

# Towards an Analysis of the Rice Mitochondrial Proteome<sup>1</sup>

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Purified rice (*Oryza sativa*) mitochondrial proteins have been arrayed by isoelectric focusing/polyacrylamide gel electrophoresis (PAGE), by blue-native (BN) PAGE, and by reverse-phase high-performance liquid chromatography (LC) separation (LC-mass spectrometry [MS]). From these protein arrays, we have identified a range of rice mitochondrial proteins, including hydrophilic/hydrophobic proteins (grand average of hydrophobicity =  $-1.27$  to  $+0.84$ ), highly basic and acid proteins (isoelectric point =  $4.0$ – $12.5$ ), and proteins over a large molecular mass range ( $6.7$ – $252$  kD), using proteomic approaches. BN PAGE provided a detailed picture of electron transport chain protein complexes. A total of 232 protein spots from isoelectric focusing/PAGE and BN PAGE separations were excised, trypsin digested, and analyzed by tandem MS (MS/MS). Using this dataset, 149 of the protein spots (the products of 91 nonredundant genes) were identified by searching translated rice open reading frames from genomic sequence and six-frame translated rice expressed sequence tags. Sequence comparison allowed us to assign functions to a subset of 85 proteins, including many of the major function categories expected for this organelle. A further six spots were matched to rice sequences for which no specific function has yet been determined. Complete digestion of mitochondrial proteins with trypsin yielded a peptide mixture that was analyzed directly by reverse-phase LC via organic solvent elution from a C-18 column (LC-MS). These data yielded 170 MS/MS spectra that matched 72 sequence entries from open reading frame and expressed sequence tag databases. Forty-five of these were obtained using LC-MS alone, whereas 28 proteins were identified by both LC-MS and gel-based separations. In total, 136 nonredundant rice proteins were identified, including a new set of 23 proteins of unknown function located in plant mitochondria. We also report the first direct identification, to our knowledge, of PPR (pentatricopeptide repeat) proteins in the plant mitochondrial proteome. This dataset provides the first extensive picture, to our knowledge, of mitochondrial functions in a model monocot plant.

The ATP-synthesizing organelles of eukaryotic cells, mitochondria, can trace their origins back to an event in which one prokaryotic cell was engulfed by another cell to form a new cellular lineage containing two genomes. These genomes became dependent over time, and mitochondria lost the ability to be viable outside the host cell. A significant net transfer of genetic information occurred from the mitochondrial to the nuclear genome during this time. It is predicted that mitochondria now synthesize only a few percent of the proteins required for their function, with the majority of their proteins being encoded in the nucleus, translated in the cytosol, and transferred back to the mitochondria as protein precursors by means of targeting information in the protein sequence (Gray et al., 1999). Biochemical investigations have revealed that isolated plant mitochondria share many features with those from animals and fungi. However, plant mitochondria also contain additional components such as non-phosphorylating bypasses of the electron transport chain, specialized metabolite carriers, and enzymes involved in the synthesis of folate, lipolic acid, and

vitamin C (Bartoli et al., 2000; Gueguen et al., 2000; Rebeille et al., 1997). Only a few of the nuclear genes encoding proteins that maintain these unique functions have been identified. The recent increased sequencing of plant genomes (Arabidopsis Genome Initiative, 2000; Goff et al., 2002; Yu et al., 2002) has encouraged the development of bioinformatic tools to predict the subcellular localization of putative plant protein sequences. Programs such as MitoProtII (Claros and Vincens, 1996), TargetP (Emanuelsson et al., 2000), and Predotar (<http://www.inra.fr/Internet/Produits/Predotar/>) predict that thousands of nuclear-encoded plant proteins are destined for mitochondria, but the veracity of such predictions still awaits confirmation.

Several reports have recently attempted to identify the protein contents of plant mitochondria from Arabidopsis (Kruft et al., 2001; Millar et al., 2001) and pea (*Pisum sativum*; Bardel et al., 2002) using isoelectric focusing (IEF)/SDS-PAGE-based separation of proteins. These three reports each identified 40 to 90 proteins using this arraying technique coupled with identification using antibodies, N-terminal sequencing, matrix-assisted laser-desorption/ionization-time-of-flight (TOF) peptide mass fingerprinting, and collision-induced tandem mass spectrometry (MS/MS) spectra of single peptides. Both these species are dicots. Increasing evidence of important differences between monocot and dicot plant lineages at a molecular level have been reported (Qiu et al., 1999; Soltis et al., 1999; Daly et al., 2001). Differences in mitochon-

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drial molecular function between monocots and dicots also have been highlighted recently (Fey and Marechal-Drouard, 1999; Thelen et al., 1999; Considine et al., 2002). Thus, we consider it important to consider the mitochondrial proteome in a model monocot species.

The gel array technique used in the dicot mitochondrial proteome reports has several limitations. In particular, very small (<10 kD) and very large (>100 kD) proteins, basic proteins (pI > 10), and hydrophobic proteins (grand average of hydropathicity [GRAVY] > 0.2) are poorly resolved by this method (Chevallet et al., 1998; Herbert et al., 1998; Santoni et al., 1999, 2000). Incremental improvements in chaotropic agents and nonionic detergents have improved the resolving power of classical denaturing two-dimensional electrophoresis (Chevallet et al., 1998; Herbert et al., 1998). However, a substantial protein set will probably always be excluded due to the physical limitations of this approach. Native gel electrophoresis coupled to second dimension SDS-PAGE provides a flexible solution to the hydrophobic protein exclusion problem. Many such proteins are not found exposed in biological membranes but are core, internalized components of multisubunit complexes. Maintenance of this complex structure avoids precipitation of hydrophobic components. Blue-native (BN) PAGE has been used previously to separate native protein complexes from mitochondria (Schagger et al., 1994). A subsequent denaturing SDS-PAGE dimension allows separation of individual components for analysis from plant mitochondria (Jansch et al., 1996; Werhahn and Braun, 2002). A different solution to the exclusion problem, with an even wider applicability, is non-gel-based separation of complex mixtures. These approaches often use liquid chromatography (LC)-based separations of peptide derived from whole-sample digests that are directly coupled to MS. One approach using a coupled strong cation affinity and reverse-phase C18 separation has been termed multidimensional protein identification technology and is being increasingly applied to a broad range of biological samples (Washburn et al., 2001).

To determine which putative proteins from the rice (*Oryza sativa*) genome sequence represent constitutively expressed proteins involved in mitochondrial function, we have used two-dimensional gel electrophoresis and LC analysis of purified rice mitochondria coupled with MS identification. Our aim was to identify proteins localized in rice mitochondria, compare and contrast protein display techniques, and identify novel mitochondrial proteins, thereby providing a substantial set of experimentally identified mitochondrial proteins from this model monocot plant.

## RESULTS

### Integrity and Function of Isolated Rice Mitochondria

The integrity of a subcellular proteome, such as that of mitochondria, is largely dependent on the

purification of the isolated compartment away from other cellular contaminants. We have adapted a two-Percoll gradient density separation technique to the isolation of these organelles from dark-grown rice shoots that yields mitochondria largely free of contamination by cytosol, peroxisomes, plastids, and other membranes (Millar et al., 2001). We have assayed the integrity and function of these purified rice mitochondria to ensure that proteins were not being lost by rupture during isolation and that key respiratory functions were maintained. The outer membrane of mitochondrial samples was found to be 96% intact based on the latency of cytochrome c oxidase activity initiated by exogenously added cytochrome c (Table I). Whole-electron transport chain activities showed that O<sub>2</sub> consumption by the mitochondria could be supported by succinate, external NADH, or a combination of malate and pyruvate. O<sub>2</sub> consumption supported by each of these substrates was significantly and transiently stimulated by the addition of ADP. These data suggested the presence and function of the electron transport chain, the F<sub>1</sub>F<sub>0</sub>-ATP synthase, the tricarboxylic acid cycle in the mitochondrial matrix, the integrity of the inner membrane, and the function of external rotenone-insensitive NADH dehydrogenase bypasses of complex I (Table I). Little or no respiration was maintained in the presence of the complex III inhibitor myxothiazol, showing that little or no alternative oxidase (AOX) was present in these mitochondria (Table I). This lack of AOX was also confirmed by addition of pyruvate and dithiothreitol to O<sub>2</sub> electrode traces without effect after myxothiazol addition. It was further confirmed by the lack of immunodetectable bands using monoclonal antibodies raised against AOX (data not shown).

