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Letter to the Editor

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A Pivotal Role of Cell Wall in Cadmium Accumulation in the Crassulaceae hyperaccumulator *Sedum plumbizincicola*

Dear Editor,

Heavy metal pollution has become a public concern (Mohammed et al., 2011), and the development of efficient phytoremediation (i.e. using plants to clean polluted environment) requires better understanding of hyperaccumulation and hypertolerance evolved in metal-hyperaccumulating plants. However, mechanistic insight into metal hyperaccumulation and hypertolerance derives exclusively from only two of a total >450 hyperaccumulators (Verbruggen et al., 2009). Whether those known mechanisms are conserved among different hyperaccumulator species or whether there exist unknown mechanisms remains to be investigated. In this study, *Sedum plumbizincicola*, a Crassulaceae hyperaccumulator recently identified specifically in China, was subjected to a genome-scale comparative study. Our results not only revealed common mechanisms conserved in a wide range of hyperaccumulating plants but also discovered that cell wall plays a pivotal role in this specific hyperaccumulator.

Previous studies made significant progresses in understanding hyperaccumulation and hypertolerance by comparative studies of two Brassicaceae hyperaccumulators, *Arabidopsis halleri* and *Noccaea caerulescens*, with their non-hyperaccumulating counterparts. Specifically, those studies revealed a common mechanism in which the expression of certain genes involved in metal uptake, long-distance transport, or vacuolar sequestration are essentially enhanced by *cis*-regulation and/or gene amplification in the genomes (Verbruggen et al., 2009), and elevated expression of those genes eventually contributes to hypertolerance by vacuolar sequestration or to hyperaccumulation by long-distance transport (Drager et al., 2004; Hanikenne et al., 2008; Shahzad et al., 2010; Deinlein et al., 2012). However, given these results derived mostly from two of a total of >450 hyperaccumulators (Verbruggen et al., 2009), whether the "common" mechanisms are conserved among different hyperaccumulator species or whether there exist unknown mechanisms remain open questions.

S. plumbizincicola is a recently identified Crassulaceae species discovered in ancient mining areas in China (Wu et al., 2013). It did not show apparent growth reduction even under 100 μM cadmium (Cd) treatment for 16 days, in contrast to dramatic growth inhibition in *Sedum alfredii* (non-hyperaccumulating ecotype [NHE]) (Figure 1A). Correspondingly, large amounts of Cd accumulated in *S. plumbizincicola*, proportionally much more Cd will accumulate in shoots than in roots (Figure 1B). In *S. alfredii* (NHE) shoots, the content of Cd was low (Figure 1B). These results indicated that *S. plumbizincicola* is a typical Cd hyperaccumulator (Verbruggen et al., 2009).

To investigate the underlying mechanisms, we then sequenced and compared the transcriptomes of *S. plumbizincicola* and *S. alfredii* (NHE) by combining the Roche-454 and Illumina sequencing platforms, because a reference genome sequence of *Sedum* plants is still unavailable. Roche-454, which generated longer fragments but fewer sequencing reads, was employed to sequence the pooled root and shoot samples of *S. plumbizincicola*, and 941 088 high-quality reads were generated with an average length of 432 bases (Supplemental Table 1). Then Illumina sequencing was used to characterize the transcriptomes of root and shoot tissues of the two *Sedum* species with or without 10 μM Cd treatment, and a total of ~24 gigabases (Gb) of valid data were obtained, representing 313 million qualified reads with an average length of 76 bases (Supplemental Table 1). Given the high similarity between the transcriptomes of two species (Supplemental Figure 1), the assembly was designed to use mixtures of reads from both species, which resulted in 58 994 uni-genes, including 4630 genes expressed specifically in *S. plumbizincicola*, 2158 in *S. alfredii* (NHE), and 52 206 in both species. All these assembled transcripts were mapped to 2447 *S. plumbizincicola* expression sequence tags (ESTs) previously generated by Sanger sequencing, and >75% ESTs were covered by more than 80% of their sequence lengths (Supplemental Figure 2), demonstrating the high quality of the assembled transcripts.

