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Phytochemistry 65 (2004) 1693–1707
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

The hydrophobic proteome of mitochondrial membranes from *Arabidopsis* cell suspensions ☆

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Received 2 February 2004; received in revised form 24 March 2004
Available online 10 May 2004

Abstract

The development of mitochondria and the integration of their function within a plant cell rely on the presence of a complex biochemical machinery located within their limiting membranes. The aim of the present work was: (1) to enhance our understanding of the biochemical machinery of mitochondrial membranes and (2) to test the versatility of the procedure developed for the identification of the hydrophobic proteome of the chloroplast envelope [Molecular and Cellular Proteomics 2 (2003) 325–345]. A proteomic analysis was performed, to provide the most exhaustive view of the protein repertoire of these membranes. For this purpose, highly purified mitochondria were prepared from *Arabidopsis* cultured cells and membrane proteins were extracted. To get a more exhaustive array of membrane proteins from *Arabidopsis* mitochondria, from the most to the less hydrophobic ones, various extraction procedures (chloroform/methanol extraction, alkaline or saline treatments) were applied. LC-MS/MS analyses were then performed on each membrane subfraction, leading to the identification of more than 110 proteins. The identification of these proteins is discussed with respect to their mitochondrial localization, their physicochemical properties and their implications in the metabolism of mitochondria.

In order to provide a new overview of the biochemical machinery of the plant mitochondria, proteins identified during this work were compared to the lists of proteins identified during previous proteomic analyses performed on plant and algae mitochondria (*Arabidopsis,* pea, *Chlamydomonas,* rice, etc.). A total of 502 proteins are listed. About 40% of the 114 proteins identified during this work were not identified during previous proteomic studies performed on mitochondria.

Keywords: *Arabidopsis thaliana*; Membrane proteomics; Mitochondria; Transport

1. Introduction

Mitochondria play a central role in eukaryotic cells by providing ATP by the process of oxidative phosphorylation. Mitochondria are also involved in many other cellular functions including numerous catabolic or anabolic reactions, apoptotic cell death (Newmeyer and Ferguson-Miller, 2003; Balk and Leaver, 2001). In humans and in animals, a growing number of severe pathologies have also been attributed to mitochondrial defects (Wallace, 1999). When compared to their animal counterparts, plant mitochondria carry additional functions (Douce, 1985; Douce and Neuburger, 1989). For example, in photosynthetic tissues, plant mitochondria participate, during the photorespiratory pathway, in the conversion of glycine into serine and accumulate in the matrix space a high amount of glycine

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2004.03.028.

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doi:10.1016/j.phytochem.2004.03.028

cleavage system and serine hydroxymethyltransferase (Douce and Neuburger, 1999; Bardel et al., 2002). Plant mitochondria possess specific biochemical properties such as NAD-malic enzyme involved in malate oxidation (Neuburger and Douce, 1977; Winning et al., 1994) or implicated in biosynthesis of essential cofactors such as vitamin C (Siendones et al., 1999), biotin (Picciocchi et al., 2001, 2003), folate (Rébeillé et al., 1997) and lipoic acid (Gueguen et al., 2000). In addition to the basic metabolite transport system found in mammalian mitochondria, specific carriers or transporters have been found in mitochondria isolated from plant tissues (Douce and Neuburger, 1989). The presence of additional NAD(P)H dehydrogenases (Moller, 2002) and an alternative oxidase (Vanlerberghe and McIntosh, 1997) in the inner membrane of plant mitochondria confer to the plant respiratory chain an increased flexibility during bioenergetic functions. The plant respiratory chain is characterized by the presence of a bifunctional cytochrome *c* reductase that is also a processing peptidase involved in removal of presequences of peptides encoded by the nuclear genome and targeted to mitochondria (Braun et al., 1992). Among the examples that highlight the functional complexity of plant mitochondria, which covers a great variety of biochemical reactions and physiological processes, is the implication of plant mitochondria in the cytoplasmic male sterility (Schnable and Wise, 1998; Bentolila et al., 2002).

More than 98% of the mitochondrial proteins are encoded in the nucleus and synthesized as precursors in the cytosol before being targeted to mitochondria (for a review, see Pfanner and Wiedemann, 2002). One way to identify the full proteome of mitochondria is to identify mitochondrial targeting sequences present in these nuclear-encoded proteins. Several bioinformatic tools have been developed that attempt to predict subcellular localization of nuclear-encoded proteins (for reviews, see Nakai, 2000; Emanuelsson and von Heijne, 2001). Each method has its strengths and weaknesses, but there are still conflicting predictions between these tools regarding which proteins are targeted to mitochondria. Since all these programs mispredict a significant proportion of proteins (Heazlewood et al., 2004), these bioinformatic predictions require additional experimental validation. Furthermore, these methods concentrate on the identification of cleavable N-terminal targeting sequences. They therefore do not correctly predict the localization of members of the mitochondrial carrier family since these proteins lack N-terminal targeting sequences (for reviews, see Pfanner and Wiedemann, 2002; Rehling et al., 2003).

The recent development of proteomics opens the path toward a deeper exploration of the mitochondrial functions and recent works dealing with mitochondria proteome analysis from *Arabidopsis thaliana* (Kruft et al., 2001; Millar et al., 2001; Giegé et al., 2003; Herald et al., 2003; Millar and Heazlewood, 2003; Heazlewood et al., 2004), pea (Bardel et al., 2002), *Chlamydomonas reinhardtii* (van Lis et al., 2003) and rice (Heazlewood et al., 2003) have proven the usefulness of the approach. The expected size of the mitochondrial proteome varies among the organism: in yeast the size has been estimated between 400 and 800 proteins, whereas in higher organisms this number seems larger, around 1000 proteins in human mitochondria and 1500–2000 in plant mitochondria (Rehling et al., 2003). A possible explanation of this larger number of proteins in plant could be due to the presence of isoforms of several enzymes and additional mitochondrial functions (see above, Kruft et al., 2001).

The aim of a subcellular targeted proteomic study, such as the present one, is to provide relevant identifications to enhance the understanding of the biochemical machinery of the purified organelle for subsequent functional studies. In a precedent work we focused our attention on the soluble complement of plant mitochondria and reported 2D-maps of soluble proteins of mitochondria isolated from different tissues and organs, thus revealing the impact of tissue differentiation at the mitochondrial level (Bardel et al., 2002). The aim of the present work was also to test the versatility of the procedure developed for the identification of hydrophobic proteins from the chloroplast envelope (Seigneurin-Berny et al., 1999; Ferro et al., 2000, 2002, 2003). To perform this analysis, we applied various extraction procedures (chloroform/methanol extraction, alkaline and saline treatments) to get a more exhaustive array of the mitochondrial membrane proteins, from the most to the less hydrophobic ones. These fractionations, based on the physicochemical properties of proteins, reduce the complexity of the protein sample and increase the representation of low abundance proteins relatively to the total protein in the original membrane fraction. The identification of these proteins is discussed with respect to their physicochemical properties, their mitochondrial location and their implications in the metabolism of mitochondria.

2. Results and discussion

2.1. Purification of Arabidopsis mitochondria and membrane subfractions

Mitochondria from *A. thaliana* cells cultured in the light were purified according to Davy de Virville et al. (1994) and Ravanel et al. (2001) with some modifications of the more recent described protocol in order to optimize yield and purity. Using this protocol, an average of 10 mg mitochondrial proteins was purified from 300 g of 6-day-old *Arabidopsis* cells (on the basis of protein, average yield of purified mitochondria from protoplasts

