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# Mechanism for the detoxification of aluminum in roots of tea plant (*Camellia sinensis* (L.) Kuntze)

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**Abstract**

To determine the mechanism of aluminum (Al) detoxification in the roots of tea plants (*Camellia sinensis* (L.) Kuntze), the amounts of Al and Al-chelating compounds (fluoride (F), organic acids and catechins) were measured and the chemical forms of Al in root cell extracts were identified by the application of $^{27}$Al-nuclear magnetic resonance (NMR) spectroscopy. Tea plants were cultivated in nutrient solutions containing 0, 4, 1.0 and 4.0 mM of Al at pH 4.2 for approximately 10 weeks. The levels of soluble Al, water-soluble oxalate and citrate, but not F, malate or catechins in young roots increased with an increase in the concentration of Al in the treatment solution. The $^{27}$Al NMR spectra of root tips and cell sap extracted from root tips that had been treated with Al were almost identical and had four signals, with two (11 and 16 ppm) apparently corresponding to the known chemical shifts of Al–oxalate complexes. In the spectra of cell sap, the resonances at 11 and 16 ppm increased with an increase in the Al contents. These results suggest that the levels of Al–oxalate complexes increased in response to an increase in the Al level, implying that oxalate is a key Al-chelating compound in the mechanism of Al detoxification in the tea root.


*Keywords:* Tea plant *Camellia sinensis*; Theaceae; Roots; Aluminum; Oxalate; $^{27}$Al-nuclear magnetic resonance

## 1. Introduction

The tea plant (*Camellia sinensis* (L.) Kuntze) is well known to be an aluminum (Al)-accumulating plant that grows well in strongly acidic soils containing high levels of $Al^{3+}$, the toxicity of which has been recognized as a major factor that limits plant growth in acidic soil (Taylor, 1991). The tea plant takes up Al throughout its life span (Chenery, 1955), and mature leaves contain up to 30,000 mg $kg^{-1}$ of Al on a dry weight basis (Matsumoto et al., 1976) without experiencing Al toxicity. These findings suggest that Al is detoxified in tea plants.

Many studies on the mechanisms of Al toxicity and Al tolerance have been carried out using mainly Al-resistant cultivars of non-Al-tolerant plant species. Recent studies have found that Al is detoxified by the exudation of Al-ligating compounds from the root tips, which form Al–ligand complexes and suppress Al uptake; a so-called exclusion mechanism. Internal tolerance mechanisms are also important for Al detoxification after plant uptake. Recently, the chemical forms of Al in Al-tolerant plant species have been identified using nuclear magnetic resonance (NMR) spectroscopy. In hydrangea (*Hydrangea macrophylla*) (Ma et al., 1997a), buckwheat (*Fagopyrum esculentum* Moench) (Ma et al., 1997b) and *Melastoma malabathricum* (Watanabe et al., 1998), Al accumulates in the form of Al–citrate (1:1), Al–oxalate (1:3) and Al–oxalate (1:1, 1:2 and 1:3) complexes, respectively. Moreover, in buckwheat (Ma and Hiradate, 2000) and *M. malabathricum* (Watanabe and Osaki, 2001), it was shown that Al is transported from root to shoot as an Al–citrate (1:1) complex. Thus, low-molecular-weight organic acids play a central role in internal detoxification in plant tissue and root-to-shoot

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transport of Al through the formation of Al–organic acid complexes in Al-accumulating plant species.

The chemical forms of Al in tea plants have also been examined using $^{27}$Al NMR spectroscopy. Nagata et al. (1992) reported that Al is mainly present as Al–catechin complexes in tea leaves, and we previously reported that Al is present as Al–citrate (1:1) complex in the xylem sap of tea plant (Morita et al., 2004a). In this study, we examined the chemical forms of Al in tea roots.

## 2. Results

### 2.1. Growth and Al content

The growth of tea plants at harvest is shown in Fig. 1. The fresh weights of whole plants at 0.4 and 1.0 mM Al were higher than those at 0 and 0.1 mM Al, while those at 4.0 mM were lower (Fig. 1a). The analysis of separated tissues indicated that the growth of young leaves and young roots was greater at 0.4 and 1.0 mM Al than at 0 and 0.1 mM (Fig. 1b). At 4.0 mM Al, however, while the growth of young leaves and roots was still greater than at 0 mM and 0.1 mM Al, such growth was only approximately 30% and 60% of those at 1.0 mM Al, respectively.

