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Short Communication

Zinc accumulation and vegetation ecology in the allotetraploid, *Arabidopsis kamchatica* ssp. *kawasakiana*

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Abstract *Arabidopsis kamchatica* ssp. *kawasakiana*, a member of the family Brassicaceae, is an endangered winter annual species that grows on sandy coasts and lakesides. *A. kamchatica* is an allotetraploid plant produced by the hybridization of two closely related diploid taxa, the Zn/Cd hyperaccumulator *A. halleri* and the non-accumulator *A. lyrata*. The heavy metal accumulation and vegetation ecology of *A. k.* ssp. *kawasakiana* were investigated by collecting leaves and rhizosphere soil samples in three natural habitats on the shore of Lake Biwa in Japan. Leaf Zn contents in almost all plants were above the level required by hyperaccumulators. Plants from one habitat preferred to grow on soils with topical high Zn levels, whereas rhizosphere soils from other populations contained basal levels of Zn suggesting that plant vegetation is more affected by soil disturbance and by soil Zn contents. *A. k.* ssp. *kawasakiana* plants were also found to be tolerant to Zn and Cd, in contrast to the nontolerant species *A. thaliana*. These findings indicated that *A. k.* ssp. *kawasakiana* is a facultative Zn hyperaccumulator, inheriting the trait from its parent *A. halleri*. Furthermore, *A. k.* ssp. *kawasakiana* is a self-compatible plant and Arabidopsis floral dip transformation might be applicable to this plant. Considering their natural diversity, *A. k.* ssp. *kawasakiana* will help the determination of the molecular mechanisms by which plants accumulate and tolerate heavy metals.

Key words: Allotetraploid, *Arabidopsis kamchatica* ssp. *kawasakiana*, endangered species, hyperaccumulator, zinc.

The leaves of plants that hyperaccumulate metals contain unusually high amounts of metals or metalloids (Baker and Brooks 1989). For example, Zn hyperaccumulators are defined as plants with leaves containing 3000 or 10,000 mg kg^{-1} Zn, concentrations 10–100 fold higher than the critical toxicity levels in ordinary plants (Krämer 2010; Pollard et al. 2014). Accumulation of metals is a defensive mechanism against environmentally toxic metals (Clemens et al. 2013; DalCorso et al. 2013; Leitenmaier and Küpper 2013). To date, approximately 500 taxa have been identified as metal hyperaccumulators, with most present in metal-rich environments. Some species, called facultative hyperaccumulators, have been observed growing on both contaminated and uncontaminated soils. It is unclear, however, whether the physiological ability of facultative hyperaccumulators to tolerate and accumulate metals occurs only in plants growing on metalliferous soils, or whether this ability is also present in plants growing on uncontaminated soils (Pollard et al. 2014).

Arabidopsis halleri is a perennial, self-incompatible member of the family Brassicaceae. Being the closest relative of *A. thaliana*, *A. halleri* is currently regarded as a facultative Zn/Cd hyperaccumulator (Krämer 2010; Weber et al. 2006). Investigations of metallicolous and non-metallicolous populations of *A. halleri* showed that high concentrations of Zn and Cd are a constitutive property of this species (Bert et al. 2002; Pauwels et al. 2006). Five subspecies of *A. halleri* have been identified to date: *A. h.* ssp. *dacica*, *A. h.* ssp. *gemmifera*, *A. h.* ssp. *halleri*, *A. h.* ssp. *ovirensis*, *A. h.* ssp. *tatrica*. All of these subspecies, except for *A. h.* ssp. *dacica*, have been shown to possess features of Zn/Cd hyperaccumulators (Bert et al. 2000; Kenderešová et al. 2012; Kubota and Takenaka 2003; Pauwels et al. 2006).

A. kamchatica is an allotetraploid Brassicaceae plant produced by the hybridization of *A. h.* ssp. *gemmifera* with non-hyperaccumulating *A. lyrata* (Shimizu et al. 2005; Shimizu-Inatsugi et al. 2009). *A. kamchatica* consists of two subspecies, ssp. *kawasakiana* and ssp. *kamchatica*, which are differ in habitat, morphology, and nucleotide allele frequencies (Higashi et al. 2012). *A. k.* ssp. *kawasakiana* is an endemic plant in Japan, mainly growing in sandy soils along sea and lake shores (Fujii 1994; Yamaguchi et al. 2010). Although Ministry of the Environment Government of Japan has classified this plant as endangered, with a very high risk of extinction, several populations were observed along the shore

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of Biwa Lake, with more than 30,000 individual plants recently found in a habitat along the east side of this lake (Yamaguchi et al. 2010).