### IEF and BN Arraying of Rice Mitochondrial Proteins

To analyze the complex mixture of proteins present in rice mitochondria, these proteins were separated in gel arrays using the apparent pI and molecular mass of individual polypeptides and the apparent native molecular mass of protein complexes. The IEF/SDS-PAGE separation used the first two of these properties and revealed a set of approximately 250

**Table I.** O<sub>2</sub> consumption by isolated rice mitochondria following addition of different substrates, effectors, and inhibitors

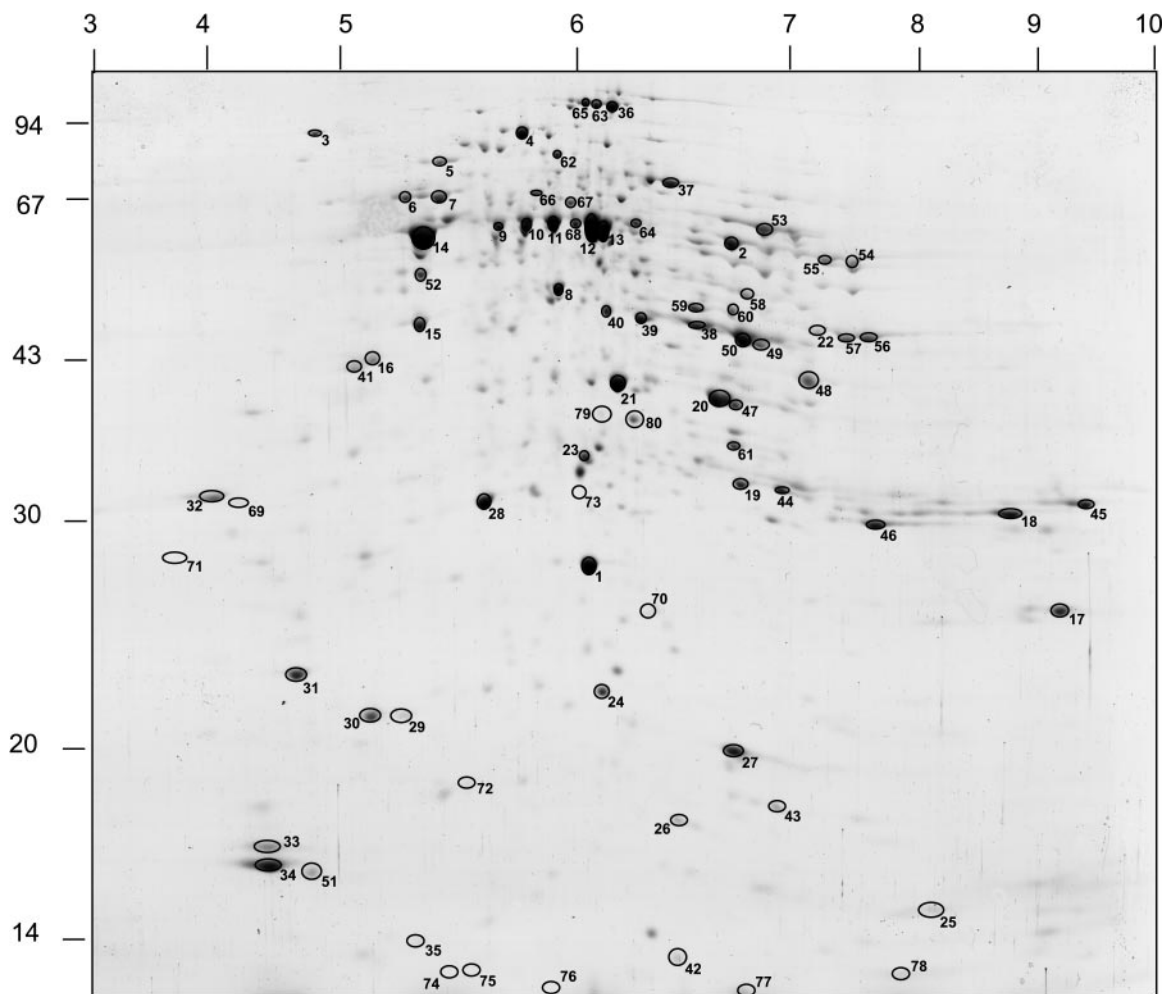
Respiratory assays were performed according to "Materials and Methods." O<sub>2</sub> consumption rates are presented as means ± SE (n > 3). Outer membrane integrity is presented as a percentage of intact mitochondria.

Substrate	-ADP	+ADP	+Myxothiazol
	<i>nmol O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein</i>		
NADH	87 ± 6	199 ± 8	3 ± 1
Succinate	99 ± 7	198 ± 17	2 ± 1
Malate and pyruvate	61 ± 9	139 ± 11	1 ± 1
Cytochrome c	438 ± 29	-	-
Membrane integrity	96.1 ± 1%	-	-

protein spots (Fig. 1). A set of 145 of the more abundant spots from this set was excised and subjected to in-gel digestion followed by MS/MS-based analysis of the resultant peptides. This analysis led to the identification of 80 of these protein spots as the products of 63 predicted gene sequences from rice (Table II).

The BN SDS-PAGE uses a combination of native masses of complexes in the first dimension and the denatured masses of polypeptides in the second dimension (Fig. 2). This analysis was only performed on the membrane fraction from rice mitochondria that contains a large number of hydrophobic proteins that were unlikely to be soluble under IEF conditions. Protein staining revealed a series of large complexes of comigrating subunits that constitute the major electron transport chain complexes: complexes I, III, and V (Fig. 2). A set of approximately 100 spots was visible, a set of 89 spots was excised, and a subset of 57 spots from this set was identified as the products of 49 rice genes. There are several examples where a

single protein sample contained peptides derived from two different and unrelated gene products; note spot 44 (cytochrome oxidase subunit I and malic enzyme), spot 57 (adrenodoxin and TIM17), and spot 42 (TOM9 and ATP synthase, 6 kD). In all these cases, the MS evidence for the presence of both proteins is very convincing. Because streaking of some proteins occurs during native gel electrophoresis, it is possible that the same proteins do exist more discretely elsewhere on the gel and that our identification point (i.e. the spot excision point) is just coincidentally the point of overlap. Comparison of these identified sets from IEF/SDS-PAGE and BN SDS-PAGE revealed that there were 21 nonredundant gene matches in common, whereas together, they represent a combined nonredundant set of 91 gene products. A set of 18 well-known components of mitochondrial inner and outer membrane complexes was identified in the BN SDS-PAGE analysis that was not identified from the IEF/SDS-PAGE analysis (Table II).



**Figure 1.** IEF/SDS-PAGE separation of rice mitochondrial proteins (pI = 3–10). A total of 145 protein spots from this map were excised, digested, and analyzed by MS/MS. Eighty were identified, and these are numbered for comparison with text and Table II. Numbers on the horizontal axis are pI, and numbers on the vertical axis are apparent molecular mass (in kilodaltons).

**Table II. Identification of proteins from purified rice mitochondria using MS/MS peptide spectra matched to rice ORFs translated by the Institute for Genomic Research (TIGR; XXXX.mXXXXX), TIGR-translated expressed sequence tags (ESTs, TCXXXXX), and rice NCBI entries**

Proteins were identified either from gel spots derived from IEF/SDS-PAGE (2-D PAGE), BN SDS-PAGE (BN-PAGE), or HPLC separation of total digested mitochondrial samples (LC-MS). Numerals in 2D-PAGE and BN-PAGE columns are spot nos. for reference to Figures 1 and 2. Numerals in the LC-MS column are the no. of peptides matches to each predicted protein sequence. The  $M_r$ , pI, and hydrophobicity (GRAVY) for each predicted protein sequence are shown along with prediction of targeting by TargetP (TP), Predotar (PT), and MitoProtII (MP). Asterisks in final columns indicate the presence of orthologs in the IEF/SDS-PAGE proteome reports from Arabidopsis (At; Kruff *et al.*, 2001; Millar *et al.*, 2001) and pea (Ps; Bardel *et al.*, 2002). Several other Arabidopsis proteins identified elsewhere by a variety of different electrophoresis techniques have also been included for comparison: a, Sweetlove *et al.* (2002); b, Millar and Heazlewood (2003); and c, Werhahn and Braun (2002). Spot 62 from IEF/SDS-PAGE was identified as bovine serum albumin, used in the preparation of mitochondria, and has not been included in the table.