The total Al contents in young leaves and roots increased with an increase in the supply of Al up to as high as around 1 and 8 mg g$^{-1}$ DW, respectively (Fig. 2). The water-soluble Al contents also increased with Al treatment but remained constant at 0.4, 1.0 and 4.0 mM Al. Consequently, the ratios of water-soluble Al to total Al in young roots gradually decreased from around 0.9 to 0.1 with an increase in the Al concentration in the culture solution. The amounts of both forms of Al in young roots were greater than those in young leaves. Although the contents of total Al in young leaves were lower than those in mature leaves, no difference were observed in the levels of water-soluble Al contents. Additionally, although the total Al levels in old roots were higher than those in young roots, the levels of water-soluble Al in old roots were smaller.

### 2.2. Amounts of Al-chelating compounds in young tea roots

The levels of F in young tea roots were not affected by the addition of Al (Fig. 3a), while the levels of malate were decreased at 4.0 mM Al (Fig. 3b). In contrast, the levels of citrate (Fig. 3c) and oxalate (Fig. 3d) increased with an increase in the Al concentration up to 0.4 mM Al and were retained at higher Al concentrations. The levels of citrate and oxalate were positively correlated with water-soluble Al contents (Fig. 4, $p < 0.05$), whereas those of F and malate were not. Catechins were not detected in tea roots.

### 2.3. Concentrations of Al and possible Al-chelating compounds in cell sap

Cell sap was extracted from 0 to 5 and 5 to 20 mm root tips treated at 0.4, 1.0 and 4.0 mM Al. Their pH values were between 5.0 and 5.3 and the Al concentrations in the cell sap from both root sections were similar but increased with an increase in the Al concentration in the culture solution (Table 1). The concentrations of F, malate and citrate in the cell sap from 0 to 5 mm root tips, but not from 5 to 20 mm root tips, increased with an increase in the Al concentration. The concentrations of oxalate in the cell sap from both root sections increased with an increase in the Al concentration, and were higher than those of the other organic acids. The molar ratio of oxalic acid to Al in the cell sap increased at 0.4, 1.0 and 4.0 mM Al: 0.63, 1.09 and 1.24 in 0–5 mm sections and 0.94, 1.24 and 1.47 in 5–20 mm sections, respectively. Catechins were not detected in the cell sap from either section.

### 2.4. $^{27}$Al NMR spectra of young roots

The $^{27}$Al NMR spectra of intact root tips (0–20 mm) and cell sap extracted from 0 to 5 and 5 to 20 mm root tips treated with 0.4 mM Al for approximately 10 weeks are shown in Fig. 5. In intact roots, three clear signals were observed at 3.1, 11.3 and 16.4 ppm (Fig. 5a). The $^{27}$Al NMR spectra of the cell sap extracted from both 0 to 5

