Comparative analysis of aluminum accumulation in leaves of three angiosperm species

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Abstract: Aluminum (Al) accumulators are widely distributed in the plant kingdom but phylogenetic implications of internal Al detoxification mechanisms are not well understood. We investigated differences in the characteristics of Al accumulation (i.e., accumulation potential, chemical form, and localization) in three woody Al accumulators, *Symplocos chinensis* (Symplocaceae, Ericales), *Melastoma malabathricum*, and *Tibouchina urvilleana* (both Melastomataceae, Myrtales). The order of Al accumulation potential under hydroponic conditions was *S. chinensis* ≈ *M. malabathricum* > *T. urvilleana*. Oxalate was at least partly involved in the internal Al detoxification mechanisms in leaves of all three Al accumulators, based on a correlation analysis between Al and organic acid in water and 0.02 M HCl extracts and the ^{27}Al nuclear magnetic resonance spectra of intact leaves. However, the Al forms in the leaves were not simple Al-ligand complexes in a specific cell structure. Al localization in leaf sections differed among the three species. Extremely high levels of Al were found in trichomes of the lower epidermis in leaves of *T. urvilleana*. These data illustrate that woody Al accumulating angiosperms have independently developed various internal Al tolerance mechanisms in which oxalate plays a significant role.

Key words: Auminum accumulators, *Melastoma malabathricum* L., oxalate, *Symplocos chinensis* (Lour.) Druce var. *leucocarpa* (Nakai) Ohwi f., *Tibouchina urvilleana* Cogn., trichomes

Introduction

Acid soils occupy approximately 30% of the world's ice-free land area (von Uexküll and Mutert, 1995). Owing to the toxic effects of Al, acid soils with high levels of soluble Al restrict plant growth (Foy et al., 1978; Kochian et al., 2004). Al tolerance is generally interpreted as the ability to exclude Al. Exudation of organic acids from the roots is considered as one of the most important strategies by which Al is excluded (Kochian et al., 2004). In contrast, some plant species developed internal Al inactivation mechanisms to avoid Al toxicity. Of these, plant species with Al levels of at least 1000 mg kg^{-1} in their leaves or shoots are defined as Al accumulators (Chenery, 1948). Metali et al. (2012), however, argue for a higher threshold of 2.3 to 3.9 mg Al g^{-1} leaf dry mass for distinguishing Al accumulators from non-accumulators for tropical plants.

Al accumulators have developed mechanisms to detoxify Al in their tissues by forming stable complexes with organic or inorganic ligands. For example, Al-citrate complexes are formed in leaves of *Hydrangea macrophylla* (Ma et al., 1997a). The formation of Al-Si complexes in leaves of *Faramea marginata* has also been suggested (Britez et al., 2002). Isolating Al from the sites that are sensitive to Al (especially the cytoplasm) is another important strategy to avoid Al toxicity in plant tissues. High levels of Al accumulate in vacuoles in buckwheat leaves (Shen et al., 2002) and epidermal cell walls in leaves of *Melastoma malabathricum*

(Watanabe et al., 1998). There are also details for *Camellia* (tea plants) and *Hydrangea macrophylla*; see for instance the nice review by Brunner and Sperisen (2013).

The phylogenetic distribution of Al accumulators has been studied by several researchers. Jansen et al. (2002) comprehensively analyzed the data in the literature, and applied recent molecular phylogenies to evaluate the systematic and phylogenetic implications of the hyperaccumulation characteristic. They found that Al accumulators are mainly eudicots, and are particularly common in basal, woody branches of fairly advanced groups, such as rosids (Myrtales, Malpighiales, Oxalidales) and asterids (Cornales, Ericales, Gentianales, Aquifoliales), but the characteristic has probably been lost in more derived, herbaceous taxa (Jansen et al., 2002).