To determine whether the mechanisms of hypertolerance and hyperaccumulation evolved in Brassicaceae hyperaccumulators are conserved in *S. plumbizincicola*, we compared the expression levels of *ZIP* family members probably involved in metal uptake between *S. plumbizincicola* and *S. alfredii* (NHE), including the *HMA* (*Heavy Metal ATPase*) genes controlling metal transport from roots to shoots, the *MTP*s (*Metal Transport Protein*) and *NRAMP*s (*Natural Resistance Associated Macrophage Protein*) regulating vacuolar sequestration capacity (VSC) of metals, and metal-nicotianamine transporter genes *Yellow-stripe-like* family (*YSL*), as well as *Nicotianamine Synthase* (*NAS*) genes (Verbruggen et al., 2009). The results indicated that, similar to those in *A. halleri* and *N. caerulescens*, genes encoding homologs of HMA2, HMA4, ZIPs, NRAMP3, YSLs, and MTL (metallothionein-like protein) showed much higher expression levels in *S. plumbizincicola* than in *S. alfredii* (NHE) (Figure 1C and Supplemental Table 2). Besides, although *NAS* and *NAS2* expressed at lower levels in *S. plumbizincicola* than in *S. alfredii* (NHE), they were strongly induced by Cd treatments in *S. plumbizincicola* roots (Figure 1C and Supplemental Table 2).

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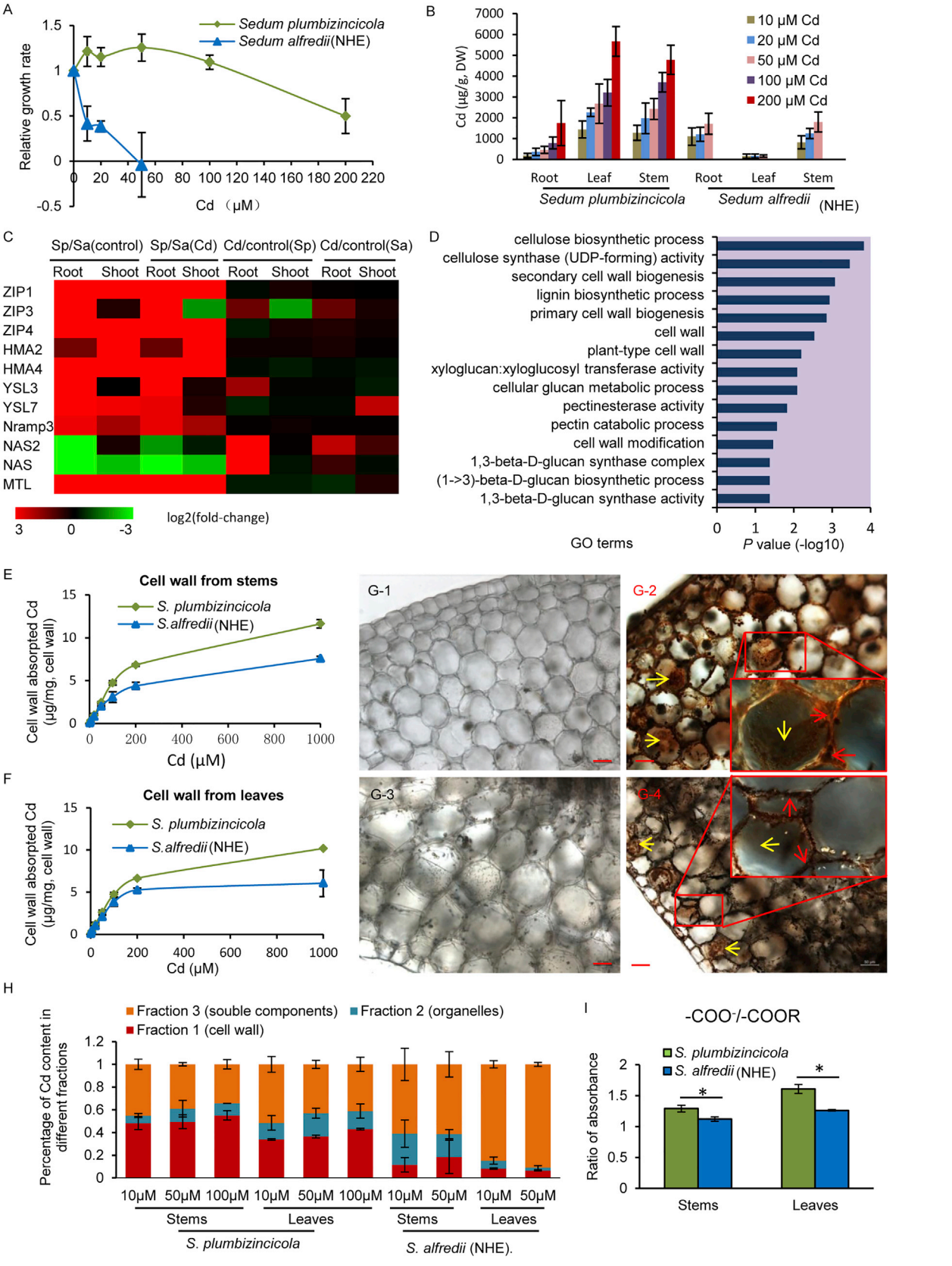

