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Proteomic identification of divalent metal cation binding proteins in plant mitochondria

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Abstract **Divalent metal binding proteins in the *Arabidopsis* mitochondrial proteome were analysed by mobility shifts in the presence of divalent cations during two-dimensional diagonal sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Tandem mass spectrometry and searches of the predicted *Arabidopsis* protein dataset were used in an attempt to identify 34 of the proteins which shifted. This analysis identified a total of 23 distinct protein spots as the products of at least 11 different *Arabidopsis* genes. A series of proteins known to be divalent cation-binding proteins, or to catalyse divalent cation-dependent reactions, were identified. These included: succinyl CoA ligase β subunit, Mn-superoxide dismutase (SOD), an Fe–S centred component of complex I and the REISKE iron–sulphur protein of the b/c_1 complex. A further set of four proteins of known function but without known divalent binding properties were also identified: the Vb subunit of cytochrome c oxidase, a subunit of ATP synthase (orfB), the acyl carrier protein, and the translocase of the outer membrane (TOM20). Three other proteins, of unknown function, were also found to shift in the presence of divalent cations. This approach has broad application for the identification of sub-proteomes based on the metal interaction of polypeptides.**

Key words: Plant mitochondrion; Divalent cation binding protein; Proteomics

1. Introduction

A range of divalent metal cations interact with proteins facilitating biological functions in enzyme catalysis and cell signalling. At one time, metalloproteins were considered distinct from metal–protein complexes, where the former contained high-affinity interactions that were not lost by isolation and dilution of proteins, while the latter's interactions were lower-affinity and were easily lost during sample handling [1]. However, it is now considered that a continuum exists of biologically relevant metal ion binding to proteins, including the often weakly interacting monovalent ions such as K^+ and Na^+, the moderately interacting divalent ions such as Mg^{2+}, Ca^{2+}, as well as the high-energy protein binding of transition metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Mo^{2+} [2,3]. Transition metal ions contain dense, small atomic radii and interact via both electromagnetic as well as electrostatic attraction, making such coordinating interactions the strongest of the metal–protein interactions known [2]. The structural rearrangements induced by all types of metal ion binding confer stability to proteins by restricting the mobility of domains via non-covalent cross-linking of charged amino acid side-groups [4]. Such interactions are able to stabilise the active conformation of proteins, to directly facilitate enzymatic reactions by active site binding and interaction with substrates of products, or to directly participate in the reaction by electron acceptance and donation [3]. Understanding the range of proteins that undertake such interactions and the biological implications of these interactions is of broad interest to researchers.

A range of methods for detecting metal binding by target proteins has been developed, including biophysical techniques for observing conformational changes or changes in absorbance spectra [4], radiolabelled metal ion overlays on electrophoresis gels or blotted membranes [5], and the use of metal-affinity columns [6]. However, these approaches generally require a purified or semi-purified target of interest, do not facilitate identification of unknown targets from complex protein mixtures, or require complex multi-step processes and very specialised equipment. The metal affinity shift assay developed by Kameshita and Fujisawa [7] was based on the simple principle that binding of metal ions to proteins changes both the charge characteristics and the conformation of proteins altering mobility during electrophoresis. These researchers found that even under the harsh denaturing conditions of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), changes in mobility of metal-binding proteins could be detected. Using combinations of either divalent cation (Ca^{2+}/Mg^{2+}) treatment followed by or preceded by chelator (ethylenediamine tetra-acetic acid (EDTA)/ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)) treatment in diagonal PAGE gels, divalent cation binding proteins have been purified from complex mixtures of rat cerebral cortex [7]. This same technique has been used by Gye et al. [8] to characterise changes in expression of divalent cation-binding proteins during sperm maturation in mice. However, this technique has yet to be used to identify metalloproteins from samples containing a mixture of proteins of unknown function.

