Identification of the tri-Al tricitrate complex in *Plantago almogravensis* by hydrophilic interaction LC with parallel ICP-MS and electrospray Orbitrap MS/MS detection†

Tomás Grevenstuk, a Paulina Flis, b Laurent Ouerdane, b Ryszard Lobinski bc and Anabela Romano* a

The identification of the ligands binding Al is essential to understand the mechanisms by which plants detoxify Al internally. However, studies concerning the speciation of Al have been frustrated by its complex chemistry. This work describes the identification of the tri-Al tricitrate (Al₃ cit₃) complex in *Plantago almogravensis*, encompassing an integrated mass spectrometry approach based on hydrophilic interaction liquid chromatography (HILIC) and parallel detection by ICP-MS and ESI-MS/MS. This work also reports that both Al and Fe are bound by tricitrate, sometimes simultaneously, and the consequences of this finding are discussed. Of the complexes separated by size exclusion chromatography, Al₃ cit₃ is the most stable occurring in *P. almogravensis* as it was the only one recovered after HILIC. This approach provided new information on the mechanism of Al detoxification in *P. almogravensis*, namely that Al is bound by the organic acid citrate and that the relative concentration of the detected complexes is affected by the organ type and internal Al concentration, and has potential for studying the speciation of Al in less tolerant plants.

Introduction

Aluminium (Al) toxicity is one of the most serious limitations to plant growth in acidic soils. Most Al exists as oxides and aluminosilicates, which are harmless to plants; however, in acidic soils, Al is solubilized as the trivalent cation Al³⁺. The precise targets at molecular and cellular levels remain elusive but it is known that free Al rapidly inhibits root growth and limits the subsequent uptake of water and nutrients.¹ The relevance of Al toxicity is aggravated by the fact that Al is the third most abundant element in the Earth’s crust and that approximately 30% of the world’s total ice-free land has a pH < 5.5.² However, some plants are capable of performing despite taking up Al from soils. This occurrence is intriguing because even knowing that only a small percentage of Al is in its free state at physiological pH, such low concentrations can still be phytotoxic because of the strong affinity of Al for oxygen donor ligands.³ *Plantago almogravensis* Franco thrives in soils with high free Al concentrations and its geographical distribution is restricted to a small area (10 ha) located at the SW of the Iberian Peninsula⁴ and has thus been included in the UICN-Red List of critically endangered species.⁵ *P. almogravensis* is known to accumulate Al,⁶ however, the complexes that bind Al internally and thus protect vital biochemical processes are unknown.

Even if, to date, ²⁷Al nuclear magnetic resonance (NMR) spectroscopy has been used almost exclusively to study the speciation of Al in plants, as it can provide structural information about Al-complexes directly from intact samples, the inherent low sensitivity and low discriminating capacity of NMR spectroscopy for Al is a drawback that has prevented accurate speciation.⁷ Mass spectrometry using electrospray ionization (ESI-MS) is a technique that offers increased sensitivity and great potential for identification of metal complexes but has found little application in the field of Al-speciation due to the lack of a suitable isotope. Because of the gentle transition from solution to the gas phase and the low collision energies, the dissociation of metal complexes is less likely and singly-charged
species with high mass resolution are generally produced, yielding simple mass spectra that facilitate assignment. The lack of an isotope to aid with localization of Al-bearing complexes in mass spectra can be circumvented by parallel detection with an elemental detection technique, such as inductively coupled plasma (ICP)-MS, which allows determining the retention times of Al-complexes and pinpointing the region of the MS chromatogram that should be examined. Biological samples are too complex for direct infusion or flow injection, so chromatographic separation prior to ESI-MS measurements is required. Chromatography can pose a hurdle to the identifi-
cation of Al-complexes because they can be dissociated as a result of the interaction with the stationary phase and the buffer content of the mobile phase can suppress ionization efficiency. These interactions are minimized when using size-exclusion chromatography (SEC) columns and hydrophilic interaction chromatography (HILIC) columns (that allow proper retention of highly polar complexes) and their corresponding buffers are compatible with ESI-MS.