Functional Class	2D-PAGE	BN-PAGE	LC-MS	$M_r$	pI	GRAVY	TP	PT	MP	Accession No.	Protein	At	Ps
ATP synthase	11, 12, 64	19, 32, 50, 51, 52	2	55,281	5.9	-0.11	-	-	-	2832.m00083, CAA35787	ATP synthase alpha-subunit	*	*
	14	18, 31	10	59,059	6.8	-0.10	M	M	M	Q01859	ATP synthase beta-subunit	*	*
	17	37	25,065	10.1	-0.16	M	M	M	M	1903.m00144	ATP synthase OSCP subunit	*	*
	29, 30	22	24,359	5.0	-0.71	-	-	-	-	TC63644, 3067.m00121	ATP synthase delta-subunit	*	*
	31	38	21,213	6.0	0.15	M	M	M	M	2119.m00096, BAC21366	ATP synthase delta-subunit	*	*
51	20, 35	42, 23	4	40,955	8.5	-0.10	-	-	-	3695.m00193, TC72968	ATP synthase gamma-subunit	b	*
	42, 23	23	6937	10.8	-0.30	M	M	M	M	BAB21526	ATP synthase 6-kD subunit		
	23	23	7877	10.9	0.84	C	C	C	C	P14863	ATP synthase subunit 9		
Complex I	28	21	2	38,217	5.0	-0.44	M	M	M	2485.m00122	ATP synthase FAD subunit	*	*
	26		2	12,492	6.7	-0.99	-	-	-	AAL67582	NADH dehydrogenase PDSW subunit		
	76		2	12,033	7.6	-0.22	M	M	M	3640.m00126	NADH dehydrogenase B22 subunit	*	*
	71	12	23,498	4.9	-0.57	M	M	M	M	3550.m00121	NADH dehydrogenase (23 kD)	*	*
	43	57		18,518	7.1	-0.90	M	M	M	387.m00162	NADH dehydrogenase B17.2		
	4	3	4	81,117	6.1	-0.17	M	M	M	AAL58200	NADH dehydrogenase 75-kD subunit	*	*
		4, 8, 9	1	44,309	9.6	-0.08	M	M	M	2451.m00084	NADH dehydrogenase (39 kD)		
		4, 5		56,301	8.2	-0.31	M	M	M	2102.m00066	NADH dehydrogenase (51 kD)		
		6, 7		44,252	7.1	-0.49	C	-	-	BAC19866	NADH dehydrogenase subunit 7		
		13		22,594	8.2	-0.78	-	-	-	BAA08794	NADH dehydrogenase subunit 9		
Complex III	14, 15	16	1	21,829	4.6	-0.24	M	M	M	TC76675, 4381.m00118	NADH dehydrogenase 13-kD subunit	*	*
	16		2	28,762	9.6	-0.30	M	M	M	TC79833, 4342.m00113	NADH dehydrogenase E 16.6-kD subunit	*	*
	24			-	-	-	-	-	-	TC79968	Mitochondrial-processing peptidase beta-subunit	*	*
	2	25		53,946	7.2	-0.10	M	M	M	2599.m00145, 2709.m00160	Mitochondrial-processing peptidase alpha-subunit	*	*
		27	1	29,820	8.6	-0.16	M	M	M	2341.m00134	Reiske Fe-S protein	c	
		28		16,616	9.8	-0.34	M	M	M	2070.m00100, 3403.m00173	Ubiquinol cytochrome c reductase (14 kD)	*	*
Complex IV	29, 23			13,086	10.8	0.41	-	M	M	3017.m00162, 3017.m00172	Ubiquinol cytochrome c reductase (8.2 kD)		
	32		2	33,293	6.2	-0.19	M	M	M	2974.m00152	Cytochrome c1 precursor	c	
	33	41		18,912	4.0	-1.01	-	-	-	BAA76393	Cytochrome c oxidase subunit Vlb-1	*	*
	77			18,047	4.8	-0.42	M	M	M	T03033	Cytochrome c oxidase subunit Vb		
Pyruvate and 2-OG DH complexes		44		13,930	9.7	0.06	M	M	M	3550.m00112	Cytochrome c oxidase subunit Via		
	53	47, 48	3	57,767	8.6	0.69	-	-	-	P14578	Cytochrome c oxidase subunit I		
			2	52,643	7.6	0.13	M	M	M	BAB39219, 2726.m00149	Dihydropyrimidine dehydrogenase (E3)	*	*
	16, 41		2	55,896	7.4	0.08	M	M	M	3313.m00119	Dihydropyrimidine dehydrogenase (E3)	*	*
66			39,950	4.8	-0.03	M	-	M	TC57558, 3493.m00141	Pyruvate dehydrogenase E1 beta-subunit	*	*	
			59,591	8.7	-0.17	M	M	M	3634.m00180	Dihydropyrimidine acetyltransferase (E2)	a	*	

(Table continues)

**Table II. Continued**

Functional Class	2D-PAGE	BN-PAGE	LC-MS	M <sub>r</sub>	pI	GRAVY	TP	PT	MP	Accession No.	Protein	At	Ps
	52		3	48,894	9.5	-0.33	M	M	M	2307.m00126	Dihydroipoamidesuccinyltransferase(E2)		a
			2	48,283	9.1	-0.30	-	-	-	CAD40552	Dihydroipoamide succinyltransferase (E2)		a
			2	123,768	6.5	-0.44	M	M	M	4591.m00151	2-Oxoglutarate dehydrogenase E1		
Succinyl-CoA ligase	48, 22		3	34,246	8.4	0.08	M	M	M	2046.m00126	Succinyl-CoA ligase alpha-subunit		*
Isocitrate DH	15	49	3	45,091	6.2	-0.03	M	M	M	2466.m00139	Succinyl-CoA ligase beta-subunit		*
Malate DH	40, 49		4	40,629	8.1	-0.06	M	M	M	2447.m00175	NAD-dependent isocitrate dehydrogenase		*
	20, 21, 47		2	35,461	8.9	0.08	M	M	M	BAB55686, 2720.m00059	Malate dehydrogenase		*
Citrate synthase	58	53	4	35,436	8.3	0.14	M	-	M	3321.m00193, 2990.m00151	Putative malate dehydrogenase		*
Malic enzyme		30	2	52,121	7.2	-0.07	M	M	M	AAG28777, 2942.m00114	Citrate synthase		*
				70,721	5.9	-0.19	M	M	M	2119.m00105	NAD-dependent malic enzyme 62-kD subunit		*
		44		57,143	8.3	-0.16	C	-	-	AAG13628	NAD-dependent malic enzyme 59-kD subunit		*
Complex II	37			59,103	6.2	-0.40	-	-	-	2338.m00147	Succinate dehydrogenase FP alpha-subunit		*
Aconitase	36, 63			107,452	7.3	-0.18	C	-	M	TC61943, 4161.m00311	Aconitate hydratase		*
	65			98,397	5.9-0.15	-	-	-	-	TC68934, 5178.m00213	Aconitate hydratase		*
GDC	34		4	17,367	4.7	-0.23	M	M	M	AAK39594	Gly decarboxylase H protein		*
	73, 80, 56, 57		3	29,224	6.5	-0.16	-	-	-	2329.m00191	Aminomethyltransferase precursor (T protein)		*
General metabolism	54, 55			54,979	8.4	-0.29	M	M	M	TC52724, 4124.m00171	Ser hydroxymethyltransferase (SHMT)		*
	59			47,875	6.6	-0.15	M	M	-	1207.m00066	Glutamate dehydrogenase 2		*
	60	33		40,631	6.7	-0.19	M	M	M	2275.m00146	Glutamate dehydrogenase		*
	70		2	43,762	6.8	-0.18	M	M	M	1904.m00155	NAD-dependent formate dehydrogenase		*
	50			41,243	7.4	-0.21	M	M	M	BAA77337	NAD-dependent formate dehydrogenase		*
	68		1	45,255	9.6	-0.21	-	-	-	1902.m00219	Aldehyde dehydrogenase		*
	13			59,307	6.8	-0.04	M	M	M	BAB19052	Aldehyde dehydrogenase		*
	67		2	87,073	8.4	-0.10	M	M	M	4976.m00233	Aldehyde dehydrogenase		*
	9, 10		1	56,106	5.8	-0.02	C	C	-	4694.m00128	Aldehyde dehydrogenase		*
				56,980	6.3	-0.03	M	M	M	AAC03055	Methylmalonate semi-aldehyde dehydrogenase		*
	79			-	-	-	-	-	-	TC59081, TC66764	Thiosulfate sulfurtransferase		*
	38			47,495	7.8	-0.25	M	M	M	JC5125	Asp transaminase		*
	39			-	-	-	-	-	-	TC54297	Isovaleryl-CoA dehydrogenase		*
	27			25,425	9.4	-0.09	C	C	-	TC62983, 4134.m00168	Inosine-5'-monophosphate dehydrogenase		*
			2	252,808	6.4	-0.28	-	-	-	4000.m00165	Acetyl-CoA carboxylase		
			2	36,812	8.9	-0.14	M	M	M	2884.m00186	Formyl transferase		
			2	80,029	6.6	-0.21	C	-	M	3385.m00173, 3006.m00143	Transketolase		
			2	29,775	10.6	-0.32	-	-	-	2541.m00142	Triacylglycerol lipase		
	46	46		35,327	8.2	-0.09	-	-	-	2329.m00193	(5)-2-hydroxy-acid oxidase		
Import	75			10,843	5.9	-0.59	-	-	-	Q9XGX7	Translocase of inner membrane 9 (TIM9)		
				15,677	9.0	0.12	-	-	-	2461.m00155 2506.m00124	Translocase of inner membrane 17 (TIM17)		
		34		37,956	6.8	-0.13	-	-	-	2733.m00140	Translocase of outer membrane 40 (TOM40)		*

