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Chemical forms of aluminum in xylem sap of tea plants (*Camellia sinensis* L.)

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Abstract

To identify the chemical forms of aluminum (Al) transported from roots to shoots of tea plants (*C. sinensis* L.), ^{27}Al-nuclear magnetic resonance and ^{19}F NMR spectroscopy were used to analyze xylem sap.

The concentration of Al in collected xylem sap was 0.29 mM, twice as high as that of F. Catechins were not detected in xylem sap. The concentration of malic acid in xylem sap was higher than that of citric acid, whereas the concentration of oxalic acid was negligible.

There were two signals in the ^{27}Al NMR spectra of xylem sap, a larger signal at 11 ppm and a smaller one at −1.5 ppm. The former signal was consistent with the peak for an Al–citrate model solution, suggesting that an Al–citrate complex was present in xylem sap. Although the latter signal at −1.5 ppm was thought to indicate the presence of an Al–F complex (at 1.7 ppm) in xylem sap, there was only one signal at −122 ppm in the ^{19}F NMR spectrum of xylem sap, indicating that the main F complex in xylem sap was F^-.

These results indicate that Al might be translocated as a complex with citrate, while Al–malate, Al–oxalate and Al–F complexes are not major Al complexes in xylem sap of tea plants.

Keywords: *Camellia sinensis*; Theaceae; Aluminum; Citric acid; Malic acid; ^{27}Al NMR; ^{19}F NMR

1. Introduction

Al is one of the most abundant elements in the earth's crust and comprises 7% of it. Under acidic conditions (pH < 5.0), Al is solubilized into a toxic trivalent cation, Al^{3+} (Foy et al., 1978; Taylor, 1991). In many agriculturally important plant species, the presence of only micromolar concentrations of Al^{3+} can result in the inhibition of root growth within minutes or hours (Kochian, 1995). Thus, Al toxicity has been recognized as a major factor that limits plant growth in acidic soil.

On the other hand, some plant species, known as "Al accumulators", accumulate Al at high concentrations in the aerial parts of the plants without showing symptoms of Al toxicity. The tea plant (*Camellia sinensis* L.) is a well-known Al-accumulating plant that grows well in strongly acidic soils that contain high levels of soluble Al. The tea plant takes up Al throughout its life (Chenery, 1955), and old leaves contain up to 30,000 mg kg^{-1} of Al on a dry weight basis (Matsumoto et al., 1976). These findings suggest that detoxification of Al occurs in tea plants. Elucidation of the mechanism of this

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detoxification may contribute to the development of procedures to enhance the tolerance toward Al in crop species that are sensitive to Al.

At present, however, little is known about Al detoxification mechanisms in tea plants. The identification of Al species present in tea plants is one possible approach to elucidate these mechanisms, but this approach is difficult due to the complexity of the aqueous coordination chemistry of Al, which results in changes in the form of Al during extraction and analytical procedures.

Recently, much research has been carried out to clarify the chemical forms of Al in Al-accumulating plants using nuclear magnetic resonance (NMR) analysis. Using ^{27}Al NMR, Nagata et al. (1991, 1992) found that Al mainly existed as Al–catechin complexes in tea leaves. In hydrangea (*Hydrangea macrophylla*) (Ma et al., 1997a), buckwheat (*Fagopyrum esculentum* Moench) (Ma et al., 1997b), and *Melastoma malabathricum* (Watanabe et al., 1998), Al has been found to accumulate in the form of Al–citrate (1:1), Al–oxalate (1:3) and Al–oxalate (1:1, 1:2, 1:3) complexes, respectively. Furthermore, in buckwheat (Ma and Hiradate, 2000) and *Melastoma* (Watanabe and Osaki, 2001), Al has been shown to be transported from the root to shoot as an Al–citrate (1:1) complex. However, in tea plants, the chemical forms in which Al is transported from the root to the shoot and subsequently to xylem sap have not yet been elucidated.

