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Cytoplasmic regulation of the accumulation of nuclear-encoded proteins in the mitochondrial proteome of maize

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Summary

Mitochondria from normal (NA)- and Texas (T)-cytoplasm maize (*Zea mays* L.) were purified from unpollinated ears via Percoll centrifugation. Approximately 300 mitochondrial proteins were resolved using two-dimensional (2-D) electrophoresis. The 197 most abundant proteins were analyzed by matrix-assisted laser desorption Ionization time-of-flight (MALDI-ToF) mass spectrometry involving overlapping pH gradients (pH 4–7 and 6–9). Database searches identified 58 genes that encode 100 of these protein spots. Functions could be predicted for 38 of the 58 genes (66%). All but one of these genes are located in the nuclear genome. Thirteen per cent of the analyzed protein spots (25 out of 197) exhibited at least a threefold difference in accumulation between the mitochondrial proteomes of NA- or T-cytoplasm maize plants that had essentially identical nuclear genomes. As most of these proteins were nuclear-encoded, these findings demonstrate that the genotype of a mitochondrion can regulate the accumulation of the nuclear-encoded fraction of its proteome. About half (27 out of 58) of the maize mitochondrial proteins identified in this study were not recovered in previous analyses of the *Arabidopsis* and rice mitochondrial proteomes.

Keywords: maize, mitochondria, proteomics, T-cytoplasm, NA-cytoplasm.

Introduction

Mitochondria are the energy-converting organelles of eukaryotic cells and contain their own genomes. Plant mitochondrial genomes are larger in size and coding capacity and show a higher variability in their organization than do the mitochondrial genomes of mammals or fungi (Fauron *et al.*, 1995). Several plant mitochondrial genomes, including the angiosperms *Arabidopsis thaliana* (Unseld *et al.*, 1997) and *Beta vulgaris* (Kubo *et al.*, 2000) with sizes of 367 and 369 kb, respectively, have been sequenced. These two genomes contain 57 and 59 genes, respectively. Although the mitochondrially encoded proteins can be defined by sequencing, during evolution many mitochondrial genes were transferred to the nuclear genome (Henze and Martin, 2001). As a consequence, analysis of a mitochondrial genome sequence will not define the complete mitochondrial proteome.

Nuclear-encoded mitochondrial proteins are directed to the mitochondria by N-terminal mitochondrial targeting presequences that are cleaved during or after import into the mitochondria (Sjöling and Glaser, 1998). Mitochondrial targeting presequences share common features, including having a positive charge, being hydroxylated, or having the capacity of forming amphiphatic α-helices (Sjöling and Glaser, 1998).

Two-dimensional (2-D) protein electrophoresis followed by mass spectrometric analysis of separated proteins is a powerful tool to identify large numbers of proteins in a proteome (Goerg *et al.*, 2000). A single-step characterization of the complete proteome of a cell, however, is beyond current technology as a consequence of the large number of cellular proteins with varying levels of abundance and diverse isoelectric points, hydrophobicities and molecular

masses (M_rs; Jung *et al.*, 2000). Subcellular fractionations reduce complexity and thereby increase the resolution of proteomic experiments. They also allow for the characterization of the proteomes of individual organelles.

Few studies have been conducted on subcellular proteomes of plants. In addition to analyses of the proteomes of plasma membranes (Santoni *et al.*, 1998, 2000), the endoplasmatic reticulum (Prime *et al.*, 2000), the leaf peroxisomes (Fukao *et al.*, 2002) of *Arabidopsis* and the chloroplast (Peltier *et al.*, 2000) of pea, two extensive studies of the *Arabidopsis* mitochondrial proteome (Kruft *et al.*, 2001; Millar *et al.*, 2001), one of the highly abundant proteins of several mitochondrial proteomes of pea (Bardel *et al.*, 2002), and an analysis of the rice mitochondrial proteome (Heazlewood *et al.*, 2003) are available.

The mitochondrial genomes of normal (NA)- and Texas (T)-cytoplasm maize can be distinguished via RFLP analysis, gene content, and by the fact that the latter is associated with cytoplasmically inherited male sterility (Fauron *et al.*, 1995). This study provides an analysis of the mitochondrial proteomes of NA- and T-cytoplasm maize and thereby identified 27 nuclear-encoded proteins that were not previously known to be imported into the mitochondria. In addition, it reveals that nuclear-encoded proteins and modified forms of proteins accumulate in a cytoplasm-specific manner, thereby demonstrating that mitochondrial genomes can differentially regulate the expression of nuclear genes or the import/stability of nuclear-encoded proteins.

Results

Purification and solubilization of mitochondrial proteins

Mitochondrial proteins of NA- and T-cytoplasm versions of the inbred line Ky21 were purified from immature, unpollinated maize ears. We used a two-step purification strategy. Mitochondrial extracts were first subjected to differential centrifugation in a buffer containing 0.4 M sucrose. Extracts were then purified on a three-step Percoll gradient (45, 21, and 13.5%) in which mitochondria accumulate at the interface between 45 and 21%. A combination of the zwitterionic amidosulfobetaines (ASB)14 and ASB16 in a mixture of thiourea and urea provided the best results for dissolving mitochondrial proteins (data not shown).