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Abbreviations: EDTA, ethylenediamine tetra-acetic acid; EGTA, ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid

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We have been investigating the proteome of plant mitochondria from *Arabidopsis thaliana*, a model plant species. Eukaryotic mitochondria contain specific transporters for divalent metal ions that are required for a large number of metal ion-dependent reactions involved in primary respiratory metabolism [9]. The exact protein subunits that bind metal ions in these reactions, many involving complex multi-subunit enzymes, are often unknown. A large number of proteins of unknown function are also found in plant mitochondria, awaiting information that may lead to their functional roles [10,11]. Using a combination of the divalent cation-shift electrophoresis approach and proteomic identification of shifting proteins, we have identified a series of 11 plant mitochondrial proteins putatively involved in divalent metal–protein interactions, some with unknown function.

2. Materials and methods

2.1. Arabidopsis *mitochondria isolation and fractionation*

A heterotrophic *A. thaliana* cell culture, established from callus of ecotype Lansberg *erecta* stem explants, was maintained on Murashige and Skoog basal media supplemented with 3% (w/v) sucrose, 0.5 mg/l naphthaleneacetic acid and 0.05 mg/l kinetin [12]. The cell cultures were grown in 250 ml conical flasks in the dark at 22°C in an orbital shaker (150 rpm). At 6–7 days after sub-culture, mitochondria were isolated from these cell suspension cultures using Percoll density gradient centrifugation according to Millar et al. [10].

2.2. *Electrophoresis*

Two-dimensional SDS–PAGE was performed primarily according to Kameshita and Fujisawa [7]. The first dimension SDS–PAGE was performed according to standard protocols, except that either $CaCl_2$ (2 mM) or EDTA (2 mM) was added to both the separating and stacking phases before polymerisation. Mitochondria samples (100 μg protein/lane) were boiled briefly before loading. Once the first dimension was completed, individual lanes were cut from the gel and incubated in an equilibration buffer (0.125 mM Tris–HCl pH 6.5, 0.1% (w/v) SDS, 20% (w/v) glycerol, 2% β-mercaptoethanol, 0.001% (w/v) bromophenol blue) containing either $CaCl_2$ (2 mM) or EDTA (2 mM) for 10 min. The equilibrated gel slices were then placed horizontally onto second-dimension SDS–PAGE gels polymerised in either 2 mM $CaCl_2$ or 2 mM EDTA. The first-dimension gel slices were sealed in place by 1% (w/v) Agarose in gel running buffer. Once the second SDS–PAGE was completed, gels were stained using colloidal Coomassie (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3% (v/v) phosphoric acid, 0.1% (w/v) Coomassie brilliant blue G250) for 16 h and destained in 0.5% (v/v) phosphoric acid. To examine the reproducibility of protein mobility shifts, various combinations of EDTA and $CaCl_2$ were used between the first- and second-dimension gel separations. Once stained, protein spots clearly shifted off the diagonal of bulk protein were sampled as gel plugs from cut pipette tip excisions, and removed for MS/MS analysis.

2.3. *Quadrupole time-of-flight mass spectrometry (Q-TOF MS)*

Q-TOF MS/MS was performed on an Applied Biosystems Q-STAR Pulsar (Q-TOF MS) using an IonSpray source. Proteins to be analysed were cut from 2-D PAGE gels, destained, dried at 50°C in a dry block heater, trypsinated according to [13] and stored at −70°C. For the sequencing analysis, resultant peptides were injected in 50% methanol/0.1% formic acid and selected doubly charged peptides fragmented by N_2 collision and analysed by MS/MS.

2.4. *Bioinformatic analysis*

Mass spectra and collision MS/MS data were analysed with BioAnalyst software (Applied Biosystems, Sydney, Australia) and Mascot (http://www.matrixscience.com) to identify matching *Arabidopsis* protein entries. Sub-cellular targeting of predicted protein sequences was performed with TargetP (http://www.cbs.dtu.dk/services/TargetP/), Predotar (http://www.inra.fr/Internet/Produits/Predotar/) and MitoProtII (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) as directed on these websites.

