Accumulation and uptake of light rare earth elements in a hyperaccumulator *Dicranopteris dichotoma*

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Abstract

Dicranopteris dichotoma, a natural perennial fern, grows in acidic soil in southern China. It hyperaccumulates several light rare earth elements La, Ce, Pr and Nd (LREEs) up to about 0.7% of its dry leaf biomass. Through electron microscopic and X-ray microanalyses, LREEs deposits were observed in the cell wall, intercellular space, plasmalemma, vesicles and vacuoles of the root endodermis and stele cells but not in the Casparian band of the fern adventitious root. In addition, LREE deposits were observed in the phloem and xylem of the fern rhizome. These results indicate that at least a portion of the LREEs can be transported symplastically. Histidine and organic acids appear to play a role in stimulating the accumulation of LREEs. This was evident in ferns cultivated in soil amended by histidine, malic and citric acids over a 60-day period. Concentrations of LREEs in *D. dichotoma* leaves increased by 21–78% as opposed to control, which only increased by 6–10%. While this suggests that histidine and organic acids promote the uptake and sequestration of LREEs in *D. dichotoma*, the enhancement mechanism of each acid appears to differ. Histidine promoted sequestration of LREEs by forming complexes with LREEs in cells. Organic acids, however, increased the release of LREE from soil, and the uptake of LREEs by fern roots. Most of the LREEs (81–89%) in the cell wall are the result of one of these mechanisms involved in LREE hyperaccumulation. Mass analysis indicated that the molecular weight of LREE-binding peptides was approximately 2000.

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1. Introduction

Interest in the phytoremediation of metal-contaminated soils is increasing although this technique is still in its early stage of application [1,2]. Approximate 400 species of metal hyperaccumulators are currently known [3,4]. Of particular physiological interests are those metallophytes that can hyperaccumulate heavy metals such as nickel (Ni), zinc (Zn), copper (Cu), cobalt (Co) and lead (Pb). These are hyperaccumulated from soils enriched with heavy metals either naturally or anthropogenically [5–8].

Considerable progress has been made to illustrate the mechanisms of translocation and sequestration of heavy met-

als in hyperaccumulators. Two transport mechanisms of metals within hyperaccumulators have been identified. These are either through the apoplastic or symplastic pathways. Most studies have identified the apoplastic transport mechanism. This is because the accumulation of Ni, Zn and Cd is found mainly in cell walls, residing on the outside of the plasma membrane of epidermal, cortical and endodermal cells up to the Casparian band [9–16]. At the same time, such accumulation is completely absent from stele [10]. However, movement of ions taken up by plant roots centripetally across the root into the xylem must be symplastic [17]. For example, a significant amount of aluminum is found to accumulate in the symplasm of the root-tip of soybean plants. This would indicate that aluminum can rapidly cross the plasma membrane and is transported symplastically [18].

The understanding of the location of rare earth elements (REEs) in plant cells is still limited and the mechanism

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remains controversial. An electron microscopic study revealed that REEs enter the apoplasm of root meristems and move from there to the stele of the root and to the shoots of intact *Hordeum vulgare* L., *Salicornia virginica* L., and *Spartina alterniflora* Loisel [19]. Lanthanum was only detected in the root cell cytosol when plants were exposed to high concentration of lanthanum for long periods of time. However, this was ascribed to be a toxic response [19]. REEs were also found in the cytoplasmic fraction of plant cells [20]. Here the nucleolus was found to be the site that firmly binds REEs.

Uptake and accumulation of LREEs in plants are influenced by plant root and soil interactions. Hyperaccumulators can solubilize LREEs from soil. This enhances the efficiency of the uptake of LREEs by roots, the translocation of LREEs to leaves, and the sequestration of LREEs within plant shoot cells. Low-molecular-weight organic acids (LMWOAs) are important root exudates. This is due to their metal chelating and/or complexing properties by which they can influence metal solubility in soils and uptake by plants [21–24]. Although organic acids participate in the metal complex in the shoots of hyperaccumulators, they are already normally present at high concentration levels in most terrestrial plants and therefore would not account for metal specificity resulting from hyperaccumulation. Of all the organic acids and proteinaceous amino acids, histidine has the highest association constant for complex formation with heavy metals at typical cytosolic pH values around 7.5 [3].