Previously, it had been suggested that Al in tea infusions might cause diseases in humans, such as Alzheimer's disease, in which high Al levels have been found in the brain (Perl and Brody, 1980; Crapper-Mclachlin et al., 1989), although at the present, it is concluded that Al is not the cause of this disease, but could well be an aggravating factor (Williams, 1999). Therefore, many studies have been also carried out to clarify the chemical species of Al in tea infusions, and several forms of Al, e.g., Al–oxalate (1:3), Al–oxalate–fluoride (F) complexes (Horie et al., 1992, 1994) and Al–polyphenol complexes (Qi et al., 1995), have been identified.

To verify the hypothesis that Al is translocated in xylem sap in the form of complexes with Al–chelating compounds, such as F, oxalate, citrate, malate and catechin, the xylem sap of tea plants was analyzed by ^{27}Al NMR and ^{19}F NMR in the present study.

2. Results and discussion

The volume of xylem sap obtained from one stem over 12 h was 4.2–32 mL. The average pH values and contents of Al, F, oxalate, malate, citrate and catechins in xylem sap are shown in Table 1. The average pH value of the sap was about 5.5. The average concentration of Al was 0.29 mM (ca. 7.9 mg L^{-1}), which is approximately twice as high as that of F. Among organic acids, the average concentration of malate (0.61 mM) was about sixfold higher than that of citrate, while the concentration of oxalate was negligible. Catechins (catechin, gallocatechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate), gallic acid and caffeine were not detected in xylem sap. The volumes of xylem sap collected in this study (4.2–32 mL) were less than those in evapo-transpiration (36–340 mL per plant) of tea plants during the collection of xylem sap according to Yanase et al. (1971), suggesting that the reduced pressure for the collection of xylem sap was moderate. Therefore, the sample collected in this study was considered to be xylem sap of the tea plant, although we cannot rule out the possibility that xylem sap was contaminated by an apoplasmic solution during prolonged reduced pressure.

Table 1
Values of pH and contents of Al, F and organic acids in the xylem sap of tea plant

Measurement	Value
Volume (mL)	24.3 ± 7.8
pH	5.46 ± 0.18
Al (mM)	0.292 ± 0.131
F (mM)	0.128 ± 0.052
Oxalate (mM)	0.015 ± 0.010
Malate (mM)	0.613 ± 0.297
Citrate (mM)	0.108 ± 0.040
Catechins[a] (mM)	
C	ND[b]
GC	ND
EC	ND
EGC	ND
EGCG	ND
ECG	ND
Gallic acid (mM)	ND
Caffein (mM)	ND

Values represent means ±SD (n = 10).
[a] C, catechin; GC, gallocatechin; EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate; ECG, epicatechin gallate.
[b] Not-detected (limits of detection are <1 mg L^{-1} for caffeine, EGCG and ECG and <2 mg L^{-1} for GA, EGC, Cathechin and EC).

^{27}Al NMR spectra of the xylem sap of tea plants and model Al solutions are shown in Fig. 1. In xylem sap, two signals, a larger signal at 10.1 ppm and a smaller signal at −1.5 ppm, were detected (Fig. 1(a)). Signals at 16–18 ppm due to Al–catechin complexes (Nagata et al., 1992) were not detected in the ^{27}Al NMR spectra of the xylem sap. This finding suggests that Al–catechin complexes were not present in the xylem sap of tea plants, and corresponds with the results of the direct analysis of catechins in xylem sap (Table 1).

A comparison with the ^{27}Al NMR spectra of model Al solutions showed that the large signal in xylem sap was identical to that of Al–citrate complex (Fig. 1(b), 10.1 ppm), indicating the presence of an Al–citrate com-