was 2.6%). Purified mitochondria were capable of oxidizing succinate (400 nmol O_2 min^{-1} mg^{-1} protein in the presence of ADP, with a respiratory control of 1.7) and NADH (430 nmol O_2 min^{-1} mg^{-1} protein in the presence of ADP, with a respiratory control of 2.8). In order to quantify the contamination of the purified mitochondria by plastids, the recovery of chlorophylls in the purified mitochondria was quantified and compared to the recovery of the activity of a mitochondrial marker. The specific activity of the mitochondrial marker fumarase (EC 4.2.1.2) was 417 nmol min^{-1} mg^{-1} proteins in purified mitochondria. This represents an enrichment of 10 when compared to the specific activity of the same marker in crude protoplast extract (41 nmol min^{-1} mg^{-1} proteins) and validates the previously described purification procedure (Ravanel et al., 2001). The chlorophyll content of purified mitochondria was 0.74 mg chlorophyll/mg proteins for an original chlorophyll concentration of 4.46 mg chlorophyll/mg of proteins in crude protoplast extract. This clearly demonstrates that the purified mitochondria still remain partially contaminated with some plastid proteins. The same was true for the peroxisomal marker hydroxypyruvate reductase (EC 1.1.1.81) since the specific activity of this marker was 67 nmol min^{-1} mg^{-1} proteins for an original specific activity of 103 nmol min^{-1} mg^{-1} proteins in crude protoplast extract. Purified mitochondria, chloroplasts and crude protein extracts were also analyzed by SDS–PAGE and Western blots in order to validate the enrichment of the mitochondrial proteins and further analyze cross-contamination with thylakoid membranes (Fig. 1). It is noticeable that the T subunit of the glycine–decarboxylase complex, known as a major matrix component in green leaf mitochondria (Douce et al., 2001), is highly enriched in purified mitochondria when compared to the crude cell extract in which it is hardly visible (Fig. 1). This is in good agreement with the 10 times enrichment obtained for the specific activity of the mitochondrial marker fumarase (see above). In order to further analyze the cross-contamination with thylakoid membrane proteins, Western blot experiments were performed using polyclonal antibodies specific for light harvesting complex proteins (LHCPs). LHCPs, which are major thylakoid proteins, were highly enriched in the chloroplast fraction when compared to crude leaf extract. On the contrary, mitochondria prepared from cultured cells appeared to contain relatively low amounts of thylakoid proteins (Fig. 1) in good agreement with the quantification of chlorophylls in the analyzed fractions (see above).

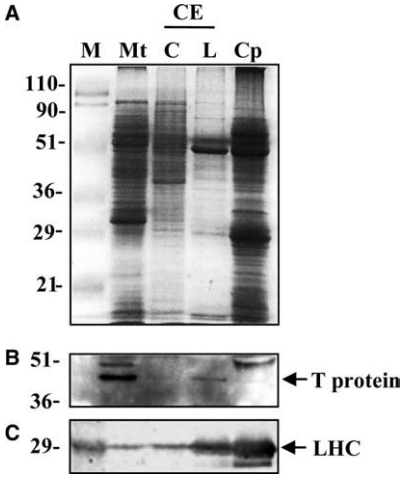

Fig. 1. Characterization of Percoll-purified *Arabidopsis* mitochondria. M, markers; Mt, mitochondria proteins extracted from *Arabidopsis* cells; C, crude extract of proteins from *Arabidopsis* cells; L, crude extract of proteins from *Arabidopsis* leaves; Cp, chloroplast proteins extracted from *Arabidopsis* leaves. (A) Fractions were analyzed on a 12% SDS–PAGE. Each fraction contained 20 μg proteins. (B,C) Western blots were performed with polyclonal antibodies raised against the T protein of the glycine decarboxylase complex (mitochondria marker) or against the LHCP (chloroplast marker), respectively. Each fraction contained 15 μg proteins.

Finally, the good purity of the membranes deriving from these Percoll-purified *Arabidopsis* mitochondria was confirmed through the proteomic analysis. Indeed, only 2% of the identified *Arabidopsis* proteins were found to correspond to known non-mitochondrial proteins (Fig. 2). Among them, one protein PIP1.1 (Table 1) appears to correspond to a previously characterized major plasma membrane component. It is also important to notice that only one identified protein, CB21 (Table 1), appears to derive from plastid, an organelle known to represent a classical contaminant of purified mitochondria. This value may be underestimated since more than 30% of the identified proteins correspond to unknown proteins that were not predicted to be associated with mitochondria using classical bioinformatic tools (Table 1 and Fig. 2). However, most of these proteins are likely to be genuine mitochondrial proteins (see below).

2.2. Identification of A. thaliana mitochondrial membrane proteins

In order to purify mitochondrial membranes, Percoll-purified intact mitochondria were lyzed in hypotonic medium and the membranes were then separated from the matrix proteins by centrifugation. From these mitochondrial preparations, an average of 50% of membrane proteins could be recovered, the other 50% corresponding to soluble proteins from the matrix. We previously demonstrated that various membrane proteins could be extracted when using complementary methods of extraction, i.e., chloroform/methanol extraction, alkaline treatment and saline treatment, to identify as many membrane proteins as possible, from the most to the less hydrophobic ones (Ferro et al., 2003). The present study further demonstrates the versatility of this method. Indeed, 83 out of 114 identified

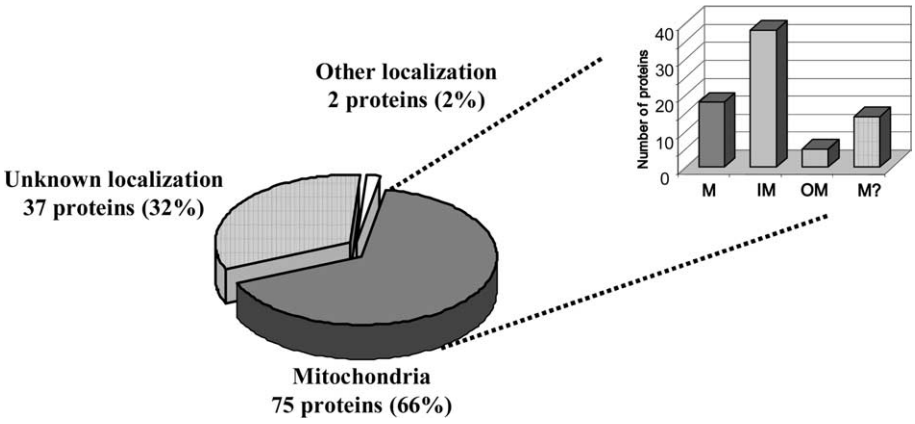

Fig. 2. Subcellular localization of the identified proteins. OM, outer membrane of mitochondria; IM, inner membrane of mitochondria; M, mitochondria; M?, predicted localization in the mitochondria.

proteins (73%) were identified using only one extraction procedure, 26 proteins (23%) were identified using two extraction procedures and only five proteins (4%) were found using all three extraction methods (Fig 3).

2.2.1. Physicochemical properties of identified mitochondrial membrane proteins

As previously observed for chloroplast envelope membrane proteins (Ferro et al., 2003), the more stringent the extraction method is, the less the number of identified proteins is. In the present study, the difference in protein dynamic range and in stringency with respect to protein hydrophobicity might explain the complementarities of the methods. Organic solvent extraction appears to select for low M_r and high hydrophobicity with a poor contamination with hydrophilic proteins (Fig. 4). It is important to note that the few proteins identified using this procedure and predicted to contain no transmembrane domain (as predicted by HMMTOP) mostly correspond to integral membrane proteins like porins (see Table 1, Transport). On the contrary, saline treatment appears to be less selective for hydrophobicity. Indeed, most of the identified proteins recovered from the saline treatment are predicted to contain no or only one transmembrane domain (Fig. 4). This difference in predicted hydrophobicity explains the low value of cross-identification using these two extraction procedures. On the other hand, alkaline treatment appears to be a good alternative to the two previous methods. Indeed, this procedure clearly selects for more intrinsic proteins when compared to saline treatment since less hydrophilic proteins are recovered (Fig. 4). However, this selection is less stringent than chloroform/methanol extraction since proteins are identified in a wide range of size and hydrophobicity (Fig. 4).

When referring to the charge of the protein, it appears that the extraction with the chloroform/methanol procedure is rather selective since most of the intrinsic proteins identified in this fraction are basic (Fig. 5). However, since intrinsic proteins identified using both alkaline and saline treatments are mostly distinct from the proteins identified with the organic solvents, it remains difficult to suggest that this bias is due to the extraction procedure and not to the nature of extracted proteins.

