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Study of metal-containing proteins in the roots of *Elsholtzia splendens* using LA-ICP-MS and LC–tandem mass spectrometry

Bei Wu[a,b,*], Iuliana Susnea[c], Yingxu Chen[b], Michael Przybylski[c], J. Sabine Becker[a]

[a] Central Division of Analytical Chemistry, Forschungszentrum Jülich, D-52425 Jülich, Germany
[b] Department of Environmental Engineering, Zhejiang University, Hangzhou 310029, China
[c] Laboratory of Analytical Chemistry and Biopolymer Structure Analysis, University of Konstanz, D-78457 Konstanz, Germany

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ABSTRACT

In the present study, the metal-containing proteins in the roots of Cu-tolerant plant *Elsholtzia splendens* were investigated. The proteins from the plant roots were extracted and separated by two-dimensional gel electrophoresis (2D GE). Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was used to screen metal-containing proteins on the dried 2D gels. Images of Cu, Zn, Na, Mg, K, Ca, Mn, Fe, P and S, and also of Ag, were obtained by scanning the gel (line by line) using the LA-ICP-MS techniques developed in our laboratory. Cu-, Zn- and Mg-containing proteins were found in the gel sections of interest via LA-ICP-MS imaging. These protein spots were then in-gel digested with trypsin and analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) for identification. The mechanisms involved in Cu tolerance and accumulation were also discussed.

1. Introduction

The study of metal-containing species is of particular interest in the research of plants, since many metals play an essential role in cellular biochemical and physiological processes, such as in photosynthesis and respiration, and in the response to stress, as well as being cofactors of enzymes that display biological activities. For instance, Cu is an important cofactor in photosynthesis, mitochondrial respiration, oxidative stress responses, and ethylene signal transduction. On the contrary, metals can also be toxic when they are excessive in the cells. The high-concentrated Cu in cells can generate reactive oxygen species (ROS), resulting in the damage of cellular constituents and disturbance metabolism process [1]. However, plants themselves, particularly those called metal hyperaccumulator or metal-tolerant plants, have developed a series of advanced and complex mechanisms for metal uptake, transportation, accumulation, resistance and detoxification. Among these mechanisms, the production of metal chelate ligands and/or proteins is prominent. Free amino acids like histidine and cysteine [2–5], organic acids like malic acid and citric acid [6–8], peptides like metallothioneins (MTs) and phytochelatins (PCs) [9–12], as well as proteins [13,14] can be induced by the elevated metal stress in the cells to chelate excessive amounts of metal. Characterizing these metal-containing species in plants, including metal content, speciation and localization, which is part of a metallomic study, provides a fundamental understanding of their interactions with the genome, transcriptome, proteome and metabolome, as well as their functions in metal tolerance and accumulation in plants [15,16]. Correspondingly, dedicated analytical approaches are required and have been developed in order to (1) separate the metal-containing species from the plants, (2) determine the concentrations of metals, (3) localize the spatial distribution of metals, and (4) visualize the metal species in the plant system. Column techniques like chromatography or capillary electrophoresis, elemental and molecular mass spectrometric methods like inductively coupled plasma mass spectrometry (ICP-MS) and electrospray ionization mass spectrometry (ESI-MS), as well as synchrotron radiation-based techniques like X-ray fluorescence (SRXRF) and X-ray absorption spectroscopy (SRXAS) are commonly combined for the purpose of analyzing metal-containing species in plants [17–20].

Among those elemental analysis techniques, laser ablation ICP-MS (LA-ICP-MS) has recently been developed as an outstanding spatially resolved analytical technique in routine research to obtain quantitative information on elemental distribution in soft biological samples, such as cryostat-cut slices of brain tissues, plant leaves, and single hair, with relatively high spatial resolution and good sensitivity [21–25]. It has also been applied in proteomic and metallomic studies, in order to investigate trace metals, metalloids

* Corresponding author at: Central Division of Analytical Chemistry, Forschungszentrum Jülich, Leo-Brandt-Strasse, D-52425 Jülich, Germany.
Tel.: +49 2461 615163; fax: +49 2461 612560.
E-mail address: b.wu@fz-juelich.de (B. Wu).