A biogeographic study of *Arabidopsis* spp. showed that the allotetraploid *A. kamchatica* grows in a broader climatic niche of temperature and precipitation than *A. lyrata* and *A. halleri* (Hoffmann 2005). Considerable amounts of functional DNA may be lost during polyploidization events (Chen 2007). Actually, the DNA contents of *A. kamchatica* were found to be slightly lower than the sum of the two parental taxa (Wolf et al. 2014). Phylogenetic analysis revealed that *A. kamchatica* originated from multiple individuals of its diploid parents (Shimizu-Inatsugi et al. 2009). To date, however, little is known about whether *A. kamchatica* inherited the trait of Zn/Cd tolerance and accumulation from its parent *A. halleri.*

Individual *A. k.* ssp. *kawasakiana* plants were sampled from three sites on the shore of Lake Biwa, in Shiga Prefecture in Japan, at which the collection of these plants was not prohibited (Table 1). To assess metal accumulation, leaf tissues and rhizospheres were collected in April 2015. Soil samples around individuals were collected from the land surface under the 0 layer for chemical analyses. In the habitat Takashima, soil samples were also collected 0.5 m, 5 m, and 50 m from colonizing *A. k.* ssp. *kawasakiana* plants.

The numbers of chromosomes in flower buds were assessed as described (Kosugi et al. 2015) to determine the ploidy of plants collected in Hikone in April 2015 and to distinguish allopolyploid species from parental diploid *A. h.* ssp. *gemmifera* and other Brassicaceae. Briefly, flower buds were harvested and immediately fixed in ethanol–acetic acid for 7 days. Petals and sepals were removed to access pollen-mother cells, and DAPI–DNA conjugates were viewed by fluorescence microscopy (BX60, Olympus, Tokyo, Japan). All plants tested were tetraploid (data not shown). Their morphological features showed that the *A. k.* ssp. *kawasakiana* plants used in the present study were not erroneously identified as other species.

Quantitative analysis of the metal contents of plants (leaf tissues, 30–150 mg) and soils were performed as described (Kosugi et al. 2015). Briefly, 1.0 g of field-collected soil samples were dried and heated in a mixture of 1 ml 60% HNO_3 and 5 ml 60% $HClO_4$. To each was added 1 g NH_4Cl, 10 ml 1 M HCl and 10 ml distilled water and then heated to boiling. The digests were filtered with filter paper to analyze total Cd and Zn contents. To extract acid-extractable Cd and Zn, 30 ml of 0.1 M HCl was added to 3.0 g of each soil sample in a 50-ml conical tube, followed by shaking at 150 rpm at 30°C for 1 h. Metal concentrations in digests and extracts were measured by inductively coupled plasma–optical emission spectroscopy (ICP–OES, SPS3100, SII Nano Technology, Tokyo, Japan).

The Zn concentrations of plant leaves are shown in Figure 1A. The leaves of *A. k.* ssp. *kawasakiana* collected in the field showed strong Zn accumulation. Zn contents

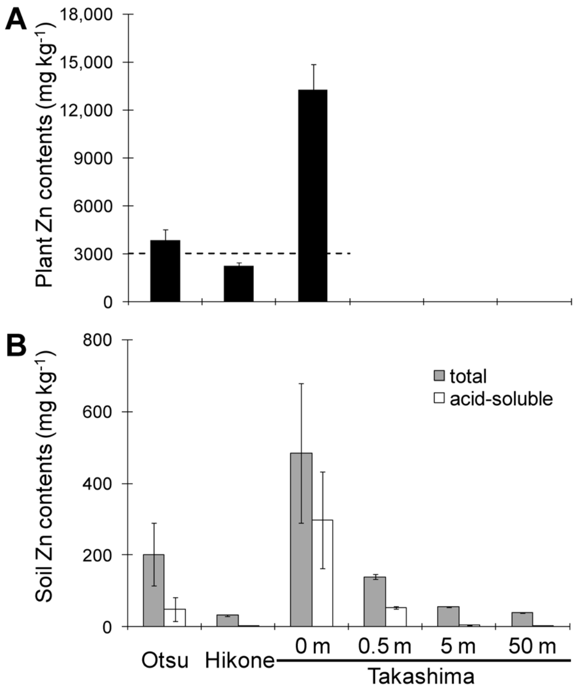

Figure 1. Zn partitions in three populations of *A. k.* ssp. *kawasakiana*. (A) Zn concentrations in leaves of plants analyzed by using ICP–OES. The horizontal dashed line indicates the threshold for Zn hyperaccumulation (3000 mg kg^{-1}) in aerial parts of plants. (B) Total and acid soluble Zn contents in rhizosphere and bulk soil samples. In habitat Takashima, the distances between the sampling sites and plant colonies are shown. Data are presented as means±SE of mean (error bars).

Table 1. *A. k.* ssp. *kawasakiana* populations used in this work.