Dicranopteris dichotoma is a natural perennial fern that hyperaccumulates LREEs. The tolerance to elevated LREEs in soils and high accumulation of LREEs in the leaves were previously reported [25]. Guo et al. [26] isolated REE-binding proteins with molecular weights of 14,100 and 38,700. Wang et al. [20,27] reported that REEs bound with DNA and with polysaccharides in the leaves of *D. dichotoma*. Lanthanides might replace magnesium of chlorophyll partly and form new metal–chlorophyll complexes. The complexes would be in sandwich-like form based on K-absorption near-edge spectra of Ce in *D. dichotoma* by extended X-ray absorption fine structure spectroscopy [28]. However, the biochemical mechanisms involved remain obscure.

Physiologically, trivalent cations, such as La^{3+} , are notoriously difficult to study because of their aggressive binding to negatively charged ligands in the plant cell wall. There has been very little convincing data on the translocation of REEs and specifically on the effects of organic acids on the uptake and accumulation of REEs by a hyperaccumulator such as *D. dichotoma*. Therefore, the main goals of the present investigation include: (1) the study of the sub-cellular location and transport mechanism of LREEs in the fern; (2) the assessment of whether histidine and organic acids have positive, negative or neutral effects on the hyperaccumulation of LREEs by the fern; (3) the observation of LREEs distribution among cell walls, cell debris, peptides, free amino acids, and pigments of the fern leaves.

2. Materials and methods

2.1. Plant material

In December 2001, the natural perennial fern, *D. dichotoma*, was sampled together with its native soil. This was done in area where there is rare earth ore deposit in southern China. The fern and the soil were then transferred to 21 culture pots and cultured in the laboratory for further electron microscopic study and X-ray microanalysis (see Section 2.2), and for the amendments of histidine and organic acids (see Section 2.4).

Additional samples of fresh fern leaves were obtained in the field and stored frozen for isolation of cellular components. The fresh fern leaves were cut off on site, cleaned with deionized water, blotted with paper tissues, and then placed in an envelope. The envelopes containing fern leaves were immediately frozen in liquid nitrogen and were kept frozen during transport. The samples were stored in a refrigerator at -80°C until isolation of cellular components took place (see Sections 2.3 and 2.5).

2.2. Electron microscopic study and X-ray elemental microanalysis

For electron microscopic analysis, an intact fern was thoroughly rinsed with tap water and distilled water, and then blotted with paper tissues. The tip root and rhizome were excised. The plant samples (1 mm^3) were fixed in 3% (w/v) glutaraldehyde and in a 0.1 M phosphate buffer (pH 7.2) for 4 h, and washed with a phosphate buffer for 1 h. Then, the samples were fixed with 1% OsO_4 (pH 7.2) for 1 h, and rinsed with a buffer for three 10 min periods. After washing, the samples were dehydrated in a graded acetone and ethanol in series, and then embedded in Spurr's resin for 3 days. Dry sections ($1\text{--}2\text{ }\mu\text{m}$) were cut on a diamond knife using an ultramicrotome and mounted on copper grids. Electron microscopic observation was made at 100 kV with a Hitachi 800 electron microscope. Elemental X-ray microanalysis was performed using a Philips EDAX 9100 microscope. A scanning transmission electron micrograph was first taken at 100 kV, followed by microanalysis for 100 s of 5.0 nm spots.

2.3. Determination of free histidine and organic acids in the fern leaves

Fresh leaf tissues (5.0 g) were ground to very fine powder with a pestle in a mortar kept chilled on an ice-bath. Precautions were taken to keep the tissue frozen throughout the grinding process with the help of additional liquid nitrogen. The tissue powder was suspended in 10 ml deionized water before centrifugation in a Beckman JA 14 rotor at $10,000 \times g$ for 10 min. The supernatant was filtered through a $0.45\text{ }\mu\text{m}$ filter membrane. The filtrate was used for

determinations of histidine and organic acids by ion chromatography and capillary zone electrophoresis (CZE), respectively.

The determination of histidine was accomplished using a DX-600 ion chromatograph (Dionex, Sunnyvale, CA, USA). The AminoPac PA 10 column set consisting of a guard column (50 mm $\times$ 2 mm i.d.) and an analytical column (250 mm $\times$ 2 mm i.d.) were used to separate amino acids and carbohydrates. The specific details of this were reported elsewhere [29].