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and/or non-metals like P and S, which are considered to be 'natural tags', present in biomolecules such as metal-containing or metalloproteins, as well as selenoproteins and phosphorylated proteins [26,27]. Because of its sensitive and direct analysis properties, LA-ICP-MS was used in several studies as the detection technique to screen such elements on gels after the separation by one- or two-dimensional gel electrophoresis (1D or 2D GE). The combination of SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and LA-ICP-MS was used to explore the speciation of selenoproteins in Se-contaminated wildlife [28]. Using laser ablation coupled to sector field ICP-MS (LA-ICP-SFMS), P and S in the protein spots in gels after 2D GE were analyzed. It was found that the concentrations of P and S decreased along with the sampling depth in gels with the maximum contents on the surface of the gels, while the P/S ratios were constant [29]. Similar to the analysis of biological tissues by LA-ICP-MS, the analysis of elements in gels could also be quantitative. For such purposes, the internal standards were added to the protein samples. The protein bands containing different concentrations of P were successfully obtained in gels after 1D GE. The calibration curve of P was then established after the analysis of P using LA-ICP-SFMS, and was applied for the quantification of LA-ICP-MS measurement on gels with respect to P content [30]. Metals such as Cu, Zn, Fe, Mn, and Pb were also investigated in gels after 1D or 2D GE [31–33]. To avoid the possible loss of metals in the denaturing processes of protein separation, non-denaturing protocols such as blue native gel electrophoresis and anodic-PAGE were proposed in several studies [32–34].

Liquid chromatography (LC) coupled with mass spectrometry (MS), especially LC–tandem mass spectrometry, has the advantages of high specificity and sensitivity and the capacity to determine multiple analytes. Each analyte in the sample is firstly separated from the LC column, and is then transported to the first MS dimension where ions of a particular mass-to-charge ratio (m/z) are isolated. In the second MS dimension, the selected precursor ion is fragmented into product ions which are used to give structural information on the precursor ion, and thus the information on each analyte [35]. The combination of the high-performance separation ability of LC and high sensitivity and low detection limits of MS provides rich information on the analyte, including molecular weight and characteristic structure fragments from a complicated matrix. LC–MS/MS has been widely applied in studies of biological metabolic processes and environmental monitoring with respect to proteome profile change due to environmental stress or chemical toxicity [36–40]. Protein and metabolite profiling analyses of cells or tissues from either model plants like *Arabidopsis thaliana* [41] or non-model plants like *Triticum tauschii* [42] were made after these plants had been exposed to environmental pollutants including heavy metals and organic compounds. However, since hardly any sequences of the non-model species are present in the available databases, it is generally difficult to identify the proteins.

Elsholtzia splendens is known as a Cu-tolerant plant, which can accumulate ~10,000 mg kg^{-1} (dry weight) Cu in its roots when grown in a Cu-contaminated environment. The plant is considered to be possibly used in the phytoremediation of Cu-polluted soils [43]. Within the plants, Cu is mainly stored on the cell walls [44]. Using synchrotron radiation X-ray absorption spectroscopy (SRXAS), Shi et al. discovered that, except for the cell walls, histidine-like ligands were bound with most of Cu via the N/O in the plants rather than the classical metal-detoxifying S ligands, such as MTs and PCs [45]. However, both free histidine and the histidine residues in proteins could provide such His-ligands for the binding of Cu. Furthermore, it was found that many of the root proteins involved in various cellular processes such as signal transduction, regulation of transcription and translation, energy metabolism, regulation of redox homeostasis and cell defense were changed upon Cu stress [46]. However, those changed proteins have not yet been studied with respect to their part in Cu binding. Nevertheless, Cu-containing and other metal-containing proteins should further be investigated in order to illustrate the mechanisms for Cu tolerance in the plant. Therefore, in the present study, we combined LA-ICP-MS for the screening of metals possibly present in the proteins separated after gel electrophoresis and LC–MS/MS for the identification of the proteins, for better understanding the metal-containing proteins in the plant roots.