Although Al accumulators are found in various vascular plant taxa, little attention has been paid to physiological, chemical, and anatomical differences of Al accumulation in leaves of various accumulating species. Therefore, this study aims to characterize Al accumulation (i.e., accumulation potential, chemical form, and localization) in representative Al accumulators of the orders Myrtales and Ericales, both of which contains many Al accumulator species (Jansen et al. 2002, 2004). *M. malabathricum* (Melastomataceae), which represents a well-studied Al accumulator, was used as control species. The diversity of Al hyperaccumulation characteristics are discussed by comprehensive analysis of the results

from this study and literature.

Materials and Methods

Estimating Al-accumulation potential in different Al accumulators

Uniform cuttings from mature plants of *M. malabathricum* L. (Melastomataceae) and *Tibouchina urvilleana* Cogn. (Melastomataceae) were rooted and precultured in a 40-L container containing an Al-free standard nutrient solution that was aerated constantly for 1 month in a glasshouse at Hokkaido University under natural conditions (13–15 h photoperiod and a day:night temperature of 25–28:18–22°C). Plants of *Symplocos chinensis* (Lour.) Druce var. *leucocarpa* (Nakai) Ohwi f. *pilosa* (Nakai) Ohwi (Symplocaceae) were purchased from a garden center. The roots were carefully washed with tap water to remove adhering soils. Subsequently, the plants were transferred to a continuously aerated 40-L container containing a standard nutrient solution for hydroponic preculture, and grown for 1 month. The standard nutrient solution contained 0.54 mM N (NH_4NO_3), 0.16 mM P ($NaH_2PO_4 \cdot 2H_2O$), 0.15 mM K (K_2SO_4:KCl = 1:1), 0.25 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.16 mM Mg ($MgSO_4 \cdot 7H_2O$), 35.8 μM Fe ($FeSO_4 \cdot 7H_2O$), 9.1 μM Mn ($MnSO_4 \cdot 4H_2O$), 46.3 μM B (H_3BO_3), 3.1 μM Zn ($ZnSO_4 \cdot 7H_2O$), 0.16 μM Cu ($CuSO_4 \cdot 5H_2O$), and 0.05 μM Mo ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$); total SO_4 = 0.21 mM. After preculturing, the plants were transferred to a phosphorus free standard nutrient solution

with 0.5 mM $AlCl_3$ ({Al^{3+}} = 0.154 mM, calculated by GEOCHEM-EZ (Shaff et al., 2010)), and cultivated for 3 months. The solution was adjusted daily to a pH of 4.0. After the Al treatment, the plants were sampled, washed with deionized water, and cut to separate roots and leaves. The fresh samples were dried at 75°C for 4 days, weighed and ground. Ground samples were digested with HNO_3-H_2O_2, and mineral concentrations were determined by inductively coupled plasma mass spectrophotometry (ELAN DRC-e, Perkin Elmer, Waltham, MA, USA).

Identifying the chemical form of Al in leaves of Al accumulators

We first examined the correlation between Al and organic acid concentrations in leaves. Leaves at different stages of development (young, mature, and old) were sampled individually from plants of three Al accumulators grown in a standard nutrient solution with 0.5 mM Al for 2 months, as described above. Fresh leaves were ground on ice using a mortar and pestle with 1 mL of water or 0.02 M HCl (sample (mg):solution (μL) = 1:10). Water and 0.02 M HCl extracts were filtered through a membrane filter (pore size = 0.45 μm), and organic acid and Al concentrations were determined by capillary electrophoresis (Quanta 4000CE, Waters, Milford, MA, USA; Watanabe et al., 1998) and inductively coupled plasma atomic emission spectroscopy (ICPS-7000, Shimadzu, Kyoto, Japan), respectively. Furthermore, liquid-state