3. Results and discussion

3.1. *Detection of metal-binding proteins in* Arabidopsis *mitochondria by 2-D SDS–PAGE*

When protein samples were subjected to 2-D SDS–PAGE without treatment, lanes excised, rotated 90° and again sub-

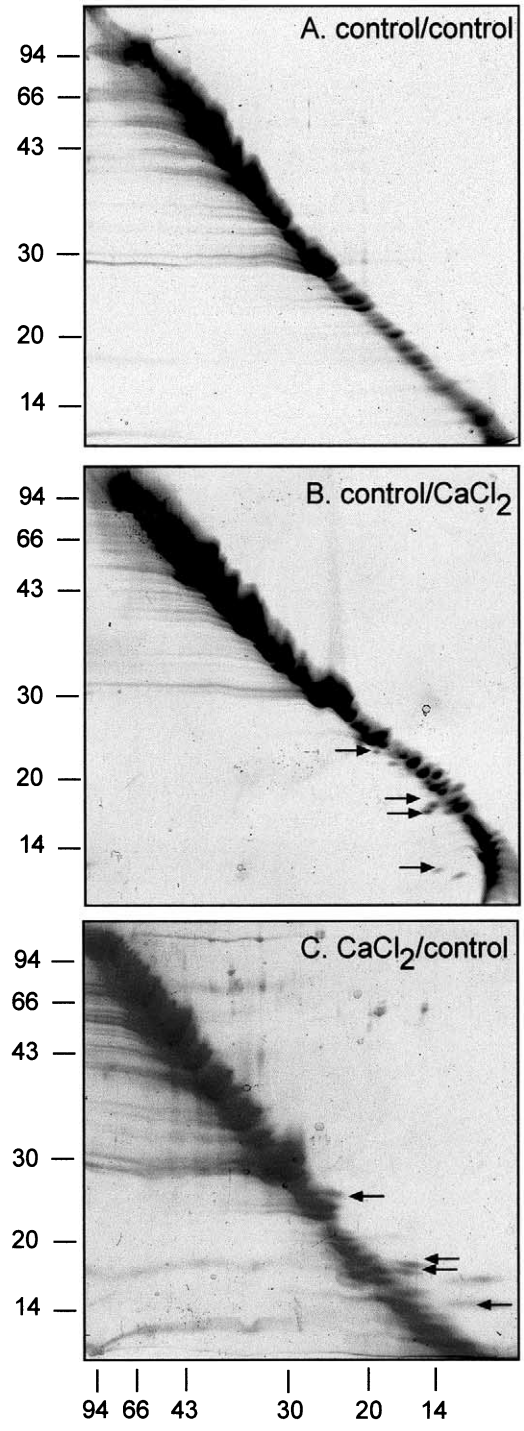

Fig. 1. Separation of *Arabidopsis* mitochondrial proteins by two-dimensional divalent metal cation shift electrophoresis. A: 1-D control/2-D control. B: 1-D control/2-D $CaCl_2$. C: 1-D $CaCl_2$/2-D control. Numbers are apparent molecular mass in kDa. Arrows indicate consistently shifting spots in panels B and C.