2. Materials and methods

2.1. Plant cultivation and protein sample preparation

The Cu-tolerant plant *E. splendens* was solution-cultivated in an environment-controlled greenhouse as described previously [24]. After 30-day of growth, the plant was treated with 100 μmol L^{-1} Cu (administered as $CuSO_4$) in the nutrient solution for 14 days. The roots of the plant were collected, immerged in 0.5 mmol L^{-1} $CaCl_2$ solution for 30 min, and then rinsed with Milli-Q water to wash off $CaCl_2$. The roots were immediately ground into fine powder in liquid N_2 and used for protein extraction by TCA-acetone precipitation.

The ground roots were first homogenized with acetone solution containing trichloroacetic acid (TCA, 10%, w/v) and DTT (0.2%, w/v). The homogenate was precipitated overnight at −20 °C and then ultracentrifuged at 35,000 × g (6 °C) for 30 min. The proteins in the pellet were concentrated by acetone precipitation and then resuspended in lysis buffer containing 9 mol L^{-1} urea (Sigma), 4% (w/v) CHAPS (Sigma), 1% (w/v) DTT (Sigma), Bio-Lyte (4–7, Bio-Rad, 0.5%), 0.2 mmol L^{-1} PMSF (Sigma) and 1 mmol L^{-1} EDTA (Sigma), and stored at −80 °C for protein separation by 2D GE.

2.2. Separation of proteins using 2D gel electrophoresis

Before electrophoresis, the protein sample was quantified by an EZQTM Protein Quantization Kit (Cat# R33200, Molecular Probes Inc.), followed by cleaning using a Clean-up Kit from Bio-Rad. The resulted pellet containing 180 μg proteins was resuspended in 500 μL rehydration buffer (8 mol L^{-1} urea, 4% (w/v) CHAPS, 65 mmol L^{-1} DTT, 0.2% (w/v) Bio-Lyte, 0.001% (w/v) bromophenol blue), and then applied to a 17 cm IPG strip (pH 4–7, nonlinear, Bio-Rad). Rehydration was run actively at 50 V for 12 h, and then the isoelectric focusing (IEF) was performed at 20 °C for 30 min at 250 V, 1 h at 250–1000 V, 5 h at 1000–6600 V, and ~9 h at 6600 V using a PROTEAN II xi (Bio-Rad). After the first dimension, the IPG strip was equilibrated twice in mixtures containing 6 mol L^{-1} urea, 2% (w/v) SDS, 37.5 mmol L^{-1} Tris–HCl (pH 8.8), 20% glycerol, a trace of bromophenol blue, and 2% (w/v) DTT or 2.5% (w/v) iodoacetamide, respectively, for 12 min. Then the proteins were separated by SDS–PAGE on 12% gel according to their molecular weight.

Before the measurement of LA-ICP-MS and LC–MS/MS, several gels of the root proteins were run to make sure that the protein spots of interest were present in all the gels. Protein separation by 2D GE was performed in duplicate. One was used for the screening the metal-containing protein by LA-ICP-MS and the other for identification by LC–MS/MS. To visualize the protein spots in the gels, different staining techniques were used. For LA-ICP-MS measurement, silver staining was used, while Sypro Ruby was applied for the gels used for LC–MS/MS studies, which was comparable in sensitivity with silver staining.

For LA-ICP-MS measurement, the 2D gels were cut into smaller pieces, in general within the area of cm^2, to allow them to be placed into the laser ablation chamber. These pieces of gels were dried on filter paper using a gel dryer (Model 583 Gel Dryer, Bio-Rad). On the other parallel-run gel, the protein spots were excised and digested

Table 1
Optimized experimental parameters of LA-ICP-MS.

ICP-MS	ICP-QMS, Agilent 7500
Rf power (W)	1600
Carrier gas flow rate (L min^{-1})	0.89
Mass resolution ($m/\Delta m$)	300
Number of passes	1
Laser ablation system	New Wave UP 266
Wavelength of Nd-YAG laser (nm)	266
Laser energy (mJ)	0.2
Repetition frequency (Hz)	20
Scan speed (μm s^{-1})	50
Spot size (μm)	160
Distance between lines (μm)	200

with trypsin, and then dissolved in the sample solvent containing 1% formic acid and 9.5% acetonitrile at pH 2.5 for LC–MS/MS identification.