The goal of this work is to study the speciation of Al in P. almogravensis plants to determine the ligands responsible for detoxifying Al using an MS-based approach. A previously established experimental design using in vitro produced plants was employed to eliminate extraneous factors and determine the intrinsic detoxification capacity of P. almogravensis without compromising natural populations.

**Experimental**

**Reagents and standards**

Analytical reagent grade chemicals such as acetonitrile, formic acid, acetic acid, nitric acid, ammonium acetate and ammoniac were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), metal standards from SCP Science (Canada) and citric acid from Acros Organics (Belgium). Ultrapure water (18 MΩ cm⁻¹) was obtained from a Milli-Q system (Millipore, Bedford, MA).

**Plant material and Al-treatment**

Shoots of a selected P. almogravensis genotype were separated from in vitro axenic cultures proliferating for 6 weeks in Murashige and Skoog’s medium supplemented with 6-benzyladenine at 0.2 mg L⁻¹. The obtained shoots were transferred to Murashige and Skoog’s medium with macronutrients reduced to half containing 0.5 mg L⁻¹ indole-3-acetic acid to induce root development. The produced plantlets were transferred aseptically to autoclaved Murashige and Skoog’s liquid medium with macronutrients reduced to one quarter at pH 4.0 supplemented with 100, 200 or 400 µM Al for a 7-day treatment according to Martins et al. The chemical speciation program Geochem-EZ (http://www.plantmineralnutrition.net/Geochem/geochem%20home.htm) was used to predict the Al³⁺ and Fe³⁺ activity values and Al and Fe speciation for each nutrient solution based on chemical equilibrium constants. In order to study the role of roots in the formation of Al-complexes, shoots without developed root system were also subjected to Al-treatment. The cultures were maintained at 25 ± 2 °C with a 16 h photoperiod (cool white fluorescent lamps, 69 µmol m⁻² s⁻¹). Samples of fresh leaves, roots and shoots (~ 20 mg) were placed in eppendorfs, frozen in liquid nitrogen and ground with a glass rod. Extraction was carried out by adding 100 µL of water and mechanically stirring for 2 min at room temperature followed by centrifugation (3 min, 14,500 rpm). The supernatants were collected and immediately analysed. Apart from biological samples, standard solutions of Al–citric and –oxalic acid (3 : 1 ratio of organic acid to Al, buffered at pH 6) at an Al concentration approximate to that of roots were prepared for speciation analysis.

**SEC-ICP-MS experiments**

The SEC separations were conducted with a Dionex UltiMate 3000 LC-system (Thermo Fisher Scientific, Bremen, Germany) coupled to a 7500cs ICP-MS instrument (Agilent) equipped with a collision cell with hydrogen as collision gas. The interface between the LC system and the ICP-MS detector consisted of a quartz double pass Scott style spray chamber, a MicroMist EzyFit nebulizer (Glass Expansion, Australia), a 2.5 mm i.d. quartz torch and a set of nickel cones (Agilent Technologies, USA). The undiluted plant extracts and standard solutions (10 µL) were injected onto a Superdex Peptide HR 10/30 column using 5 mM ammonium acetate buffer at pH 6.2 as a mobile phase at a flow rate of 0.7 mL min⁻¹. The ICP-MS conditions were optimized daily for highest intensities and lowest interferences using a standard built-in software procedure. The H₂ collision cell mode was used to exclude polyatomic interferences that may occur for Fe and Al isotopes.