To be useful for analyzing the mitochondrial proteome, protein preparations must be free of contaminating proteins from other subcellular compartments. Although there are several ways to detect such contamination, the most common is via assays for enzymes that are known to be located in other subcellular compartments. Cytochrome *c* oxidase was used as a marker for the mitochondrial fraction. The mitochondrial protein preparations from the NA- and T-cytoplasm versions of Ky21 both contained cytochrome *c* activity (Table 1). Alcohol dehydrogenase (ADH) and catalase were used as markers for cytosolic and microsomal contamination, respectively (Table 1). In both cases, enzyme activities were similar to the no-protein negative controls. Hence, these enzyme assays demonstrated that our mitochondrial protein preparations were not significantly contaminated by either cytosolic or microsomal proteins.

Chloroplast contamination can be a major concern in mitochondrial preparations. We therefore decided to work with non-green maize ears, which should not contain differentiated chloroplasts. Nevertheless, the samples were assayed for chloroplast contamination via the method of Kruft *et al.* (2001). This approach involves assaying for the presence of the most abundant chloroplast proteins (i.e. those in the two photosystems and the b_6f complex) on 2-D gels. If our mitochondrial preparations had been contaminated with chloroplasts or broken thylakoids, it would be expected that many of the abundant chloroplastic proteins identified in the extensive studies of the proteome of green maize leaves conducted by Porubleva *et al.* (2001) and of the pea chloroplast proteome by Peltier *et al.* (2000) would have been recovered in this study. As none of these abundant chloroplastic proteins was identified (see Supplementary Material), we conclude that our mitochondrial protein preparations were free of chloroplast contamination.

Table 1 Purity of mitochondrial preparations

Marker enzyme	(NA) Ky21	(T) Ky21	Negative control[a]	Positive control[b]
ADH	0.003 ± 0.001[c]	0.002 ± 0.001	0.002 ± 0.001	0.23 ± 0.05
Catalase	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.19 ± 0.04
Cytochrome *c* oxidase	0.10 ± 0.01	0.22 ± 0.08	0.005 ± 0.003	ND[d]

Purity of mitochondrial preparations was assayed using marker enzymes. ADH, catalase, and cytochrome *c* oxidase activities are markers for the cytosol, microsome, and mitochondria, respectively. Enzyme activities are expressed as arbitrary units per minute per milligram of protein. Each value represents the average of three enzyme assays.

[a]Reaction conducted without the addition of the mitochondrial preparation.

[b]Reaction conducted with commercial enzyme substituted for the mitochondrial preparation.

[c]SD.

[d]Not determined.

Although immature ears do not contain chloroplasts, they do contain proplastids. Even though proplastids do not co-purify with mitochondria, we assayed for the presence of the very abundant angiosperm etioplast protein protochlorophyllide oxidoreductase (NADPH:POR) found in the prolamellar body (Ougham *et al.*, 2001) and for PHYA, another abundant etioplast protein (von Arnim and Deng, 1996). The purity of our samples was confirmed by the absence of these proteins from our samples.

Two-dimensional separation of mitochondrial proteins

Isoelectric focusing of purified mitochondrial protein extracts was performed using two overlapping pH gradients: pH 4–7 and pH 6–9. This approach increased the probability of separating proteins with similar isoelectric points (p*I*) and therefore enhanced resolution. After the separation of proteins according to their M_rs in a second dimension and staining with Coomassie blue, it was possible to detect about 250 protein spots on the acidic maps and about 50 on the basic maps. Three independent mitochondrial protein extracts from each genotype were subjected to 2-D gel electrophoresis under both acidic and basic conditions.

The computer program PDQUEST (Bio-Rad Laboratories, Hercules, CA, USA) was used to filter and smoothen the original scans of the gels, then 'Gaussian spots' were obtained from the clarified spots. Gaussian modeling is used because the image profile of an ideal protein spot conforms to a Gaussian curve (Brandle *et al.*, 2000). The maps in Figure 1 are synthetic match sets created using PDQUEST. Each map was generated using data from the three gels made for each pH gradient/cytoplasm combination and represents average normalized spot intensities as calculated by PDQUEST (Experimental procedures). Average normalized spot intensities from the three gels are provided in the Supplementary Material. Overall, excellent reproducibility of spot intensities was obtained among the three replicates of a cytoplasm; the average percentage coefficient of variance (C.V.) ((SD/arithmetic mean value) $\times$ 100) per spot for NA- and T-cytoplasm gels was 38 and 39%, respectively, which are similar to those observed by Chang *et al.* (2000). As expected, less abundant proteins often had higher C.V.s.

Identification of mitochondrial proteins

Protein spots were eluted and digested with trypsin, and the resulting peptide mixtures were analyzed via MALDI-ToF mass spectrometry and database searches. Peptide mixtures that did not yield good spectra were concentrated with a speed vac and analyzed again. Proteins that were not identified in this manner were eluted from a different gel and analyzed again. In combination, these procedures resulted in the identification of 51% of the protein spots analyzed via MALDI-ToF mass spectrometry (100 out of 197 spots).

Automated MS-FIT software was used to search protein databases with the resulting spectra. A protein identified via the MS-FIT database search was accepted as correct only if criteria similar to those used by Porubleva *et al.* (2001) were met. First, the deviations between experimentally determined and predicted M_rs of peptides were required to be less than 50 p.p.m., and the difference between the smallest and largest deviations was not allowed to exceed 30 p.p.m. Second, at least four peptides were required to match the expected M_rs and only one missed cleavage was allowed. Third, the matching tryptic fragments (peptides) were required to represent $>$15% of the protein. Fourth, the molecular weight search (MOWSE) score (Pappin *et al.*, 1993), which indicates that the probability of a true positive identification was required to be higher than 1000. Finally, the experimentally determined M_r of a protein was required to be within 20% of the predicted M_r of its protein match.