No.	Population	Description	GPS coordinates	Altitude (m)	Density of colony	Area of habitat (m^2)	Soil pH (H_2O)[a]	Number of plants[b]
1	Otsu	Sandy place near storage shed	N 35°12′ E 135°55′	87	Moderate	6	5.39±0.07	4
2	Hikone	Sandy open beach	N 35°13′ E 136°9′	85	Moderate	9000	6.06±0.06	6
3	Takashima	Sandy beach in pine forest	N 35°18′ E 136°2′	86	High	2	5.26±0.11	5

Altitudes at the sampling sites were estimated by GSI maps (http://www.gsi.go.jp/ENGLISH/index.html) provided by The Geospatial Information Authority of Japan. [a] Values are mean±SE. [b] Number of plants used for the analyses of metal contents.

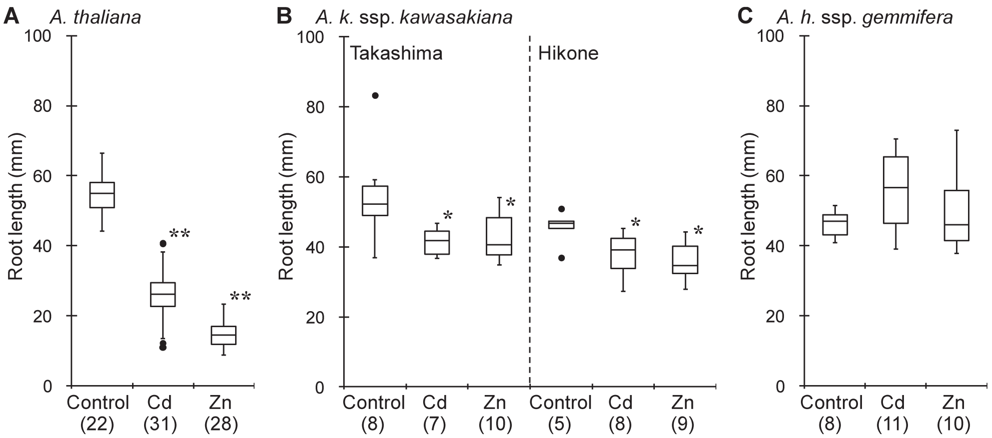

Figure 2. Zn and Cd tolerance of *A. k.* ssp. *kawasakiana*. Box plots of the growth of seedling roots of *A. thaliana* (A), two populations of *A. k.* ssp. *kawasakiana* (B) and *A. h.* ssp. *gemmifera* (C) on agar medium with or without 25 μM Cd or 250 μM Zn. The boxes represent the inter-quartile range, with medians indicated by horizontal lines. Vertical lines connect the nearest observations within 1.5 times the inter-quartile ranges of the lower and upper quartiles. The outliers are represented by solid circles. The number in parentheses following each treatment represents the number of samples. Statistical comparisons were by one-way ANOVA, followed by Dunnett's test, using R software version 3.0.1 (R Core Team 2013) with add-in package multcomp (Hothorn et al. 2008). $*p<0.05$; $**p<0.01$ comparing treated and control plants.

ranged from the leaves of plants collected in Otsu and Takashima ranged from 2680 to 5720 mg kg^{-1} and from 8940 to 16,900 mg kg^{-1} dry biomass, respectively, greater than the recently proposed nominal threshold for Zn hyperaccumulation of 3000 mg kg^{-1}. Zn contents in the leaves of plants collected in Hikone ranged between 1580 and 3230 mg kg^{-1}. None of these plants showed visible signs of metal toxicity or nutrient deficiency.

The total and acid-soluble soil Zn contents are shown in Figure 1B. Because high-density colonization of *A. k.* ssp. *kawasakiana* was observed in Takashima, we presumed the topical precipitation of Zn in soil and therefore collected and analyzed soils located 0.5 m, 5 m and 50 m from plant communities. Mean total and acid soluble Zn contents in soils from Takashima were 485±195 mg kg^{-1} and 297±135 mg kg^{-1}, respectively, with both strongly associated with the density of *A. k.* ssp. *kawasakiana* plants (Figure 1B). Zn concentrations in these soils decreased as the distance between plant colonies and sampling points increased. The pH values of three soil samples from Takashima also decreased as the distance between plant colonies and sampling points increased, with mean pHs at 0.5 m, 5 m, and 50 m of 5.65±0.04, 5.21±0.10, and 5.03±0.02, respectively, indicating that local Zn condensation was not strongly associated with soil pH. Relative to the metal profile of soils in Japan (https://gbank.gsj.jp/geochemmap/zenkoku/gazou_san/japan_sanZn.jpg), however, soil Zn contents were moderate in Otsu and low in Hikone (Figure 1B). Nevertheless, the largest community of these plants, totaling 11,000 individuals, was observed in *ca.* 9000 m^2 of sandy beach in Hikone (Kosugi et al., *in press*). According to the investigation by Lake Biwa Environmental Research Institute, the drastic topographical change on the east shore of a lake have been induced by the coastal current in Lake Biwa (Kaneko et al. 2011).