**HILIC-ICP-MS/ESI-MS/MS experiments**

The HILIC separations were performed using an Agilent 1100 capillary pump (Agilent, Tokyo, Japan) as a delivery system equipped with a 100 µL min⁻¹ splitter module. The HILIC column was a TSK gel amide 80 (250 × 1 mm i.d.) (Tosoh Bioscience, Germany). The binary mobile phase consisted of (A) 10 mmol L⁻¹ ammonium formate buffer at pH 5.5 and (B) acetonitrile. The optimized HILIC gradient started with 10% of A and was increased to 50% of A in 45 min. Samples were diluted with acetonitrile to obtain a 1 to 2 ratio (sample : aceto-
nitrile), centrifuged and 7 µL of the supernatant was analyzed at the flow rate of 50 µL min⁻¹. The HPLC unit was coupled either to an ICP-MS or an ESI-MS/MS instrument. The interface between the LC module and the ICP-MS detector consisted of a glass Cinnabar cyclonic spray chamber (Glass Expansion, Australia), a 50 µL min⁻¹ Micromist U-series nebulizer (Glass Expansion, Australia), a 1 mm i.d. injector torch (Agilent Technologies, Japan), a T-connector allowing the introduction of 11% O₂ and a set of platinum cones (Agilent Technologies, USA). The ICP MS conditions were optimized in the same way as for SEC-ICP-MS measurements. The ESI-MS/MS instrument was an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source (H-ESI II) (Thermo Fisher Scientific).
The instrument was operated in the positive ion mode with the electrospray voltage set at 3 kV. The source and capillary temperature were 120 °C and 280 °C, respectively. The ESI-MS spectra were obtained in the 150–1200 m/z range with the resolution set at 100 000. The ions were fragmented by collision-induced dissociation (CID) at the energy level of 45%.

**Total metal concentration determination**

Triplicates of dried leaf and shoot samples were ground with a pestle and mortar, digested in 70% nitric acid for 6 h at 65 °C and then diluted before analysis 10 times with 2% HNO₃. The acidified sample and standard solution of metallic elements were analysed by ICP-MS (Agilent 7500ce, Tokyo, Japan).

**Results**

Analysis of *P. almogravensis* samples exposed to Al by SEC-ICP-MS allowed for the separation of five Al species numbered according to elution time (Fig. 1). The same complexes were observed in root and leaf samples of plants grown under controlled conditions exposed to 400 μM Al, albeit at lower concentrations in leaves (Fig. 1A). However, when plantlets with excised root systems (shoots) were subjected to the same concentration of Al, the complexes 1 to 4 could not be observed. Instead, a poorly resolved broad signal was detected at longer elution times. Complexes 1 to 4 were detected in plantlets exposed to the lowest concentration of Al (100 μM) as well, however variations in their relative concentrations were observed between plants exposed to 100 μM and 400 μM of Al (Fig. 1A and B). Monitoring of other elements during SEC-ICP-MS measurements allowed determining that Fe co-elutes with the detected Al complexes 1 to 4 and that phosphor co-elutes with the complex detected in shoots (Fig. 1C and D; respectively). Oxalate is a well-described ligand for Al in accumulating plants, therefore an Al-oxalic acid standard solution was analyzed by SEC-ICP-MS. However, the elution time of the detected complex did not match any of the previously detected complexes, indicating that oxalate is not a major ligand for Al in *P. almogravensis* (Fig. S1A in the ESI†).

High-resolution MS experiments were employed in an attempt to identify the Al-complexes occurring in *P. almogravensis*. Because the chromatographic separation provided by SEC is not sufficient to obtain precise MS measurements, the separation was conducted by HILIC. The Al species identified so far in accumulating plants are complexes with organic acids. HILIC is an adequate method for the separation of these complexes considering that the resins guarantee a strong retention of highly polar complexes and the corresponding buffers are compatible with ESI-MS. However, our results show that although a better resolved signal was obtained, as opposed to SEC, only one sharp Al peak could be detected after HILIC separation (Fig. 2). This result can be explained by the fact that the other minor complexes are eluting in less defined peaks because of a probably lower stability of these forms and higher interaction with chromatographic material.