2.2.2. Submitochondrial localization of identified proteins

As very few known proteins from other compartments were detected in the present proteomic study (Table 1), most of the identified proteins are likely to be located in mitochondria. Our aim being the identification of mitochondrial membrane proteins, the identified proteins were classified according to their known (IM, OM and M) or putative (M?) localization within the mitochondria (see Table 1 and Fig. 2). The known localizations of the proteins, or their association with the mitochondrial membranes, were essentially retrieved from the literature. The putative localizations were assessed using software programs. Moreover, some of these proteins have putative functions which are compatible with their localization in the mitochondrial membranes. Localization of contaminant proteins (referred to Th and PM in Table 1) were deduced from their strong homology with proteins known to be localized in other subcellular compartments. According to this classification, about two-third (75/114) of the identified proteins can be considered as genuine mitochondrial membrane proteins (Table 1). However, one cannot exclude that some of the proteins without proposed localization could actually lack a predictable targeting peptide as previously described for proteins from both outer and inner mitochondrial membranes (for reviews, see Pfanner and Wiedemann, 2002; Rehling et al., 2003).

When considering the subcellular localization of the 44 proteins that were not identified through previous proteomic studies, it appears that only 36% (16/44) are known (8/44) or predicted (8/44) to be associated with mitochondria, 5% (2/44) are known to be associated with other subcellular compartments and 59% (26/44)

Table 1
List and general characteristics of the proteins identified in mitochondrial membranes (classification according to protein function)

Protein name	N° SP/ TrEMBL	N° AGI	Description	Loc	TM	Res / TM	Gravy index	M_r	pI	Pept nb	Extraction Metthod		
											C/M	Na OH	NaCl
Tranport													
HP165	Q9XIE2	At1g59870	ABC transporter-like	??	15	98	-0.062	165082	8.08	2	-	+	-
HP52	Q9LP58	At1g29310	Putative protein transport protein SEC61 alpha SU	M ?	12	40	0.529	52171	9.12	1	+	-	-
STA1	Q9LF78	At5g58270	Half-ABC transporter	IM (M)	7	104	-0.020	80395	9.16	3	-	+	-
ADT2	P40941	At5g13490	ADP, ATP carrier protein 2 (ANT 2)	IM (M)	6	64	-0.111	41746	9.79	2	-	-	+
MCAT	Q93XM7	At5g46800	Carnitine/acylcarnitine carrier-like protein	IM (M)	5	60	0.173	31023	9.56	2	+	-	+
HP40	Q9FMU6	At5g14040	Phosphate translocator	IM (M)	5	75	0.145	40090	9.29	2	-	+	-
HP31	Q8SFL2	At5g19760	Dicarboxylate/tricarboxylate carrier-like	IM (M)	4	75	0.129	31912	9.35	3	+	+	+
ADT1	P31167	At3g08580	ADP, ATP carrier protein 1 (ANT 1)	IM (M)	4	95	-0.120	41476	9.84	10	-	+	+
PUMP2	O81845	At3g54110	Uncoupling protein PUMP2-like	IM (M)	3	102	0.176	32662	9.62	4	-	+	+
HP29	Q9FKM2	At5g57490	Porin-like protein	OM (M)	1	274	-0.166	29505	9.23	3	+	-	-
HP20	Q8LE12	At5g58070	Outer membrane lipoprotein lipocalin-like	??	0	-	-0.626	20687	5.96	1	-	-	+
VDAC2	Q9SMX3	At5g15090	Porin 2 (VDAC2)	OM (M)	0	-	-0.190	29080	7.98	14	+	+	+
VDAC1	Q9SRH5	At3g01280	Porin 1 (VDAC1)	OM (M)	0	-	-0.122	29294	8.77	6	+	-	+
HP29b	Q9FJX3	At5g67500	Porin-like protein	OM (M)	0	-	-0.138	29595	8.88	4	+	+	+
Protein targeting													
TIM17	Q9SP35	At2g37410	TIM17	IM (M)	3	81	-0.267	25440	5.24	1	-	+	-
HP37	Q9M288	At3g44370	low homology to mitochondrial inner membrane	IM (M)	2	169	-0.119	37547	7.75	1	-	+	-
MPA1	Q9ZU25	At1g51980	Mitochondrial processing peptidase alpha SU1-like	IM (M)	2	251	-0.126	54402	5.94	6	-	-	+
HP42b	Q8VYE2	At1g55900	TIM50-like	IM (M)	1	376	-0.475	42640	9.28	1	-	-	+
MPA2	O04308	At3g16480	Mitochondrial processing peptidase alpha SU2-like	IM (M)	1	499	-0.123	54053	6.04	4	-	-	+
TOM40	Q9LHE5	At3g20000	TOM40 homolog	OM (M)	0	-	-0.220	34116	6.36	1	-	-	+
HP59	Q9SGA7	At3g02090	Mitochondrial processing peptidase-like	IM (M)	0	-	-0.342	59160	6.03	8	-	-	+
ATP synthases													
ATP9*	P92895	ATMG01080	ATP synthase C chain	IM (M)	2	37	1.414	7571	8.28	1	+	-	+
HP06b	Q9SN96	At3g46430	ATP synthase-like	IM (M)	1	55	-0.351	6585	10.17	1	+	+	-
ATP5	Q96253	At1g51630	ATP synthase epsilon chain	IM (M)	1	70	-0.406	7832	9.39	1	+	-	-
AT21	P83483	At5g08670	ATP synthase beta chain 1	IM (M)	1	556	-0.155	59671	6.18	17	-	+	+
ATPQ	Q9FT52	At3g52300	ATP synthase D chain	IM (M)	0	-	-0.913	19455	5.09	2	-	-	+
ATP4	Q96252	At5g47030	ATP synthase delta chain	IM (M)	0	-	0.017	21548	6.20	1	+	-	-
ATP0_b	Q96251	At5g13450	ATP synthase delta chain	IM (M)	0	-	-0.088	26322	9.25	1	-	-	+
ATP7	Q9SJ12	At2g21870	ATP synthase-like 24 kDa SU	IM (M)	0	-	-0.656	27597	6.27	4	-	-	+
ATP3	Q96250	At2g33040	ATP synthase gamma chain	IM (M)	0	-	-0.054	35448	9.01	2	-	+	+
ATP0	P92549	At2g07698	ATP synthase alpha chain	IM (M)	0	-	-0.029	55045	6.23	13	-	+	+
Respiratory chain													
CYB	P42792	ATMG00220	Cytochrome-*b*	IM (M)	9	44	0.637	44291	6.65	2	-	+	+
HP33	Q9LK29	At3g27240	Cytochrome-*c*1	IM (M)	2	154	-0.184	33650	5.74	2	-	+	+
AX1A	Q39219	At3g22370	Alternative oxidase 1a	IM (M)	2	177	-0.327	39978	8.56	1	-	-	+
HP43b	Q9SK66	At2g20360	NADH-ubiquinone oxidoreductase 43 kDa SU (complex I)	IM (M)	2	201	-0.059	43936	9.26	1	-	+	-
HP65	Q9SKT7	At2g20800	NADH dehydrogenase 65 kDa	IM (M)	2	291	-0.363	65372	9.03	3	-	+	+
HP05	Q94K78	At2g40765	Ubiquinol-cytochrome-*c* reductase complex 6 kDa protein	IM (M)	1	57	0.228	5976	8.14	1	+	-	-
HP29c	Q9LYR2	At5g13440	Ubiquinol-cytochrome-*c* reductase iron sulfur SU	IM (M)	1	274	-0.157	29902	8.86	1	-	+	-
HP08	Q8LDP7	At1g15120	Ubiquinol-cytochrome-*c* reductase complex 8 kDa protein	IM (M)	0	-	-0.638	8013	7.57	1	+	-	+
UCR6	Q9SUU5	At4g32470	Ubiquinol-cytochrome-*c* reductase complex 14 kDa protein	IM (M)	0	-	-0.633	14527	9.73	2	-	+	+
HP11b	Q9SKC9	At2g02050	NADH-ubiquinone oxidoreductase 11 kDa SU (complex I)	IM (M)	0	-	-0.428	11740	7.65	2	+	-	-
NUCM	P93306	ATMG00510	NADH dehydrogenase SU 7 (complex I)	IM (M)	0	-	-0.408	44578	7.70	1	-	-	+
NUAM	Q9FGI6	At5g37510	NADH-ubiquinone oxidoreductase (Complex I-75 kDa)	IM (M)	0	-	-0.154	81182	6.24	2	-	-	+
HP31e	Q9G3M0	At3g27380	Succinate dehydrogenase iron-sulphur SU	IM (M)	0	-	-0.412	31227	8.78	1	-	-	+
DHSA	O82663	At5g66760	Succinate dehydrogenase [ubiquinone] flavoprotein SU (complex II)	IM (M)	0	-	-0.355	69656	5.86	5	-	+	+
Carbon metabolism													
HP49b	Q8H107	At4g26910	Putative dihydrolipoamide succinyltransferase	M	4	116	-0.255	49988	9.08	1	-	+	-
HP50	Q9FLQ4	At5g55070	2-oxoglutarate dehydrogenase E2 SU	M	4	116	-0.251	50133	9.19	2	-	+	+
MDHM	Q9ZP06	At1g53240	Malate dehydrogenase, mitochondrial precursor	M	1	341	0.165	35804	8.54	3	-	+	+
ODPB	Q38799	At5g50850	Pyruvate dehydrogenase E1 beta SU	M	0	-	-0.002	39176	5.67	2	-	-	+
HP58	Q8RWN9	At3g13930	Dihydrolipoamide acetyltransferase	M	0	-	-0.332	58438	7.55	1	-	-	+
Chaperones													
HP71	Q9SZJ3	At4g37910	HSP70 like protein	M	1	666	-0.298	71174	5.31	6	-	+	+
HSP60	O49314	At2g33210	Chaperonin (HSP60)	M	0	-	-0.023	55254	5.31	5	-	+	+
HP60b	Q9LRW0	At3g13860	Chaperonin CH60-like	M	0	-	-0.023	60466	5.85	1	-	-	+
CH60	P29197	At3g23990	Chaperonin CPN60	M	0	-	-0.069	61281	5.66	4	-	-	+
Other functions													
UMP2	Q9ZUX4	At2g27730	F1F0-ATPase inhibitor protein-like	IM (M)	2	57	-0.467	11948	9.64	2	+	+	+
SODB	P21276	At4g25100	Superoxide dismutase [Fe], chloroplast precursor (EC 1.15.1.1)	??	2	117	-0.300	26507	6.87	1	+	-	-
C983	O22203	At2g40890	Cytochrome P450 98A3	??	2	254	-0.263	57927	8.74	2	-	+	-
CAX1	P29402	At5g61790	Calnexin homolog 1 precursor	??	2	265	-0.758	60486	4.81	4	-	+	+
HP30	O04331	At5g40770	Prohibitin	??	1	277	-0.013	30400	6.99	8	-	+	-
HP30e	Q9LK25	At3g27280	Prohibitin	??	1	279	-0.083	30638	6.94	1	-	-	+
HP31f	Q9SIL6	At2g20530	Putative prohibitin	M ?	1	286	-0.140	31637	9.65	1	+	+	+
HP31b	Q9ZNT7	At1g03860	Putative prohibitin 2 protein	??	1	286	-0.155	31811	9.39	7	-	-	+
APX3	Q42564	At4g35000	Ascorbate peroxidase (EC 1.11.1.11)	??	1	287	-0.365	31572	6.47	1	-	-	+
HP31d	Q8LA39	At4g28510	Prohibitin-like protein	??	1	288	-0.116	31711	9.26	4	-	+	+
C726	O65787	At2g24180	Cytochrome P450 71B6	??	1	503	-0.115	57009	7.70	1	-	-	+
HP59b	O49650	At4g22690	Cytochrome P450-like	??	1	524	-0.172	59236	6.23	1	-	+	-
HP60	Q9SVG4	At4g20830	Reticuline oxidase-like protein	??	1	540	-0.172	60287	9.55	1	-	+	-
QUA1	Q9LSG3	At3g25140	Glycosyltransferase QUASIMODO1 (EC 2.4.1.-)	??	1	558	-0.370	64368	8.87	1	-	+	-
THH3	Q42403	At5g42980	Thioredoxin H-type 3 (TRX-H-3)	??	0	-	0.163	13109	5.06	1	+	-	-
GSHH	O48646	At4g11600	Probable phospholipid hydroperoxide glutathione peroxidase	??	0	-	-0.253	18609	6.59	1	+	-	-
KADA	O82514	At5g63400	Adenylate kinase 1	??	0	-	-0.393	26932	6.91	3	-	-	+
KADB	Q9FK35	At5g50370	Adenylate kinase 2	??	0	-	-0.382	27335	6.91	2	-	-	+
HP30b	Q9C5S7	At1g47260	Transcription factor APFI	M	0	-	-0.146	30053	6.24	2	-	+	+
HP30c	Q8LDU0	At4g28360	50S ribosomal protein L22, putative	M ?	0	-	-0.541	30456	10.92	1	-	+	-
RT04	Q31708	ATMG00290	Ribosomal protein S4	M	0	-	-0.512	43163	10.90	1	-	+	-

