S. Alfredii*

Ecotype	Metal concentration in shoots (mg/kg dry weight)			
	Zn	Cd	Cu	Pb
HE	12532.98 ± 408.01	1023.8 ± 197.11	27.94 ± 1.08	222.86 ± 15.98
NHE	526.77 ± 7.83	10.08 ± 0.34	13.19 ± 5.13	43.37 ± 3.49

The HE was sampled on mined soil and NHE on non-mined soil, respectively

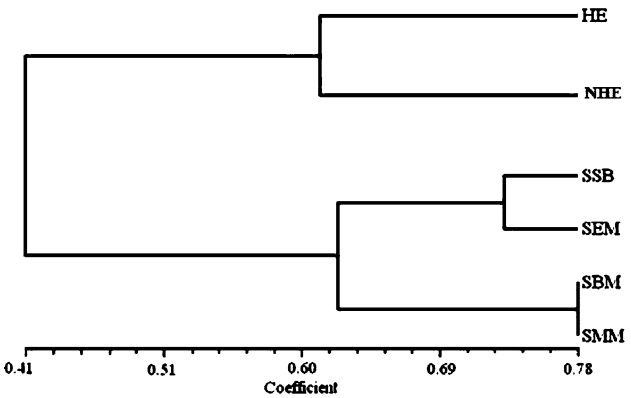

Fig. 1 The dendrograms of the six *Sedum* populations based on RAPD analysis

six *Sedum* populations can be clearly divided into two groups. Subgroup I included the HE and NHE, indicated that HE may be a mutant from NHE. Subgroup II included the other four species. The branch length of HE and NHE were even longer than that of other interspecies, indicated that environment has great influence on the genetic diversity.

Although the relationship of HE and NHE is closer than HE with other species, the genetic diversity of HE and NHE is notable. Moreover, HE and NHE *S. alfredii* was collecting from an old Zn/Pb mined area and non-contamination tea planting area, respectively. Even in the same hydroponics condition or pot experiment, the differences of heavy metal accumulation ability and phenotypes between HE and NHE was significant, too, which indicated that a relatively long-term (more than 1,000 years) exposure of heavy metal induce significant genomic variation of the plant and produce the genetic stable new ecotype–HE. Similar results were also found in *Thlaspi caerulescens* and *Arabidopsis halleri* (Escarré et al. 2000; Bert et al. 2002; Rossens et al. 2003).

The RAPD bands, observed only in HE as compared with other species of *Sedum*, were selected for clones and sequencing. The sequences of 10 DNA fragment were obtained and compared with those presented in the GenBank database (Table 3). Among the 10 DNA fragments, three showed significant homology to genomics with known or putative function, whereas the remaining seven DNA fragments have no significant matches.

Of the three DNA fragments shown significant homology to genomics, the No. 1 clone was homology to *Arabidopsis thaliana* chromosome 1 genomic sequence; No. 2 clone showed homology to *A. thaliana* ATP sulfurylase gene and vacuolar H^+-pumping ATPase 16 kDa proteolipid gene; and the No. 3 clone was homologs to *A. thaliana* dehydration-responsive protein-related gene. Moreover, there

Table 3 Homologies of sequences of unique marked DNA fragments from HE as compared with other species of *Sedum* by BLAST in the databases

Sequence no.	Length (bp)	Homology[a]	BLAST score
1	877	*Arabidopsis thaliana* chromosome 1 BAC F28P22 genomic sequence	3e−11
2	971	*Arabidopsis thaliana* ATP sulfurylase (APS2) gene, and vacuolar H^+-pumping ATPase 16 kDa proteolipid	6e−46
3	826	*Arabidopsis thaliana* dehydration-responsive protein-related	3e−30 1e−23 2e−13
4	595	No homology[b]	
5	588	No homology[b]	
6	639	No homology[b]	
7	505	No homology[b]	
8	628	No homology[b]	
9	674	No homology[b]	
10	1090	No homology[b]	

[a] Search database nr/nt using discontiguous Megablast

[b] No significant sequence homology found in genome, EST, and protein database

are seven fragments belonging to unknown genomic fragments, suggesting that Zn/Cd hyperaccumulation in HE appears to be related to many other unknown genes.

We used 100 random primers only in this study, 10 DNA fragments was obtained that shown only in HE as compared with other species of *sedum*. Of course, whole genomic difference could be marked by using adequate random primers, but such study will cost excess time and money. Moreover, it was difficult to functionally analyze the differences among the genomic fragments.

In conclusion, the HE is a natural mutant of the NHE due to long-term exposure to high heavy metal pollution stress by RAPD analysis. The character of Zn/Cd hyperaccumulation in HE appeared to be related to SH-containing compounds and resist osmotic stress, and also many unknown genes.

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