All CZE experiments were performed using a Beckman P/ACE System 2050 capillary electrophoresis system controlled by a computer equipped with Gold software. Separation was carried out in fused-silica capillaries (50 μ m i.d. $\times$ 75 cm total length, 70.4 cm effective length) at a voltage of 20 kV with the cathode on the detector side. The capillary was kept at a constant temperature of 25 °C by a thermostat. Sample injection was accomplished by voltage (5 s) or mainly by pressure injection at 0.5 psi for 5 s. The signal was measured by the indirect UV detection mode at 254 nm.

2.4. Histidine and organic acid amendments

Eight culture pots containing the fern with the original adjacent acidic soils were used to investigate the effects of histidine and organic acid amendments on LREEs uptake. Two pots each were used for control and for amendments. An amendment with 100 ml of 0.01 M histidine, 0.01 M citric acid, and 0.01 M malic acid was added daily for 60 days, respectively. The growth temperature during cultivation was about 15 °C at night and about 25 °C during the day. After 60 days, the fern leaves were harvested from each pot for determination of LREE concentrations (see Section 2.7).

2.5. Isolation of cellular components

Only the frozen fern leaves (see Section 2.1) were used for the isolation of cell wall, cell debris and peptides following a protocol of Sugaya et al. [30] with modification (Fig. 1). Briefly, 20 g of frozen leaves were ground for at least 30 min with a pestle in a large mortar, which was kept chilled by an ice-bath. Liquid nitrogen was also added throughout the grinding process to keep the tissue frozen. After the tissue was ground to a very fine powder, it was suspended in a 10 volume (w/v) peptide extraction buffer (0.2 M Tris-HCl, pH 8.0) containing 1 mM EDTA, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and sodium dodecyl-sulphonate (SDS). The mixture was homogenized with a Polytron. The homogenate was filtered through four-layer cheesecloth to remove the residue. The filtrate was centrifuged in Beckman JA 14 rotor at 20,000 $\times$ g for 20 min to yield the crude extract and to remove cell debris. The resulting supernatant (crude extract) was precipitated with 30–85% ammonium sulfate by stirring for 120 min at 4 °C under nitrogen atmosphere inside a glove box. The suspension was centrifuged at 6000 $\times$ g for 10 min in a JA 14 rotor. The precipitated fraction was re-suspended in 20 ml 0.05 M Tris-HCl (pH 8.0), after which the mixture was stirred for 45 min at 4 °C. After centrifugation at 10000 $\times$ g for 10 min, the newly resulting supernatant was saved, which was designated as crude LREE-binding peptides.

The crude LREE-binding peptides obtained were loaded on a Sephadex G-25 column (1.8 cm i.d. $\times$ 30 cm) equilibrated with 0.05 mM Tris-HCl buffer (pH 8.0). Then, the column was washed with the same buffer at a flow rate of 1 ml min⁻¹ until the eluate was peptide-free by determination of absorbance at 280 nm. The first peak was collected at every 5-ml fraction. All fractions were combined together

Fig. 1. A schematic diagram of isolation of fern leaf into cell wall, cell debris, crude proteins and peptides, free amino acids and pigments.

and then concentrated to about 5 ml for HPLC-ESI-MS analysis.

2.6. Characterization of LREE-binding peptides by HPLC-ESI-MS

Separation of LREE-binding peptides was performed on an Agilent 1100 series HPLC (Palo Alto, CA, USA) equipped with an autosampler. All connections were made with 0.127 mm i.d. polyether ether ketone (PEEK) tubing and finger tight fittings (Upchurch Scientific). Chromatographic analysis was performed with a reversed-phase Vydac C₁₈ column (150 mm × 4.6 mm i.d.; particle size 5 μm; pore size 300 Å). A flow-rate of 0.5 ml min⁻¹ was used for all samples in HPLC-ESI-MS analysis. Elution was carried out with a gradient from 0 to 90% B during a 30 min period. Gradient elution was performed with solvent A (0.05%, v/v, aqueous TFA) and solvent B (0.05%, v/v, TFA in acetonitrile). An injection of 20 μl samples was used according to ref. [31] and the UV absorption of peptide fraction was measured at 280 nm.