Table 1 (*continued*)

Protein name	N° SP/ TrEMBL	N° AGI	Description	Loc	TM	Res / TM	Gravy index	M_r	pI	Pept nb	Extraction Metthod		
											C/M	Na OH	NaCl
Unknown function													
HP25	Q9SX96	At1g47640	HP	??	7	33	0.600	25309	8.57	1	+	-	-
HP11c	O64847	At2g26240	HP	??	4	27	0.620	11108	10.07	1	+	-	-
HP26	Q9LEW9	At3g61070	HP	??	3	79	0.045	26299	9.89	2	-	+	-
HP26b	Q9LTX0	At3g12650	HP	M ?	3	79	0.031	26887	9.80	1	-	+	-
HP27	Q9SU27	At4g12590	HP	??	3	82	0.037	27915	9.48	1	+	-	-
HP43	O23281	At4g14230	HP	M ?	3	136	-0.012	43844	5.70	1	-	+	-
HP74	Q93Z16	At4g21150	HP	M ?	3	230	0.166	74668	7.16	3	-	+	-
HP85	Q9M1Z2	At3g59820	HP (calcium binding protein)	M	3	252	-0.512	85735	6.31	1	-	-	+
HP11	Q8LD96	At5g25940	Nodulin-like protein	??	2	55	0.043	11522	10.49	2	+	-	-
HP12b	Q949R9	At5g20090	HP	M ?	2	55	-0.182	12438	9.34	1	-	+	+
HP14	Q8LDV0	At5g18250	HP	??	2	66	-0.002	14157	9.26	1	+	-	-
HP15	Q941A6	At1g08480	HP	??	2	71	-0.305	15813	6.28	2	+	+	-
HP61	Q9SVK6	At3g50930	HP (BCS1 mitochondrial protein like)	M	2	267	-0.548	61288	5.83	1	-	-	+
HP62	Q9M292	At3g44330	HP	??	2	283	0.031	62202	5.99	2	-	+	-
HP06	Q9ZPY5	At2g46540	HP	M ?	1	65	0.551	6803	9.03	1	+	-	-
HP08b	O64725	At2g02510	HP	??	1	72	-0.503	8051	10.01	1	+	-	-
HP10	O65262	At4g00530	HP	??	1	94	0.382	10677	6.04	1	+	-	-
HP10b	Q9MAL8	At1g01170	HP	??	1	97	0.382	10579	9.52	1	+	-	-
HP10d	Q8LBU0	At5g62575	HP	??	1	99	-0.101	10731	9.95	1	-	-	+
HP10c	Q8GWC7	At1g72170	HP	??	1	102	-0.091	10752	4.98	1	-	-	+
HP12	Q8L3S7	At3g57785	HP	M ?	1	114	-0.521	12662	9.51	1	+	-	-
HP13	Q9M0D5	At4g29480	HP	??	1	122	-0.366	13940	9.02	1	-	-	+
HP18	P93303	At2g07707	HP (mitochondrial genome)	M	1	158	-0.074	18211	9.69	1	-	-	+
HP20b	Q9C542	At1g55160	HP	??	1	188	-0.529	20828	9.78	1	-	-	+
MI25	S34401	ATMG00640	HP (mitochondrial genome)	M	1	192	0.271	21693	9.53	1	-	+	-
HP25c	Q9SIV6	At2g16460	HP	M ?	1	230	-0.317	25790	9.16	1	-	-	+
HP30d	Q9C8N0	At1g53760	HP	M?	1	272	-0.266	30607	9.52	1	-	-	+
HP49	Q9ZR98	At5g39410	HP (low homology to SLL1601 *Synechocystis*)	??	1	454	-0.178	49681	8.54	2	-	+	+
HP54	Q9FZ33	At1g54990	HP	??	1	473	-0.234	52419	5.63	1	-	+	-
HP63	Q9LRP6	At3g15590	HP (pentatricopeptide repeat (PPR) containing protein)	M ?	1	610	-0.355	69394	8.50	1	-	-	+
HP72	Q8RXF8	At5g27540	HP (calcium binding protein)	M	1	648	-0.249	72322	5.17	1	-	-	+
HP07	Q9FN38	At5g53880	HP	??	0	-	-1.564	7782	9.19	1	-	+	-
HP11d	Q8LB46	At4g22310	HP	??	0	-	-0.082	11906	9.37	1	+	-	-
UMP6	Q9SX77	At1g47420	HP	M	0	-	-0.323	28106	6.19	1	-	-	+
HP31c	Q9SRH6	At3g01290	HP (hypersensitive-induced response protein)	??	0	-	-0.069	31321	5.67	4	-	+	-
UMP1	Q9LKA5	At3g15000	HP	M	0	-	-0.949	42869	9.38	1	-	+	-
HP70	Q9ASV5	At4g39690	HP	M ?	0	-	-0.514	70553	5.12	1	-	-	+
Other localization than mitochondria (experimental validation)													
CB21	P04777	At1g29910	Chlorophyll A-B binding protein	Th	3	89	0.022	28227	5.29	1	-	-	+
WC1A	P43285	At3g61430	Aquaporin PIP1.1 (PIP1a) (Aquaporin 1)	PM	6	48	0.367	30689	9.14	1	-	+	+