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These observations suggested that increased active uptake, long-distance transport, and efficient chelation of heavy metals may be conserved mechanisms contributing to various hyperaccumulators, including *S. plumbizincicola* used in the current study and those Brassicaceae hyperaccumulators in previous studies. Interestingly, *MTP*s and *CAX5*, genes possibly mediating vacuolar Cd sequestration, did not show apparently enhanced expression in *S. plumbizincicola* compared with the non-hyperaccumulating control (Supplemental Table 3), indicating that other mechanisms contributing more essentially than vacuolar sequestration might have evolved.

To provide further support to this hypothesis, we compared the transcriptomes of *S. plumbizincicola* and *S. alfredii* (NHE), and found that 4597 and 4921 genes showed constitutively higher expression in *S. plumbizincicola* roots and shoots, respectively (Supplemental Figure 3A and 3B). Interestingly, gene ontology (GO) analysis showed that these genes are significantly enriched in processes related to cell wall metabolism, and this observation was more prominent in shoots (Supplemental Figure 3C and 3D). Further analysis of these cell wall-related genes indicated that they get involved in biosynthesis/modification of cellulose, lignin, pectin, glucan, and other cell wall-related components (Figure 1D and Supplemental Table 4). Quantitative RT-PCR determination of 20 of these genes showed high accordance with RNA-sequencing data (Supplemental Figure 4). Taken together, our results suggested that these dramatic changes in gene expression might have made the cell wall an essential player to tackle the challenge imposed by excessive Cd in *S. plumbizincicola* shoots.