(Table continues)



Table II. Continued

Functional Class	2D-PAGE	BN-PAGE	LC-MS	M <sub>r</sub>	pI	GRAVY	TP	PT	MP	Accession No.	Protein	At	Ps
Transport	46	42		9665	10.2	-0.01	M	-	-	2494.m00117, 3845.m00238	Translocase of outer membrane 9 (TOM9)		
	45			29,220	7.9	-0.16	-	M	-	TC48593, CAB82853	Voltage-dependent anion channel	*	
	18	39,36,26,54		-	-	-	-	-	-	TC65294	Voltage-dependent anion channel	*	
		45		27,815	8.7	-0.05	-	C	-	CAC80850, 2981.m00134	Voltage-dependent anion channel	*	
		36		24,534	7.7	0.01	C	C	M	5186.m00209	Voltage-dependent anion channel	*	
Ox stress				33,663	9.8	-0.12	-	-	-	3497.m00176	Voltage-dependent anion channel	*	
				41,510	10.3	-0.10	C	-	-	2506.m00136, 2986.m00110	Adenine nucleotide translocator ANT	b	
				33,021	9.4	0.14	C	-	-	3698.m00164, 3631.m00202	Phosphate transporter	b	
	1	55		32,802	10.1	0.10	-	-	-	3317.m00075	2-Oxoglutarate/malate translocator	b	
	24			24,947	7.0	-0.10	S	M	M	AAA57130, Q43008	Manganese superoxide dismutase	*	*
Chaperones				20,874	8.2	0.01	M	M	M	BAA90363	Peroxisome protein	*	
				17,146	7.5	-0.03	M	M	M	3606.m00327, 3602.m00254	Adrenodoxin precursor	*	
	5	57		83,380	8.1	-0.42	M	M	M	TC51015, 4256.m00159	Heat shock protein 70	*	*
	6, 7			60,850	5.6	-0.08	M	M	M	1379.m00089, 5187.m00183	Chaperonin 60	*	*
				65,276	4.8	-0.10	-	-	-	4781.m00152	Chaperonin 60	*	
Transcription/translation				10,643	9.0	-0.09	M	C	M	3664.m00136, T03585	Chaperonin 10	*	
				13,996	10.2	-0.39	M	M	M	5140.m00090	Chaperonin 10	*	
	19			30,643	7.1	0.03	S	-	-	2952.m00055	Prohibitin	*	
				31,413	10.1	-0.24	S	-	-	4127.m00167, BAC07170	Prohibitin	*	
	8	1, 2		48,423	6.4	-0.06	M	M	M	AAL5626t	Translational elongation factor T0	*	*
Miscellaneous function	35			15,547	8.9	-0.59	C	-	M	CAA05729	Gly-rich RNA-binding protein	*	
				97,919	10.8	-0.89	-	-	-	3373.m00119	DEAD box RNA helicase	*	
				127,634	6.3	-0.53	C	-	M	3952.m00081	DEAD box RNA helicase	*	
				172,773	9.1	-0.58	-	M	-	AAK54292	Putative helicase	*	
				31,230	9.2	-0.46	C	-	-	5028.m00163	C2H2-type zinc finger protein	*	
Unknown function protein				62,012	8.8	0.04	M	M	M	AAL59047	PPR protein		
				79,219	8.5	-0.06	M	M	M	BAB64813	PPR protein		
				184,237	8.1	-0.70	-	-	-	2474.m00123, 5076.m00090	CREB-binding protein		
	69			29,437	4.2	-0.53	M	-	M	2772.m00163	Mitochondrial glycoprotein		
	23			27,499	7.9	0.12	M	M	M	TC65865, 4358.m00206	Ferriyochoelin-binding protein	*	
Unknown function protein	44			26,455	6.4	-0.02	M	M	M	2030.m00175	Ferriyochoelin-binding protein	*	
	61	11		23,214	6.4	-0.19	M	-	-	TC48467, 2868.m00167	Ferriyochoelin-binding protein	*	
	3	10		67,681	5.0	-0.60	M	M	M	2249.m00144	Mitofilin motor protein	*	
		43		60,729	4.4	-0.74	S	-	-	2296.m00078	Calnexin-like protein		
		47		72,778	4.8	-0.46	C	-	-	2673.m00127	ADP-ribosylation factor binding protein		
Unknown function protein				53,280	6.5	-0.30	-	-	-	2500.m00126	Actin		
				39,196	6.1	-0.27	S	-	-	AAM01174	Wall-associated kinase 2-like protein		
				28,078	8.3	-0.37	-	-	-	BAB92895	sec14-like protein		
				98,221	6.6	-0.28	-	-	-	2251.m00196	Disease resistance protein		
				94,632	7.6	-0.27	S	-	-	4193.m00176	Disease resistance protein		
Unknown function protein		42, 23		10,945	9.4	-0.40	-	M	-	2419.m00150	Unknown protein		*
		56		12,914	7.3	-0.21	C	C	-	4127.m00174	Unknown protein		
		17		7,466	10.7	-0.55	M	M	-	2468.m00122, 2520.m00121	Unknown protein		
	72			27,479	9.2	-0.03	M	M	M	2284.m00104	Unknown protein		*
	74			13,273	8.7	-0.13	M	C	M	32.m00149	Unknown protein		*
	42		-	-	-	-	-	-	TC48363	Unknown protein			

(Table continues)

