Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
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Response of ATP sulfurylase and serine acetyltransferase towards cadmium in hyperaccumulator *Sedum alfredii* Hance*

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Received May 21, 2008; Revision accepted Sept. 17, 2008; Crosschecked Jan. 6, 2009

Abstract: We studied the responses of the activities of adenosine-triphosphate (ATP) sulfurylase (ATPS) and serine acetyltransferase (SAT) to cadmium (Cd) levels and treatment time in hyperaccumulating ecotype (HE) *Sedum alfredii* Hance, as compared with its non-hyperaccumulating ecotype (NHE). The results show that plant growth was inhibited in NHE but promoted in HE when exposed to high Cd level. Cd concentrations in leaves and shoots rapidly increased in HE rather than in NHE, and they became much higher in HE than in NHE along with increasing treatment time and Cd supply levels. ATPS activity was higher in HE than in NHE in all Cd treatments, and increased with increasing Cd supply levels in both HE and NHE when exposed to Cd treatment within 8 h. However, a marked difference of ATPS activity between HE and NHE was found with Cd treatment for 168 h, where ATPS activity increased in HE but decreased in NHE. Similarly, SAT activity was higher in HE than in NHE at all Cd treatments, but was more sensitive in NHE than in HE. Both ATPS and SAT activities in NHE leaves tended to decrease with increasing treatment time after 8 h at all Cd levels. The results reveal the different responses in sulfur assimilation enzymes and Cd accumulation between HE and NHE. With increasing Cd stress, the activities of sulfur assimilation enzymes (ATPS and SAT) were induced in HE, which may contribute to Cd accumulation in the hyperaccumulator *Sedum alfredii* Hance.

Key words: *Sedum alfredii* Hance, Cadmium (Cd), Adenosine-triphosphate (ATP) sulfurylase (ATPS), Hyperaccumulator, Serine acetyltransferase

doi:10.1631/jzus.B0820169 **Document code:** A **CLC number:** X24

INTRODUCTION

Cadmium (Cd) is a highly toxic metal, and has been ranked No. 7 among the top 20 toxins mainly due to its negative influence on the enzymatic systems of cells (Al-Khedhairy *et al*., 2001). Large areas of land in many countries have been contaminated by Cd and other heavy metals due to the application of sludge or urban composts, pesticides, fertilizers, emissions from waste incinerators, wastewater irrigation, and residues from metalliferous mining and the metal smelting industry (Yang *et al*., 2002a). Cd contamination in agricultural soils is unlikely to affect plant growth; however, as Cd is easily transferred to human food chain from the soils, Cd contamination is a great threat to human health. These effects limit the marketing of agricultural products and reduce the profitability of the agricultural industry. The residence time of Cd in soil is over thousands years (Alloway, 1995). Phytoremediation is a novel technique to clean up contaminated soils using green plants, which offers the benefits of being in situ, low cost, and environmentally sustainable (Yang *et al*., 2005). Successful implementation of phytoremediation depends on the identification of suitable plant species that are capable of not only

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* Project supported by the National Natural Science Foundation of China (No. 30630046) and the Hi-Tech Research and Development Program (863) of China (No. 2006AA06Z386), and the Program for Changjiang Scholars and Innovative Research Team in University, China (No. IRT0536)

growing on soils containing high levels of metals, but also accumulating much higher concentrations of metals in their shoots than normal species. These plants are termed hyperaccumulator. Up to now, more than 450 species of hyperaccumulators belonging to 45 families have been identified (Baker *et al*., 2000). However, most of the metal-accumulating plants identified so far grow slowly and have small biomass, failing to meet the need of remediation on a large scale. Understanding mechanisms of Cd hyperaccumulation is of importance for improving metal hyperaccumulators in successful phytoremediation of the contaminated soils with Cd and other metals.

A few Cd-hyperaccumulators have been reported, including *Thlaspi caerulecens*, *Arabidopsis halleri*, and *Brassica junica* (Brown *et al*., 1995; Küpper *et al*., 2000; Su and Huang, 2002). *Sedum alfredii* H. growing in a Pb/Zn mine area has also been identified as a Zn/Cd co-hyperaccumulator native to China (Yang *et al*., 2002b; 2004). The ability of hyperaccumulating Cd by *S. alfredii* H. has been demonstrated (Yang *et al*., 2004; 2006; Xiong *et al*., 2004). It also has characteristics of large biomass, fast growth, asexual propagation, and perennial. Therefore, it is an ideal plant to study the mechanism responsible for hyperaccumulation and could be applied for practice of phytoremediation.