From the acidic map (pH 4–7), the 163 most abundant protein spots were picked and analyzed spectrometrically. Of these proteins, 156 yielded high quality MALDI-ToF spectra, and 85 of these spectra were identified by matching known proteins or translated plant expressed sequence tags (ESTs). From the basic map (pH 6–9) the 34 most abundant proteins were similarly analyzed. Thirty of these proteins yielded high quality spectra and 15 were identified via database searches.

In summary, a total of 197 mitochondrial proteins were analyzed and 100 (51%) were identified via mass spectrometry and database searches. These 100 proteins are encoded by 58 different GenBank accessions. It was possible to identify 83% (49 out of 58) of the accessions via comparisons to known maize proteins or the 138 000 maize ESTs available from http://www.zmdb.iastate.edu/. The remaining proteins were identified via their similarity to proteins or ESTs from other plant species. Thirty-six of the protein spots, representing 13 different GenBank accessions, were identified via comparisons to the annotated protein database NCBInr.8.17.2002. Sixty-four, encoded by 45 GenBank accessions, were identified via comparisons to the EST databases dbEST.others.5.30.2002 and zmdb.TUC and TUS as of 4.7.2003.

Consistent with their mitochondrial localization, MITOPROT (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) predicted that most of these proteins contain N-terminal mitochondrial matrix import sequences (see Supplementary Material). The few exceptions that did not contain mitochondrial targeting presequences include proteins encoded by the mitochondrial genome such as the F1-ATPase alpha subunit (spots 8, 10–15, 18–21, and 24) and several proteins that are known to be imported into

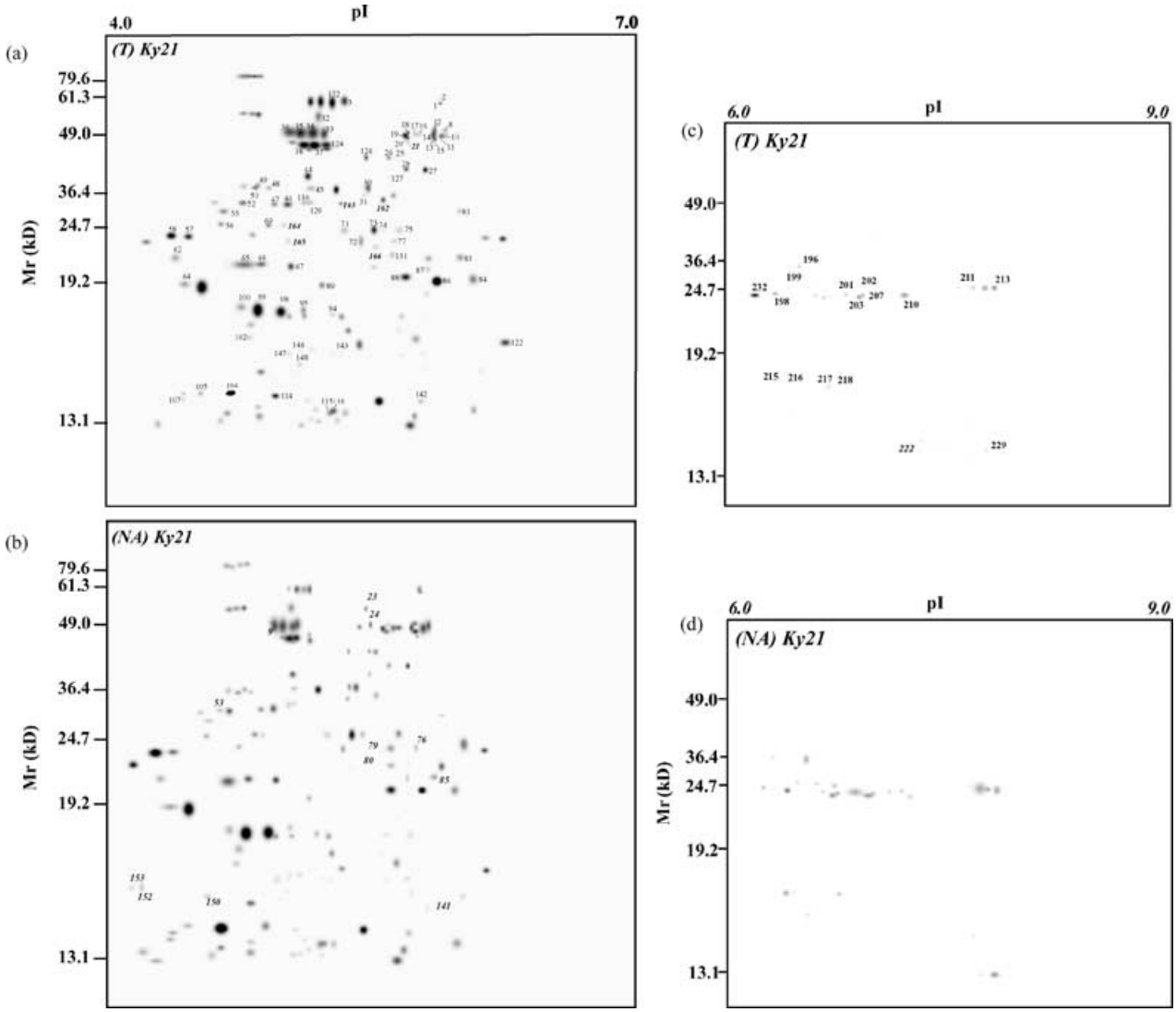

Figure 1. Two-dimensional maps of proteins isolated from mitochondrial extracts from immature, unpollinated ears of NA- and T-cytoplasm versions of the inbred line Ky21.