To confirm this hypothesis, we first extracted the cell wall of aerial tissues from *S. plumbizincicola* and *S. alfredii* (NHE). *In vitro* Cd binding assay (see Supplemental Methods for more details) showed that cell wall extracted from *S. plumbizincicola* stems (Figure 1E) and leaves (Figure 1F) absorbed more Cd than cell wall did from *S. alfredii* (NHE), indicating that cell wall binding capacity for Cd increased in the hyperaccumulator *S. plumbizincicola*. Then we performed histochemical analysis to determine the cellular distribution of Cd in shoots of both species. Apparent staining was observed in both stems and leaves of *S. plumbizincicola* when treated with 50 μM Cd (Figure 1G-2, G-4), while staining was not detected in control conditions (Figure 1G-1, G-3) and in the non-hyperaccumulator *S. alfredii* (NHE) (Supplemental Figure 5). Moreover, staining was most obvious in cell walls (Figure 1G-2, G-4, red arrows), and also observed in central parts of cells which are occupied by central vacuoles (Figure 1G-2, G-4, yellow arrows). These results suggested that the majority of Cd is sequestrated to the cell wall in *S. plumbizincicola*. This postulation was further supported by direct determination of Cd contents in subcellular fractions of shoot tissues from Cd-treated *S. plumbizincicola* and *S. alfredii* (NHE), which showed that 48.34–55.06% and 33.82–43.00% of total Cd in stems and leaves, respectively, accumulated in the cell wall fraction from *S. plumbizincicola* (Figure 1H). In contrast, 61.08%–61.44% and 84.72%–90.93% of the total Cd in *S. alfredii* (NHE) stems and leaves, respectively, were localized in the soluble fraction, which consists primarily of vacuoles and cytoplasmic solutes. Further analysis with Fourier transform infrared (FT-IR) spectroscopy showed that the absorbance ratio of $-COO^-$ (about 1419 cm^{-1}, Zhang et al., 2008) against –COOR (about 1735 cm^{-1}, Zhang et al., 2008) was significantly higher in *S. plumbizincicola* cell wall than in that of *S. alfredii* (NHE) (Figure 1I and Supplemental Figure 6), implying lower esterification in pectins, which enables more efficient binding of divalent and trivalent metal ions with increased free carboxyl groups (Krzeslowska, 2011; Meyer et al., 2015). This postulation was further supported by the observation that genes with elevated expression in *S. plumbizincicola* shoots were significantly enriched in pectinesterase activity and pectin catabolic process (Figure 1D and Supplemental Table 4).

In summary, our present study demonstrated that elevated expression of some genes likely represents a common mechanism evolved in a wide range of hyperaccumulators. Moreover, we revealed the crucial role of cell wall in Cd storage in *S. plumbizincicola* (Figure 1D–1H) and proposed that cell wall modification occurred in the process of evolution, hence contributing to hyperaccumulation and hypertolerance observed in this newly identified Cd hyperaccumulator. The cell wall-related genes showing elevated expression identified in this study are good candidates for further investigation of specific

Figure 1. Cell Wall Contributes Essentially to Cd Storage in the Hyperaccumulator *S. plumbizincicola*.

(A and B) Cd tolerance **(A)** and accumulation **(B)** in *S. plumbizincicola* and the non-hyperaccumulating control *S. alfredii* (non-hyperaccumulating ecotype, NHE). Plants were grown in hydroponics for 6 weeks and then transferred to solution supplemented with indicated concentrations of $CdCl_2$ for 16 days. Data are means ± SD, n = 4 in **(A)**, n = 7–15 in **(B)**. The growth rate was defined as (FWAT-FWBT)/FWBT, where FWAT is fresh weight after treatments, and FWBT is fresh weight before treatments. Relative growth rates are growth rates relative to those under control condition.

(C) Comparative expression pattern of genes homologous to known genes contributing to hypertolerance/hyperaccumulation in Brassicaceae hyperaccumulators. ZIP, zinc-regulated transporter, iron-regulated transporter-related protein (ZIP1, c33410_g1. ZIP3, c31670_g1. ZIP4, c36833_g1). HMA, heavy metal ATPase (HMA2, c44920_g2. HMA4, c45293_g1). YSL, yellow stripe-like (YSL3, c33770_g1. YSL7, c37441_g3). NRAMP3, natural resistance-associated macrophage protein 3 (c45598_g1). NAS, nicotianamine synthase (NAS2, c35564_g3. NAS, c35472). MTL, metallothionein-like (c32140_g1). Sp, *S. plumbizincicola*. Sa, *S. alfredii* (NHE).

(D) Cell wall-related GO terms enriched in genes with higher expression in *S. plumbizincicola* shoots with or without Cd treatment ($P < 0.05$).

(E and F) Cd absorption ability of extracted cell wall from stems **(E)** and leaves **(F)**. Cell wall extract was incubated with $CdCl_2$ for 40 h. Data are means ± SD, n = 3–4.