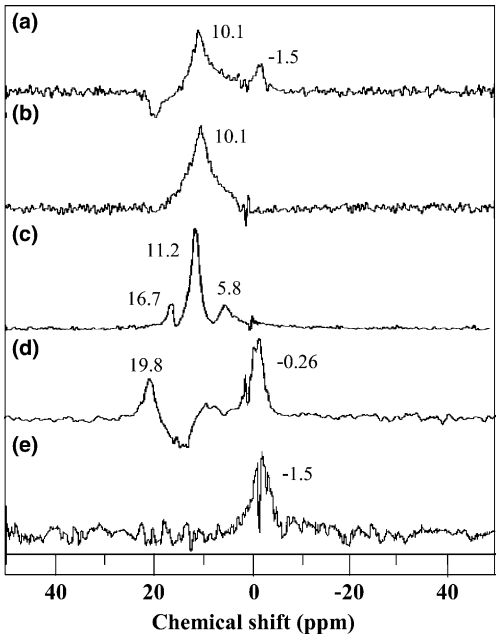

Fig. 1. Chemical shift of ^{27}Al NMR spectra of xylem sap (a) and Al model solutions (b) Al/citrate 2/2 mM, (c) Al/oxalate 2/2 mM, (d) Al/malate 2/2 mM, (e) Al/F 2/2 mM. Numerical values represent chemical shift (ppm) referenced to $AlCl_3$ (0 ppm).

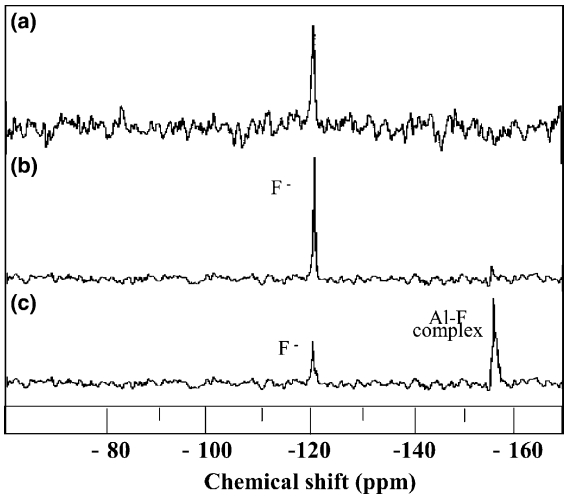

Fig. 2. Chemical shift of ^{19}F NMR spectra of xylem sap (a) of tea plant and Al–F model solution (b) NaF 2 mM, (c) Al/F=2/2 mM).

plex in xylem sap. A dominant peak of Al at a chemical shift of 10–12 ppm, which is in good accordance with the chemical shift of the Al–citrate complex, was also detected in intact leaves and cell sap of hydrangea (Ma et al., 1997a), in xylem sap of buckwheat (Ma, 2000), and in xylem sap of melastoma (Watanabe and Osaki, 2001).

It has been suggested that Al is transported from roots to shoots of tea plants in the form of an Al–oxalate complex, since the content of oxalate in tea leaves was higher than those of other organic acids (Morita and Tsuji, 2002). However, the amount of oxalate in the xylem sap was very low. The ^{27}Al NMR spectrum of a model Al–oxalate solution (1:1, pH 5.5) showed three signals, one at 5.8, one at 11.2 and the other at 16.7 ppm (Fig. 1(c)), which were assigned to Al–oxalate, Al–$(oxalate)_2$ and Al–$(oxalate)_3$, respectively, according to the report by Kerven et al. (1995). However, no signals were detected at 5.8 or 16.7 ppm in xylem sap. These findings show that the Al–oxalate complex is not a major Al species in xylem sap of tea plants.