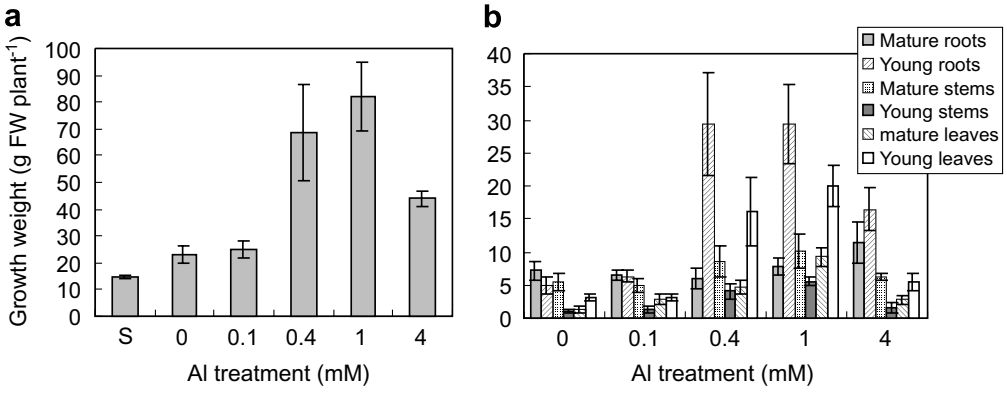


Fig. 1. Effect of Al treatment on the growth of tea plants: (a) total fresh weights of tea plants at the end of Al treatment, (b) fresh weights of each part of tea plants at the end of Al treatment. S: at the start of Al treatment. Values are the average ± SD ($n = 4$).

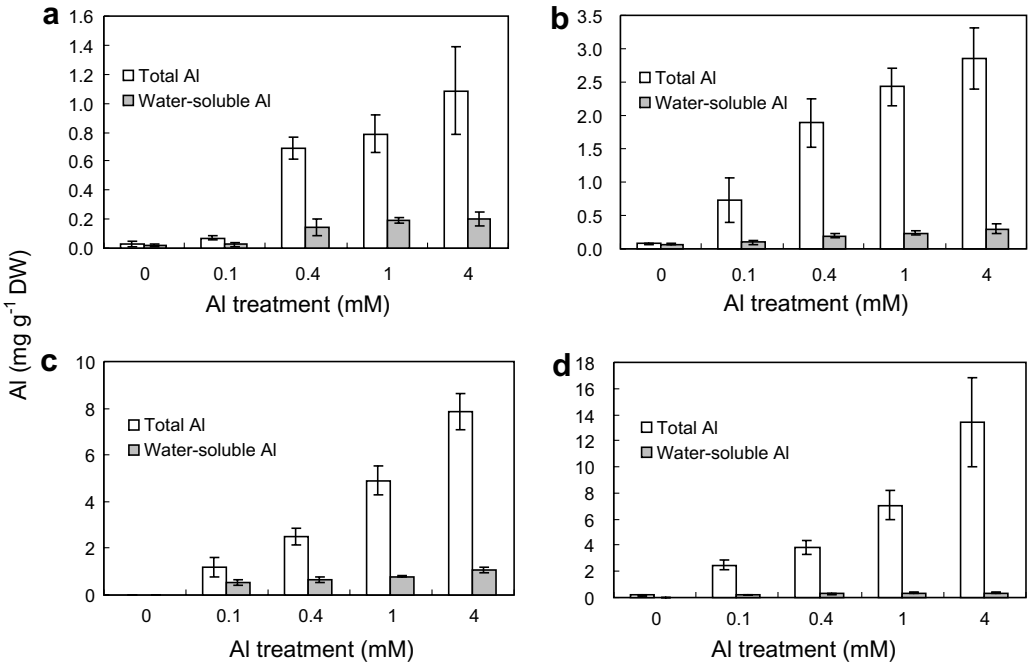


Fig. 2. Effects of Al treatment on the total and water-soluble Al contents in leaves and roots of tea plants: (a) young leaves, (b) mature leaves, (c) young roots and (d) mature roots. Values are the average ± SD ($n = 4$).