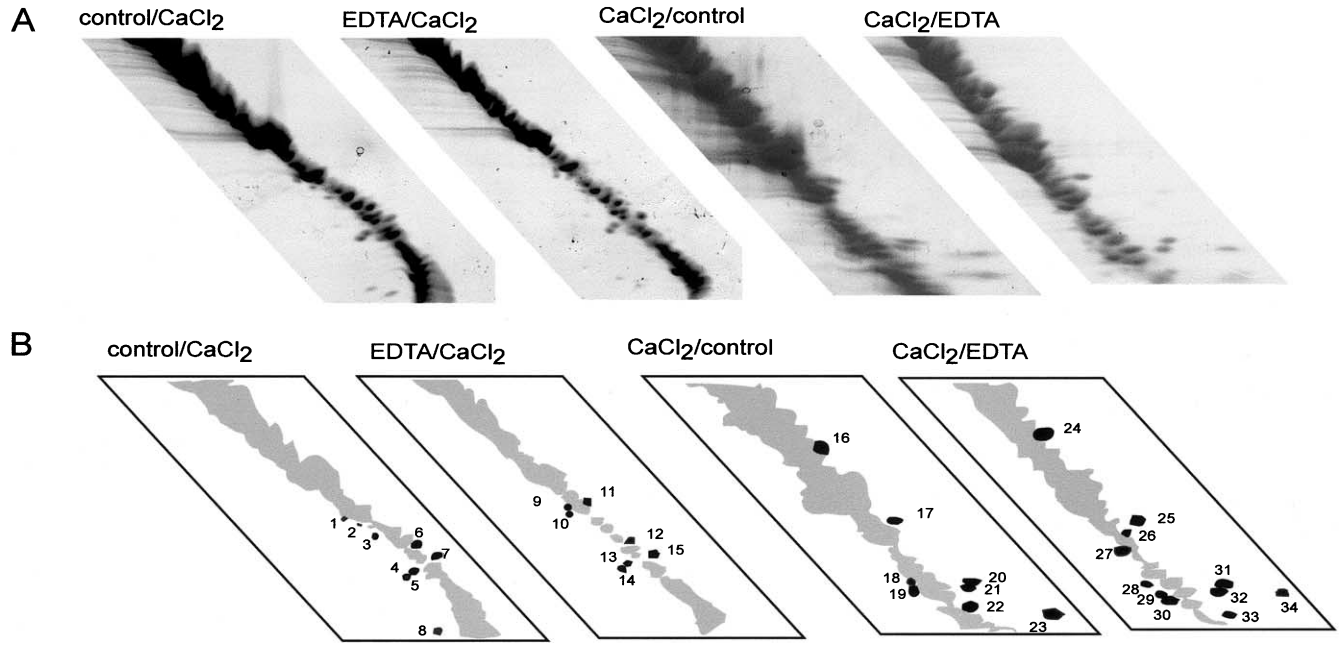

Fig. 2. Divalent cation-shifted *Arabidopsis* mitochondrial proteins separated for analysis by mass spectrometry. A: Coomassie stained diagonal gel sections showing shifting protein profiles in control/$CaCl_2$, EDTA/$CaCl_2$, $CaCl_2$/control, $CaCl_2$/EDTA. B: Cartoon of gel sections in panel A. The 34 spots indicated in black were characterised by mass spectrometry (Table 1); bulk protein on diagonals is shown in grey.

jected to SDS–PAGE, they gave an expected diagonal straight line of protein products observed in Fig. 1A. On the other hand, when electrophoresis was carried out without treatment in the first dimension, but with $CaCl_2$ in the second dimension, a series of protein spots were apparent that had shifted from the straight diagonal (Fig. 1B). These protein spots represent polypeptides whose mobility in SDS–PAGE changes with the availability of $CaCl_2$. Clearly, most proteins present in mitochondrial samples were unaffected by this treatment and only a very small number of discrete proteins shift in response to increased metal cation concentration. When electrophoresis was carried out first in the presence of $CaCl_2$ and then in its absence (Fig. 1C), again protein spots not aligned to the diagonal were detected. When treatments with $CaCl_2$ or EDTA were performed in both first and second dimensions, no protein spots migrating off the diagonal were observed (data not shown). Kameshita and Fujisawa [7] showed that many of the spots that migrate differentially in the presence of Ca^{2+}, also do so in the presence of Mg^{2+}. Many functional studies have shown that the endogenous divalent metal centres of proteins can often be extensively interchanged in vitro [4]. Such changes may or may not adversely affect the function of these proteins. This strongly suggests that while the shifts induced by $CaCl_2$ in this study are probably reflective of the presence of divalent metal-binding sites, they may not indicate that Ca^{2+} is the specific endogenous ligand of all the proteins.