Maps for each genotype were prepared from three 2-D gels conducted using three independent mitochondrial preparations. Proteins were separated in the first dimension according to their p*I*s on an IPG strip pH 4–7 (a,b) or pH 6–9 (c,d) and in the second dimension according to their masses (M_r kDa) on a linear 12–18% SDS-polyacrylamide gel. Proteins were stained with colloidal Coomassie blue. Proteins spots that were identified via MALDI-ToF spectrometry or that showed at least a threefold difference in accumulation between NA- and T-cytoplasm are numbered on the maps. Data from these maps are summarized in Table 2 and in the Supplementary Material. (a), T-cytoplasm Ky21, pH 4–7; (b), NA-cytoplasm Ky21 pH 4–7; (c), T-cytoplasm Ky21, pH 6–9; (d), NA-cytoplasm Ky21, pH 6–9.

mitochondria but that do not utilize cleavable presequences (e.g. Laloi, 1999).

The experimentally determined values of M_r and p*I* of each spot (Figure 1) were compared to the predicted M_rs and p*I*s of each spot's best database match (see Supplementary Material). Differences between the experimentally determined and predicted M_r of nuclear encoded-mitochondrial proteins are expected because mitochondrial targeting presequences are cleaved following their import into mitochondria (Sjöling and Glaser, 1998). Hence, predicted presequences were removed prior to predicting M_rs. Only those protein identifications that exhibited M_r differences of less than 20% were accepted.

In contrast to M_r, which can be used as a criterion for the correct identification of proteins, predicted and experimentally determined p*I*s of a correctly identified protein can differ substantially. This is because post-translational modifications can have major impacts on p*I*s and most proteins are subjected to post-translational modifications (Van Wijk, 2001) that are themselves affected by physiological conditions (Verner *et al.*, 2001). Consistent with this, in several cases, multiple protein spots were identified as being derived from the same gene (Table 2). As maize is a segmental allotetraploid (Gaut and Doebley, 1997), its genome contains many small gene families. In most instances, not all members of a particular gene family have

Table 2 Identified proteins that accumulate predominantly or exclusively in T- or NA-cytoplasm maize[a]

In T-cytoplasm mitochondria			In NA-cytoplasm mitochondria		
Spot no.[b]	Specificity[c]	Function (AC)	Spot no.[d]	Specificity[e]	Function (AC)
21	∞	ATPase alpha, F1 (P05494)	24	∞	ATPase alpha, F1 (P05494)
162	∞	Probable enoyl-[acyl-carrier-protein] reductase (NADH) (T03735)	80	∞	Putative ATP synthase (NM_127756)
163	∞	Probable enoyl-[acyl-carrier-protein] reductase (NADH) (T03735)	85	∞	Mn-superoxide dismutase (Sod3.4) (AAA72022)
164	∞	Inorganic pyrophosphatase like protein (AY087275)	141	∞	Unknown protein (AW574213)
165	∞	Unknown protein (AW289451)	153	∞	Probable cytochrome *c* oxidase Vb chain precursor (T03033)
12	3.29	ATPase alpha, F1 (P05494)			
34	3.29	F1-ATPase subunit 2 (AAA70268)			
143	3.25	Unknown protein (NM_111626)			

[a]Proteins were only considered to be differentially expressed if they accumulated to levels that were at least threefold different and their SDs did not overlap.
[b]The unidentified spot 166 also accumulated exclusively in T-cytoplasm and the unidentified spots 124, 129, 148, and 210 accumulated preferentially in T-cytoplasm.
[c]Ratios of protein accumulations in T-cytoplasm mitochondria versus NA-cytoplasm mitochondria.
[d]The unidentified spots 9, 23, 53, 76, 79, 150, and 152 also accumulated exclusively in NA-cytoplasm.
[e]Ratios of protein accumulations in NA-cytoplasm mitochondria versus T-cytoplasm mitochondria.

been characterized. Therefore, it is also possible that gene family members that encode proteins with similar tryptic peptides could be falsely identified as having been encoded by the single gene family member that is available in public databases.

Functional categorization of mitochondrial proteins

It was possible to assign functions to 66% (38) of the 58 different genes identified via National Center for Biotechnology Information (NCBI) and EST database searches. Predicted functions were available for all 13 proteins identified via searches of the annotated NCBI database (see Supplementary Material). In contrast, functions could not immediately be predicted for most of the proteins identified via searches of EST databases. In an effort to determine the functions for this latter group of proteins, each EST sequence was subjected to a BLASTX database search (Altschul *et al*., 1997). These analyses provided putative functions for 56% (25 out of 45) of the ESTs.

Mitochondrial proteins with known or predicted functions were functionally categorized according to the system used by Kruft *et al*. (2001) and Millar *et al*. (2001) (see Supplementary Material). Only one of these proteins is encoded by the mitochondrial genome.