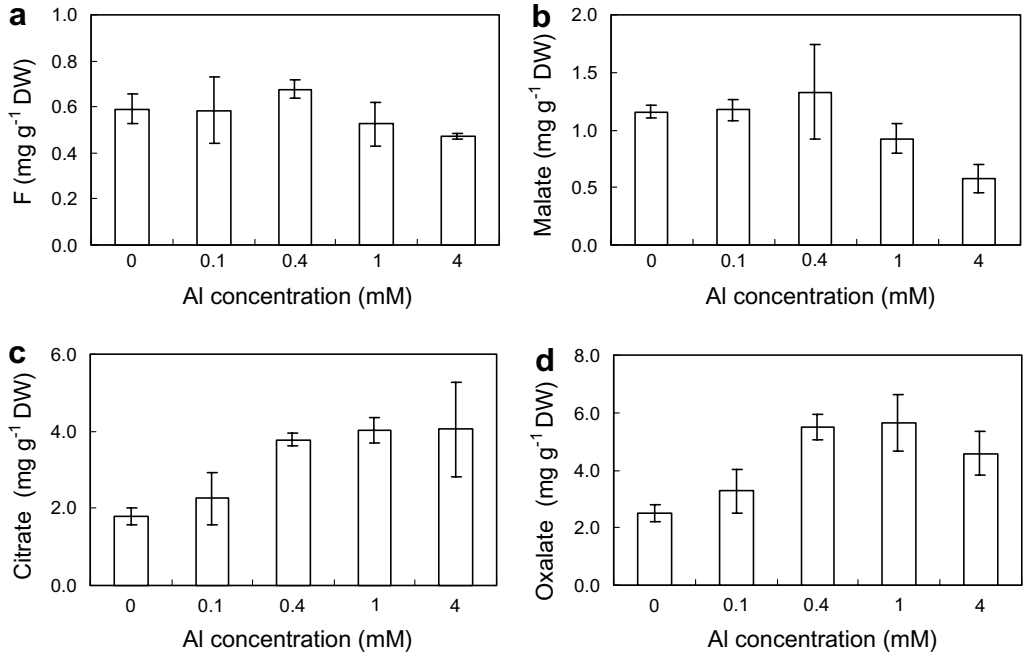


Fig. 3. Effects of Al treatment on F (a), malate (b), citrate (c) and oxalate (d) contents in young roots of tea plants. Values are the average ± SD ($n = 4$).

and 5 to 20 mm root tips treated with 0.4 mM Al (Fig. 5b and c) showed four signals at chemical shifts of −1.1, 3.1, 10.9 and 16.1 ppm. The relative heights of the resonances at three peaks were comparable. These results indicated that the chemical forms of Al in intact root tips, which showed peaks at 3.1, 11.3, and 16.4 ppm in $^{27}$Al NMR spectra, did not change during the extraction of cell sap. This estimation was confirmed by comparing the $^{27}$Al NMR spectra of intact root tips and cell sap extracted from root tips that had been treated with 1.0 mM (data not shown).

The $^{27}$Al NMR spectra of the cell sap and the 1:1 Al–oxalate solution are shown in Fig. 6. The $^{27}$Al NMR spectra in the cell sap extracted from 0 to 5 and 5 to 20 mm root sections treated with 1.0 mM Al showed four signals at around −1, 3, 11 and 16 ppm (Fig. 6c and d). The peak