The HPLC effluent was analyzed with an Agilent ESI-mass spectrometer. Agilent ChemStation software controlled HPLC and mass spectrometer.

A post-column addition of glacial acetic acid was delivered at a flow rate of 100 μl min⁻¹ in order to negate the signal suppressing properties of TFA in the mobile phase. The glacial acetic acid was added to the column effluent by using a PEEK “Y” connector (Upchurch) and a syringe pump (Cole Parmer). The “Y” was connected to the electrospray interface with 30 cm of PEEK tubing. The length of the “Y” tubing and point of entry in the ESI source were crucial for adequate mixing of the acid with the column effluent.

A variable fragmentation voltage was used during the ESI detection. The optimized voltage ramp was found to be m/z 500 = 60 V, m/z 1000 = 120 V, and m/z 3000 = 220 V. In addition, the following parameters were used for MS detection of the chromatographic eluent: gain, 5; step size, 0.15; drying gas, 10 l min⁻¹; drying gas temperature, 350 °C; nebulizer pressure, 25 psi (1 psi = 6894.76 Pa); capillary voltage, 4500 V.

2.7. Determination of LREEs

Before a determination of the concentration of LREEs was made, soil and fern tissue samples were subjected to pre-treatment procedures. Soil samples were air-dried, ground and passed through a 2-mm screen to remove rocks, roots, and other large particles. Before analysis, precautions were taken to avoid contamination during sampling, drying, and grinding. The root, leaf and ground stem (rhizome) of the fern were washed with tap water and with deionized water successively. They were then blotted with paper tissues. The cleaned fern tissue was cut into small pieces with stainless steel knife and dried at 80 °C for 48 h. It was then

ground utilizing an agate mortar and pestle until it could pass through a 0.85-mm (20 mesh) sieve.

A portion (0.100 g) of air-dried soil or dried fern tissue was decomposed with a mixture of 3 ml HNO₃ + 1 ml HClO₄ + 1 ml HF under high-pressure conditions. This has been detailed previously [32]. A portion (2 ml) of the peptide, fractionated by Gel filtration, was accurately taken into a Teflon vessel. It was then decomposed with mixture of 3 ml HNO₃ + 1 ml HClO₄. The determination of LREEs in soil and plant samples was accomplished by inductively coupled plasma mass spectrometry (ICP-MS) (Plasma Quad III, UK) [33]. During the determination, a solution (¹¹⁵In at a concentration level of 10 ng ml⁻¹ in 2% HNO₃) was also used as an internal standard to compensate for matrix suppression and signal drifting.

3. Results and discussion

3.1. Distribution of LREEs among soil, root and different components of *D. dichotoma* leaves

The concentration of LREEs was determined in the soil, as well as the adventitious root, rhizome and leaf of the fern collected from the field. The concentration of LREEs in fern dry leaves was approximately 0.7%. The ratios of the concentrations of La, Ce, Pr and Nd in leaves to those in roots were 12–15, 25–26, 3–12 and 1–7, respectively (Table 1). However, no hyperaccumulation phenomenon was observed for the medium and heavy REEs (data not shown).

Fig. 1 depicts a schematic program for the isolation of the frozen fern leaves to cell wall, cell membrane debris, crude peptides, free amino acids and pigments. The total concentration of LREEs in leaves and the distribution of LREEs among individual component are shown in Table 2. Most of the LREEs (81–88%) were found in the cell wall portion of the leaf (Table 2). This is consistent with previous studies [33,34]. Cell membrane debris contained about 10% of the total LREEs. The percentages of the LREEs found in the free amino acids and pigments and in crude peptides were about 0.4–1% and about 1–3%, respectively (Table 2).

These observations support the notion that the high accumulation of LREEs in cell wall is responsible for the tolerance of LREE hyperaccumulation in *D. dichotoma*. The most likely explanation is that there are many carboxylate groups in cell wall, and those radicals can bind with LREE ions effectively [35].