^{27}Al nuclear magnetic resonance (NMR) was used to determine the Al form in the leaves. Fresh leaves of three Al accumulators were individually placed in a NMR tube (10 mm diameter). The Al concentrations in leaves of *S. chinensis*, *M. malabathricum*, and *T. urvilleana* used for ^{27}Al NMR analysis were 6890, 7920, and 8680 mg kg^{-1} DW, respectively. The ^{27}Al NMR spectrum was recorded at 156.3 MHz (JNM-α600 spectrometer; JEOL, Tokyo, Japan). The parameters used were as follows: frequency range, 62.5 kHz; data point, 131 k; and acquisition time, 0.6 s. An aluminum chloride solution (1 mM $AlCl_3$ in 0.1 M HCl) was used as an external reference for calibration of the chemical shift (0 ppm). The spectrum of the leaves was compared with that of a mixture of $AlCl_3$ (1 mM) and oxalate (1 or 3 mM), malate (2 mM), or citrate (2 mM) (pH4.0), which were added to a 5-mm tube and subjected to ^{27}Al NMR analysis using the following parameters: frequency range, 62.5 kHz; data point, 33 k; and acquisition time, 0.52 s.

Localization of aluminum in leaves of Al accumulators

Mature leaves of the three Al accumulators grown in a standard nutrient solution with 0.5 mM Al as described above were sampled to examine Al localization. After washing with deionized water, the leaves were sectioned (20 µm thickness) with a cryo-microtome (CM-3050S, Leica Biosystems, Wetzlar, Germany). After sectioning, the transverse sections were stained on a

glass slide with pyrocatechol violet (PCV) according to the method of Watanabe et al. (1998). In brief, the sections were stained with 0.02% (w/v) PCV and 2.5% (w/v) hexamine-NH_4OH buffer (pH 6.2) for 15 min, washed with 2.5% hexamine-NH_4OH buffer (pH 6.2), and observed under a light microscope (BX51, Olympus, Tokyo, Japan).

Results

Al accumulation

Al concentration in leaves of *S. chinensis* and *M. malabathricum* grown for 3 months in a nutrient solution with 0.5 mM Al exceeded 8000 mg kg^{-1}, whereas that in *T. urvilleana* was less than half that of these two species (Table 1).

Al and organic acid concentrations in water and 0.02 M HCl extracts

Young, mature, and old leaves were separately extracted with water or 0.02 M HCl, and Al and organic acid concentrations in both extracts were determined (Table 2). A significant positive correlation was observed between Al and oxalate concentrations in water and 0.02 M HCl extracts of *S. chinensis* and *M. malabathricum*, respectively (Table 3). No significant correlation was observed between Al and organic acid concentrations in *T. urvilleana*, and the molar ratios of oxalate to Al in both extracts were low (Tables 2 and 3).

^{27}Al NMR

When comparing the ^{27}Al NMR spectrum of intact leaves with a mixture of Al and organic acid, the resonance peaks in *M. malabathricum* corresponded almost to those obtained for the Al-oxalate mixture (Fig. 1). The chemical shifts of the minor downfield peaks corresponded to those of the mixture of Al-oxalate, but shifted greatly downfield in both *S. chinensis* and *T. urvilleana* (Fig. 1).

Al localization in leaves

Staining with PCV yielded a blue color by generating a chelating complex with Al. PCV staining of the leaf transverse section indicated that the distribution of Al in *S. chinensis* and *M. malabathricum* was similar; high levels of Al were found in epidermal cells but not in mesophyll cells (Fig. 2A and 2B). In *T. urvilleana*, Al localized in the thick lower epidermis, particularly in trichomes (Fig. 2C).

Discussion

Al accumulators are defined as plants containing more than 1000 mg Al kg^{-1} DW in their leaves or shoots (Chenery, 1948). However, some Al accumulators adapted to strongly acid

soils, particularly in the tropics, may contain more than 5000 mg Al kg^{-1} in their leaves or shoots (de Medeiros and Haridasan, 1985; Masunaga et al., 1998; Osaki et al., 2003). Strong Al accumulators are often observed in the genus *Symplocos* (Watanabe et al., 2007), including *S. chinensis* used in this study. *S. spicata* was reported to contain more than 72000 mg Al kg^{-1} in its leaves by von Faber (1925). *M. malabathricum* is also a strong Al accumulator containing $\geq$ 10000 mg Al kg^{-1} in leaves.