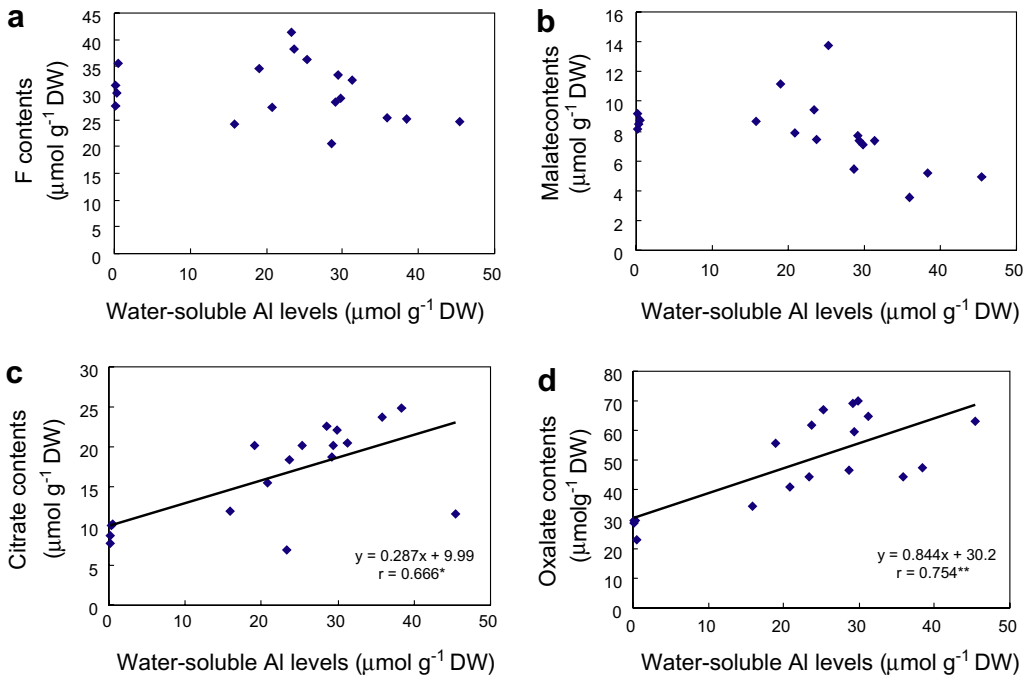


Fig. 4. Correlations between the levels of water-soluble Al and F (a), malate (b), citrate (c) and oxalate (d) in young roots of tea plants. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 1
Effects of Al treatment on the pH and concentrations of Al, F, organic acids and catechins in cell sap from 0–5 and 5–20 mm root sections

| Root section Al treatment | 0–5 mm | | | 5–20 mm | | |
|---|---|---|---|---|---|---|
| | 0.4 mM | 1.0 mM | 4.0 mM | 0.4 mM | 1.0 mM | 4.0 mM |
| pH | 5.0 | 5.2 | 5.1 | 5.1 | 5.3 | 5.0 |
| Al (mM) | 1.42 | 1.96 | 2.17 | 1.64 | 1.93 | 2.19 |
| F (mM) | 1.48 | 4.09 | 3.65 | 2.10 | 1.25 | 2.49 |
| Malate (mM) | 0.270 | 0.305 | 0.234 | 0.441 | 0.185 | 0.148 |
| Citrate (mM) | 0.68 | 1.72 | 1.84 | 1.40 | 0.59 | 1.96 |
| Oxalate (mM) | 0.89 | 2.13 | 2.68 | 1.54 | 2.60 | 3.21 |
| Catechins (mM)[a] | ND | ND | ND | ND | ND | ND |

ND: not detected (<0.001 mM).
[a] catechin, epicatechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate.

in cell sap with 4.0 mM Al appeared at around −1, 11 and 16 ppm (Fig. 6e and f). The signal at 16 ppm in the 5–20mm cell sap was higher than that in the 0–5 mm cell sap. In 1:1 Al–oxalate solution, three signals (6.9, 11.6 and 16.1 ppm) were detected (Fig. 6g), and these were assigned to 1:1, 1:2 and 1:3 Al–oxalate complexes, respectively.

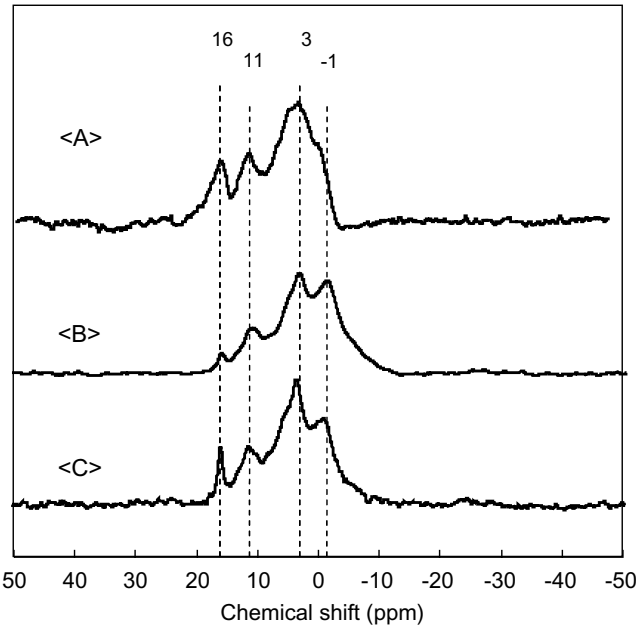


Fig. 5. $^{27}$Al NMR spectra of intact root tips (0–20 mm) (a) and cell saps extracted from two root tips (0–5 mm (b) and 5–20 mm (c)) The plants were treated with 0.4 mM Al. Chemical shifts were calibrated using AlCl3 (0.2 mM AlCl3 in 0.1 N HCl) as a reference (0 ppm).