No. SP/TrEMBL: accession number in Swiss-Prot or TrEMBL. No. AGI: accession number in the TAIR protein database. The gray AGI numbers correspond to proteins that were not previously associated with mitochondria through proteomic studies. Loc, subcellular localization; ??, unknown localization; M, mitochondria; M?, probably associated with mitochondria (at least one of the programs TargetP, Psort and Predotar indicated a location in the mitochondria); IM (M), inner membrane of the mitochondria; OM (M), outer membrane of the mitochondria; Th, thylakoids; PM, plasma membrane. TM, number of transmembrane domains according to HMMTOP (http://www.enzim.hu/hmmtop/); Res/TM, ratio between the number of residues and the number of transmembrane domains. The GRAVY (grand average of hydropathicity) index was calculated using the ProtParam tool available on the Expasy server (http://au.expasy.org/tools/protparam.html). Pept nb, number of unique peptides that allowed the identification of the corresponding protein (LC-MS/MS described in the paper were considered all together); ATP9*, this protein was identified with respect to P92895, a protein from *Lolium perenne*. The ATMG01080 accession number corresponds to the similar *Arabidopsis* protein. SU, subunit; HP, hypothetical protein.

are of unknown and unpredictable subcellular localization. Most (81%; 21/26) of these proteins are predicted to contain at least one transmembrane helice. Since some mitochondrial proteins from both the inner and outer membrane are known to lack a classical (and thus predictable transit sequence), and because bioinformatics tools are not always reliable, mitochondrial localization of these proteins is highly likely. These 26 proteins would then correspond to previously unknown and unpredictable mitochondrial proteins.

2.3. A functional survey of identified mitochondrial membrane proteins

The possible function of the identified proteins was also assessed using bioinformatic tools such as Blast or InterProScan (Fig. 6, Table 1). Most of the identified mitochondrial proteins are proteins involved in actual or putative mitochondrial functions. Almost one-third of these mitochondrial proteins have unknown function and none of the bioinformatic tools we used were able to suggest any function.

2.3.1. Plant mitochondrial respiration: beyond the control of the protonmotive force

Plant mitochondria are characterised by the presence of both phosphorylating (cytochrome) and non-phosphorylating (alternative) respiratory pathways, the relative activities of which directly affect the efficiency of mitochondrial energy conservation (for a review, see Affourtit et al., 2001). Some components of all complexes of the plant mitochondrial respiratory chain were identified during this study (Table 1). From all these proteins, only one subunit (At1g15120, Table 1) of the

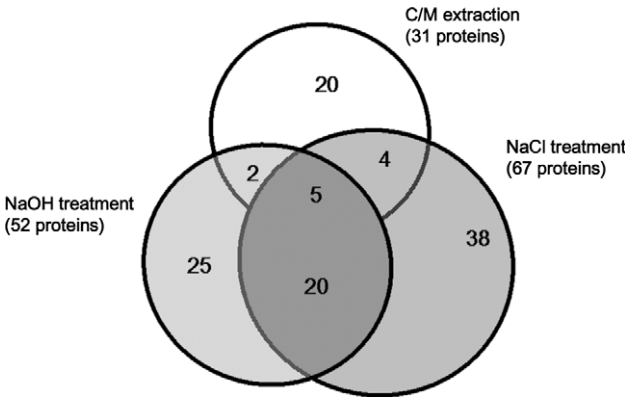

Fig. 3. Proteins identified in mitochondria membrane extracts according to the extraction method. As previously observed during the work performed on the chloroplast envelope (Ferro et al., 2003), the use of several extraction procedures allows identifying various proteins. Note that 83 out of 114 proteins were identified using only one extraction procedure, 26 proteins were identified using two extraction procedures and only five proteins were identified using all three extraction procedures.

complex III and only one subunit of the ATPase (At1g51630, Table 1) were not identified through previous targeted proteomic studies (see supporting information, Table 2).

2.3.2. Members of the mitochondrial carrier family

Screening of protein sequence database for proteins homologous to several mitochondrial carrier lead to the identification of a large mitochondrial carrier family (MCF) showing conserved residues, six transmembrane

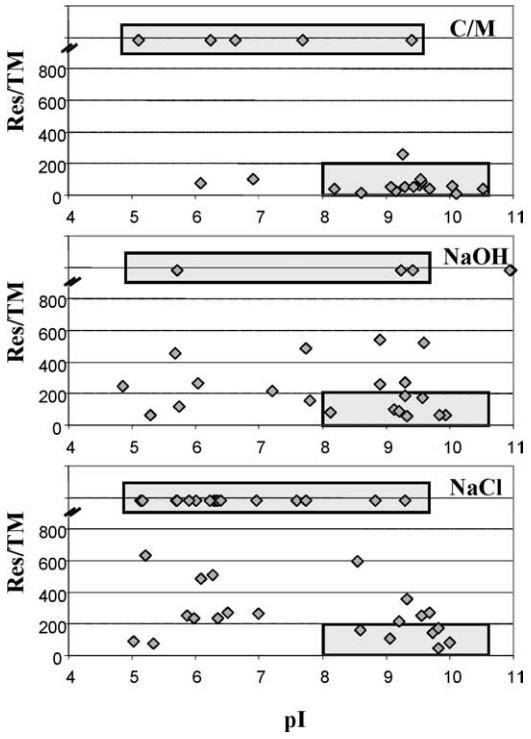

Fig. 5. Physicochemical properties (charge and hydrophobicity) of identified proteins in mitochondria membrane extracts according to the extraction method. As previously observed in Fig. 4, chloroform/methanol extraction selects for high hydrophobicity. Alkaline and saline treatments are less selective for hydrophobicity. None of the extraction procedure appears to be selective for the charge of the protein. Res/TM indicates the ratio of residue number on predicted transmembrane helices.