**Table II. Continued**

Functional Class	2D-PAGE	BN-PAGE	LC-MS	M <sub>r</sub>	pI	GRAVY	TP	PT	MP	Accession No.	Protein	At	Ps
Unknown function protein (continued)	1		1	134,187	5.6	-0.20	-	-	-	3273.m00129	Unknown protein		
	1		1	8786	9.2	-0.17	S	-	-	3018.m00158	Unknown protein		
	1		1	8101	9.8	-0.50	C	-	-	1987.m00120	Unknown protein		
	1		1	19,438	5.9	-0.33	-	-	-	4958.m00111	Unknown protein		
	3		3	126,829	6.7	-0.21	-	-	-	4983.m00113	Unknown protein		
	2		2	100,723	9.2	-0.41	M	M	-	4638.m00135	Unknown protein		
	2		2	78,515	10.7	-0.72	M	M	M	BAA84788	Unknown protein		
	2		2	12,966	12.1	-0.30	-	C	-	AAI58157	Unknown protein		
	3		3	75,587	5.8	0.01	-	-	-	AAI06831	Unknown protein		
	2		2	57,805	5.7	-0.46	M	C	-	2366.m00159, 2275.m00133	Unknown protein		
	2		2	48,104	8.7	-0.07	-	-	-	2284.m00111	Unknown protein		
	2		2	171,963	7.4	-0.63	-	M	-	4183.m00149, 3872.m00127	Unknown protein		
	2		2	69,967	8.7	-0.37	C	-	-	2475.m00089	Unknown protein		
2		2	48,779	8.7	-0.21	M	M	M	2631.m00097	Unknown protein			
2		2	129,607	8.7	-0.87	M	-	-	BAC20757	Unknown protein			
3		3	84,126	5.0	-0.65	M	-	-	5113.m00174	Unknown protein			
3		3	21,831	12.5	-1.27	M	-	-	AAI22746	Unknown protein			

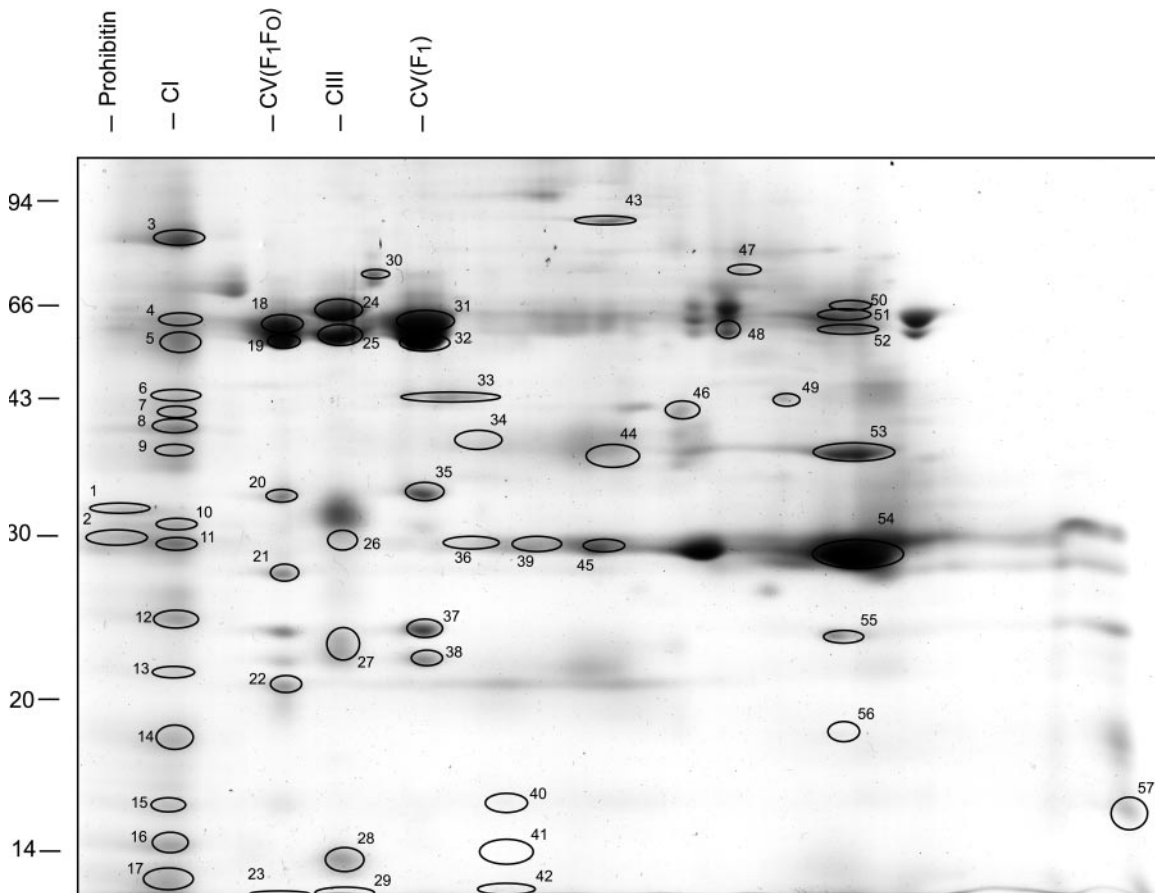
Matching of MS/MS spectra from peptides from IEF/SDS-PAGE and BN SDS-PAGE spots to predicted gene products was performed using ProID software (Applied Biosystems, Sydney) with custom-made databases using TIGR rice open reading frame and EST data. Many of the rice proteins identified were not represented at National Center for Biotechnology Information; thus, Mascot on-line (<http://www.matrixscience.com>) could not be used routinely for analysis. ProID was used with mass accuracy cutoffs of peptide mass  $\pm 0.15$  and MS/MS ions  $\pm 0.05$ , which are significantly more stringent than Mascot default settings. In most cases, more than three matching MS/MS spectra were identified by ProID, but in the case of a number of proteins with low masses (<15 kD), single or pairs of matching MS/MS spectra with very high probabilities of matching (>95%) derived by ProID were evaluated and included in Table II.

**LC-MS/MS Analysis of Whole Mitochondrial Protein Extracts**

Gel-based arraying of protein samples can exclude proteins from analysis due to hydrophobicity, size, and pI that physically prevents separation in these media. Gel arraying also prevents analysis of peptides that are not easily eluted from polyacrylamide gel samples. The use of reverse-phase chromatography using C18 media provides a gel-free separation of peptides from complex samples. Coupled with on-line MS/MS analysis, the gradual elution of peptides from reverse-phase C18 with organic solvent can be used as a powerful proteomic identification tool. We have taken this approach with a whole-rice mitochondrial protein extract. The identified peptides are matched back to predicted gene products, and the total number of peptides matched provides increasing confidence that the intact gene product was present in the original sample before digestion. Matching of peptides to predicted gene products was again performed using ProID (Applied Biosystems) using mass accuracy cutoffs of peptide mass  $\pm 0.15$  and MS/MS  $\pm 0.05$ . This analysis matched a set of 170 MS/MS spectra back to a set of 72 predicted rice gene products (Table II). For multi-peptide matches to a single gene product, a probability cutoff of 85% in ProID was used to filter matches; for single peptide matches, a probability cutoff of 99% in ProID was used for filtering.

**A Wide Range of Mitochondrial Functions Identified in Rice**

Extensive discussions of many of the components identified here and their biological roles can be found in the earlier reports on the proteomes of Arabidopsis and pea (Kruft et al., 2001; Millar et al., 2001; Bardel et al., 2002). Here, we have largely restricted any



**Figure 2.** BN SDS-PAGE separation of rice membrane mitochondrial proteins. A total of 89 protein spots from this map were excised, digested, and analyzed by MS/MS. Fifty-seven were identified, and these are numbered for comparison with text and Table II. Numbers on vertical axis are apparent molecular mass of protein subunits (kilodaltons), and annotations on horizontal axis are known mitochondrial protein complexes identified in this study.

extensive comment to components that were not identified in these earlier studies (Table II). The protein sequences for each entry were subjected to BLAST-based similarity analysis to confirm annotation or propose appropriate putative functional annotation for each entry.