To evaluate heavy metal tolerance, seedlings of *A. thaliana* wild-type (Col-0) and metallicolous (Takashima) and non-metallicolous (Hikone) populations of *A. k.* ssp. *kawasakiana*, and *A. h.* ssp. *gemmifera* were grown in metal-containing plant culture medium containing 1% sucrose and solidified with 1.5% Agar, Powder (for Plant Culture Medium, Wako, Japan), as described in Weber et al. (2006). The seeds were surface sterilized, germinated on one-tenth-strength modified Hoagland medium (0.4 mM $Ca(NO_3)_2$, 0.6 mM KNO_3, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 5 μM Fe-EDTA, 0.03 μM $CuSO_4$, 0.2 μM $ZnSO_4$, 0.5 μM $MnCl_2$, 4.6 μM H_3BO_3, 0.01 μM MoO_3, pH 5.7, and transferred to the same medium containing 25 μM $Cd(NO_3)_2$ or 250 μM $ZnSO_4$. The plates were incubated vertically with a temperature cycle of 22 : 18°C, day : night and a photoperiod of 16 : 8-h, light : dark. After 14 days root length in each plant was measured. Figure 2 shows that the seedlings of *A. k.* ssp. *kawasakiana* were less affected by 25 μM Cd or 250 μM Zn than the seedlings of *A. thaliana*. The relative root lengths of seedlings of *A. thaliana*, *A. k.* ssp. *kawasakiana* from Takashima and Hikone and *A. h.* ssp. *gemmifera*, expressed as a percentage of growth in control medium, were 46.9%, 75.9%, 82.6% and 119.7%, respectively, in the presence of Cd, and 26.4%, 78.8%, 79.9% and 107.9%, respectively, in the presence of Zn. No significant differences were apparent between metallicolous and nonmetallicolous populations of *A. k.* ssp. *kawasakiana*. Under these metal conditions, the tolerance of *A. k.* ssp. *kawasakiana* was comparable to that of *A. h.* ssp. *gemmifera*.

Overall, these results showed that *A. k.* ssp. *kawasakiana* is a facultative Zn hyperaccumulator, inheriting this trait from its parent *A. halleri*. Although these plants were also tolerant to Cd, the Cd contents in both plants and soils at all sampling sites were under the limits of quantification (data not shown).

Zn accumulation and vegetation of *A. k.* ssp. *kawasakiana* showed that these plants have adopted to diverse environmental conditions. Colonization was observed in Zn-enriched soil in Takashima. However, the large population in Hikone suggests that the vegetation of *A. k.* ssp. *kawasakiana* does not directly indicate a high Zn concentration in soil. These findings suggest that *A. k.* ssp. *kawasakiana* plants prefer to grow under conditions of environmental disturbance, including high metal contents in the rhizosphere, to achieve a competitive advantage over other plants.

Interspecific crosses between *A. h.* ssp. *halleri* and *A. lyrata* ssp. *petraea* were performed in the laboratory to obtain progenies to identify the loci that control the segregation of Zn and Cd tolerance (Bert et al. 2003; Courbot et al. 2007). Shoot metal contents and tolerance thresholds were significantly lower in the F1 progeny than in the hyperaccumulating ancestor (Bert et al. 2003; Isaure et al. 2015; Sarret et al. 2009), indicating that metal tolerance and accumulation are likely regulated by several major genes. The natural variation of Zn/Cd tolerance and metal accumulation have been investigated in *A. halleri* to determine the mechanisms of metal detoxification (Meyer et al. 2015). Allotetraploids *A. kamchatica* plants containing genes that regulate metal tolerance and accumulation.

Agrobacterium-mediated stable transformation of *A. h.* ssp. *halleri* was established on tissue culture–based procedure and have been performed to produce RNA interference (RNAi) lines targeting potential key genes for metal hyperaccumulation (Deinlein et al. 2012; Hanikenne et al. 2008). *Agrobacterium*-mediated plant transformation via floral-dip method is a simple and efficient approach, and has been successfully used to several plant species, including *A. thaliana* (Clough and Bent 1998), *Medicago truncatula* (Trieu et al. 2000), *Linum usitatissimum* (Bastaki and Cullis 2014). Unlike its parent species, *A. k.* ssp. *kawasakiana* is a self-compatible plant (Sugisaka and Kudoh 2008). The transformation procedure for *A. thaliana* that involve the infiltration of flowering plants might be applicable to *A. k.* ssp. *kawasakiana*. *A. k.* ssp. *kawasakiana* plants are potential resources to elucidate molecular mechanism of plant metal accumulation and environmental adaptation.

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