Differential accumulation of proteins in NA- and T-cytoplasm mitochondria

The average number of pixels attributed to each individual protein spot on three replicate normalized gels (Experimental procedures) containing mitochondrial proteins from NA-cytoplasm Ky21 was compared to the average number of pixels attributed to the corresponding spots on the three gels containing proteins from T-cytoplasm Ky21. Several proteins accumulated exclusively in NA- or T-cytoplasm Ky21 (Table 2). A protein was classified as accumulating in a cytoplasm-exclusive manner if it was found in all three gels of one cytoplasm but in none of the gels of the other cytoplasm. Out of the 197 protein spots analyzed in this study, 6 accumulated exclusively in T-cytoplasm (spots 21 and 162–166) and 12 accumulated exclusively in NA-cytoplasm (spots 9, 23, 24, 53, 76, 79, 80, 85, 141, 150, 152, and 153). Five of the six T-cytoplasm-specific proteins (spots 21 and 162–165) and five of the 12 NA-cytoplasm-specific proteins (spots 24, 80, 85, 141, and 153) were successfully identified via mass spectrometry. Several spots accumulated preferentially in T-cytoplasm Ky21 (Table 2). Proteins were classified as accumulating in a cytoplasm-preferential manner if the accumulation in one cytoplasm was more than three times as much as that in the other cytoplasm and if the error bars of their SDs did not overlap. By this criterion, seven proteins (spots 12, 34, 124, 129, 143, 148, and 210) accumulated preferentially in T-cytoplasm. Three of these proteins (spots 12, 34, and 143) were identified via mass spectrometry. None of the proteins accumulated preferentially in the NA-cytoplasm. Hence, 13% (25 out of 197) of the analyzed protein spots accumulated to at least 3-fold higher levels in either NA- and T-cytoplasm mitochondria relative to the mitochondria from other cytoplasm.

Comparisons among the mitochondrial proteomes of maize, Arabidopsis*, and rice*

The sequences of the maize mitochondrial proteins identified in this study were compared to the sequences of the *Arabidopsis* and rice mitochondrial proteins identified by Kruft *et al.* (2001), Millar *et al.* (2001), and Heazlewood *et al.* (2003) (Experimental procedures).

Out of 58 GenBank accessions in the maize mitochondrial proteome, 31 (53%) exhibited a high degree of similarity with the *Arabidopsis* and rice mitochondrial proteins (see Supplementary Material). Twenty-seven proteins encoded by distinct GenBank accessions identified in the maize mitochondrial proteome were not detected in either the *Arabidopsis* or rice experiments.

Discussion

Analysis of a grass mitochondrial proteome

Relatively few proteomic analyses have been performed in grasses. In addition to a few studies conducted in rice (e.g. Heazlewood *et al.*, 2003; Imin *et al.*, 2001; Komatsu *et al.*, 1999) and barley (e.g. Finnie *et al.*, 2002; Ostergaard *et al.*, 2002), only two other analyses of the maize proteome have been published (Chang *et al.*, 2000; Porubleva *et al.*, 2001). The studies on maize analyzed the total proteomes of root tips (Chang *et al.*, 2000) and leaves (Porubleva *et al.*, 2001). Chang *et al.* (2000) identified 46 spots representing 33 different proteins and Porubleva *et al.* (2001) identified 205 spots representing 109 proteins.

Based on the sequence of the *Arabidopsis* genome, it is estimated that the proteome of angiosperms comprises about 25 000 proteins organized in about 11 000 gene families. A total of 2897 or 11% of the *Arabidopsis* proteins contain predicted mitochondrial targeting presequences (The Arabidopsis Genome Initiative, 2000; http://www.inra.fr/predotar/Arabidopsis_mit.html).High-throughput immunolocalization experiments establish that 13% of the total yeast proteome accumulates in the mitochondria (Kumar *et al.*, 2002). With the exception of the few proteins encoded by the mitochondrial genome (Kubo *et al.*, 2000; Unseld *et al.*, 1997), little is known about those proteins that accumulate in plant mitochondria.

This study of the mitochondrial proteome of the model grass species maize resulted in the identification of 100 protein spots that are encoded by at least 58 genes.

Even though it would be expected that highly abundant mitochondrial proteins would be observed in unfractionated protein extracts, only 1 of the 58 mitochondrial proteins (spot 203) identified in this study was detected in the extensive study of the maize leaf proteome conducted by Porubleva *et al.* (2001). This demonstrates the value of subcellular fractionations in proteomic analyses.

Differences in the NA- and T-cytoplasm mitochondrial proteomes

As 25 spots (13% of all identified spots) exhibited at least threefold differences in accumulation between the NA- and T-cytoplasm mitochondrial proteomes, this study demonstrates that mitochondrial genotypes can affect the composition of the mitochondrial proteome. Twelve protein spots were detected in the NA-cytoplasm proteome but not in the T-cytoplasm mitochondrial proteome. Similarly, six spots were detected in the T-cytoplasm mitochondrial proteome but not in the NA-cytoplasm mitochondrial proteome. Seven additional spots accumulated to higher levels (>threefold) in one proteome than those in the other. No database hits were found for 12 proteins that showed an exclusive or preferential accumulation in T- or NA-cytoplasm Ky21. As excellent MALDI-ToF spectra were obtained for these proteins, it should be possible to identify them once the maize genome is sequenced (Bennetzen *et al.*, 2001).