## 3. Discussion

Generally, $Al^{3+}$ inhibits plant root elongation under acidic conditions (Matsumoto, 2001). However, in this study, the growth of tea roots was enhanced rather than suppressed with the addition of Al to the culture solution (Fig. 1). This confirms a previous report (Konishi, 1992) that Al was beneficial for tea plant growth. The contents of total and water-soluble Al in young roots increased dose-dependently up to 8 and 1 mg $g^{-1}$ DW at 4 mM Al, respectively (Fig. 2c). This showed that tea roots possess a huge capacity for Al accumulation in the form of water-soluble and insoluble Al complexes. Matsumoto

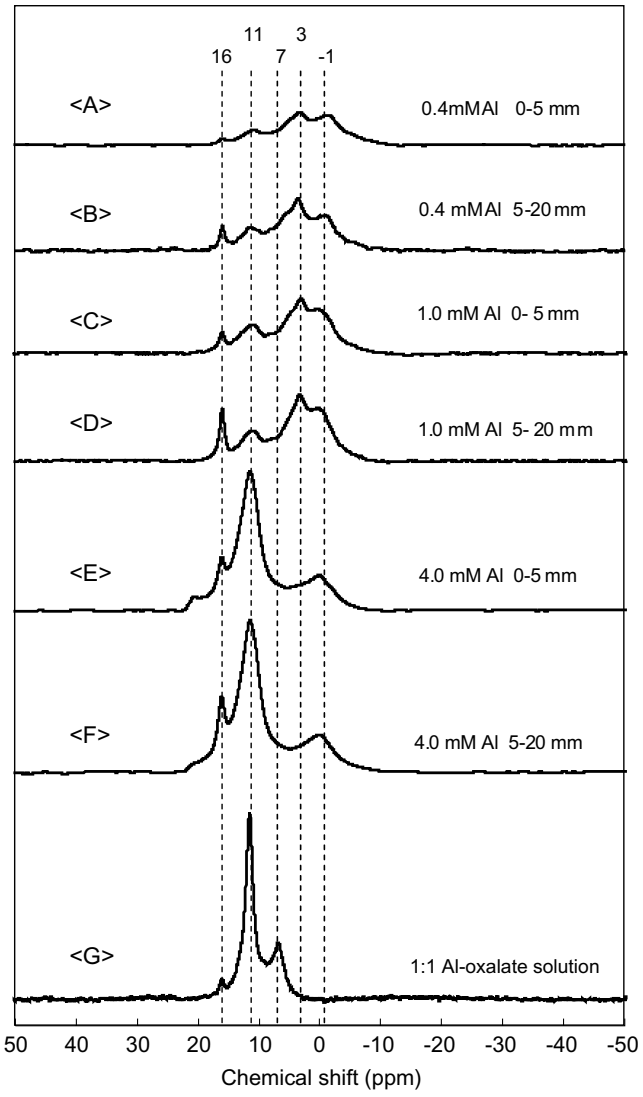


Fig. 6. $^{27}$Al NMR spectra of cell sap extracted from root tips (0–5 mm and 5–20 mm) treated with 0.4, 1.0 and 4.0 mM Al and of 1:1 Al–oxalate solution. Chemical shifts were calibrated using AlCl3 (0.2 mM AlCl3 in 0.1 N HCl) as a reference (0 ppm).

et al. (1976) proposed, based on electron microprobe X-ray analysis, that Al in tea leaves was accumulated in the epidermal cell walls. In tea roots, Al would also be precipitated on the surfaces of epidermal cells, which would not experience Al toxicity. Moreover, the contents of both total and water-soluble Al were much higher in tea roots than in leaves (Fig. 2). Therefore, in tea plants, the roots might play important roles in the mechanisms of Al tolerance, i.e., detoxification of Al through the formation of non-toxic soluble and insoluble complexes.