Among the three Al accumulators examined, *S. chinensis* and *M. malabathricum* showed comparably higher Al accumulation in leaves than *T. urvilleana* (Table 1). This result indicates that characteristics of Al accumulation are quantitatively different between *M. malabathricum* and *T. urvilleana*, despite the fact that *Tibouchina* and *Melastoma* belong to the Melastomataceae family and are therefore phylogenetically closely related.

Formation of stable complexes with organic and inorganic ligands is an important mechanism for internal Al detoxification in Al accumulators (Watanabe and Osaki, 2002). Organic acids are common ligands for Al in many Al accumulators. In our previous studies, for example, we showed that soluble Al forms in leaves and roots of *M. malabathricum* are Al-oxalate complexes and inorganic monomeric Al ions (Watanabe et al., 1998; 2005). To elucidate the species-specific formation of Al complexes in leaves, the relationship between Al and organic acid concentrations was investigated. Only oxalate showed a comparable concentration to Al

in all three accumulators (Table 2), and a significant positive correlation was found between Al and oxalate concentrations in water and 0.02 M HCl extracts of *S. chinensis* and *M. malabathricum*, respectively (Table 3). The fact that we found no significant correlation for the water extract of *M. malabathricum* may be because of a higher concentration of free oxalate in the extract (Table 2). In contrast, oxalate concentration in both extracts was low in comparison with Al concentration, and no significant correlation was observed between Al and organic acid concentrations in *T. urvilleana* (Tables 2 and 3). These results imply that the primary forms of soluble Al are Al-oxalate complexes in *S. chinensis* and *M. malabathricum*, but not in *T. urvilleana.*

Although determining the correlation between Al and possible ligands is an effective method to estimate Al forms in a plant, additional method(s) such as the ^{27}Al NMR technique, which can non-destructively estimate Al species in intact leaves, must be employed because it is possible that the extraction process changes the forms of Al. The chemical shift of each peak in intact leaves of *M. malabathricum* corresponded fairly well to the peaks of the Al-oxalate mixture (Fig. 1), suggesting that the primary forms of soluble Al in leaves of *M. malabathricum* are Al-oxalate complexes and inorganic monomeric Al as shown in our previous report (Watanabe et al., 1998). Likewise, the chemical shifts of the minor downfield peaks corresponded to those of the mixture of Al-oxalate in both *S. chinensis* and *T. urvilleana*

but shifted greatly downfield (Fig. 1). This result suggests that the pH and/or ion composition in the environment, where Al-oxalate complexes exist in leaves of *S. chinensis* and *T. urvilleana*, could differ substantially from those in the standard solution.

The ^{27}Al NMR spectrum of intact leaves of all three species showed octahedral Al-oxalate complexes with empty coordination positions (coordinated with H_2O or OH^-, Hiradate 2004) (Fig. 1). If oxalate concentration is high enough to bind to Al at cytosolic pH (pH7.0-7.5), the major resonance peak must appear at around 18 ppm (Al-(oxalate)$_3$). Even if the oxalate concentration is not high enough, proteins and phospholipids could easily bind to these Al complexes with empty coordination positions at cytosolic pH. Thus, these results suggest that Al may primarily occur at acidic sites, such as vacuoles, in leaves of these Al accumulators. The major resonance peak in leaf samples at approximately 0 ppm, presumably mainly derived from inorganic monomeric Al species, also indicates Al accumulation in acidic sites, particularly in *T. urvilleana* (Fig. 1). A comparison of the ^{27}Al NMR spectrum of the isolated protoplasts and vacuoles with that of intact leaves is necessary for more exact speciation of Al in leaves. Moreover, an Al complex composed of multiple inorganic and/or organic ligands as well as the coexistence of different Al-ligand complexes in leaf tissues should also be considered.