3.2. *Isolation and identification of metal-binding proteins from* Arabidopsis *mitochondria*

We have used the mobility shift approach outlined above, in the presence and absence of $CaCl_2$ or the chelator EDTA, to probe for metal-binding proteins. EDTA provided more stringent stripping of metals before first-dimension electrophoresis or after initial electrophoresis in the presence of metal ions. The patterns of spots which shifted during the four treatments are shown in Fig. 2A and the spots excised from

Table 1
Identification of peptides derived from shifted proteins by MS/MS analysis

Spot number	ID	MM	Description	MP	MOWSE	Percentage coverage
1, 10, 25	At1g79010, At1g16700	25	NDUFS8 complex I subunit	2–4	24–62	11–14
16, 24	At5g08300	36	succinyl–CoA ligase α subunit	4–8	150–243	25–32
9, 26	At5g13440, At5g13430	30	REISKE iron–sulphur protein	4	39–96	14–16
11, 27	At3g10920	25	Mn–superoxide dismutase	15	291–297	38–43
4, 21	At3g15640	19	Cytochrome c oxidase Vb subunit	2	29–38	7–8
8, 23	At1g65290	14	Acyl carrier protein	2–3	58–60	
7, 15	AtMg00031	18	OrfB	3–5	52–71	13–17
2	At1g27390	23	Translocase outer membrane 20	3	49	12
3	At2g16460	26	Unknown protein	3	80	13
22	At3g15660	19	Unknown protein	5	62	26
6, 12, 19, 29, 30	At3g62530	24	Unknown protein	8–15	188–273	28–50

Protein spots from Fig. 2 were excised, digested with trypsin and multiply charged peptides fragmented and analysed by MS/MS. The predicted molecular mass of the matched protein (MM) is shown. Significance of matches is supported by the number of matching peptides (MP), the combined MOWSE score of the matching peptides (MOWSE) and the percentage of the predicted protein covered by the matched peptides (percentage coverage).

Table 2
Expression and predicted mitochondrial targeting of putative metal-binding unknown function proteins

ID	MM	ESTs	Predotar	TargetP	MitoPII	*E* value	BLAST match
At2g16460	26	11	M	M	–	2.00E−20	24 kDa yeast hypothetical protein (P43557)
At3g15660	19	4	C	M	M	1.00E−21	12 kDa Rickettsia glutaredoxin-like protein (O05957)
At3g62530	25	9	M	M	–	1.00E−14	36 kDa yeast hypothetical protein (P47120)

The number of ESTs for each protein in the TIGR TC assignments is shown along with the predicted targeting of each hypothetical protein by the Predotar, TargetP and MitoProt II (MitoPII) prediction programmes. The highest-similarity BLAST match to the NCBI database along with the *E* value for this similarity is shown.

gel pieces for analysis are highlighted in Fig. 2B. After in-gel digestion and peptide elution, samples from each protein spot were subjected to tandem mass spectrometry and MS/MS data matched to databases of theoretical *Arabidopsis* proteins. This analysis identified 23 of the 34 shifting protein spots as shown in Table 1. While 23 protein spots were identified, these only represented 11 different *Arabidopsis* gene loci, indicating that some proteins were found more than once. Three proteins were only identified once, six proteins were identified twice, and two proteins were identified three or five times in the analysis. Spots 5, 13, 14, 17, 18, 20, 28, 31, 32, 33 and 34 could not be unambiguously identified due to lack of sufficient peptide matches to single *Arabidopsis* protein entries, and are not included in Table 1.

3.3. Known metal-binding mitochondrial proteins identified

Succinyl–CoA synthetase hydrolyses the high-energy compound succinyl–CoA with the coupled synthesis of GTP in mammals and ATP in plants. The plant enzyme has received little attention and the only detailed work on its kinetics dates back to a study by Palmer and Wedding [14]. These researchers highlighted the metal-ion cofactor required for this enzyme and showed its function by addition of Mg^{2+}, Mn^{2+} or Co^{2+}. Succinyl–CoA synthetase is a heterodimer and our results suggest that the α subunit is most likely responsible for the metal binding (Table 1). The mitochondrial form of superoxide dismutase (SOD) contains a transition metal centre with a preference for Mn^{2+}. The coordination of this metal by cysteine residues most likely enables other divalent ions to occupy this site and thus explains the shift in in the presence of Ca^{2+} observed in Fig. 2 and identified in Table 1. The 23 kDa subunit of complex I is one of the eight predicted Fe–S centre proteins of this complex in eukaryotes [15]. The RE-ISKE Fe–S protein of complex III contains a metal centre vital for the electron transport function of this complex [16]. The strong coordination centre of these Fe–S centres involves clusters of cysteine residues that will likely coordinate other divalent ions, as suggested by the shifts shown in Fig. 2. These four proteins were consistently observed on metal ion shift gels in our hands and provide confirmation that this approach identifies a subset of metal-binding proteins.