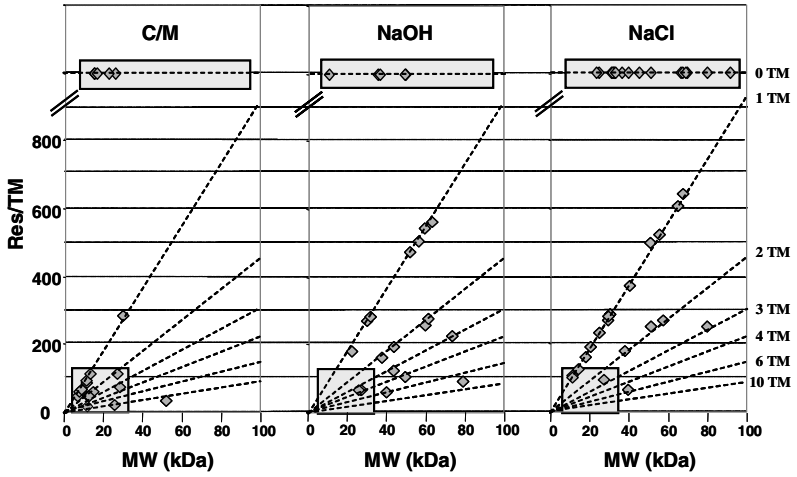

Fig. 4. Physicochemical properties (hydrophobicity and M_r) of identified proteins in mitochondria membrane extracts according to the extraction method. C/M extraction selects for low M_r and high hydrophobicity with a poor contamination with hydrophilic proteins. The few proteins containing no predicted TM (as predicted by HMMTOP) are true membrane proteins like the four identified porins (see Table 1). On the contrary, salt extraction is less selective and allows recovery of less hydrophilic proteins. Many identified proteins contain no or only one predicted TM. Saline treatment appears to be more selective for hydrophobicity (less hydrophilic proteins when compared to alkaline treatment). However, this selection is less stringent than chloroform/methanol extraction, since proteins are identified in a wide range of M_r and hydrophobicity. Res/TM indicates the ratio of residue number on predicted transmembrane helices.

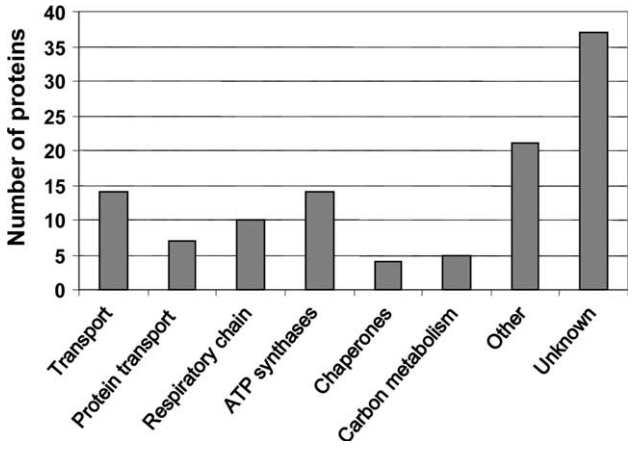

Fig. 6. Survey of the functions of the proteins identified in the membranes of *A. thaliana* mitochondria (according to Table 1). Contaminants from subcompartments other than mitochondria were not considered.

α-helical domains, a fairly uniform size of about 300 residues as well as phylogenetic relationships (For a review, see Kuan and Saier, 1993). Members of the family are present exclusively in eukaryotic organelles, mostly in mitochondria, and were found in our study. The model for such proteins is the mitochondrial ATP/ADP exchange carrier.

ATP, the principal energy currency of the cell, fuels most biosynthetic reactions in the cytoplasm by its hydrolysis into ADP and inorganic phosphate. ATP synthesized in the mitochondrial matrix is then exported into the cytoplasm, while ADP is imported into the matrix. This exchange is accomplished by proteins of the ADP/ATP mitochondrial carrier family. Since some very rare structures at atomic resolution were reported for membrane proteins implicated in transport processes, it is important to note that the structure of the bovine mitochondrial ADP/ATP carrier was recently solved at a resolution of 2.2 Å by X-ray crystallography (Pebay-Peyroula et al., 2003). Two previously known members of this family (At3g08580 and At5g13490, Table 1) were identified during this study. Surprisingly, while highly hydrophobic and basic, these integral membrane proteins were not identified during the chloroform/methanol extraction procedure.

We also identified a carnitine/acylcarnitine carrier-like protein (At5g46800, Table 1). By similarity with animal homologues, this protein may transport carnitine or acylcarnitine from the cytosol to the mitochondrial matrix as an alternative or a complement to the succinate-producing glyoxylate cycle. It was recently demonstrated to be involved in the transition from the embryonic stage to the juvenile autotrophic stage of *Arabidopsis* plants (Lawand et al., 2002).

A member of the dicarboxylate/tricarboxylate (DTC) mitochondrial carrier family was also identified during this study (At5g19760, Table 1). These proteins catalyze the transport of 2-oxoglutarate across the inner mitochondrial membrane in an electroneutral exchange for malate or other dicarboxylic acids and play an important role in several metabolic processes. Functional analyses of this plant mitochondrial carrier family have demonstrated that these proteins are capable of transporting both dicarboxylates (such as malate, oxaloacetate, oxoglutarate and maleate) and tricarboxylates (such as citrate, isocitrate, *cis*-aconitate and *trans*-aconitate) (Picault et al., 2002).

The phosphate translocator (At5g14040, Table 1) that was identified is a close homologue to mitochondrial proton/phosphate transporters from animal cells. These proteins are known to co-transport phosphate groups with H^+ from the cytosol to the mitochondrial matrix. The isolation and characterization of cDNAs from several plants (soybean, maize, rice and *Arabidopsis*) have been reported (Takabatake et al., 1999). The current state of research covering the molecular regulation and biochemical mechanisms involved in the transport of phosphate in plant has been reviewed recently. The protein identified here was classified as ARAth;Pht3;1 in the Pht3 family of plant phosphate transporters from mitochondria (Rausch and Bucher, 2002).

2.3.3. Members of the ABC transporters family

Two ABC transporters were identified during this study. The first protein (At5g58270, Table 1) is classified as a half-ABC transporter since it contains only seven predicted transmembrane helices. The role of this protein was already investigated *in planta*. A mutation in the *Arabidopsis* gene coding for this protein induces mitochondrial accumulation of more non-heme, non-protein iron than do wild-type organelles (Kushnir et al., 2001). Furthermore, this protein can substitute for the maturation of cytosolic Fe/S protein in Atm1p-deficient yeast. These results suggests that plant mitochondria possess an evolutionarily conserved Fe/S cluster biosynthesis pathway, which is linked to the intracellular iron homeostasis by the function of this Atm1p-like ABC transporters.

The second putative ABC transporter (At1g59870, Table 1) we identified is classified as the PDR8 ABC transporter in the plant PDR family of ABC transporters (Van Den Brule and Smart, 2002). This protein is predicted to contain 15 transmembrane helices. Its function and its role *in planta* still remain to be identified. This transporter was not associated to mitochondria in previous targeted proteomic studies (see supporting information Table 2). However, as it is the case for several inner membrane proteins, the lack of any predictable mitochondrial transit peptide does not exclude that this protein is a genuine mitochondrial protein.

2.3.4. Members of the porin family

Porins are a group of β-barrel proteins having the electrophysiological property of symmetric voltage gat-

ing. This property led to their identification as voltage-dependent anion channels (VDACs). Based on our knowledge of yeast porins (see for instance Dihanich et al., 1987), VDACs are generally considered as the main pathway for the transport of small molecules (<8–10 kDa) across the mitochondrial outer membrane and are essential for mitochondrial respiration. Therefore, it is not surprising to find a series of VDACs (At5g15090 and At3g01280) or porin-like proteins (At5g57490 and At5g67500) in mitochondrial membranes (Table 1). However, yeast has only two VDAC genes, both encoding proteins of the outer mitochondrial membrane, whereas the number of plant and animal homologues is much larger, some of them being extra-mitochondrial. For instance, not all animal VDAC reside to the mitochondria, they also appear to be located in the caveolae domains of the plasma membrane (Bathori et al., 2000; Buettner et al., 2000). The same is probably true in plants (Wandrey et al., 2004). Since VDAC do not have any targeting sequences, their subcellular localization should be further validated.

2.3.5. A lipocalin-like protein

We identified a lipocalin-like protein (At5g58070, Table 1) that is classified as a transport protein. Lipocalins are a large and diverse group of small proteins that bind and transport small, principally hydrophobic molecules such as steroids, lipids, pheromones and odorant molecules. They share a conserved tertiary structure of eight β-strands forming a barrel configuration and are found in vertebrates and invertebrate animals, plants and bacteria (Sanchez et al., 2003). Lipocalins were early established as transport proteins, but it is becoming increasingly clear that some of them may be implicated in many other important functions. The first plant enzymes identified as members of the lipocalin family are localized in the thylakoid lumen: the violaxanthin de-epoxidase and zeaxanthin epoxidase, two enzymes of the xanthophyll cycle, responsible for the protection against photo-oxidative damage (Bugos et al., 1998). To date, we have no idea of a possible role for a lipocalin-like protein in mitochondria. Furthermore, since this protein was not previously reported to be associated to mitochondria, the subcellular localization of this protein remains to be validated.