Sulfur assimilation has an important effect on Cd accumulation. Adenosine-triphosphate (ATP) sulfurylase (ATPS; EC 2.7.7.4) catalyzes the first reaction in the assimilation of inorganic sulfate (Osslund *et al*., 1982), and it activates SO_4^{2-} via an ATP-dependent reaction, which leads to the formation of APS and pyrophosphate (PPi). ATPS is considered to be an excellent candidate for the pathway-regulating, rate-limiting enzyme (Leustek, 1996). Serine acetyltransferase (SAT; EC 2.3.1.30) that catalyzes the formation of *O*-acetyl-L-serine (OAS) from L-serine and acetyl-CoA is responsible for the entry step from L-serine metabolism to cysteine biosynthesis (Saito *et al*., 1995). OAS then reacts with hydrogen sulfide to yield L-cysteine through the action of cysteine synthase. SAT plays a regulatory role in cysteine biosynthesis in plants (Noji *et al*., 1998). Many research indicated that both ATPS and SAT played important roles in heavy metal tolerance and accumulation (Wangeline *et al*., 2004; Hawkesford, 2003; Freeman *et al*., 2004).

Our previous studies showed that antioxidant defense system was enhanced in hyperaccumulating ecotype (HE) when exposed to Cd, as compared with non-hyperaccumulating ecotype (NHE) (Jin *et al*., 2008). We raised the hypothesis that sulfur metabolism, especially the pathway of glutathione synthesis, might play an important role in Cd tolerance and hyperaccumulation. In this study, hydroponically experiment was carried out to investigate response of these two sulfur assimilation enzymes (ATPS and SAT) to Cd in HE and NHE *S. alfredii*.

MATERIALS AND METHODS

Plant collection and culture

The HE of *S. alfredii* H. was obtained from an old Pb/Zn mine area in Zhejiang Province, China, while the NHE of *S. alfredii* H. was obtained from a tea garden near Hangzhou, China. Healthy and uniform shoots of *S. alfredii* H. were chosen and grown for 2 weeks in the basal nutrient solution, containing (in mmol/L) $Ca(NO_3)_2{\cdot}4H_2O$ 2.00, KH_2PO_4 0.10, $MgSO_4$ 0.50, KCl 0.10, and K_2SO_4 0.70; and (in μmol/L) H_3BO_3 10.00, $MnSO_4{\cdot}H_2O$ 0.50, $ZnSO_4{\cdot}7H_2O$ 1.0, $CuSO_4{\cdot}5H_2O$ 0.20, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.01, and Fe-ethylene diamine tetraacetic acid (EDTA) 100. The pH of nutrient solution was adjusted daily to 5.5 with NaOH or HCl. Plants were grown under glasshouse conditions with natural light, day/night temperature of 26/20 °C and day/night humidity of 70%/85%. The nutrient solution was aerated continuously and renewed every 4 d.

Cd treatment and sample preparation

After preculturing for 4 weeks, healthy and uniform seedlings were selected for 4 Cd treatments: control, 10, 25, and 100 μmol/L Cd. Each treatment was applied in triplicates in a completely randomized design. Cd was applied as $CdCl_2$. Nutrient solution was aerated continuously and renewed every 4 d with the pH maintained at 5.5. Plants were harvested at 0, 4, 8, 16, 24, and 168 h after treatments. At the time of harvest, roots were soaked in 20 mmol/L Na_2EDTA for 15 min to remove excess metal ions adhering to the root surfaces. Fresh samples (1 g) of leaves were immediately weighted, then frozen in liquid nitrogen,

and stored at −80 °C until analysis. Roots and shoots were separated and dried at 65 °C for metal concentration analysis.