Al localization in leaves may also be related to mechanisms of Al tolerance because

sequestration of Al from sensitive tissues is also important for Al accumulators. In *S. chinensis* and *M. malabathricum*, high levels of Al accumulation were observed in epidermal cells but not in mesophyll cells, which are responsible for photosynthesis (Fig. 2A and 2B). Al localizes in leaf epidermal cells of many Al accumulators including *Camellia sinensis* (Tolrà et al., 2011), *Faramea marginata* (Britez et al., 2002), and *Richeria grandis* (Cuenca et al., 1991). Meanwhile, Al localized in the thick lower epidermis but not in upper epidermis of *T. urvilleana*, particularly in trichomes (Fig. 2C). In addition to Al, it has been reported that some plants also accumulate Mn (e.g., sunflower; Blamey et al., 1986, pumpkin; Iwasaki and Matsuura 1999), Cd (e.g., Indian mustard; Salt et al., 1995), or Ni (e.g., *Alyssum* L., Broadhurst et al., 2009) in trichomes. Choi et al. (2001) found large crystals formed on head cells of trichomes contain high concentrations of Cd and Ca. They expected that these crystals were composed of Ca-oxalate, in which Cd was embedded.

In conclusion, our results suggest that oxalate is commonly used as a ligand for a portion of Al in leaves of three woody Al accumulators, whereas other Al forms could be dominant, particularly in *T. urvilleana*. Oxalate is a major organic ligand for detoxification of Al in many Al accumulators, such as *Camellia sinensis* (Theaceae, Ericales, Morita et al., 2008) and *Fagopyrum esculentum* (Polygonaceae, Caryophyllales, Ma et al., 1997b). Oxalate has often been regarded as an end product that is not further metabolized or only slowly metabolized.

Many Al accumulators may effectively use oxalate, which is less metabolically important, for internal Al detoxification, irrespective of the plant phylogeny. However, because the results of the ^{27}Al NMR analysis using intact leaves suggested that oxalate does not simply form a complex with Al in leaf cells, further investigations are necessary. Localization of Al in a leaf section was not similar between *M. malabathricum* and *T. urvilleana*, despite both being in the same family of Melastomataceae, suggesting that the phylogenetic effect is relatively small for tissue compartmentalization of Al in leaves. However, we investigated only three Al accumulators in this study. Because Al accumulators are widely distributed in vascular plants (Jansen et al., 2004; Watanabe et al., 2007; Metali et al. 2012), a more extensive survey is needed.

Acknowledgments

This study was supported financially by Grants-in-Aid for Scientific Research (No. 24580088) from the Japanese Society for the Promotion of Science (TW), a grant from the Sapporo Agricultural Alumni Association (EM), and a grant from the Juniorprofessorenprogramm MWK (Baden-Wurttemberg) (SJ).

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Table captions

Table 1. Total Al concentration (mg kg^{-1}) in leaves of three Al accumulators grown in a standard nutrient solution with 0.5 mM Al

Table 2. Average concentration (mM) of Al, oxalate, malate, and citrate in the extract of leaves

Table 3. Correlation coefficient between Al and each organic acid concentrations

Figure captions

Fig. 1 ^{27}Al NMR spectra of the standard solution (1 mM $AlCl_3$ + 1 mM/3 mM oxalate, pH 4.0) and the intact leaves of *Symplocos chinensis*, *Tibouchina urvilleana*, and *Melastoma malabathricum* grown in a nutrient solution containing 0.5 mM.

Fig. 2 Transverse sections of *Symplocos chinensis* (A), *Tibouchina urvilleana* (B), and *Melastoma malabathricum* (C) after pyrocatechol violet staining. Blue-colored tissues indicate localization of Al. Bar = 100 μm

Table 1

Symplocos chinensis	8309 ± 282
Tibouchina urvilleana	3431 ± 262
Melastoma malabathricum	8540 ± 1330