2.3.6. Members of the mitochondrial protein import machinery

If one excludes the very few proteins that are encoded by the mitochondrial genome, most proteins residing in this organelle are nuclear-encoded and synthesized in the cytosol before being delivered to their final destination. This last step depends on a network of specialised import components that form at least four main translocation complexes (for a review, see Truscott et al., 2003). Several members of TIM and TOM complexes and mitochondrial processing peptidase subunits were identified during this study.

The mechanism of protein export from the matrix to the inner membrane (i.e., mitochondrial-encoded inner membrane proteins) is still poorly understood (Truscott et al., 2003). Interestingly, and as a first evidence for its mitochondrial localization, we identified a protein (At3g44370, Table 1) which shares homology with the precursors of the chloroplast ALBINO3 protein and the inner mitochondrial membrane protein OXA1. The OXA1 protein is a member of an evolutionary conserved protein family with homologs in the inner membrane of bacteria (YidC) and the thylakoid membrane of chloroplasts (Alb3). It is part of the "export machinery" and is required for the insertion of some integral membrane proteins into the mitochondrial inner membrane. The composition and mode of action of the export machinery is however far from being solved and thus should provide rapid developments in mitochondrial protein transport in the near future.

Finally, and surprisingly, a SecY-like protein was also identified (At1g29310, Table 1). The bacterial Sec genes encode a generalized protein export machinery. Although the mitochondria present in eukaryotic cells are derived from bacterial ancestors, a comprehensive search of the complete genomic sequence for the yeast *Saccharomyces cerevisiae* did not reveal any close homologues of the bacterial Sec genes (Glick and Von Heijne, 1996), strongly suggesting that yeast mitochondria lack a generalized bacterial-type export system. This finding originally raised the question of how were mitochondrial-encoded and nuclear-encoded inner membrane proteins that are assembled via the conservative sorting mechanism, integrated into the inner membrane in the absence of a prokaryotic-type Sec-machinery (Luirink et al., 2001). Recently, compelling evidence has been obtained suggesting that Oxa1p constitutes or contributes to a novel translocon for inner membrane proteins (see above). Since the SecY-like protein identified during this study was not previously reported or even suspected to be associated to mitochondria, the subcellular localization of this protein remains to be validated and contamination of the purified mitochondrial membranes used for this analysis with membranes of the ER cannot be excluded.

2.3.7. Chaperones and prohibitins

Together, the chaperones of the mitochondrial matrix form a complex interdependent chaperone network that is essential for most reactions of mitochondrial protein biogenesis. While some chaperones mediate the prevention of aggregation under stress conditions and eventually the degradation of mitochondrial proteins, the major Hsp70 of the mitochondrial matrix (At4g37910, Table 1) is essential for the translocation of cytosolic precursor proteins across the two mitochondrial membranes (for a

review, see Voos and Rottgers, 2002). All four chaperones identified during this work (Table 1) were identified through previous targeted proteomic studies (see supporting information Table 2).

An ubiquitous and conserved proteolytic system regulates the stability of mitochondrial inner membrane proteins. Two proteases with catalytic sites at opposite membrane surfaces form a membrane-integrated quality control system and exert crucial functions during the biogenesis of mitochondria. Their activity is modulated by another mitochondrial inner membrane complex, which is composed of prohibitins (for a review, see Nijtmans et al., 2002). The five prohibitins identified during this study (At5g40770, At3g27280, At2g20530, At1g03860 and At4g28510, Table 1) were already reported to be associated to mitochondria in previous targeted proteomic approaches (see supporting information Table 2).

A calnexin homologue (At5g61790) was also found in our preparations (Table 1). Calnexin is considered as an endoplasmic reticulum-localized, calcium-binding protein that is believed to function as a molecular chaperone (Bergeron et al., 1994). For instance, Li et al. (1998) proposed that calnexin act as molecular chaperones in the folding and assembly of newly synthesized vacuolar H^+-ATPase at the endoplasmic reticulum from oat seedlings. The presence of a calnexin homologue in *Arabidopsis* mitochondrial membranes questions the functional significance of this observation, but this subcellular localization requires further validation.

2.3.8. Cytochrome P450s

Plants use a diverse array of cytochrome P450 monooxygenases (P450s) in their biosynthetic and detoxificative pathways. The P450s involved in biosynthetic pathways play critical roles in the synthesis of lignins, pigments, fatty acids, defence compounds, UV protectants, hormones and signaling molecules. Those that are involved in catabolic pathways participate in the breakdown of endogenous compounds and toxic compounds encountered in the environment. In general, P450s occur predominantly in microsomal membranes, but they have also been described in chloroplasts where they are involved in the formation of pigments and lipid-derived signaling molecules (see for instance Blee and Joyard, 1996; Helliwell et al., 2001; Tian et al., 2004). For instance, Ferro et al. (2003) identified CYP74A (allene oxide synthase) in chloroplast envelope membranes by proteomics. However, while previously demonstrated to be targeted to, and folded in, the inner membrane of animal mitochondria (Robin et al., 2002), members of this superfamily were never reported, to our knowledge, to be associated with plant mitochondria. Table 1 shows the presence, within membranes from *Arabidopsis* mitochondria, of three cytochrome P450 (At2g40890, At2g24180 and At4g22690), all containing at least one predicted transmembrane domain. These results provide further evidence for the wide diversity of P450s active in various plant cell compartments.

2.3.9. Carbon metabolism and TCA Cycle

We identified a series of components of the mitochondrial 2-oxoglutarate dehydrogenase complex and pyruvate dehydrogenase complex (Table 1), both being essential to cellular metabolism within the tricarboxylic acid cycle. The 2-oxoglutarate dehydrogenase complex catalyses the oxidative decarboxylation of 2-oxoglutarate (2-OG) to form succinyl-CoA and NADH by the sequential operation of three separate enzymes: 2-oxoglutarate dehydrogenase (OGDC-E1, EC 1.2.4.2), dihydrolipoamide succinyltransferase (OGDC-E2, EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4). E3 is also present in the pyruvate dehydrogenase complex. This complex catalyses the oxidative decarboxylation of pyruvate to form acetyl-CoA and NADH by the sequential operation of three separate enzymes: pyruvate dehydrogenase (E1; EC 1.2.4.1), dihydrolipoamide transacetylase (E2; EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4). The 2-oxoglutarate dehydrogenase complex (OGDC) is largely associated with the membrane fraction of osmotically ruptured mitochondria, whereas most of the other tricarboxylic acid cycle enzymes are found in the soluble matrix fraction (Millar et al., 1999). In the same experiments, Millar et al. (1999) found only a small proportion of the pyruvate dehydrogenase complex to be associated with mitochondrial membranes. The question thus raised is whether the pyruvate dehydrogenase complex only resides to the mitochondrial matrix or whether it is functionally associated to the inner membrane.

2.3.10. Other functions

We identified a series of proteins with putative functions, but which have not yet been localized within the cell (Table 1). Therefore, they can be either true mitochondrial proteins or contaminants from other cell membranes, or even present in several compartments. In all cases, proper subcellular localization should be validated. Among the proteins with putative mitochondrial function, two adenylate kinases (At5g63400 and At5g50370) were identified. Such proteins reside within the intermembrane space and their activity can affect both the amount of ADP available to ATP synthase and the level of ATP regulating electron transport (Roberts et al., 1997).

We also identified a series of proteins that could be involved in oxidative stress responses: Indeed, mitochondria are the sites of vital cellular functions such as respiration (oxidative phosphorylation) that can result in the formation of active oxygen species. The production of such harmful molecules results in lipid peroxidation, in protein inactivation and degradation, etc.