Assay of ATPS activity

In the ATP sulfurylase activity assay, the method of Lappartient and Touraine (1996) was used. Frozen tissues from leaves were ground at 4 °C using a pre-chilled mortar and pestle in a pre-clod buffer consisting of 10 mmol/L Na_2EDTA, 20 mmol/L Tris-HCl (pH 8.0), 2 mmol/L DL-dithiothreitol (DTT), and approximately 0.01 g/ml insoluble polyvinyl pyrrolidone (PVP), using a 1:4 (w/v) tissue-to-buffer ratio. The homogenate was centrifuged at 17000×*g* for 20 min at 4 °C. The supernatant (crude extract) was used for in vitro ATPS assay. ATPS activity was measured using molybdate-dependent formation of pyrophosphate. The reaction was initiated by adding 0.1 ml of crude extract to 0.5 ml of the reaction mixture, which contains 7 mmol/L $MgCl_2$, 5 mmol/L Na_2MoO_4, 2 mmol/L Na_2ATP, and 0.032 units/ml of sulfate-free inorganic pyrophosphatase in 80 mmol/L Tris-HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture without Na_2MoO_4. Incubation was carried out side by side at 37 °C for 15 min, after which phosphate was determined calorimetrically by the method of Fiske and Subbarow (1925). A unit of enzyme was defined as the amount that produces 1 μmol/L inorganic phosphate in 1 min at 37 °C.

Assay of SAT activity

The protocol of Nakamori *et al*.(1998) was followed in the SAT activity assay. Frozen tissues from leaves were ground at 4 °C using a pre-chilled mortar and pestle in a pre-clod buffer consisting of 10 mmol/L Na_2EDTA, 20 mmol/L Tris-HCl (pH 8.0), and approximately 0.01 g/ml insoluble PVP. The supernatant obtained after centrifugation was used as enzyme sources. SAT activity was assayed by monitoring the decrease in absorbance at 232 nm of the reaction mixture (final volume 1 ml), the reaction mixture containing 50 mmol/L of Tris-HCl (pH 7.6), 1 mmol/L L-serine, 0.1 mmol/L acetyl coenzyme A, and 0.05 ml enzyme solution at 30 °C. A unit of enzyme was defined as the amount that produces 1 μmol/L inorganic phosphate in 1 min at 37 °C.

Cd concentration determination

Cd content was estimated in the stems and leaves. Dry plant samples (0.1 g) of each treatment were digested with 5 ml HNO_3 and 1 ml $HClO_4$ in closed Teflon vessels until clear. The digested material was washed into a 50-ml flask and made up to volume using de-ionized water. Metal concentrations in plant samples were determined using an Integrated Couple Plasma Mass Spectrophotometer (Agilent 7500a, USA). The Cd content absorbed was expressed as mg/kg dry weight.

RESULTS AND DISCUSSION

Effects of Cd on plant growth and biomass accumulation

Cd treatments did not cause visible phytotoxicity to the growth of HE; however, NHE displayed obvious toxic symptoms under Cd treatment for 168 h, such as stunted and dark roots, cracked and brownish stems, and wilted leaves, and some leaves fell off when the Cd concentration raised up to 100 μmol/L.

Exposure to different Cd levels caused variable effects on the biomass of both ecotypes of *S. alfredii* (Fig.1). The biomass of HE was significantly increased with exposure to Cd for 168 h. However, the biomass of NHE did not increase but declined in the three Cd treatment levels. Results show that Cd inhibited NHE growth but promoted HE growth. HE can continually grow and have a great biomass under Cd stress, which is an excellent character for hyperaccumulator.

Cd concentration and accumulation

Cd concentrations (on dry weight basis) in leaves increased rapidly in response with increasing Cd levels in HE, reaching 2546 mg/kg dry weight when exposed to 100 μmol/L Cd for 168 h (Fig.2). Cd concentrations in leaves of NHE also increased with raising Cd levels, but at much lower rates than HE. For instances, Cd concentrations in HE leaves were 11.6, 10.2, and 33 times higher than those in the NHE, respectively, when grown at Cd levels of 10, 25, and 100 μmol/L.

Cd accumulation was higher in HE than in NHE at all Cd treatments (Fig.3). Under 10 and 25 μmol/L

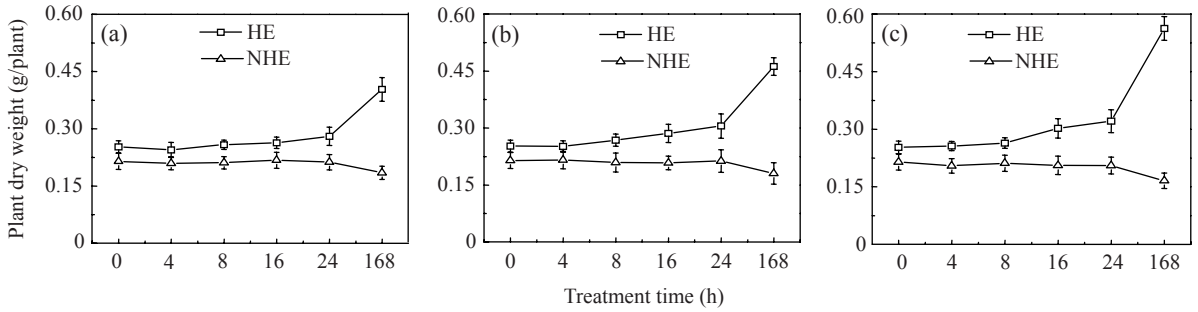

Fig.1 Biomass of two ecotypes of *Sedum alfredii* under different Cd treatments. (a) 10 μmol/L Cd; (b) 25 μmol/L Cd; (c) 100 μmol/L Cd