2.3. LA-ICP-MS instrumentation and measurement procedure

A quadrupole-based ICP mass spectrometer (ICP-QMS, Agilent 7500ce) was coupled to a laser ablation system (New Wave UP 266) for the screening of metals in the protein spots separated by 2D GE. The protein spots on 2D gels were scanned line by line by a focused Nd-YAG laser beam (wavelength: 266 nm, repetition frequency: 20 Hz, energy output: 0.3 mJ, laser spot size: 160 μm). The laser-ablated materials were transported by Ar as carrier gas to the inductively coupled plasma. The ion intensities of $^{63}Cu^+$, $^{64}Zn^+$, $^{23}Na^+$, $^{25}Mg^+$, $^{39}K^+$, $^{43}Ca^+$, $^{55}Mn^+$, $^{56}Fe^+$, $^{107}Ag^+$, $^{31}P^+$ and $^{34}S^+$ were then measured by the quadrupole-based mass spectrometer. The experimental parameters of the LA-ICP-MS were optimized with respect to the maximum ion intensity of $^{103}Rh^+$ using an Rh standard solution of 2 μg g^{-1}. Several areas of interest on the 2D gels were investigated to obtain the information on the metal contents in the protein spots under the operation conditions as shown in Table 1.

2.4. Liquid chromatography/electrospray ionization ion trap mass spectrometry

The HPLC system consisted of an Agilent 1100 Series binary pump system and was operated at a flow rate of 50 μL min^{-1}. The solvents consisted of (A) 0.2% aqueous formic acid and (B) 0.2% formic acid in acetonitrile.

The 10 cm × 1 mm Discovery Bio Wide Pore C18 column (Sigma–Aldrich, Germany) with a particle size of 3 μm was used to achieve peptide separation and was equilibrated for 30 min with 2% solvent B before sample injection. The digested samples were dissolved in 6–7 μL 1% formic acid, 9.5% acetonitrile, 89.5% water (pH 2.5), of which 5 μL were injected into the column.

Electrospray ionization mass spectrometry was performed on an Esquire 3000+ ion trap instrument (Bruker Daltonik, Bremen, Germany), equipped with an atmospheric pressure ionization electrospray ion source. The electrospray ion source was operated in the positive ion mode at a potential difference of 4.0 kV. Nitrogen was used as both the nebulizing and drying gas. Other source parameters were as follows: capillary exit 120 V, nebulizing gas pressure 20 psi, drying gas 9 L min^{-1}, desolvation temperature 300 °C. The instrument was operated in the data-dependent mode, such that if an ion reached a predetermined signal threshold, the MS/MS of the ion was acquired.

The ion trap was set for automatic gain control of all experiments. Six microscans were collected for each full MS scan and 20 microscans were collected for each MS/MS scan, while the maximal accumulation time for the ions entering the trap was 200 ms. The intensity threshold triggering the ion selection for MS/MS was set at 2×10^4 and the instrument was set to acquire a single parent ion per MS scan. For the MS^2 acquisitions, the SmartFrag function of the ion trap was used, which ramps the fragmentation amplitude from 30% to 200% of the preset value (1.10 V).

2.5. MS data analysis and database search

The MS data were externally calibrated, visualized and processed using the Data Analysis software, version 3.3 (Bruker Daltonik, Germany). Each data set was saved in a *.mgf file. These data were searched against the NCBInr database by means of the Mascot MS/MS Ion Search engine (www.matrixscience.com), using a precursor tolerance of 1.2 Da and an MS/MS tolerance of 0.6 Da. The taxonomy was set to all entries. The fixed and variable modifications were set to carbamidomethyl (C) and oxidation (M), respectively. The maximum missed cleavages were 2.