Out of the 13 identified proteins that exhibit cytoplasm-specific differences in accumulation, 9 were associated with multiple protein spots. For example, 3 of the 14 protein spots identified as the alpha subunit of ATPase (P05494, spots 12, 21, and 24) accumulated to greater than threefold higher levels in the mitochondrial proteomes of one of the cytoplasms than the other. Two accumulated exclusively (spot 21) or predominantly (spot 12) in T-cytoplasm and one accumulated exclusively in the NA-cytoplasm (spot 24). Hence, some of the cytoplasm-specific differences in protein accumulation are likely the result of differential protein modification or differential expression of gene family members.

In contrast, not all of the differences in protein accumulation between the two cytoplasms can be attributed to differential modification or expression of related proteins. For example, a probable cytochrome *c* oxidase Vb chain precursor (spot 153) accumulated specifically in the NA-cytoplasm mitochondrial proteome and no other cytochrome *c* oxidase Vb chain precursors were detected in either mitochondrial proteome.

Enoyl-acyl carrier protein (ACP) reductases, two of which preferentially accumulate in the T-cytoplasm mitochondrial proteome (spots 162 and 163), catalyze the last step of *de novo* fatty acid synthesis and belong to the family of short chain ADHs (Ohlrogge and Browse, 1995). ADHs catalyze the reversible conversion of aldehydes to alcohols and can play critical roles in plant development. For example, the *tasselseed2* gene of maize encodes an ADH and plays a pivotal role in determining the sexual fate of floral meristems (DeLong *et al.*, 1993). The physiological role of the T-cytoplasm-specific ADH identified in this study, however, remains to be established. Interestingly, another putative enoyl-ACP reductase (spot 31) that is encoded by a different accession accumulated in both proteomes.

The 540- and the 700-kb mitochondrial genomes of NA- and T-cytoplasms contain many of the same genes (Fauron *et al.*, 1995). Although, these genomes also contain cytoplasm-specific open-reading frames (ORFs), the significant differences in the mitochondrial proteomes of NA- and T-cytoplasm plants observed in this study cannot be attributed solely to unique mitochondrial ORFs or differences in the expression of genes that are present in both mitochondrial genomes. This is because most of the proteins that differentially accumulate in the two cytoplasms are encoded by nuclear genes. Hence, because the two stocks used in this study have nearly congenic nuclear genomes as the result of extensive backcrossing into the inbred line Ky21, the differences in the composition of the nuclear-encoded fraction of these mitochondrial proteomes must be regulated by the mitochondria themselves.

In *Saccharomyces cerevisiae*, mitochondrial dysfunction signals the expression of particular nuclear genes, thereby altering metabolism to compensate for the mitochondrial dysfunction (Poyton and McEwen, 1996). This process, retrograde regulation of nuclear genes occurs by yet undefined mitochondrial signals and has also been observed for several plant genes. The nuclear-encoded alternative oxidase (AOX) is upregulated by mitochondrial signals when electron transfer through the normal mitochondrial (cytochrome) pathway is blocked (McIntosh *et al.*, 1998). In another study, electron transfer through the cytochrome pathway was blocked by antimycin A, which allowed for the identification of seven additional nuclear genes induced by blocking the normal flow of electrons in the mitochondria (Maxwell *et al.*, 2002). Analyses of the DNA sequences of the mitochondrial genomes of NA- and T-cytoplasm maize (Newton *et al.*; NSF Plant Genome Program award 0110168) may begin to define the mitochondrial proteins that participate in regulating the accumulation of the nuclear-encoded fraction of the mitochondrial proteome.

Comparisons among the mitochondrial proteomes of maize, Arabidopsis*, and rice*

A comparison of the proteins identified via this maize mitochondrial proteome project to those identified via the analyses of the mitochondrial proteome of *Arabidopsis* (Kruft *et al.*, 2001; Millar *et al.*, 2001) and rice (Heazlewood *et al.*, 2003) revealed that the mitochondrial proteomes of these species contain many similar proteins. Fifty-three per cent of the maize mitochondrial proteins identified in the current study exhibited a high degree of similarity to proteins in the *Arabidopsis* and/or rice mitochondrial proteomes. The current study extends these prior analyses of plant mitochondrial proteomes, however, in that it identified 27 mitochondrial proteins that were not detected in prior analyses of plant mitochondrial proteomes. Most of the novel proteins identified in the current study are involved in nucleotide or carbon metabolism or 'other' processes (see Supplementary Material). One of the novel proteins was a succinyl-CoA synthetase. Unlike in animals, the tri-carboxylic acid (TCA) cycle in plants uses a succinyl-CoA synthetase (EC 6.2.1.5) that generates ATP rather than GTP. The novel mitochondrial succinyl-CoA synthetase identified in this study is the GTP-generating form of succinyl-CoA synthetase (EC 6.2.1.4) whose physiological function is not known in plants.

The differences in proteins detected in these four analyses of mitochondrial proteomes can be explained in part by the different technical approaches used to analyze the proteomes of *Arabidopsis*, rice, and maize mitochondria. Current technology does not allow for the quantitative solubilization and detection of all proteins from a proteome. Our approach (Experimental procedures) mainly led to the identification of soluble proteins with molecular weights between 13 and 50 kDa and p*I*s between 4 and 7. This, explains why only one TCA enzyme, an NAD-dependent malate dehydrogenase (spot 30), was identified in this study as only 2 of 15 TCA enzymes have M_rs and p*I*s that would allow them to be resolved with our gel system. The hydrophobicity and small size (13 kDa) of the T-cytoplasm-specific membrane protein T-URF13 may explain why this protein was not detected in our study.