Data are means of three replications and bars depict ±*SE*

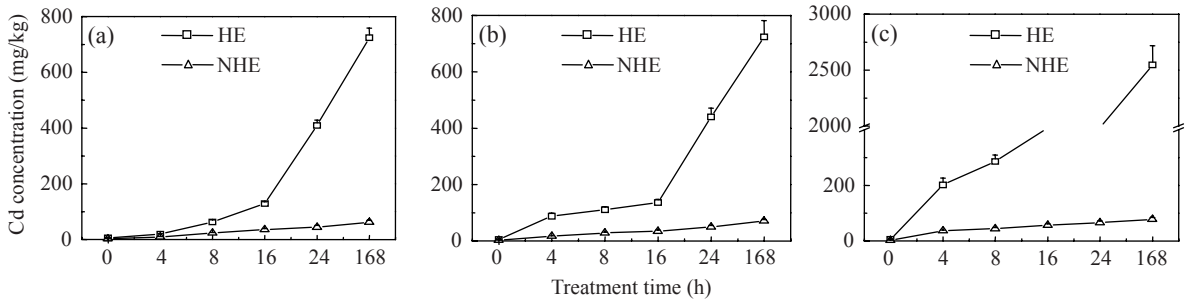

Fig.2 Cd concentrations in leaves of two ecotypes of *Sedum alfredii* under different Cd treatments. (a) 10 μmol/L Cd; (b) 25 μmol/L Cd; (c) 100 μmol/L Cd

Data are means of three replications and bars depict ±*SE*

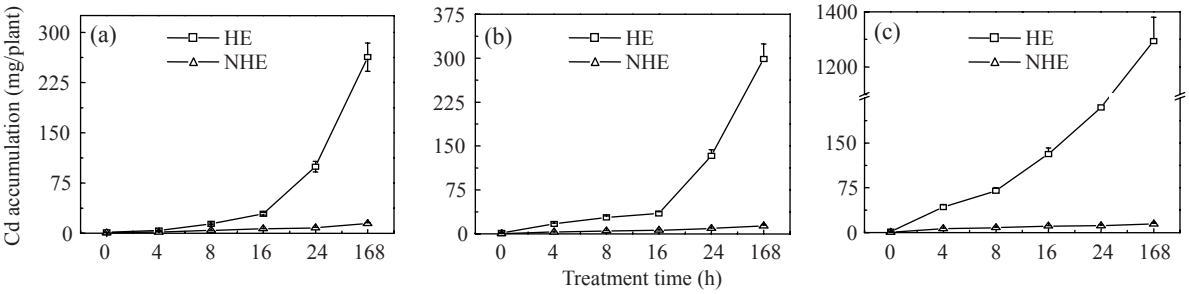

Fig.3 Cd accumulation in shoots of two ecotypes of *Sedum alfredii* under different Cd treatments. (a) 10 μmol/L Cd; (b) 25 μmol/L Cd; (c) 100 μmol/L Cd

Data are means of three replications and bars depict ±*SE*

Cd, the maximum Cd accumulation reached 250~350 mg/plant in HE. When at 100 μmol/L Cd, Cd accumulation increased faster in HE than in NHE, with the maximum Cd accumulation of 1350 mg/plant. In NHE, Cd accumulation was much lower than that in NH, reaching 12, 17, and 27 mg/plant, respectively, when grown under Cd treatments with 10, 25, and 100 μmol/L.

These results show that Cd was transported rapidly to the leaves of HE after Cd treatment, and such transporting seemed to be enhanced by increasing Cd supply levels. By contrast, Cd concentration in NHE leaves and Cd accumulation in NHE shoots increased slowly after Cd treatment. Results indicate that HE has extraordinary ability to hyperaccumulate Cd in the shoot tissues, which is a superior character for phytoremediation of heavy metal polluted soil.