The obtained HILIC-ICP-MS chromatogram indicates the approximate retention time of the detected Al-complex, however, because Al is a mono-isotopic element it is not possible to examine the mass spectra obtained by HILIC-ESI-MS for molecular ions containing metals with specific isotopic signatures, as is the case for nickel and cadmium complexes for instance. A multitude of molecular ions is typically observed for...
each specific retention time and it is therefore unfeasible to identify de novo an Al-bearing complex without additional information. This is one of the limitations when pursuing the identification of metal complexes of mono-isotopic elements, especially in samples for which no prior information is available. In our case, because the Al-complex detected after HILIC also co-elutes with Fe (Fig. 2), the MS spectra were examined for molecular ions bearing the characteristic isotopic pattern of Fe at the retention time of the detected Al-complexes (Fig. 3). Two molecular ions with exact masses of $m/z$ 677.92 and 695.93 were identified following this approach. Two other ions were observed at the same retention time with an exact mass difference of Δ 28.95 $m/z$, which corresponds to the difference in atomic weight between $^{56}$Fe and $^{27}$Al. This result indicates that the $m/z$ 648.96 and 666.97 molecular ions bear at least one Al atom and that it is interchangeable with Fe. Exact mass determination indicated that the $m/z$ 648.96 and 666.97 molecular ions are consistent with a tri-Al tricitrate ($A_3c_3$) and an $A_3c_3 + H_2O$ complex, respectively. The assignment of these complexes was confirmed by analyzing a standard solution of Al and citric acid under the same conditions (Fig. S2 in the ESI†). This allowed for the assignment of the molecular ions at $m/z$ 677.92 and 695.93 as $A_2Fe_c3$ and $A_2Fe_c3 + H_2O$ (Fig. 3). Interestingly, the molecular ions corresponding to the $A_2Fe_c3$ and $Fe_3c_3$ complexes could also be detected at lower intensities (Fig. S3 in the ESI†). An extracted ion chromatogram indicated that the identified tricitrate complexes have the same retention time as the Al-complex detected by HILIC-ICP-MS (inset Fig. 2). To confirm the role of citric acid in Al complexation, a citric acid–Al standard solution was analyzed by SEC-ICP-MS. The resulting chromatogram shows a very good match between the complexes 2, 3 and 4 from root extract and the Al–citric acid standard solution (Fig. 1A and Fig. S1B in the ESI†) indicating that they are different forms of Al–citrate complexes, with $A_3c_3$ (peak 2) corresponding to the major peak as this complex was identified as the main Al complex by HILIC ESI MS. Regarding the remaining Al signals
detected in Fig. 1, peak 1 corresponds to the void due the Al entrapped with high molecular weight biomolecules and nanoparticles while peak 5 (detected exclusively in shoots) is likely to correspond to Al phosphate (matching peak shapes and migration times between Fig. 1A and D).

The total concentration of Al and Fe was determined in leaf samples and plotted together with the calculated Al and Fe activity in the culture media (Fig. 4). According to Parker et al.,15 the activity of a free uncomplexed metal is in most cases the most relevant parameter to determine plant responses to changes in nutrient solution composition. The low pH and reduced macronutrients concentration in culture media allowed obtaining a significant amount of free Al in solution (34.6%, Table 1) because less Al is bound to SO4 and EDTA. Our results also demonstrate that the concentration of Al found in plantlet leaves is two orders of magnitude higher than the Al3+ activity in culture solution (Fig. 4), indicating that P. almogravensis is able to concentrate Al in its upper tissues. Also, the determination of total Al indicates that its uptake is concentration-dependent and that the uptake capacity has not reached saturation at 400 μM Al (Fig. 4).