The major peaks of Al-complexes did not change during the extraction of cell sap from root sections (Fig. 5). In all of the Al treatments, the $^{27}$Al NMR spectra of cell sap showed three signals at around −1, 11, and 16 ppm (Fig. 6). The resonances at 11 and 16 ppm in cell sap were identified as those of 1:2 Al–oxalate and 1:3 Al–oxalate complexes, respectively. Although Al–citrate solution showed a peak at around 10.1 ppm (Morita et al., 2004a), the present sharp peak at 11 ppm in root cell sap could not be Al–citrate. This supposition was evident from the finding that, in cell sap of 0–5 and 5–20 mm sections, the NMR signal peaks at 11 ppm increased with the concentration of soluble A (Fig. 6), in addition to an increase in oxalate, but not citrate (Table 1). According to a report by Kerven et al. (1995), small but broad signals in Al–oxalate and Al–citrate solutions were detected at around 0 ppm, which were identified as Al monomeric species. Therefore, the resonances at around −1 ppm suggest the presence of Al monomeric species that are affected by anions coexisting in the solution. It has also been reported that the substitution of one aquo/hydroxo ion of octahedral Al with one carboxyl group leads to an increase in the $^{27}$Al–NMR chemical shift value (ca. 3 ppm) (Hiradate, 2004). Thus, the signal at around 3 ppm suggested the presence of Al binding to a carboxyl group of an organic acid, such as malic acid. In the $^{27}$NMR-spectra of Al–malate solution, a broad peak was detected at 3.3 ppm (data not shown). The signals at around 3 ppm were predominant at 0.4 and 1.0 mM, but were greatly decreased at 4.0 mM Al (Fig. 6). On the other hand, the resonances at 11 and 16 ppm increased with an increase in the concentration of Al in the treatment solution. These findings suggest that, in response to the concentration of Al in root cells sap (Table 1), the chemical forms of Al might change from an Al–one carbonyl group complex to 1:2 and 1:3 Al–oxalate complexes. The stability constants ($K$) of 1:2 and 1:3 Al–oxalate complexes have been reported to be 11.9 and 15.12, respectively (Vance et al., 1996), which are higher than the values for 1:1 Al–citrate complex ($K = 8.32$) and Al–ATP complex ($K = 10.9$) (Martin, 1988). The formation of highly stable Al–oxalate complexes in both the vacuole and cytosol might result in an increase in the Al–accumulating capacity of tea roots under excess Al conditions. This speculation is supported by a report that almost all of the oxalate in tea roots was present as a water-soluble form, rather than as a crystal form (Morita et al., 2004b). Additionally, it has been reported that oxalate is secreted from tea roots in response to the addition of Al to the nutrient solution (Morita et al., 2001). Consequently, oxalate plays a central role in the detoxification of Al in tea plants through the formation of Al–oxalate complexes in root cells and/or at the root–soil interface.

It has been reported that Al–citrate complex (1:1) is the chemical form of Al in xylem sap of tea plants (Morita et al., 2004a). Nagata et al. (1992) reported the presence of Al–catechin complexes in tea leaves using $^{27}$Al NMR spectroscopy. These results suggest that the chemical forms of Al in tea plants change during uptake into root cells, translocation in xylem and accumulation in leaf cells. $Al^{3+}$ in plants may be detoxified by chelating with oxalate in tea root cells upon uptake. Next, when Al is loaded to the xylem, the ligand is changed to citrate to form Al–citrate complexes. As Al–citrate complex is unloaded from the xylem to leaf cells, another ligand exchange occurs to

form Al–catechin complexes, which might then be stored in the vacuole of the leaves. In roots, xylem and leaves of buckwheat (Ma et al., 2001), Al was present as Al–oxalate, Al–citrate and Al–oxalate complexes, respectively. In *Melastoma malabathricum* (Watanabe et al., 1998; Watanabe and Osaki, 2001), Al–citrate and Al–oxalate were detected in xylem and leaves, respectively. Thus, a complex mechanism may be present for detoxifying and accumulating Al in Al-accumulating plant species, although the reasons for these variations in the forms of Al are still not clear.