Effects of Cd on ATPS activity and SAT activity

Intracellular chelation by either glutathione (GSH) or phytochelatins (PCs), or both (Zenk, 1996; Cobbett, 2000; Mutoh and Hayashi, 1988; Clemens, 2001; Rea *et al*., 1998) is one of the important pathways for plant metal detoxification (Reed and Gadd,

1990; Kaplan *et al*., 1995; Zenk, 1996; Macfie and Welburn, 2000; Cobbett, 2000; Hall, 2002). Starting from inorganic sulfate, GSH synthesis requires the sulfur assimilation and the cysteine biosynthetic pathways, which are affected by different stress situations such as heavy metal exposure and sulfur or nitrogen deficiency (Xiang and Oliver, 1998; Domínguez-Solís *et al*., 2001; Koprivova *et al*., 2000). Enhancement of the sulfur assimilation and the amount of thiol compound can make the plant accumulate more heavy metal. Wangeline *et al*.(2004) reported that the overexpression of ATPS in Indian mustard made plant tolerate and accumulate 12 metals, and Freeman *et al*.(2004) reported that the overexpression of SAT in *Thlaspi* made the plant accumulate more nickel.

In this study, we found that ATPS activity was higher in HE than in NHE at all Cd treatments. ATPS activities were enhanced both in HE and in NHE with Cd treatments within 8 h (Fig.4a). The obvious difference of ATPS activity between HE and NHE was found at 168 h, when this enzyme activity was increased in HE but reduced in NHE (Fig.4a). Harada *et al*.(2002) reported that in *Arabidopsis* under Cd stress, the expression of three sulfur assimilation pathway enzyme genes including ATPS increased significantly, and that the total thiol compounds increased 3-fold. These may be due to the need of thiol for the increased chelation. Our results indicate that the difference between HE and NHE in ATPS activity may be one of the reasons for their difference in Cd accumulation (Figs.4a and 4b).

SAT activity had similar trends to ATPS activity under Cd treatments; it was higher in HE than in NHE in all treatments (Fig.5), but was more sensitive in NHE than in HE. With Cd treatment for 8 h, SAT activity increased rapidly in NHE, but had minimal change in HE (Fig.5a). Under Cd treatment for 168 h, the difference of SAT activity between NHE and HE became larger than that for 8 h (Fig.5a), showing a similar change trend to ATPS activity. This may be due to the fact that SAT and ATPS are both in the thiol compounds synthesis pathway, which responds to the changes of thiol compounds synthesized.

Our results indicate that sulfur assimilation and cysteine synthesis increased in HE with increasing Cd levels. Previous studies proved that GSH contents were markedly higher in HE than in NHE under Cd stress (Jin *et al*., 2008; Chao *et al*., 2008), and that the total sulfur accumulation increased rapidly in HE after