On the other hand, the chemical shifts of Al–malate solution (−0.26 ppm) (Fig. 1(d)) and Al–F complex (1.5 ppm) (Fig. 1(e)) were similar to that of the small signal in the xylem sap (−1.5 ppm). The signal at −0.26 ppm for model Al malate solution might have resulted from an Al monomeric species (Bertsch et al., 1986). Moreover, the signal at 19.8 ppm for Al–malate solution (Fig. 1(d)) which corresponded to the Al–malate complex was not detected in xylem sap (Fig. 1(b)). Therefore, xylem sap and Al–F (1:1, pH 5.5) solution were analyzed by ^{19}F NMR spectroscopy to determine whether the xylem sap of tea plants contains an Al–F complex. Although a ^{19}F NMR spectrum of the model Al–F solution showed two signals, one at −122 ppm derived from F^- and one at −155 ppm derived from AlF^{2+}, AlF_2^+ and AlF_3 complexes, there was only one signal at −122 ppm in xylem sap (Fig. 2), suggesting that the main form of F in xylem sap of tea plants is F^- and that little, if any, Al–F complex exists in the xylem sap of tea plants. However, this speculation does not agree with the results obtained by ^{19}F NMR spectroscopic analysis, which showed that the migrating Al species in xylem sap of tea plants after the exposure of detached stems to Al–F solution (pH 5.0) were Al–F complexes (Nagata et al., 1993). The discrepancy between these results may have been caused by the difference between the pH values of the sample solutions, since the pH value of a solution containing Al strongly affects the form of Al. The signal at −2 ppm in the ^{27}Al NMR spectrum was also detected for Al–succinate and Al–gallic acid complexes (Nagata et al., 1992). However, neither succinatic acid nor gallic acid was detected in the xylem sap of tea plants (data not shown).

These results show that Al in the xylem sap of tea plants forms complexes with citrate and translocates from roots to shoots, which supports the speculation that tea plants internally detoxify Al by chelating Al with organic acids and/or polyphenols (Sivasubramaniam and Talibudeen, 1972). Furthermore, these findings also support the hypothesis proposed by Ma (2000, 2001) that organic acids play important roles in the mechanism of Al transport in Al-accumulating plants by forming nontoxic Al–organic acid complexes. Moreover, when the correlation between the contents of Al and Al–ligand compounds was examined, only the citrate contents tended to increase with an increase in

Table 2
Correlation analysis between the contents of Al and Al–ligand compounds (F, oxalate, malate and citrate) in xylem sap

Variable	F	Oxalate	Malate	Citrate
Al	−0.206	−0.434	0.211	0.609

the Al contents in xylem sap (Table 2, $P < 0.1$). However, this speculation must be evaluated based on integration of the resonance peaks or by an equilibrium calculation using GEOCHEM. Additionally, solutions consisting of Al and F, oxalate, malate, and citrate at the ratios in xylem sap should be examined using ^{27}Al NMR. Recently, chemical species of Al in plants and tea infusions were examined by fast protein liquid chromatography (FPLC) equipped with inductively coupled plasma–atomic emission spectrometry (ICP–AES) or electrothermal atomic absorption spectrometry (ETAAS) (Bantan et al., 1998, 1999, 2001) and by ion chromatography with an eluent containing pyrocatechol violet (Liang et al., 1999). The chemical species in tea plants should be examined not only by NMR spectroscopy, but also by these new techniques.

Yamada and Hattori (1977, 1980) suggested that tea plants take up complexes such as AlF^{2+} and AlF_2^+ from a soil solution. Based on the results of ^{27}Al NMR spectroscopy, Nagata et al. (1992) suggested that an Al–catechin complex is the major form of Al in old tea leaves. Matsumoto et al. (1976) observed the accumulation of Al in the epidermal layers of old tea leaves, suggesting that Al localization contributes to the mechanism of Al accumulation in tea plants. These findings suggest that tea plants have an internal tolerance, i.e., Al ions are detoxified in the symplasm by compartmentation, immobilization, and chelation with organic or inorganic compounds. Comprehensive studies are needed to elucidate the mechanisms by which tea plants tolerate and accumulate Al as well as to identify the chemical species of Al in tea plants.

3. Experimental

3.1. Collection of xylem sap

Xylem sap was collected from six-year-old plants (*C. sinensis* cv. Yabukita) in the tea field of Shizuoka University, Shizuoka, Japan, according to the methods described by Selvendran and Sabaratnam (1972) and Nakamura (1988). The stem (about 8 mm diameter) of each tea plant was cut with scissors. The cut surface was trimmed with a sterilized knife and then the cut end was connected to the apparatus shown in Fig. 3. This apparatus consisted of a test tube (50 mL) to collect the xylem sap, a trap (5 L) to maintain the pressure, a vacuum pump to reduce the pressure, and rubber or Teflon tubes to connect different parts. No sap could be collected without a reduction in pressure. The collection of sap was started by applying a vacuum with the pump at 8:00 p.m. After a vacuum was applied, tap B was closed and the unit was disconnected from the pump and left in the field until 8:00 a.m. the next morning. During the suction procedure, the test tube used to collected the sap was kept on ice. The volume and pH of the sap were measured immediately after suction was complete. The sap was then stored at −20 °C after filtration on a millipore filter (0.45 μm, Advantec Toyo, Tokyo).