3.2. Electron microscopic study and energy-dispersive X-ray microanalysis

Scanning electron microscopic and energy-dispersive X-ray microelemental analyses revealed no LREEs deposits in the Casparian band of the fern adventitious root (Fig. 2a). Instead, the deposition of LREEs was confirmed

Table 1

Concentrations of rare earth elements in the fern leaves, roots and soil ($n = 5$, $\mu\text{g g}^{-1}$, dry mass)

	La	Ce	Pr	Nd
Leaf	1503.4–2148.4	2829.7–3956.6	463.5–609.5	1023.1–1358.7
Rhizome	213.3–544.4	233.7–243.0	105.1–126.3	337.8–352.4
Adventitious root	118.8–141.5	108.4–157.7	36.9–201.5	146.3–1247.9
Soil	150.5–453.1	244.9–281.1	33.1–137.1	119.2–474.9

Table 2

Concentrations of rare earth elements in different components of fern leaves ($n = 5$, $\mu\text{g g}^{-1}$, dry mass)

	La		Ce		Pr		Nd	
	$\mu\text{g g}^{-1}$	%	$\mu\text{g g}^{-1}$	%	$\mu\text{g g}^{-1}$	%	$\mu\text{g g}^{-1}$	%
Crude peptides	20.8 ± 1.3	1.0	102.4 ± 13.6	2.7	7.0 ± 0.6	1.2	17.9 ± 4.7	1.4
Free amino acids and pigments	11.3 ± 0.8	0.5	36.8 ± 3.8	1.0	2.6 ± 0.1	0.4	6.4 ± 0.2	0.5
Cell debris (membrane)	212.6 ± 15.1	10.4	577.9 ± 45.8	15.4	57.1 ± 10.3	9.6	125.9 ± 12.9	10.1
Cell wall	1802.6 ± 87.3	88.1	3031.6 ± 265.4	80.9	527.6 ± 23.1	88.8	1094.1 ± 53.7	87.9
Leaflet	2047.3 ± 165.3	100.0	3748.3 ± 312.5	100.0	594.5 ± 45.7	100.0	1244.9 ± 86.5	100.0

in the plasmalemma, vesicle and vacuole of the endodermis cells of the adventitious root of the fern (Fig. 2a). These results demonstrated that the LREE permeated the Casparian band. This significantly differed from other studies, which considered Casparian band as a barrier to the movement of lanthanum from cortex to stele [36].

LREE deposits were also observed in the cell wall, plasmalemma, vesicle, vacuole and intercellular space of root stellar cell (Fig. 2c), rhizome phloem (Fig. 2d) and rhizome xylem (Fig. 2e). These suggest that at least a partial cell-to-cell transport mechanism is taking place. These results indicate that both symplastic and apoplastic transport mechanisms co-exist in this natural perennial fern.

3.3. Effect of free histidine and organic acids on uptake of LREEs

D. dichotoma cultivated in soil amended with organic acids or histidine (see Section 2.4) was used to examine the assumption that organic ligands can stimulate the uptake or translocation of heavy metals in hyperaccumulators. Specifically, the intent was to evaluate whether uptake and translocation of LREEs were stimulated by histidine and organic acids.

3.3.1. Hyperaccumulation of LREEs enhanced by histidine complexation in cells

After the fern soil was amended for 60 days by L-histidine, the concentrations of LREEs in leaves increased by 21–34%, while those in control leaves only increased by 6–10% (Table 3). This modest increase of LREEs in fern leaves amended with histidine suggests that free histidine was involved in the uptake and translocation of LREEs from root to leaves and the sequestration of LREEs in fern leaves. This apparent importance of histidine in LREE hyperaccumulation in *D. dichotoma* is in agreement with their role in

nickel uptake by *A. lesbiacum* [3,4,10,12], as well as zinc uptake by *Thlaspi caerulescens* [3,12].

High concentrations of histidine in leaf cells also indicate that histidine could facilitate plant cell loading of LREEs. This may be possible because the LREE complex can easily form in the presence of histidine [3,12]. Through ion chromatography (see Section 2.4), histidine was identified as the most enriched component of the fern sap (Fig. 3). The concentration of free histidine in the leaf cell sap was estimated to be about 128 mM. Several other amino acids such as arginine, threonine, serine and leucine were detected in the fern leaves but at lower concentrations (Fig. 3). Histidine can readily complex with LREEs due to high stability constants between LREEs and histidine (Table 4) [37,38] and the high protonation constant of histidine [39]. At the pH values commonly found in the cytoplasm (pH 7.5), histidine is an ideal ligand for LREEs.