### Electron Transport Chain

Over 30 components of the five electron transport chain complexes from rice were identified. Many of these samples were from BN SDS-PAGE (Fig. 2). This has now allowed the clear identification of the native complexes separating by this technique and noted on the horizontal axis of Figure 2. A significant number of these identifications, although clear orthologs to components of these complexes in other eukaryotic species, have not been reported previously in plant proteome mapping studies. These newly identified components include two subunits of ATP synthase, seven subunits of complex I, and three subunits of complex IV (Table II).

### Carbon Metabolism

At least one component of each of the seven tricarboxylic acid cycle enzymes were identified in Table II along with components of the associated pyruvate dehydrogenase complex and the NAD-malic enzyme. The two distinct NAD-malic enzyme subunits have been identified by antibody reaction on IEF/SDS-PAGE from potato (*Solanum tuberosum*; Jenner et al., 2001) but were not revealed by proteome mapping studies in Arabidopsis or pea. Here, the two subunits were both found on BN SDS-PAGE and appear to be present in separate high-molecular mass complexes. The larger subunit is in a complex with a mass over 300 kD based on its presence between complex III and separated  $F_0$  of the ATP synthase (Fig. 2). The E1 subunit of 2-oxoglutarate dehydrogenase complex was not identified in the Arabidopsis or pea reports, perhaps due to its large mass (Table II). Three components associated with Glyc metabolism in mitochondria, the H and T subunits of Gly decarboxylase and the Ser hydroxymethyltransferases, were identified, even though these rice mi-



tochondria were isolated from dark-grown shoots and the photorespiratory requirement for Gly metabolism was absent. A series of 18 enzymes involved in amino acid metabolism (Glu dehydrogenase, Asp transaminase, aldehyde dehydrogenases, and methylmalonate semi-aldehyde dehydrogenase), C1 metabolism (formate dehydrogenase), nucleotide metabolism (inosine-5-monophosphate dehydrogenase and formyl transferase), and miscellaneous metabolic functions were identified. Based on sequence similarity, the formyl transferase is mostly likely a phosphoribosylglycinamide formyl transferase (GART) involved in purine biosynthesis. The potential for plant mitochondria to facilitate nucleotide metabolism has been reported previously (Atkins et al., 1997; Smith et al., 1998). The acetyl-CoA carboxylase is normally involved in long-chain fatty acid synthesis, and although this is commonly considered to be a plastidic function in plants, both mammalian and yeast (*Saccharomyces cerevisiae*) mitochondria contain this enzyme (Schon, 2001).

#### Protein and Metabolite Transport

Four components of the import apparatus were identified, two TIM components (subunits 9 and 17) and two from the outer membrane TOM complex (subunits 9 and 40). The latter comigrated during BN electrophoresis (Fig. 2). In Arabidopsis, the TOM complex has to be extensively purified to see its subunits on BN-PAGE (Werhahn and Braun, 2002). Interestingly, the TOM9 and TOM40 subunits represent the core of the TOM complex in yeast, to which other subunits are peripherally attached. A series of specific gene products for the outer membrane voltage-dependent anion channels (porins) were identified, along with three members of the carrier family on the inner mitochondrial membrane responsible for the exchange of inorganic phosphate, ATP/ADP, and dicarboxylates. The Arabidopsis ortholog of the dicarboxylate carrier has been shown recently to be a broad dicarboxylate/tricarboxylate carrier capable of transporting many of the tricarboxylic acid cycle intermediates that enter and exit plant mitochondria (Picault et al., 2002). These carriers were identified mainly through LC-MS/MS, which is in agreement with the difficulty of identifying these proteins in two-dimensional gel separations from Arabidopsis (Millar and Heazlewood, 2003). None of these carrier proteins were identified in the IEF/SDS-PAGE approaches in Arabidopsis or pea (Millar et al., 2001; Bardel et al., 2002).

#### Miscellaneous Functions

The mitochondrial-specific superoxide dismutase and the redox carriers, peroxiredoxin and adrenodoxin, identified in Arabidopsis mitochondrial preparations, were also identified here in rice. Members

of the major mitochondrial molecular chaperone classes (70, 60, and 10) and the prohibitins were also found. A series of transcription/translation-related proteins were identified, including a translation elongation factor, a Gly-rich RNA-binding protein, and a set of RNA helicases, like those observed in Arabidopsis (Kruft et al., 2001; Millar et al., 2001). In addition, two members of the PPR (pentatricopeptide repeat) family have been found, along with members of the CH<sub>2</sub>H<sub>2</sub> zinc finger and CREB-binding families putatively involved in protein-RNA interactions. Proteins from the PPR class have been implicated in nuclear restoration of cytoplasmic male sterility in plants (Bentolila et al., 2002) and belong to a large family of proteins that may be responsible for the control of RNA stability and editing in plant mitochondria (Small and Peeters, 2000). To our knowledge, this is the first time these proteins have been definitively identified in plant mitochondrial isolates. A series of miscellaneous function proteins include homologs of the ferripyochelin-binding proteins that were also identified in Arabidopsis (Millar et al., 2001). Homologs of the known mitochondrial acid glycoprotein in yeast, Mam33p (Seytter et al., 1998), the coiled-coil mitofilin motor protein for cytoskeleton attachment to mitochondria (Odgren et al., 1996), along with actin itself, were also identified. A set of 23 unknown function proteins have been found for which no clear similarity based on sequence can be proposed, but a very significant number contain predicted mitochondrial-targeting presequences (Table II).

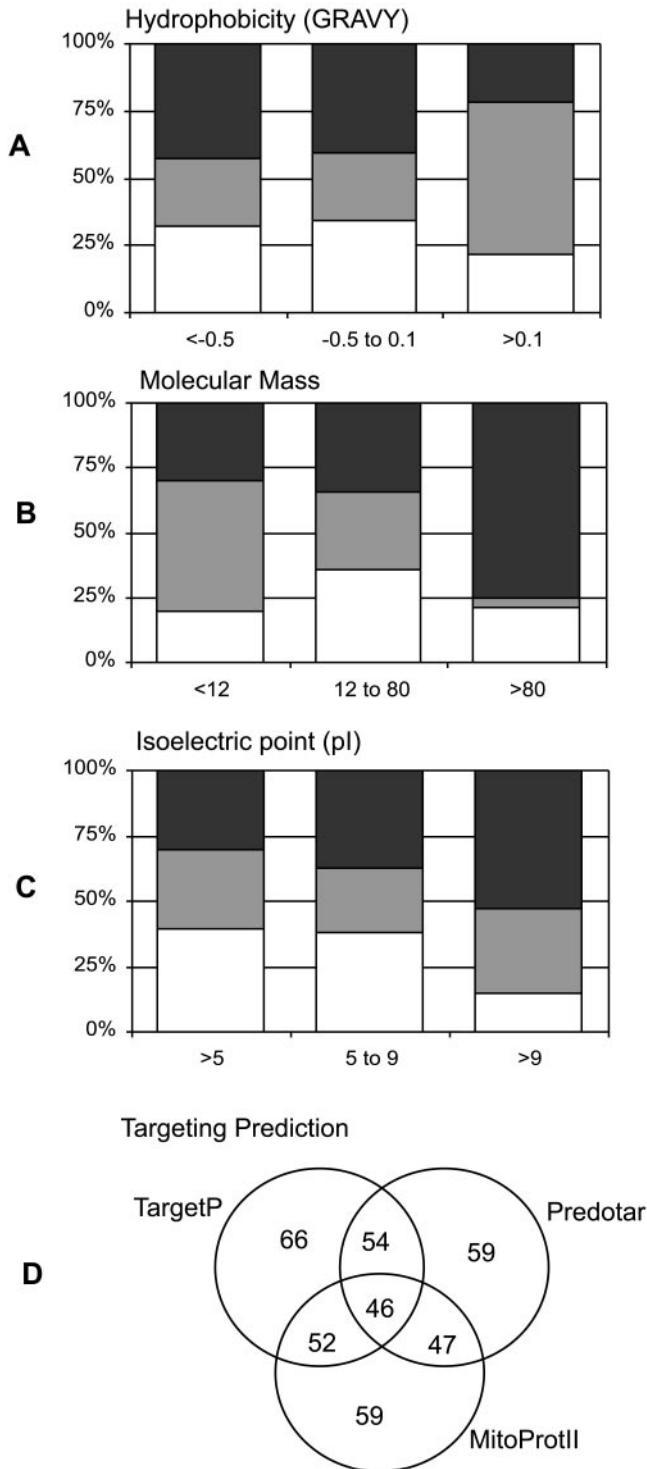
## DISCUSSION

### Comparison of the Three Proteome Datasets

Each separation technique identified a series of proteins contributing to a total of 136 nonredundant rice proteins (Table II). Within the total set, each separation technique matched 38% to 53% of the identified proteins. Using the predicted protein sequences, a series of parameters were calculated that included physical characteristics such as molecular mass, pI, and hydrophobicity (GRAVY), and predicted intercellular targeting based on N-terminal sequence as determined by Predotar, TargetP, and MitoProtII. These data were then used for further analysis of the proteome set as outlined below.