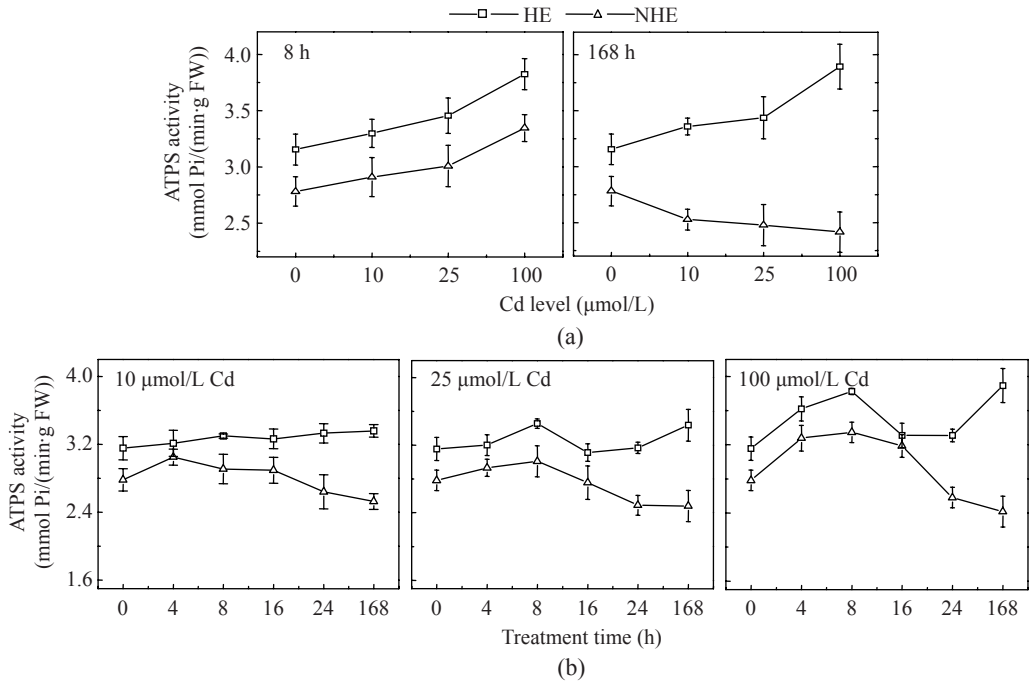

Fig.4 ATPS activities in leaves of two ecotypes of *Sedum alfredii* under Cd treatments. (a) Treatment for different time (8 and 168 h); (b) Treatment with different Cd concentrations (10, 25, and 100 μmol/L Cd)

Pi: pyrophosphoric acid; Data are means of three replications and bars depict ±*SE*

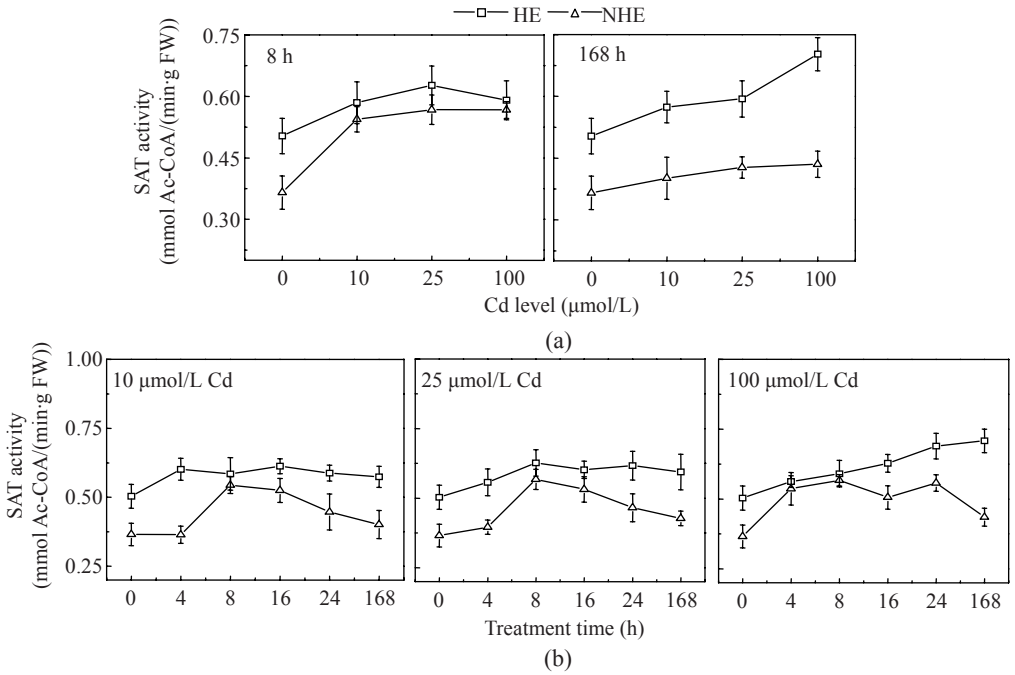

Fig.5 SAT activities in leaves of two ecotypes of *Sedum alfredii* under Cd treatments. (a) Treatment for different time (8 and 168 h); (b) Treatment with different Cd concentrations (10, 25, and 100 μmol/L Cd)
Data are means of three replications and bars depict ±*SE*

Cd treatment (Chao *et al*., 2008). Our results imply that HE enhanced sulfur assimilation pathway enzymes activities, which could be the source of sulfur for GSH synthesis and related to heavy metal accumulation. More investigations are needed to clarify the relation between sulfur assimilation pathway gene expression and Cd hyperaccumulation, and to clarify the key step in thiol compound synthesis.

CONCLUSION

Our results revealed the different responses in sulfur assimilation enzymes and Cd accumulation between HE and NHE. With increasing Cd stress, the sulfur assimilation enzyme (ATPS and SAT) activities were increased in HE, which may contribute to Cd accumulation in HE.

ACKNOWLEDGEMENT

Authors thank Miss Xiao-feng Jin for her valuable comments on the manuscript and Mr. Guo-xiang Hu for his help with Cd analysis using inductively coupled plasma mass spectrometry (ICP-MS).

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