3.3.2. Hyperaccumulation of LREEs by malic and citric acids promoted desorption from soil and enhanced uptake by *D. dichotoma*

Malic and citric acids were separated from the fresh fern leaves and determined by capillary zone electrophoresis (see Section 2.4). The determination of citric acid was hindered because of matrix interference, and only malic acid was de-

Table 3

Effects of free histidine, malic and citric acids on accumulation of LREEs in *D. dichotoma* ($n = 2$, $\mu\text{g g}^{-1}$, dry fern leaf mass)

	Control		Histidine		Malic acid		Citric acid	
	Before	After	Before	After	Before	After	Before	After
La	63.4	67.6	72.4	95.5	136.0	163.9	62.8	111.9
Ce	223.0	239.6	222.2	299.2	460.1	541.6	236.4	430.2
Pr	7.0	7.7	8.4	10.7	16.3	20.1	9.7	16.5
Nd	15.5	17.1	16.4	20.0	33.1	38.0	21.1	31.0

Fig. 2. Scanning transmission electron micrographs of fern adventitious root (a), root stellar cell (c), rhizome phloem (d), rhizome xylem (e), and X-ray microelemental analysis of adventitious root (b) and rhizome xylem (f). A 5.0 nm spot of plasmalemma of endodermis cell (a) and rhizome xylem (e) were used for the X-ray microelemental analysis of accompanying scanning transmission electron micrographs. Arrows refer to the deposit of rare earth elements. CB: Casparian band, cw: cell wall, ics: intercellular space, P: plasmalemma, T: tonoplast, v: vacuole, vs: vesicle. Expansion scale of (a), (c), (d) and (e) is 12,000, 9000, 12,000 and 9000, respectively.

tected. The electrophoretogram is shown in Fig. 4. The concentration of malic acid detected was 0.65 mM. In addition, a quantitative recovery of 109% was obtained when standard malic acid was added before grinding the leaf.

By supplying malic and citric acids to fern soils for 60 days, the concentrations of LREEs in fern leaves increased by 21–28% and by 48–78%, respectively (Table 3). The enhanced uptake in the presence of citric or malic acids is most

likely caused by the increase in LREEs being release from soil, thus making LREEs easily available to plants [40]. The desorption of LREEs from soil by organic acids, in which the natural perennial fern was grown, followed the order: citric acid > malic acid. This order is consistent with the stability constants of complexes formed between LREEs and organic acids (Table 4) in which citric acid displayed the highest value. This order also suggests that the formation

Table 4
pK_a values of organic acids and stability constants of LREE–ligand complexes

Organic acid	pK _a	Stability constants			
		La ³⁺	Ce ³⁺	Pr ³⁺	Nd ³⁺
Citric acid	3.13, 4.78, 6.43	7.17 ± 0.08 ^a , 3.00 ± 0.20 ^a	7.40 ± 0.08 ^a , 3.00 ± 0.20 ^a	7.95 ± 0.04 ^b	7.96 ± 0.08 ^b
Malic acid	3.46, 5.10	4.37 ± 0.01 ^a , 2.79 ± 0.03 ^a		4.65 ± 0.01 ^a , 3.09 ± 0.03 ^a	4.77 ± 0.01 ^a , 3.17 ± 0.02 ^a
Histidine	2.16, 5.36, 6.70, 7.92	4.10 ± 0.12 ^c	4.6 ± 0.2 ^d	4.36 ± 0.03 ^c	4.40 ± 0.09 ^c

pK_a values were adopted from ref. [37]. Stability constants were adopted from ref. [38].

^a Ionic strength *I* = 0.1, 25 °C.

^b Ionic strength *I* = 0.05, 25 °C.

^c Ionic strength *I* = 3, 25 °C.

^d Ionic strength *I* = 0.1, 20 °C.

Fig. 3. Chromatogram of histidine by ion chromatography: (A) standard amino acids and (B) amino acids in fern cell sap.

of aqueous LREE–organic ligand complexes is one of the important reasons for the uptake of LREEs by this fern hyperaccumulator.