Discussion
Identification of a tri-Al tricitrate complex following a mass spectrometry based approach

This work reports the first direct and unequivocal identification of an Al-complex in an accumulating plant using ESI-MS without prior information. Al speciation studies in plants have been frustrated by the technical constraints limiting the separation and identification of Al complexes.16 The complex aqueous coordination chemistry of Al, the lack of a suitable multi-isotopic profile and the typical instability of metal complexes that renders concentration procedures unfeasible have slowed the understanding of the physiology of Al detoxification in tolerant plants. Most studies have been based on 27Al NMR spectroscopy with a consequence that only plants accumulating extremely high concentrations could be examined. A 1:1 complex of Al and citrate has been detected in the xylem of several Al accumulating plants by 27Al NMR spectroscopy.17 ESI-MS experiments confirmed that in Hydrangea macrophylla cell sap an Al–citrate complex (m/z 215) occurs.18 In this work we report that Al can be complexed by a tricitrate ligand. High-resolution MS experiments rendered a molecular ion at 648.96 m/z after separation by hydrophilic interaction LC. Exact mass determination and comparison with an Al-citrate standard solution allowed assigning the complex as tri-Al tricitrate. This complex is the most stable one occurring in P. almogravensis as it remained intact after both HILIC and SEC separation. The structure of a tri-Al tricitrate complex obtained by chemical synthesis has been characterized by NMR spectroscopy and X-ray crystallography19 and a stability constant of 16.3 was determined by potentiometric studies,20 which indicates that it is a quite stable complex. The results obtained for fragmentation of the m/z 648.96 molecular ion after CID-MS experiments

| Table 1 | Speciation of Al and Fe in Murashige and Skoog medium with macronutrients reduced to one quarter supplemented with 400 μM Al at pH 4.0. Values were calculated using Geochem-EZ and are presented as a percentage of the total Al and Fe present in solution |
|---------|----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Species  | [Al]3+ (%) | [Fe]3+ (%) |
| Free     | 34.60      | 92.38      |
| Complexed with: |         |         |
| PO4-     | 27.67      | 3.96      |
| EDTA     | 23.16      | 0.15      |
| SO4-     | 12.18      | 2.64      |
| OH-      | 1.94       | 0         |
Al and Fe are complexed by tricitrate

The relation between Fe and Al metabolism is interesting considering that Fe is essential for development and Al toxic for most plants, yet their similar chemical properties pose a significant challenge for selective uptake and incorporation into metabolism.23 More interestingly, a tri-Fe(m) tricitrate complex involved in Fe transport has been recently identified in xylem sap of tomato plants24 and our results show that Fe(m) and Al are interchangeable in this complex indicating that one of the ligands binding Al is the same ligand responsible for Fe transport. The Fe complex was modelled as having an oxo-bridged tri-Fe(m) (Fe(Ocitr)3) core and owing to its compact molecular geometry it is expected to permeate the plasmodesmata directly.24 The authors also reported that while both Fe3cit3 and Fe3Ocit3 complexes could be identified in Fe-citrate standard solutions, only Fe3Ocit3 was found in xylem samples. However, the Fe3cit3 complex was detected in P. almogravensis root samples and represents the first report of this complex in biological samples (Fig. S3 in the ESI†). The fact that, of the biologically relevant elements, Fe has the closest ionic radius to the one of Al might explain their binding by the same ligand considering that the ionic radius is one of the most important predictive parameters relating to the biological behavior of metal ions.25 The relative concentration ratios between the Al- and Fe-complex determined using the relative 27Al and 56Fe signal intensities (depicted in Fig. 1C) combined with the sensitivity of the instrument for these isotopes and the fact that the estimated free activity of Fe in the culture medium supplemented with 400 μM is higher than that of Al (Fig. 4) suggest that Al can outcompete Fe(m) for the tricitrate ligand. This observation has consequences concerning the effect of free Al concentration in soils on the Fe homeostasis in P. almogravensis. In fact, the results determined for the total Fe and Al concentrations indicate that the concentration of Al in the culture media affects the concentration of 56Fe found in leaves (Fig. 4). While the concentration of 56Fe increases when the concentration of Al in the culture solution is increased from 100 μM to 200 μM, an increment of the Al concentration from 200 μM to 400 μM leads to a reduction in 56Fe concentration. In fact, a reduction of Fe uptake as a result of Al supplementation was also observed in M. malabathricum.26 The authors observed that Al supplementation ameliorated Fe toxicity and speculated that, in M. malabathricum, the accumulation of Al could be an adaptive advantage to Fe-rich acid sulphate soils. The fact that P. almogravensis colonizes soils enriched in Fe and free Al,4 makes it an interesting model plant to study the interaction of the uptake of both metals. However, further experiments will have to point out to what extent Al can compete with Fe for binding with citrate and how it affects Fe uptake. A recent study on Fe uptake in Bacillus cereus demonstrated for the first time that a protein, FctC, could act as a specific binder for the Fe3cit3 complex.27 Even if the structure of Al3cit3 seems to be slightly different from the one of Fe3cit3,27 it could be hypothesized that similar proteins could occur in higher organisms such as plants and for other metal complexes such as Al3cit3.