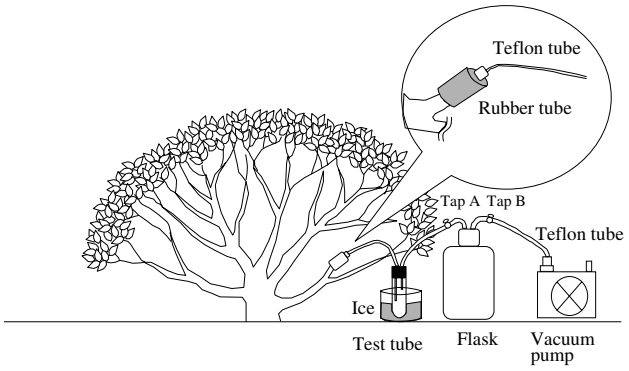

Fig. 3. Schematic drawing of the apparatus for the collection of xylem sap from tea plant.

3.2. Amounts of Al, F, organic acids and catechins in xylem sap

The Al content of xylem sap was measured using an atomic absorption spectrometer with a graphite furnace (Spectra AA with Zeeman correction, Varian, Australia). F and oxalate were analyzed using an ion chromatograph (IC 500S, Yokogawa Hokushin Dennki Co., Tokyo) equipped with a guard column (IonPac AG4A-SC (4 × 50 mm), Dionex, Sunnyvale, CA) and an analysis column (IonPac AS4-SC (4 × 250 mm) Dionex, Sunnyvale, CA) (Ma and Miyasaka, 1998). The contents of malic acid and citric acid were measured by HPLC (Ma et al., 1997a). The contents of catechins, gallic acid and caffeine were measured by HPLC using a standard solution containing catechin (C), gallocatechin (GC), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallic acid and caffeine (Goto et al., 1996).

3.3. Model Al solutions

As model Al solutions for ^{27}Al NMR and ^{19}F NMR measurements, Al–citrate (2/2 m M), Al–oxalate (2/2 mM), Al–malate (2/2 mM), Al–F (2/2 mM) and F (2 mM) solutions were prepared by mixing 10 mM $AlCl_3$ with 10 mM malic acid, 10 mM oxalic acid, 10 mM citric acid or 10 mM NaF in the prescribed ratios. The pH of each model solution was then adjusted to 5.5, corre-

sponding to the pH of xylem sap, with diluted HCl or NaOH.

Xylem sap of tea plants and model Al solutions were analyzed by ^{27}Al NMR and ^{19}F NMR spectroscopy according to the method described below, and the ^{27}Al NMR and ^{19}F NMR spectra obtained from the sap were compared with those of model Al solutions.

3.4. ^{27}Al NMR measurement

Following the method of Nagata et al. (1991), ^{27}Al NMR spectra were obtained at 130.4 MHz with a JML-LA500 spectrometer (JEOL, Tokyo) using a 5-mm ∅ auto-tuning probe. The parameters used were as follows: frequency range, 100 kHz; data point, 16 k; sampling point, 1224; 10.0 μs; 8000–1,330,000 scans; total time for acquisition, 2–12 h. The spectra are presented on a ppm chemical shift scale referenced to $AlCl_3$ (0 ppm).

3.5. ^{19}F NMR measurement

^{19}F NMR spectra were obtained at 470.5 MHz according to the method of Nagata et al. (1993). The following parameters were used: frequency range, 94 kHz; data point, 16 k; sampling point, 1.2 k; 0.90 μs; 7000–3572000 scans; total time for acquisition, 10 min to 24 h; delay time, 500 μs. The spectra are presented on a ppm chemical shift scale referenced to CF_3COOH (−76.5 ppm).

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