### More Hydrophobic, Large, and Basic Proteins Were Identified by BN SDS-PAGE and LC-MS Than by IEF/SDS-PAGE Separations

When the protein identifications made by each technique are considered in the context of physical properties of the predicted proteins, such as hydrophobicity, molecular mass, and pI, an interesting pattern emerges (Fig. 3, A–C). A GRAVY score is a single-value indication of the overall hydrophobicity



**Figure 3.** Comparison of identified rice protein sets. Proportion of rice proteins identified by the three separation techniques (IEF/SDS-PAGE, clear; BN SDS-PAGE, gray; and LC-MS, black) in different classes of hydrophobicity (A), molecular mass (B), and pI (C). D, Overlapping of targeting prediction of the rice proteins identified by TargetP, Predotar, and MitoProtII. Numbers in each circle indicate numbers predicted either by one, two, or all three predictors.

of a protein sequence based on the Kyte and Doolittle algorithms; the more positive the score, the more hydrophobic the overall sequence (Kyte and Doolittle, 1982). The addition of thiourea as a chaotropic agent, the advent of new nonionic sulfobetaine detergents, and the use of the non-charged reductant, tributylphosphine, have all been credited with allowing more hydrophobic proteins to enter two-dimensional gels for separation and analysis (Chevallet et al., 1998; Rabilloud, 1998). To date, proteins with GRAVY scores of approximately +0.25 to +0.30 are considered to represent the upper limit of these two-dimensional gel solubility improvements (Herbert, 1999). Our analysis has revealed that the highest GRAVY score for a rice mitochondrial protein identified from IEF/SDS-PAGE was +0.14 for the ATP synthase delta subunit. In BN SDS-PAGE, GRAVY scores reached +0.84 in the case of subunit 9 of the ATP synthase. Overall, there was little difference in the proportion of identifications achieved by the three separation techniques at GRAVY < +0.1, but above this level, more than 60% of the identifications were achieved from BN SDS-PAGE (Fig. 3A).

Proteins with large molecular masses can be separated by single-dimension SDS-PAGE. Proteins as large as 200 to 300 kD can be routinely separated using gradient gels. However, such large proteins are not very soluble during IEF and as a result, large proteins (>100 kD) are rarely seen on IEF/SDS-PAGE gels. The proportion of identifications of proteins greater than 80 kD was very significantly shifted toward the LC-MS/MS separations, where size of the parent protein was not an impediment to separation and analysis (Fig. 3B). The basicity of proteins has often restricted identification from IEF/SDS-PAGE due to the difficulty of preparing stable gradients in excess of pH 10 for separation of such proteins. Clearly, in our analysis, it is both the non-IEF gel-separating technique of BN SDS-PAGE and the non-gel LC MS/MS analysis that dominate the identifications of proteins with pI values greater than pH 9 (Fig. 3C).

These analyses clearly indicate that to fully characterize mitochondrial proteomes, a series of experimental approaches will be required to maximize the representation of all proteins without bias based on physical characteristics of size, charge, and hydrophobicity. LC MS/MS does not appear to be the complete answer, although when many biases are eliminated, a great deal of information about the parent protein from which peptides are derived is lost. Furthermore this technique appears to selectively not identify some proteins present in two-dimensional gel separation of the same material (Table II). Using different gel-based approaches, information on possible posttranslational modifications (such as the multiple spots for malate dehydrogenase, T protein of GDC, alpha-subunit of ATP synthase, and E1 $\beta$ -subunit of pyruvate dehydrogenase subunit; Fig. 1;

Table II) and protein-protein interactions (such as components in the electron transport chain and import translocase complexes; Fig. 2; Table II) can be uncovered.

### Targeting Prediction of the Rice Proteome Set

The predicted protein sequences of the identified rice proteome were analyzed by three targeting prediction programs commonly used to assess mitochondrial localization in plants (Table II). Five entries in Table II were matched only to rice TCs. It is unknown whether these represent full or partial sequences; thus, they have been excluded from the analysis. Each prediction program assessed that approximately 50 to 60 of the protein products contained N-terminal extensions for mitochondrial targeting. In total, 77 of the 130 protein sequences were predicted to be mitochondrial by at least one targeting program. Looking at the overlapping predictions (Fig. 3D), TargetP identified the largest number of proteins, and the combination of TargetP and Predotar provided the best pair for prediction in rice. In the list of 39 proteins not assessed to be mitochondrial targeted by any program, a large number (19) are well-known mitochondrial proteins involved in the electron transport chain, tricarboxylic acid cycle, import apparatus, and mitochondrial transport. This lack of prediction may be explained either by the absence of targeting presequences (as has been documented for carrier and import components; Millar and Heazlewood, 2003), cryptic presequences not recognized by these program, and/or the lack of presequences due to incorrect annotation of the exons making up N-terminal sequences of these predicted proteins (Frishman et al., 1999). A further three proteins are products of mitochondrial-encoded genes ( $\alpha$ -subunit ATP synthase, COXI, and NAD9); thus, they do not require targeting presequences. A total of 11 of the unknown function proteins identified were predicted as mitochondrial targeted by at least one prediction program.

### Comparison of Rice, Arabidopsis, and Pea Mitochondrial Proteomes

A comparison between the proteins found here in rice mitochondria and those previously discovered in Arabidopsis (Kruff et al., 2001; Millar et al., 2001) and pea mitochondria (Bardel et al., 2002) is presented in the final two columns of Table II. An asterisk in the second-to-last column indicates that an ortholog was identified in Arabidopsis among the 91 nonredundant set defined by the two Arabidopsis proteome reports and several recent publications containing small sets of proteins. Overall, this analysis indicates that 71 proteins have been found in the mitochondrial proteomes of both species, including two of unknown function. A further set of 28 of the identi-

fied rice proteins represent orthologs of well-known mitochondrial proteins in other eukaryotic systems but have not been identified in the published Arabidopsis proteome to date. An asterisk in the last column indicates that an ortholog was found in the nonredundant set of 48 proteins found to date in pea mitochondria. A total of 28 proteins were found in both rice and pea mitochondria. Six of these proteins were not observed in Arabidopsis mitochondria. This leaves a set of 35 proteins, of which 21 have no predicted function, that have been exclusively identified in rice mitochondria to date. This demonstrates the necessity to compare mitochondrial proteomes from different plant species, and most certainly between the major lineages of higher plants, to better understand the complex role of these organelles in the plant cell. However, until a much more complete survey of the proteomes of mitochondria in several plants is conducted in similar tissue types using more similar protein arraying and identification technology, it will be difficult to truly discuss the presence and/or absence of specific proteins between plant species with any accuracy. Currently, many absences in the published Arabidopsis and pea sets reflect technical issues rather than biological absence. Here, we highlight the rice mitochondrial protein complement and present the tools that will be required for such comparative plant proteome investigations in the future.

## MATERIALS AND METHODS

### Growth of Rice Seedlings

Batches of 200 g of rice (*Oryza sativa* cv Amaro) seed were washed in 1% (w/v) bleach for 10 min, rinsed in distilled water, and grown in the dark in vermiculite trays (30 × 40cm) at a constant 30°C, watered daily, and shoot tissue harvested at 7 d for mitochondrial isolation.