Speciation of Al is concentration-dependent

The results presented in Fig. 1 indicate that Al speciation in P. almogravensis is dependent on internal concentration. The Al3cit3 complex (complex 2) was not detected in leaves of plants exposed to the lowest concentration of Al while it was detected in roots (Fig. 1B) and in leaves of plants exposed to 400 μM Al (Fig. 1A). The Al3+ cation has a great affinity for negatively charged pectin28 making it difficult to distinguish intracellular Al from Al adhered to the root cell wall. Therefore, instead of determining total concentration of Al in roots, a more precise measure of uptake is to consider the accumulation of Al in the upper organs. The quantification of total Al confirms that plants exposed to 400 μM Al accumulate more Al in leaves than plants exposed to 100 μM (165.4 ± 16.6 μM and 65.8 ± 1.8 μM, respectively). Shen et al.29 reported that in buckwheat (F. esculentum) leaves different Al species were detected depending on the concentration of internal Al. At lower Al concentrations only Al-oxalate was detected while at higher concentrations Al–citrate complexes could also be observed. In buckwheat, Al that is bound to citrate in xylem undergoes a
ligand exchange in leaves, where it is converted to Al-oxalate. The authors proposed that when oxalate is limiting Al-citrate cannot be converted to Al-oxalate completely and both complexes are therefore detected simultaneously. Considering that an Al–citrate complex also occurs in P. almogravensis, the observed results could be an indication that a similar ligand exchange process is present and that it is halted at higher internal Al concentrations. However, it is more likely that the speciation of Al is dependent on the internal Al : citrate ratio considering that complexes 3 and 4 are also citrate complexes and that no Al–oxalate complexes could be detected (Fig. S1 in the ESI†). Also the Al–citrate speciation diagrams presented by Fukushima et al.19,27,30 show that at typical physiological pH values the speciation of Al is dependent on the Al : citrate ratio and the formation of Al3cit3 is favoured as the Al : citrate ratio increases. Our results are consistent with the predicted speciation diagrams as the intensity of peak 2 (corresponding to the Al3cit3 complex) increases drastically between the extract of leaves exposed to 100 μM Al (Fig. 1B) and 400 μM Al (Fig. 1A) as a result of increasing internal Al concentration (Fig. 4). Accordingly, it could also be hypothesized that peaks 3 and 4 could respectively be related to Alcit2 and Alcit complexes,27 which would explain the presence of three main peaks in the Al–citric acid standard solution chromatogram (Fig. S1B, ESI†).

Finally, as stated above, competition with Fe certainly occurs during Al homeostasis. Actually, even if the structures of Al3cit3 and Fe3cit3 complexes have some dissimilarities,27 they have comparable stability constants,31 which would clarify why Al and Fe seem to enter into competition when present at similar concentration levels (Fig. 4). The actual equilibrium between Al, Fe and citrate (and other minor ligands) is therefore related to the Al(III) : Fe(III) : citrate ratio, to their total amount, to the pH and to the presence of competing metals/organic acids/salts in the different specific plant cell compartments, which means that further investigation would be needed to get a detailed and simultaneous view of Al and Fe speciation in P. almogravensis.