3. Results and discussion

3.1. LA-ICP-MS imaging of 2D gels

The 2D gel electrophoresis of the root proteins of *E. splendens* was run in duplicate. Both gels for LA-ICP-MS and LC–MS/MS measurement displayed good reproducibility (Fig. 1). The sensitivities for protein visualization of the two staining techniques, silver staining of the gel for LA-ICP-MS and staining with Sypro Ruby for LC–MS/MS, were comparable in the present study. Five areas of interest were selected in one of the 2D gels and investigated by LA-ICP-MS for the purpose of screening metal-containing proteins, as shown in Fig. 1A. Although the scanning mode in LA-ICP-MS is time-consuming (time intervals between 0.5 h and 12 h were needed for each gel piece, according to the size area), it provides lively information on elemental distribution and avoids ambiguous signal spikes that could occur in single-shoot analysis mode [47]. Since silver staining was applied for the visualization of protein spots on the gel used for LA-ICP-MS analysis, ion intensity of $^{107}Ag^+$ was also monitored together with other metals such as Cu, Zn, and Mg, to localize the proteins in the images obtained by LA-ICP-MS. The metal distributions in the selected areas *I* to *V* are shown in Fig. 2*I*–*V*, respectively. In all the images of these five areas, the distribution of Ag was the same as in the gel visualized by silver staining. The protein content of each spot could be given by the shade of color of Ag in the images. Therefore, the images of other metals (Cu, Zn, Na, Mg, K, Ca, Mn, Fe, P and S) could be used to investigate the metal distributions in these areas. However, only Cu, Zn and Mg were observed in these five areas. This could be due to the possible loss of metals during the denaturing procedure when the proteins were separated by SDS–PAGE as well as during the staining process. Therefore, non-denaturing separation processes have been proposed in the study of metal-containing proteins [32–34].

The protein spot *a* in the area *I* (Fig. 2*I*) was present after Cu treatment while it was absent in the control plants (data not shown), which could be a response to Cu stress and/or responsible for Cu tolerance. Using LA-ICP-MS imaging, Cu and Zn were shown in enrichment in this protein (Fig. 2*I*), indicating that the protein *a* could be a Cu- and Zn-containing protein. Fig. 2*II* shows three of the proteins (*b*, *c* and *e*) enriched in Zn, among which the two left-hand spots, *b* and *c*, were up-regulated after Cu treatment, suggesting that some of the Zn-containing proteins participated in the metabolism of the plant. Note that the amount of Cu in these proteins was much less, so that no clear distribution of Cu was found. However, in area *III* (Fig. 2*III*), a remarkable content of Cu was detected in the protein spot *h*, while the Zn content was con-

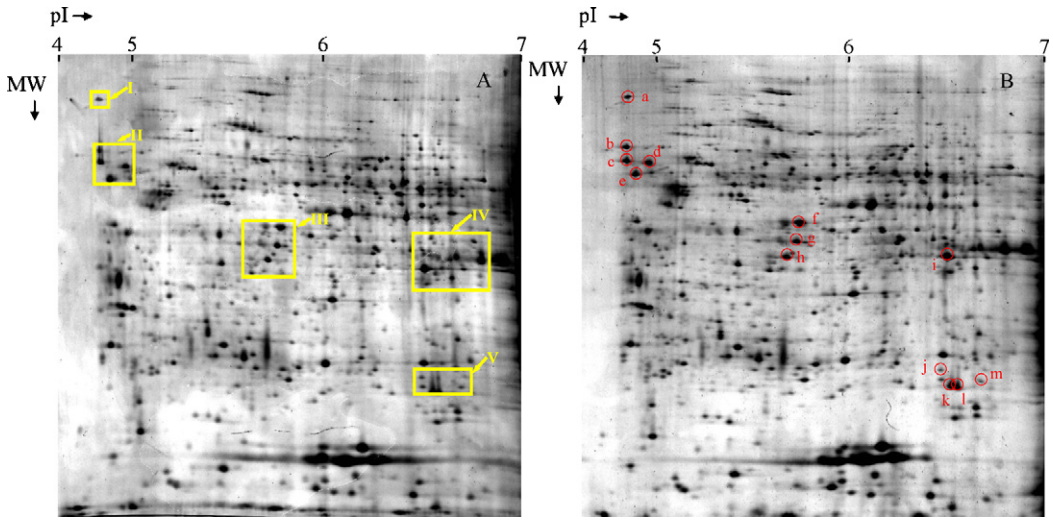

Fig. 1. 2D-PAGE gels of the root proteins of *E. splendens* after 100 μM Cu treatment (pH 4–7, non-linear). A and B were run in parallel. (A) Silver staining, used for LA-ICP-MS analysis and (B) stained with Sypro Ruby, used for LC–MS/MS studies. The yellow panes marked as Roman *I* to *V* in gel A indicate the LA-ICP-MS scanning areas, while the red circles marked with *a* to *m* in gel B are the proteins to be identified using LC–MS/MS. MW: molecular weight; p*I*: isoelectric point.