Technical differences probably do not account for all of the differences in the proteins detected in these studies. As the mitochondrial proteomes of *Arabidopsis* and rice analyzed by Kruft *et al.* (2001), Millar *et al.* (2001), and Heazlewood *et al.* (2003) were isolated from vegetative tissue, suspension culture cells, and young shoots, and we analyzed the mitochondrial proteome of immature, unpollinated maize ears, these studies involved not only different species but also different 'organs'. Hence, the observed differences are consistent with the observations that even within a species, proteome composition can be affected by not only nuclear genotype (Leonhard *et al.*, 1988) but also the type of tissue or organ being analyzed (Conley and Hanson, 1994).

Protein localization

Fewer than 100 of the estimated 2000–3000 proteins that accumulate in mitochondria are encoded by the mitochondrial genome (Peeters and Small, 2001); the vast majority is encoded by the nuclear genome. These proteins are synthesized by cytosolic ribosomes and are subsequently imported into the organelles via active protein transport systems. Of the 58 different proteins identified in this study, 57 are encoded by the nuclear genome; only a small portion of these were previously known to be imported into the mitochondria. Many of the proteins that were not previously known to accumulate in the mitochondria have only predicted functions or completely lack functional

assignments. Access to this subcellular localization data represents a first step in determining the functions of these proteins.

Five of the mitochondrial proteins identified in this study exhibited similarities to chloroplastic proteins. Two of the five (P05494 and AAA70268) belong to protein families known to accumulate in both mitochondria and chloroplasts and were also detected in the mitochondrial proteome of *Arabidopsis* (see Supplementary Material). Each of the three remaining proteins with similarities to chloroplast proteins (HSP 70, spot 3; plastid division protein FtsZ, spot 49; and a chaperonin, spot 67) contains a predicted mitochondrial targeting presequence. One (spot 3) was also detected in the mitochondrial proteome of *Arabidopsis* (see Supplementary Material). Hence, we hypothesize that these proteins are either imported into both the mitochondria and the chloroplast (Peeters and Small, 2001), or that highly related proteins are imported into both compartments.

Spot 49 (similar to FtsZ) is of particular interest because no mitochondrial fission components have been described to date (Arimura and Tsutsumi, 2002). However, a gene that encodes a protein similar to FtsZ and that localizes to the constriction sites of dividing mitochondria has been cloned from a red algae (Beech *et al.*, 2001), which shares a common ancestry with green algae and plants. Hence, even though an FtsZ gene has not been identified in the *Arabidopsis* genome (Unseld *et al.*, 1997; The Arabidopsis Genome Initiative, 2000), we hypothesize, based on the detection of spot 49, that the mitochondrial proteomes of at least some green plants contain an FtsZ homolog.

Experimental procedures

Plant materials

The inbred line Ky21 carries the NA-cytoplasm (personal communication, Christiane Fauron, University of Utah) and is homozygous for both restorers of cmsT (i.e. *Rf1-Ky21* and *Rf2a-Ky21*). A T-cytoplasm version of Ky21 was generated by backcrossing a T-cytoplasm version of the inbred line R213 to Ky21 for seven generations (Liu *et al.*, 2001). As Ky21 carries both restorers of cmsT, the T-cytoplasm version of Ky21 is male fertile. NA- and T-cytoplasm maize (*Zea mays* L.) plants of the inbred line Ky21 were grown under field conditions in Molokai, HI, USA during the winter of 2000/2001 and shipped overnight on ice to Ames for the isolation of mitochondria. The pedigree numbers the NA-cytoplasm Ky21 plants were 00g 1113, 1121, 1150, and 1169 and those of the T-cytoplasm Ky21 plants were 00g 1090, 1095, and 1103.

Purification of mitochondrial proteins

Mitochondria were isolated from immature, unpollinated maize ears via differential centrifugation followed by discontinuous Percoll gradient centrifugation using a three-step Percoll gradient (45, 21, and 13.5%; Jackson and Moore, 1979). Each mitochondrial preparation was conducted with 200 g of fresh plant material (i.e. approximately 10–15 unpollinated ears).

Catalase, ADH, and cytochrome *c* oxidase (all obtained from Sigma-Aldrich, St Louis, MO, USA) were used as markers to demonstrate the purity of the mitochondrial fractions according to procedures by Vigil (1983; catalase), Freeling and Schwartz (1973) and Quail (1979) (ADH), and Errede *et al.* (1967; cytochrome *c* oxidase).

The mitochondrial pellet was dissolved in a solution containing 7 M urea, 2 M thiourea, 2 mM Tris (2-carboxyethyl) phosphine hydrochloride (Pierce, Rockford, IL, USA), 1% ASB14 (Calbiotech, Spring Valley, CA, USA), 1% ASB16 (Calbiotech), 0.5% Bio-Lytes 3/10 (Bio-Rad, Hercules, CA, USA), 40 mM Tris, and 0.001% orange G dye. The samples were then ultrasonicated for 5 min, followed by treatment with endonuclease and subjected to five additional minutes of ultrasonication before being incubated at room temperature for 15 min. The insoluble fraction was removed via ultracentrifugation at 25 000 ***g*** for 60 min. The resulting supernatant was immediately subjected to 2-D protein separation. Aliquots were stored at −70°C for subsequent enzyme activity tests.