## 4. Experimental

### 4.1. Hydroponic culture and sampling

One-year-old rooted tea cuttings (var. Yabukita) were transplanted into 10-L plastic pots with continuously aerated nutrient solution in a greenhouse. The nutrient solution was prepared according to Konishi et al. (1985), except that the P concentration was 0.1 mM. After 6 weeks, the plants were transplanted to the nutrient solutions containing 0, 0.4, 1.0 or 4.0 mM of Al. At the start of Al treatment, the fresh weight of the tea plants was $14.6 \pm 0.7$ g. Each treatment was carried out with four replicates. The Al solution was prepared from Al2(SO4)3 · 14–18H2O (Wako, Tokyo, Japan). The nutrient solutions were adjusted to pH 4.2 with 1 M H2SO4 and renewed every week. Al treatments were applied for approximately 10 weeks from April to July in 2004.

At harvest, roots of tea plants were immersed in dilute H2SO4 solution at pH 3.0 for 3 min to remove Al adsorbed on the root surface. The plants were separated into leaves, stems and roots, and each part was re-separated into young and mature parts: young parts were those that had emerged during Al treatment and mature parts were those that were present at the initiation of Al treatment. Each sample was washed with distilled water and weighed. The leaves and roots were freeze-dried, ground into a fine powder, and stored at −20 °C until analysis.

### 4.2. Extraction of cell sap from root tips

Before freeze-drying, some of the young roots that had been treated with Al were excised from root tips at 0–5 and 5–20 mm with a razor on ice (Ma et al., 1998). The cut root tips were centrifuged at 600 g for 15 min at 4 °C to remove free space solution. The remaining root tips were frozen in liquid N2, thawed, and then centrifuged at 10,000*g* for 20 min at 4 °C to obtain cell sap. The pH of the cell sap was measured using a pH meter (F-23, Horiba, Kyoto, Japan), and the concentrations of Al, fluoride (F), organic acids and catechins were determined immediately after cell sap extraction as described below. An aliquot of root tip (0–20 mm) from 0.4 and 1.0 mM Al treatment was immediately subjected to $^{27}$Al NMR analysis.

### 4.3. Analysis of Al, F, organic acids and catechins

Fine powders (50 mg) of freeze-dried samples were digested with HClO4:HNO3 (2 mL:2 mL) and diluted 5- to 10-fold with distilled $H_2O$ for the measurement of total Al. The powdered samples (50 mg) were agitated in deionized $H_2O$ (10 mL) for 1 h after the addition of 100 mg polyvinylpolypyrrolidone (PVPP). The supernatant obtained by centrifugation (3000*g*, 10 min) was passed through a fine filter (0.45 ‘‘m, Toyo Roshi, Tokyo, Japan) and the filtrate was used for the analysis of water-soluble Al, F, malate, citrate and oxalate. Catechins were also extracted from powder samples according to the above procedure without the addition of PVPP.

The amount of Al was determined by graphite furnace atomic absorption spectrometry (Spectra AA Zeeman 220, Varian, Palo Alto, CA, USA). F, malic acid, citric acid and oxalic acid were analyzed by ion chromatography (ICS 2000, Dionex, Sunnyvale, CA, USA) equipped with a guard column (Ion Pac AG17, Dionex) and an analytical column (Ion Pac AS17, Dionex). Catechins were determined by HPLC (SCL-10Avp, Shimadzu, Kyoto, Japan) according to the method of Goto et al. (1996), using a standard containing (+)-catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and epigallocatechin (EGC).

### 4.4. $^{27}$Al NMR measurement

$^{27}$Al NMR spectra were obtained at 156.3 MHz (Alpha FT NMR 600, JEOL, Tokyo, Japan). The root tips and cell sap were placed in NMR tubes (5mm in diameter). The parameters used were: frequency range, 62.5 kHz; data point, 33 K; acquisition time, 0.52; and sampling point, 32768. AlCl3 (0.2 mM AlCl3 in 0.1 M HCl) was used as an external reference to calibrate the chemical shift (0 ppm). The $^{27}$Al NMR spectra of Al–oxalate, Al–citrate and Al–malate solutions, prepared by mixing equimolar AlCl3 and oxalate, citrate or malate (10 mM), were also examined as standards. The pH of the solution was adjusted with 0.2N HCl to 5.0, which corresponded to the pH of cell sap.

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