siderably less than in the neighbor spots like *g* and *f*. In area *IV* (Fig. 2*IV*), no metal was associated with the proteins, except for a little Cu concentrated in the protein *i*, although the resolution of the Cu image was relatively lower than in the area *III*. Mg was also found in some of the proteins in the areas *III* and *V*. Contrary to area *V* (Fig. 2*V*), the distribution of Mg in area *III* was similar to that of Zn. In area *V*, the most abundant proteins *k* and *l* contained a considerable quantity of Mg, while amounts of Zn and Cu were relatively lower, indicating these proteins could be Mg-containing proteins. Proteins *j* and *m* in area *V* were found Zn-enriched protein spots. Table 2 summarizes the metals detected in the protein spots in these five areas in 2D gel.

3.2. Identification of proteins by LC–MS/MS

To illustrate the relation between those metal-containing proteins found by LA-ICP-MS imaging and the Cu tolerance of the plant, characterization of the proteins was carried out using LC MS/MS. However, as the genome of *E. splendens* has not been chartered yet, the sequence information of the plant *E. splendens* is limited, resulting in a great difficulty for protein identification. In Fig. 3, the LC-ESI fragmentation mass spectrum of a peptide sequence from the protein spot *h* in area *III* is shown as an example. The protein–protein BLAST searches were performed using *Elsholtzia haichowensis* (taxid: 331068) as organism, since *E. haichowensis* is the most closely related species to *E. splendens*. Most of the protein samples did not yield significant matches, while three proteins (spot *h* in area *III*, spot *i* in area *IV* and spot *j* in area *V*) seemed to have similarities with metallothionein-like protein in the plant *E. haichowensis* which is also a Cu-tolerant plant [48], although with relatively low coverage. Nevertheless, the protein identification should be further conducted when the genomic data for *E. splendens* become available in databases.

Table 2
Relative contents of the detected metals in protein spots in the five areas of interest in 2D gel (–: not detected; +: detected with low content; ++: relative higher content; +++: significantly high content of metals detected).

Area	*Spot*	Ag	Cu	Zn	Mg
I	*a*	++	++	++	–
II	*b*	++	–	++	–
	c	+++	+	++	–
	d	+++	+	–	–
	e	+++	–	+++	–
III	*f*	+++	–	+++	++
	g	+++	–	++	+
	h	+++	+++	+	+
IV	*i*	+++	+	–	–
V	*j*	+	–	++	–
	k	+++	+	–	+++
	l	+++	+	–	+++
	m	+	–	++	++

3.3. Mechanisms of Cu tolerance and accumulation in E. splendens

By investigating the global proteome of *E. splendens* [46], it was found that the root proteins were clearly responsive to Cu toxicity with either up- or down-regulation expression. Those changed proteins were classified in various functional groups including cellular metabolic process, primary metabolic process, macromolecule metabolic process, response to stress, biosynthetic process, establishment of localization, and regulation of biological process and transport, indicating that the response to Cu in *E. splendens* was a complex biological process [46]. It was also suggested in this fundamental research that there were germin-like proteins (GLPs) which might possibly have been involved in binding the Cu. Germins and GLPs are a class of developmentally regulated glycoproteins and play an important role during embryogenesis, pathogen elicitation, as well as in the response to salt and heavy metal stress [49]. Despite having histidine-containing motifs, they are hardly found to be bound with heavy metals, as was also the case in the present study. With respect to Cu, metallothioneins (MTs) and phytochelatins (PCs), both cysteine-rich peptides, have been reported in many studies as potential candidates for Cu ligands via S in the thiol groups, and are thus responsible for Cu tolerance [50–53]. In germinating embryos of *Oryza sativa*, metallothionein-like protein, together with membrane-associated protein-like protein, putative wall-associated protein kinase, pathogenesis-related proteins and the putative small GTP-binding protein Rab2, were up-regulated by Cu stress [54]. In another Cu-tolerant plant from the same genus of *Elsholtzia*, *E. haichowensis*, Cu tolerance involved the production of Cu-induced thiol peptides [55]. In *E. splendens*, we found that the thiol peptides or metallothionein-like proteins similar to those in *E. haichowensis* seemed to play a part in binding Cu. However, since the coverage of the protein identification was very low, we would not consider such proteins as the main ligands acting in the Cu tol-