Enhancement of LREEs uptake by fern amended by organic acids is also supported by a previous study [32]. In that study, a good correlation (correlation coefficient 0.7253–0.9635) was found between concentrations of LREEs by malic and citric acids extraction from soil and those of plants. The results suggested that malic–citric acid extracted LREEs were bioavailable for plants. In a hydroponic culture study, organic acids enhanced the uptake

of lanthanum although a different plant species was used [41].

In addition to ligand-promoted desorption of LREEs from soil, citric and malic acids may also enhance LREEs uptake by shuttling metal from root to shoot. High concentrations of malic acid were found in the shoot tissues of *T. caerulea* [42,43]. This suggests that organic acids such as malic and citric acids may play a key role in the cells. It is also likely that organic ligands help to facilitate the long-distance transport of LREEs in the xylem and might be instrumental in sequestering LREEs in the leaf

Fig. 4. Electrophoretogram of malic acid in fern leaf cell sap.

cell wall. Both citrate and malate are intermediates in glycometabolism and are involved in the energy production in the tricarboxylic (TCA) cycle. Citric and malic acids are also strong ligands for LREEs [38]. Therefore, it is conceivable that citric and malic acids can take part in glucose synthesis metabolism, resulting in LREEs being deposited on the cell wall. Further study of the complexation between citric and malic acids and LREEs in hyperaccumulators is required to test this hypothesis.

In summary, although the mechanisms of enhancement differ, both histidine and organic acids (e.g. citric and malic

Table 5

Concentrations of rare earth elements in HPLC eluates with different retention times ($n = 5$, ng g^{-1} , fresh leaf mass)

Retention time (min)	La	Ce	Nd	Pr
8.325	0.62	1.42	0.54	0.14
9.118	0.48	2.06	0.26	0.09
9.491	0.55	1.23	0.33	0.11
9.965	0.42	1.37	0.32	0.09
10.611	0.47	1.09	0.29	0.09
12.265	0.66	1.94	0.45	0.12
12.832	0.69	1.34	0.25	0.10
13.371	0.98	3.10	0.54	0.18
13.978	1.43	4.31	0.86	0.26
17.551	0.55	1.21	0.42	0.08

acids) stimulate the uptake of LREEs in soil by hyperaccumulator *D. dichotoma* and sequestration of LREEs in fern leaves. Histidine promoted sequestration of LREEs by forming complexes with LREEs in cells. Organic acids increased LREE desorption from soil, and also enhanced the uptake of LREEs in the soil solution by fern roots.

3.4. Characterization of LREE binding peptides

Hyperaccumulators such as *D. dichotoma* often utilize several defense strategies against metal stress. Several mechanisms for such a defense include: compartmentation of metals in vacuoles [33,44,45], complexing of metal ions with organic ligands [46], and coordination with histidine [3]. Another mechanism of detoxification is the formation of peptides [3,9,47–50]. We demonstrate below that LREEs are associated with peptides in leaves of *D. dichotoma*.

Fig. 5. HPLC chromatogram of purified peptides through Sephadex G-25. The peptides were applied to the RP-C₁₈ column and eluted with solvent A (0.05% TFA, v/v, in water) and B (0.05% TFA, v/v, in acetonitrile) in gradient mode with flow-rate of 0.5 ml min^{-1} .

Fig. 6. Mass spectra of LREE-binding peptides with HPLC-ESI-MS. HPLC conditions were the same as that in Fig. 5.

Although this may not be sufficient to prove that formation of LREE binding peptides is engaged in the detoxification and homeostasis of LREEs, it does indicate that LREE-binding peptides contribute to rendering the metal ineffective.

The crudely purified peptides isolated from leaves (Fig. 1) were analyzed by reversed-phase (C_{18} HPLC and ESI-MS), and the chromatograms and mass spectrum of peptides are shown in Figs. 5 and 6. Each eluted component (e.g. a

specific peptide) as shown in Fig. 5 was collected, and the concentrations of LREEs (Table 5) in an individual component were determined by ICP-MS (see Section 2.7). Most peptides contained sub-ng g⁻¹ to a maximum of 4.3 ng g⁻¹ LREEs (Table 5). This suggests that the LREEs were bound to plant peptides although further purification and characterization of peptides were required. ESI-MS spectra (Fig. 6) indicated that the molecular weight of LREE-binding peptides was about 2000.

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