Two-dimensional separation of mitochondrial proteins

Isoelectric focusing of mitochondrial proteins was performed with 1 mg of protein extract using an IPG Phor isoelectric focusing unit (Amersham Pharmacia Biotech, Uppsala, Sweden) and 18 cm immobilized, linear pH gradients (Imobiline drystrips, Amersham Pharmacia Biotech). To increase resolution two overlapping 18 cm gradients were utilized (pH 4–7 and pH 6–9). Basic pH gradients (pH 6–9) were pretreated for 24 h in 100 mM ascorbic acid pH 4.5, then rinsed two times for 15 min in water followed by a 30-min treatment in 2% glycerol. The strips were then dried O/N (Chan *et al.*, 1999). The voltage settings of the isoelectric focusing were according to Porubleva *et al.* (2001) to a final setting of 90 000 Vh for pH 4–7 gradients and 130 000 Vh for pH 6–9 gradients. Equilibration of the strips was performed according to Porubleva *et al.* (2001) for 2 × 15 min in disposable 25 ml pipettes sealed on both ends with parafilm on an orbital shaker. Proteins in the equilibrated strips were then separated on the basis of their M_rs in 12–18% gradient gels using the DALT electrophoresis system (Amersham Pharmacia Biotech).

After electrophoresis, proteins were stained with a modified colloidal Coomassie blue stain (Neuhoff *et al.*, 1988) consisting of 17% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 34% methanol, 0.15% Coomassie blue G-250 for 48–72 h on an orbital shaker. Stained gels were scanned with a Bio-Rad GS 710 scanner. The resulting images were analyzed with Bio-Rad Quantity One and normalized using PDQUEST software. During normalization, 1 000 000 pixels were distributed among all visible spots on a given gel in proportion to each spot's intensity.

MALDI-ToF mass spectrometry

The most abundant proteins from a representative stained gel containing mitochondrial proteins from T-cytoplasm Ky21 were excised and transferred into 96 well plates containing 50% methanol. Protein spots that were present only on NA-cytoplasm Ky21 gels (i.e. spots 9, 23, 24, 53, 76, 79, 80, 85, 141, 150, 152, and 153) were excised from a representative gel containing mitochondrial proteins from NA-cytoplasm Ky21. To remove Coomassie dye, protein-containing gel pieces were washed two times for 10 min in 50% acetonitrile in 20 mM ammonium bicarbonate and then dried for 10 min in a speed vac. In-gel digestion of proteins was performed in 10 μl of 10 μg ml^{-1} trypsin in 20 mM ammonium bicarbonate (pH 8.5) at 37°C for at least 16 h. Elution of tryptic fragments was performed by diffusion into 10 μl of 50% (v/v) acetonitrile and 0.5% (v/v) trifluoracetic acid and facilitated by 10 min of ultrasonification. Prior to MALDI-ToF mass spectrome-

try, eluted peptides were concentrated using a speed vac. Two microliters of each sample was mixed with 2 μl of 1% α-cyano-4-hydroxycinnamic acid as a matrix and transferred to a target plate where they were crystallized prior to being analyzed with a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Spectra were obtained in a reflectron-delayed mode over a mass range of 600–4000 Da. Spectra from 100 to 200 shots at various plate positions were combined to generate a representative mass fingerprint of each protein sample. Autolytic peptide ions of trypsin (m/z 842.51$^+$ and m/z 2211.10$^+$) were used for internal calibration of the spectra.

Analysis of spectrometric data

Proteins were identified via peptide mass fingerprints using the MS-FIT program of the Protein Prospector package (Clauser *et al.*, 1999; http://prospector.ucsf.edu/) and the annotated non-redundant NCBInr protein database and the non-human pdbEST.others EST database, as well as the maize EST contig database available at http://www.zmdb.iastate.edu. Database searches were performed on all available higher plant proteins as the maize genome has not been completely sequenced and many proteins are well conserved among higher plants. Mass Spectrometry Utilities (MSU) software was used to automate the use of MS-FIT identification tools (Porubleva *et al.*, 2001).

Comparisons of maize, Arabidopsis*, and rice proteins*

Maize protein sequences identified from the NCBI protein database (see Supplementary Material) were directly compared to the *Arabidopsis* and rice proteins. The nucleotide sequences of the genes that encode proteins identified via ESTs were first translated into all six reading frames. Those ORFs that encoded fewer than 20 amino acids were discarded. The remaining ORFs were compared to the two *Arabidopsis* data sets. Data by Kruft *et al.* (2001) were downloaded from http://www.gartenbau.uni-hannover.de/genetik/Page3.html. The mitochondrial protein sequences by Millar *et al.* (2001) were downloaded from the TrEMBL (TE), SWISS-PROT (SP), GenBank (GB), and the *Arabidopsis* Information Resource (AT) databases using the protein accessions provided in the manuscript. The rice data set was downloaded from The Institute for Genomic Research (TIGR) website according to the accession numbers given by Heazlewood *et al.* (2003). The ORF translations of the rice genome have recently been revised; only 105 of the 136 sequences published by Heazlewood *et al.* (2003) are still available. These 105 sequences were used for the rice–maize comparisons. The maize protein data set was set as the query file and used to search the *Arabidopsis* and rice data sets using BLASTP with an *E*-value cut-off of 1e^{-10}.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1955/TPJ1955sm.htm

Table S1 Maize (T) and (NA) Ky21 mitochondrial proteins identified via two-dimensional separation of purified maize mitochondria using MALDI-ToF spectra of trypsin digested proteins matched against NCBInr.dbEST.others and zmdb contig database entries

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