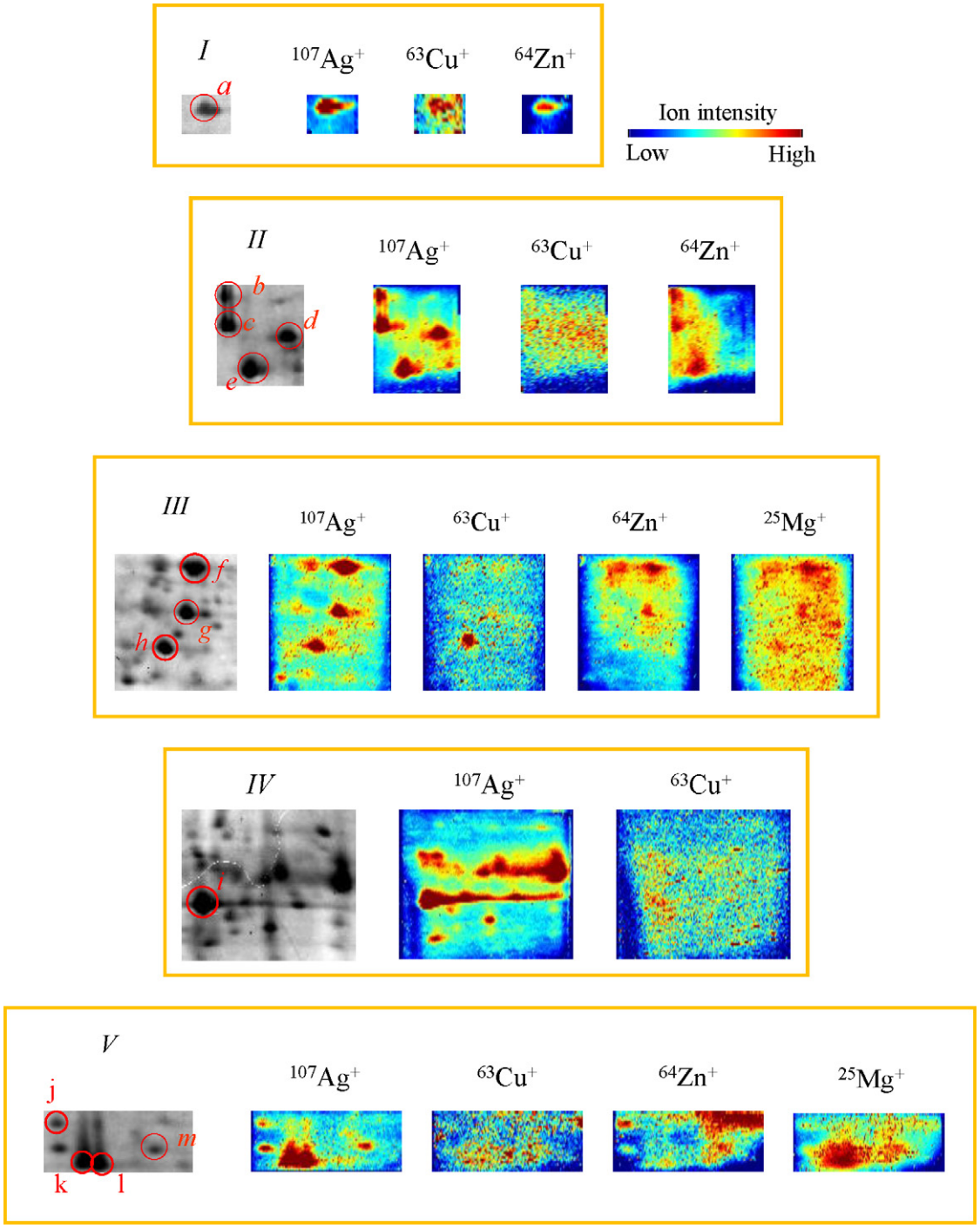

Fig. 2. Metal distributions in the selected areas *I* to *V* of the 2D gel of plant root proteins. The red circles marked *a* to *m* are the corresponding proteins to be identified in gel B using LC–MS/MS (*cf.* Fig. 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