**P. almogravensis roots and the uptake of Al**

When cultured in vitro, P. almogravensis plants grow normally even in the absence of developed root systems. Consequently, the importance of the root system in the speciation of Al in the upper tissues could be examined. In terms of Al uptake, no differences were observed between plantlets and shoots, considering that similar amounts were quantified in leaves (165.4 ± 16.6 μM in plantlets and 171.8 ± 14.0 μM in shoots; Fig. 4). Because the root systems were excised in shoots, the conducting vessels are directly exposed to the culture solution and Al uptake is a consequence of co-transport of culture solution driven by transpiration. The fact that shoots and plantlets accumulate the same amount of Al in leaves suggests that the root cell membrane does not pose a barrier to Al uptake. However, the speciation analysis provided interesting results. The obtained SEC-ICP-MS chromatograms showed that the complexes 1 to 4 were absent from shoot samples, yet a broad Al signal eluting at longer retention times was observed, suggesting that it is bound to a ligand with lower molecular weight. By comparing the elution time of this Al-complex with the signal of other elements a correlation was observed with 31P (Fig. 1D). In fact, according to the calculated speciation values, over 25% of Al is complexed by PO4 in the culture solution (Table 1), suggesting that Al does not undergo chemical transformation once taken up by shoots. The fact that the 31P signal detected in shoots at 26.2 min was not observed in plantlet leaves (Fig. 1D) supports the hypothesis that Al is taken up directly as an AlPO4 complex in shoots. The reason why Al found in shoots is not converted to Al3cit3 is unclear, considering that large amounts of citrate can be found in shoots3a and the Al3cit3 complex is formed spontaneously when Al and citrate are in solution. One hypothesis is that the Al3cit3 complex is formed at the roots because Al is absorbed as Al3+ and is available for binding with citrate. Information on the molecular form of Al that is able to permeate the plasma membrane is still fragmentary, but evidence has been presented indicating that Al3+ is the most membrane mobile species.32 Because it was not expected for plants to have specific transporters for a non-essential element, it was hypothesized that Al3+ could permeate divalent cation channels.25 However, a specific Al transporter named Nrat1 (Nramp aluminum transporter 1) was recently discovered in rice.33 The protein is located on the plasma membrane of root cells and is specific for the Al3+ cation. Therefore it is possible that in P. almogravensis Al is taken up as Al3+ as well and immediately complexed by citrate. The Fe3cit3 complex has been identified only recently,24 so it is yet unknown if it is a universal ligand for Fe transport in plants. If this is the case, the fact Al-accumulation has only been described for a limited amount of plants indicates that the Al-permeability at roots seems to be the main difference between accumulating and non-accumulating plants.

**Conclusions**

An integrated mass spectrometry approach encompassing parallel LC ICP-MS and ESI-MS measurements using the Fe signal as an internal tracer in MS spectra allowed for the direct identification of the Al3cit3 complex based on exact mass determination and retention time comparison. Our results also demonstrate that the same tricitrate ligand complexes Al and Fe and that the relation between Al accumulation and Fe homeostasis should be further investigated. In P. almogravensis, Al is mainly bound to complexes related to citrate, of which Al3cit3 is the major form. In a later stage the subcellular localization of these complexes will be crucial to better understand the mechanisms behind Al tolerance in P. almogravensis and in plants in general. The high sensitivity of the employed methodology allowed studying the speciation of Al using minute samples. Therefore it is envisioned that the described methodology may provide new insights not only into the mechanisms regarding Al tolerance because it provides a means to study the speciation of Al with greater organ specificity but it may also provide information about Al species occurring in susceptible plants and its relation to toxicity.
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Notes and references