3.4. Known mitochondrial proteins newly identified as putative metal-binding proteins

OrfB is a putative component of the plant mitochondrial F_1F_O ATP synthase [17]. To our knowledge, the data from Table 1 represent the first evidence of metal ion binding for this protein. The acyl carrier protein is involved in fatty acid biosynthesis and in mitochondria is located within complex I of the respiratory chain, where it functions to deliver lipoic acid to the reaction centres of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes, as well as the H subunit of the glycine decarboxylase complex in plants [18,19]. If lipoic acid is attached to ACP, the sulphdryls present under reducing conditions may allow metal coordination and could explain the shift of this protein. Alternatively, a distinct metal-binding site may be present. The cytochrome oxidase (COX) subunit Vb is nuclear encoded and while its exact function in the electron transport chain complex is unknown, it is not regarded as a metal-binding site in the complex. In mammals, COXVb has been reported to bind the regulatory subunit of c-AMP-dependent mitochondrial protein kinase and may be involved, therefore, in the ATP regulation of COX [20]. Whether a similar process occurs in plants has yet to be established. The TOM20 protein is a receptor on the outer mitochondrial membrane involved in import of precursor proteins from the cell cytosol [21]. We have not found any literature citation of a metal requirement for this receptor. It is possible that the proteins found here do not all bind metals in their native state as our experiments were all performed under denaturing conditions. Determination of whether these findings translate to a metal-binding characteristic of the native protein will require further experimentation but this research has highlighted candidates for such analysis.

3.5. Novel proteins identified as putative metal-binding proteins

The three proteins of unknown function presented in Table 1 represent potentially novel metal-binding proteins in plant mitochondria. Similarity searches with protein sequences yielded best matches for two of the proteins to other unknown function proteins in yeast (Table 2). One of the three has some similarity to a protein in Rickettsia, the closest related living bacteria to the likely progenitor of modern mitochondria, which is similar to the thioltransferase proteins known as glutaredoxins. All three proteins are expressed in *Arabidopsis* based on our analysis (Table 1) and their apparent representation in EST databases (Table 2). Targeting prediction programmes clearly reveal that all three are likely to be authentic mitochondria proteins and not contamination from other cellular sources.

3.6. Metal shift electrophoresis for divalent ion-binding protein discovery

Kameshita and Fujisawa [7] showed, perhaps surprisingly,

that conformational or charge differences induced by metal ion binding to SDS-denatured polypeptides were sufficient to significantly shift their apparent molecular mass during electrophoresis. Kameshita and Fujisawa [7] and later Gye et al. [8] used this assay to show a pattern of shifting polypeptides in protein samples from different mammalian tissues. However, apart from the use of known divalent cation-binding proteins as controls, these authors did not identity these shifting proteins. Here we have used the shifting property to purify small quantities of proteins and then identified them with mass spectrometry. The analysis revealed a selection of known and unknown putative divalent metal ion-binding proteins in plant mitochondria. Clearly a range of known metal-binding proteins in the mitochondrial electron transport chain, metabolism and proteolysis machinery were not identified in our analysis. This suggests their metal-binding sites were either not present following detergent denaturation or they did not shift mobility by metal binding. In order to identify these proteins alternative metal affinity methods need to be exploited and/or developed. However, in our hands, this simple electrophoretic approach provides a valuable initial tool for the enrichment of putative metal-binding proteins from complex mixtures and could be broadly and easily applied to other biological systems.

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