erance in the plant. On the other hand, in some plants, other small molecular ligands like free amino acids, rather than MTs and/or PCs, have been found to chelate the Cu in plant cells [56–58,3]. In previous studies [45], it was revealed by synchrotron radiation X-ray absorption spectroscopy (SRXAS) that N/O ligands were dominant in the plant *E. splendens*, while S ligands were rare, suggesting that Cu bound by N/O ligands plays a key role in Cu detoxification of *E. splendens*. Furthermore, most Cu was bound with cell wall and histidine-like ligands, while a minor proportion of the Cu was bound to oxalate and glutathione-like ligands [45] which have been considered to be the precursor of PCs [59]. It was also found that the level of free histidine in the plant tissues was significantly elevated when excessive Cu was supplied in the nutrient solution (unpublished data), indicating free histidine could probably be a major candidate bound with Cu. This could be one of the reasons why few Cu-containing proteins were observed in 2D gels. Nevertheless, the affinity of free histidine and metallothionein-like proteins bound with Cu in *E. splendens* and their roles should be further investigated in order to fully illustrate the mechanisms for Cu tolerance and accumulation in the plant *E. splendens*.

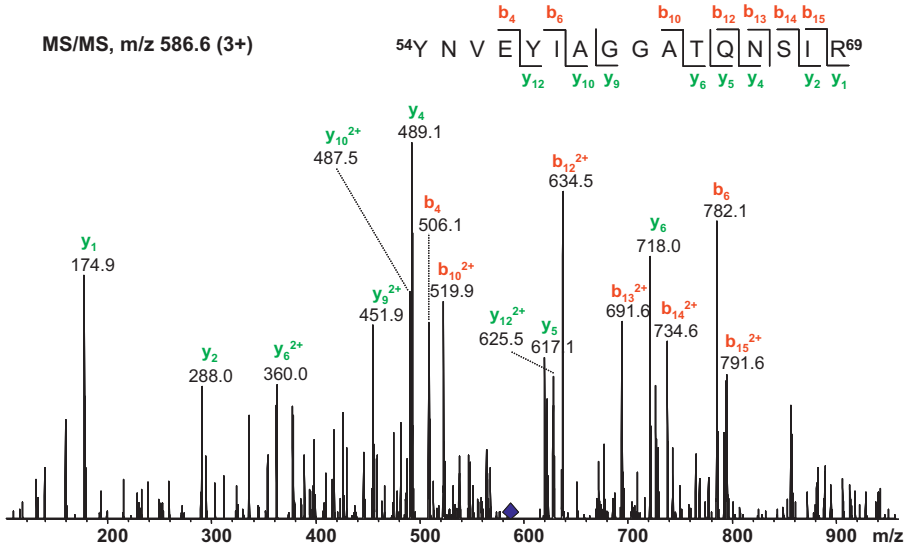

Fig. 3. LC-ESI fragmentation mass spectrum of a peptide from the spot h in area III (*cf.* Fig. 2.).

4. Conclusion

In the present study, metallomic strategies including LA-ICP-MS for screening metal-containing proteins on 2D gels and LC–MS/MS for characterizing the detected metal-containing proteins were used. It was shown that LA-ICP-MS imaging technique, via the images of Ag, was able to distinguish almost all the protein spots in 2D gels, even those with relative low abundance. The metal images were correlated very well with those obtained by Ag staining. Owing to the fact that the knowledge on the genome of *E. splendens* is limited, the protein identification was difficult to achieve. Only a few spots were found to be similar to metallothionein-like proteins in *E. haichowensis*. However, since only a few Cu-containing proteins were found and identified, we would not attribute these metallothionein-like proteins to the major chelators for Cu. Other ligands like free histidine would be the main candidates for Cu and would be considered responsible for Cu tolerance and accumulation in *E. splendens*. The mechanisms for Cu tolerance and accumulation in the plant will be further studied with respect to Cu chelators and species.

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