(G) Histochemical detection of Cd in stems (G-1, G-2) and leaves (G-3, G-4) of *S. plumbizincicola* with 0 μM (G-1, G-3) or 50 μM $CdCl_2$ (G-2, G-4) for 16 days. Plants were grown in hydroponics for 6 weeks before treatments. Bar, 50 μm. Red arrows in G-2 and G-4 indicate Cd-dithizone sediments in cell wall. Yellow arrows indicate Cd-dithizone sediments in vacuoles.

(H) Subcellular distribution of Cd in stems and leaves of *S. plumbizincicola* and *S. alfredii* (NHE). Plants were grown in hydroponics for 6 weeks and then transferred to solution supplemented with indicated concentration of $CdCl_2$ for 16 days. Data are means ± SD, n = 3.

(I) The absorbance ratio of $-COO^-$ (about 1419 cm^{-1}) against –COOR (about 1735 cm^{-1}) in cell wall of *S. plumbizincicola* and *S. alfredii* (NHE). Cell wall was extracted from stems and leaves of 6-week-old plants and subjected to FT-IR spectroscopy. Data are means ± SD, n = 3. $^*P < 0.05$ (Student's *t*-test).

molecular mechanisms involved in the Cd hyperaccumulation and hypertolerance of *S. plumbizincicola*.

ACCESSION NUMBERS

The RNA-seq data of this study have been deposited in Gene Expression Omnibus (GEO) under the accession number GEO: GSE92529.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

J.G. and J.P. designed the research. J.P., G.D., and H.M. performed the experiments. Y.Z., Y.W., and J.P. analyzed the RNA-seq data. J.G., Y.Z., J.P., and Y.W. wrote the paper.

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REFERENCES

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T.H., Husted, S., Schjoerring, J.K., Talke, I.N., Kramer, U., et al. (2012). Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. Plant Cell **24**:708–723.

Drager, D.B., Desbrosses-Fonrouge, A.G., Krach, C., Chardonnens, A.N., Meyer, R.C., Saumitou-Laprade, P., and Kramer, U. (2004). Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. Plant J. **39**:425–439.

Hanikenne, M., Talke, I.N., Haydon, M.J., Lanz, C., Nolte, A., Motte, P., Kroymann, J., Weigel, D., and Kramer, U. (2008). Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature **453**:391–395.

Krzeslowska, M. (2011). The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy. Acta Physiol. Plant. **33**:35–51.

Meyer, C.L., Juraniec, M., Huguet, S., Chaves-Rodriguez, E., Salis, P., Isaure, M.P., Goormaghtigh, E., and Verbruggen, N. (2015). Intraspecific variability of cadmium tolerance and accumulation, and cadmium-induced cell wall modifications in the metal hyperaccumulator *Arabidopsis halleri*. J. Exp. Bot. **66**:3215–3227.

Mohammed, A.S., Kapri, A., and Goel, R. (2011). Heavy metal pollution: source, impact, and remedies. In Biomanagement of Metal-Contaminated Soils, S.M. Khan, A. Zaidi, R. Goel, and J. Musarrat, eds. (Dordrecht: Springer), pp. 1–28.

Shahzad, Z., Gosti, F., Frerot, H., Lacombe, E., Roosens, N., Saumitou-Laprade, P., and Berthomieu, P. (2010). The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. PLoS Genet. **6**:e1000911.

Verbruggen, N., Hermans, C., and Schat, H. (2009). Molecular mechanisms of metal hyperaccumulation in plants. New Phytol. **181**:759–776.

Wu, L.H., Liu, Y.J., Zhou, S.B., Guo, F.G., Bi, D., Guo, X.H., Baker, A.J.M., Smith, J.A.C., and Luo, Y.M. (2013). *Sedum plumbizincicola* X.H. Guo et S.B. Zhou ex L.H. Wu (Crassulaceae): a new species from Zhejiang Province, China. Plant Syst. Evol. **299**:487–498.

Zhang, X.B., Liu, P., Li, D.T., Xu, G.D., and Jiang, M.J. (2008). FTIR spectroscopic characterization of chromium-induced changes in root cell wall of plants. Spectrosc. Spect. Anal. **28**:1067–1070.