Values are means of 3 replicates ± standard error

Table 2

		Al		Oxalate		Malate		Citrate	
Water soluble	*Symplocos chinensis*	2.84	(0.286)	2.92	(0.310)	0.348	(0.239)	0.441	(0.738)
(n=8)	*Tibouchina urvilleana*	1.00	(0.196)	0.67	(0.451)	0.233	(0.263)	0.392	(0.753)
	Melastoma malabathricum	3.14	(0.166)	6.49	(0.228)	0.205	(0.483)	0.744	(0.335)
0.02 M HCl soluble	*Symplocos chinensis*	4.67	(0.156)	7.44	(0.239)	0.357	(0.599)	0.381	(0.877)
(n=8)	*Tibouchina urvilleana*	2.14	(0.587)	1.88	(0.502)	0.211	(0.646)	0.188	(0.548)
	Melastoma malabathricum	6.04	(0.153)	23.11	(0.257)	0.412	(0.303)	1.874	(0.554)
Water soluble /	*Symplocos chinensis*	0.61		0.39		0.98		1.16	
0.02M HCl soluble[a]	*Tibouchina urvilleana*	0.47		0.35		1.10		2.08	
	Melastoma malabathricum	0.52		0.28		0.50		0.40	

Values in brackets are coefficients of variation.

[a]Concentration of each component in the water extract / that in the 0.02 M HCl extract

Table 3

		Oxalate	Malate	Citrate
Water soluble	*Symplocos chinensis*	0.924 **	0.624	0.647
	Tibouchina urvilleana	0.531	0.277	-0.007
	Melastoma malabathricum	-0.168	-0.552	-0.143
0.02 M HCl soluble	*Symplocos chinensis*	0.364	-0.236	0.397
	Tibouchina urvilleana	0.659	-0.080	0.508
	Melastoma malabathricum	0.858 *	0.157	0.590

** and *, significant at $P < 0.01$ and 0.05, respectively.

Fig. 1

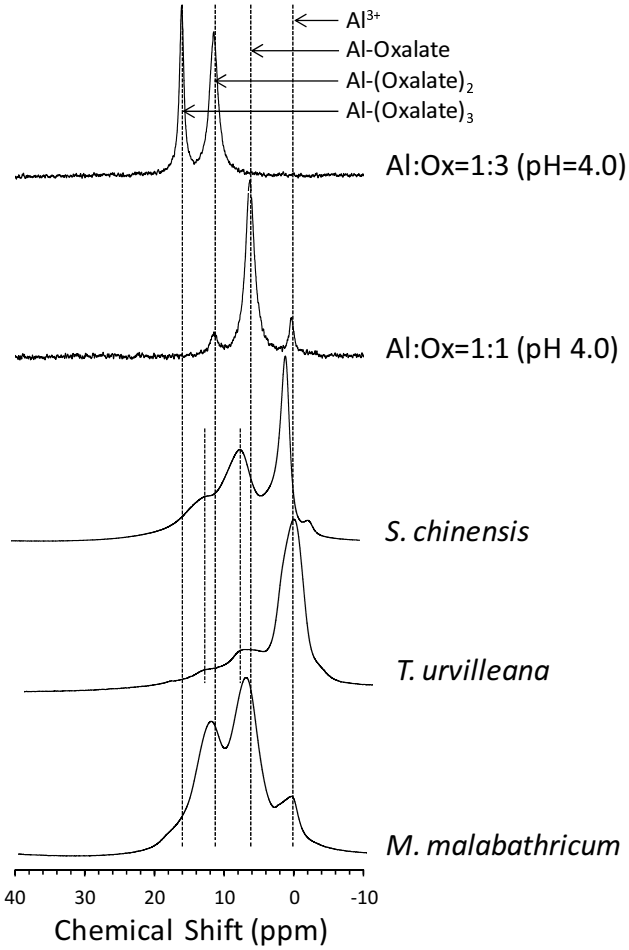
Al3+
Al-Oxalate
Al-(Oxalate)2
Al-(Oxalate)3
Al:Ox=1:3 (pH=4.0)
Al:Ox=1:1 (pH 4.0)
S. chinensis
T. urvilleana
M. malabathricum
40
30
20
10
0
-10
Chemical Shift (ppm)

Fig. 2