### Rice Mitochondrial Isolation

Rice shoots were harvested using scissors and cut into 5- to 10-mm lengths into a beaker in ice. Cut shoots (100 g) were ground in a precooled mortar and pestle using acid-washed sand in 300 mL of homogenization solution (0.3 M Suc, 25 mM tetrasodiumpyrophosphate, 2 mM EDTA, 10 mM  $\text{KH}_2\text{PO}_4$ , 1% [w/v] polyvinylpyrrolidone-40, 1% [w/v] bovine serum albumin, and 20 mM ascorbate [pH 7.5]). After filtering homogenate through four layers of Miracloth (Calbiochem, San Diego), it was centrifuged for 5 min at 1,000g, and the supernatant was centrifuged again at 20,000g for 20 min. The resultant organelle pellet was resuspended in wash buffer (0.3 M Suc, 10 mM TES-KOH [pH 7.5], and 0.1% [w/v] bovine serum albumin), and the two centrifugations steps were repeated to produce a washed organelle pellet. This resuspended pellet was layered over 0% to 4.4% (w/v) polyvinylpyrrolidone-40 preformed gradient in a 28% (v/v) Percoll self-forming gradient in wash buffer and centrifuged at 30,000g for 45 min. Mitochondria formed a band toward the bottom of the gradient, the upper plastid material was discarded, and the mitochondrial band was removed in an attempt to minimize contamination of peroxisomal material from the bottom of the gradient. After two wash centrifugation steps at 20,000g for 15 min, the pellet was layered over a second self-forming gradient consisting of 45% (v/v) Percoll in wash buffer and centrifuged for 30 min at 30,000g. Mitochondria remained near the top of the gradient, whereas peroxisomal material migrated to the bottom of the gradient. The mitochondrial layer was aspirated and concentrated by wash centrifugation steps at 20,000g for 15 min.



## Respiratory Measurements

O<sub>2</sub> consumption was measured in a Clark-type O<sub>2</sub> electrode in 1 mL of reaction medium containing: 0.3 M mannitol, 10 mM TES-KOH (pH 7.5), 5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM NaCl, 2 mM MgSO<sub>4</sub> and 0.1% (w/v) bovine serum albumin. Pyruvate (5 mM), malate (0.5 mM), succinate (10 mM), NADH (1 mM), ADP (0.5 mM), and myxothiazol (5 μM) were added as indicated to modulate O<sub>2</sub> consumption rates. Cytochrome c oxidase activity was measured as ascorbate (5 mM) and cytochrome c (25 μM)-dependent O<sub>2</sub> consumption in the presence of 0.05% (w/v) Triton X-100. Outer membrane integrity was assayed as the latency of cytochrome c oxidase activity (Neuberger, 1985).

## Two-Dimensional Gel Electrophoresis

Mitochondria protein samples (500 μg) were acetone extracted by addition of acetone to a final concentration of 80% (v/v) at -80°C. Samples were stored at -20°C for 4 h and then centrifuged at 20,000g for 15 min. The pellets were resuspended in an IEF sample buffer consisting of: 6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) ampholytes (pH 3–10), 2 mM tributylphosphine, and 0.001% (w/v) bromophenol blue. Aliquots of 360 μL were used to reswell dried 180-mm, nonlinear immobilized pH gradient strips (pH 3–10; Immobiline DryStrips, APBiotec, Sydney) overnight, and then IEF was performed for 19.5 h, reaching a total of 49 kV h<sup>-1</sup> at 20°C on a flat-bed electrophoresis unit (Multiphor II, APBiotec). Immobilized pH gradient strips were then transferred to an equilibration buffer consisting of 50 mM Tris-HCl (pH 6.8), 4 M urea, 2% (w/v) SDS, 0.001% (w/v) Bromophenol blue, and 100 mM mercaptoethanol and incubated for 20 min with rocking. The equilibrated strips were then slotted into central single wells of 4% (w/v) acrylamide stacking gels above 0.1- × 18.5- × 20-cm, 12% (w/v) acrylamide, 0.1% (w/v) SDS-polyacrylamide gels. Strips were overlaid with 0.5% (w/v) agarose in SDS-PAGE running buffer. Gel electrophoresis was performed at 25 mA per gel with circulating cooling and completed in 5 h. Proteins were visualized by colloidal Coomassie (G250) staining. M<sub>r</sub> and pI standards from APBiotec were used to confirm fixed pH gradient positioning on first dimension separation and to identify apparent molecular masses on second dimension separation.

## BN PAGE/SDS-PAGE

BN PAGE was performed largely according to Jansch et al. (1996). Gels consisted of a separating gel (5%–15% [w/v] acrylamide) and a stacking gel (4% [w/v] acrylamide) were formed in a solution of 0.25 M ε-amino-*n*-caproic acid and 25 mM Bis-Tris-HCl (pH 7.0). The anode buffer consisted of 50 mM Tricine, 15 mM Bis-Tris-HCl, 0.02% (w/v) Blue G250 (pH 7.0), and the cathode buffer of 50 mM Bis-Tris-HCl (pH 7.0). Aliquots of 1 mg of membrane protein were suspended in 75 μL of an amino caproic acid buffer solution (containing 0.75 M ε-amino-*n*-caproic acid, 0.5 mM Na<sub>2</sub>EDTA, and 50 mM Bis-Tris-HCl [pH 7.0]) followed by the addition of 15 μL of a freshly prepared solution of 10% (w/v) *n*-dodecylmaltoside. Afterward, 10-min centrifugation at 20,000g was performed. Fifteen microliters of 5% (w/v) Blue G250 was added to the supernatants (dissolved in amino caproic acid buffer). Gels were run at 4°C in a precooled apparatus with all samples, and buffers were precooled to 4°C. Approximately 30 min before the run, 0.03% (w/v) *n*-dodecylmaltoside was added to the cathode buffer only. Electrophoresis was commenced at 100-V constant voltage for 45 min and then increased to 15 mA without voltage limitation for 5 h. BN lanes were cut from gels, equilibrated in standard SDS-PAGE sample buffer for 30 min, laid horizontally on 12% (w/v) acrylamide SDS-PAGE separating gels, sealed in with 0.5% (w/v) agarose, and electrophoresis was continued for 5 h under standard conditions.

## Trypsin Digestion of Gel Plugs and Whole Mitochondrial Extracts

Protein spots to be analyzed were cut from two-dimensional PAGE gels, destained, dried at 50°C, and digested with trypsin according to Sweetlove et al. (2001). Aliquots of 50 μg of purified rice mitochondrial protein were acetone precipitated, and the protein pellets were air dried. A digestion solution consisting of 100 mM Tris-HCl (pH 8.6) and 50 μg mL<sup>-1</sup> trypsin was added to a total volume of 45 μL, and the digestion mixture was incubated for 16 h at 37°C.

## Quadrupole TOF MS

Quadrupole TOF MS/MS was performed on an Applied Biosystems Q-STAR Pulsar using an IonSpray source. Samples from gel plugs were directly injected into the electrospray source in 50% (v/v) methanol/0.1% (v/v) formic acid, and selected doubly charged peptides, identified in MS-TOF mode, were fragmented by N<sub>2</sub> collision and analyzed by MS/MS using IDA software (Applied Biosystems, Foster City, CA). Samples from digestion of whole extracts were bound onto a microbore HPLC C18 column (Agilent, Palo Alto, CA) and eluted over 6 h with a linear acetonitrile gradient from 2% to 80% (v/v) in water. Mass spectra and collision MS/MS data were analyzed with Analyst QS and BioAnalyst software (Applied Biosystems).

## Identification of Database Entry Matches and Sequence Analysis

TIGR draft rice genome and rice EST gene indices and rice National Center for Biotechnology Information entries were used for sequence matches. Predotar (<http://www.inra.fr/Internet/Produits/Predotar/>), TargetP (<http://www.cbs.dtu.dk/services/TargetP/>), and MitoProtII (<http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter>) predictions were determined using full-length predicted protein sequences. Molecular mass, pI, and GRAVY scores were determined using the ProtParam program on an ExPASy Web site (<http://au.expasy.org>).

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