Among the proteins found in membrane from *Arabidopsis* mitochondria, we identified a phospholipid hydroperoxide glutathione peroxidase (At4g11600), an ascorbate peroxidase (At4g35000) and a superoxide dismutase (At4g25100). The actual function of such enzymes in plants is unknown, but some clues are provided by the demonstration that mitochondrial phospholipid hydroperoxide glutathione peroxidase is involved in the protection from inactivation of the adenine nucleotide translocator during hypoglycemia-induced apoptosis (Imai et al., 2003). Another point is that enzymes like ascorbate peroxidases are distributed in distinct compartments (for a review, see Shigeoka et al., 2002). In fact, key components of the ascorbate–glutathione cycle in *Arabidopsis* cell organelles are encoded by single organellar targeted isoforms that are dual localized in the chloroplast and the mitochondria (Chew et al., 2003). Indeed, we found the same ascorbate peroxidase (At4g35000) in mitochondrial membranes (Table 1) and in envelope membranes from *Arabidopsis* chloroplasts (Ferro et al., 2003). Together, these results are in agreement with the model of an integrated ascorbate–glutathione antioxidant defense common to plastids and mitochondria that is linked at the level of the genome in *Arabidopsis* (Chew et al., 2003).

Thioredoxins, a class of small 12-kDa redox proteins, are also present in all plant cell compartments and involved in oxidative stress responses. We identified in *Arabidopsis* mitochondria a thioredoxin h3 (At5g42980), an animal-like thioredoxin known to occur in multiple cell compartments (Marcus et al., 1991). Divergent results were obtained concerning the localization of thioredoxin h isoforms: soybean proteins were reported to be bound to the plasma membrane, whereas *Arabidopsis* h2 and pea h4 thioredoxins were both extracted as soluble proteins (discussed in Montrichard et al., 2003).

Rather unexpected was the identification of a reticuline oxidase-like protein (At4g20830). Reticuline oxidase (or berberine-bridge-forming enzyme) is a flavoprotein of the pathway leading to benzophenanthridine alkaloids. Such an enzyme has been extensively analyzed in Papaveracea and was reported to be essential in plant responses to pathogenic attack (Dittrich and Kutchan, 1991). They have never been reported to be associated to mitochondria.

We also found a glycosyltransferase (At3g25140) previously identified as the protein encoded by the *QUASIMODO1* gene (Bouton et al., 2002). This putative membrane-bound protein is required for normal pectin synthesis and cell adhesion in *Arabidopsis* and was therefore not expected to be a mitochondrial protein.

2.4. Conclusion

Table 2 (see supporting information) contains a list of 502 non-redundant proteins and shows a comparison between the proteins found in this study of mitochondrial membranes from *A. thaliana* and those previously discovered in mitochondria from several plant species: *A. thaliana* (Kruft et al., 2001; Millar et al., 2001; Giegé et al., 2003; Herald et al., 2003; Millar and Heazlewood, 2003; Heazlewood et al., 2004), pea (Bardel et al., 2002), *C. reinhardtii* (van Lis et al., 2003) and rice (Heazlewood et al., 2003). The main difference between the different previous analyses and our work on *Arabidopsis* mitochondria is that (a) we restricted this study to membranes and (b) we combined different treatments of mitochondrial membranes, i.e., chloroform/methanol extraction, alkaline or saline treatments, to get a broader view of the membrane proteome. Despite the large number of previous proteomic studies of plant mitochondria, the strategy that was shown to be efficient to identify new membrane proteins from the chloroplast envelope membranes (Ferro et al., 2002, 2003) was also efficient for mitochondrial membranes. In the present study, 44 out of 114 (39%) identified proteins were not previously identified in plant or algae mitochondria (Table 1 and supporting information Table 2). Again, combination of different extraction procedure proved to be important for membrane proteomics: out of the 44 new proteins, 12 and 17 were only identified from chloroform/methanol extraction or alkaline treatment, respectively. Since 20 and 25 proteins were identified exclusively using these two respective procedures, this strengthens the importance of combining various extraction procedures to gain access to previously unidentified proteins from a membrane system.

When considering the physicochemical properties of these 44 previously unidentified mitochondrial membrane proteins, 37 of them (84%) were predicted (using HMMTOP) to contain at least one transmembrane domain, as expected for a membrane proteome. It is also important to note that 20 of them (45%) correspond to proteins of unknown functions. This again provides additional evidence for the interest to use a wide set of methods to get the most exhaustive repertoire of a complex protein mixture.

Finally, the high proportion of putative proteins that were identified in this study requires further validation by relevant functional studies. The task promises to be difficult, especially for proteins belonging to the same family, as the expression of other gene products with redundant function may lead to uninformative reverse genetic experiments.

3. Experimental

3.1. Plant cell growth conditions

Arabidopsis thaliana (ecotype Columbia) cell suspension cultures were grown under continuous white light

(40 μmol m^{-2} s^{-1}) at 23 °C with rotary agitation at 125 rpm in Gamborg's B5 medium supplemented with 1 μM 2-naphtalene acetic acid and 1.5% (w/v) sucrose (Axelos et al., 1992). Cells were grown 6 days before harvesting.

3.2. Purification and fractionation of Arabidopsis mitochondria

3.2.1. Purification of Arabidopsis mitochondria

Mitochondria were isolated from *Arabidopsis* cell suspension cultures and purified on discontinuous density Percoll gradients using a procedure adapted from Davy de Virville et al. (1994) and Ravanel et al. (2001). Protoplasts were prepared by enzymatic digestion of 6-day-old cell suspension cultures. The cells (approx. 150–180 g of fresh weight) were washed twice in culture medium containing 0.5 M D-mannitol instead of sucrose, then submitted to enzymatic digestion for 2 h at 25 °C in the presence of 1% (w/v) cellulase Y-C and 0.1% (w/v) pectolyase Y-23 (Kikkoman Corporation, Japan). All subsequent procedures were carried out at 4 °C. Protoplasts were collected by centrifugation at 150*g* for 10 min and washed twice with rupture medium (0.45 M D-mannitol, 20 mM sodium pyrophosphate, pH 7.5, 2 mM EDTA, 1% (w/v) polyvinylpyrolidone 25, 0.2% (w/v) BSA and 1 mM $CaCl_2$). Protoplasts were broken using a hand Potter homogenizer, centrifuged at 700*g* for 10 min to remove heavy cellular particles, and mitochondria were collected by centrifugation at 11,000*g* for 20 min. Mitochondria were resuspended in washing buffer (0.45 M D-mannitol, 20 mM potassium phosphate, pH 7.2, and 1 mM EDTA) containing 0.1% (w/v) BSA and centrifuged successively at 500*g* for 5 min and 12,000*g* for 15 min. Crude mitochondria were resuspended in 2 ml of washing buffer, homogenized using a Potter homogenizer and layered on two discontinuous gradients consisting of 18% (v/v), 23% and 40% Percoll layers in 0.3 M sucrose, 10 mM potassium phosphate, pH 7.2, 1 mM EDTA and 0.1% (w/v) BSA. Gradients were centrifuged at 39,000*g* for 40 min and mitochondria collected at the 23/40% Percoll interface. Mitochondria were diluted in washing buffer without BSA, centrifuged twice at 6000*g* for 5 min to remove residual thylakoid membranes and concentrated at 12,000*g* for 15 min.

3.2.2. Purification of membranes from Arabidopsis mitochondria

In order to separate soluble and membrane proteins, purified intact mitochondria were lysed in hypotonic medium in the presence of protease inhibitors (10 mM MOPS–NaOH, pH 7.8, 4 mM $MgCl_2$, 1 mM PMSF, 1 mM benzamidine and 0.5 mM ε-amino caproic acid). Membranes were purified from the lysate by centrifugation at 70,000*g* for 1 h (Beckman SW41-Ti rotor) and washed two times in the same buffer. Mitochondrial membrane preparations were stored in liquid nitrogen in 10 mM MOPS–NaOH, pH 7.8 (in the presence of protease inhibitors).

3.3. Differential extractions of mitochondrial membrane proteins

Protein contents of membrane fractions were estimated using the Bio-Rad protein assay reagent (Bradford, 1976). In order to remove most of the soluble matrix proteins contaminating the mitochondrial membrane vesicles, membrane preparations were first treated by sonication as previously described (Miras et al., 2002). The resulting mixture was stored for 15 min on ice before centrifugation (4 °C, 20 min, 12,000*g*) and proteins recovered in the pellet (membrane proteins) were further analyzed, while solubilized proteins (most of the matrix proteins) were discarded.

The more hydrophobic proteins of the mitochondrial membrane were extracted from crude membrane preparations using a 5/4 (v/v) chloroform/methanol mixture as previously described (Seigneurin-Berny et al., 1999; Ferro et al., 2000, 2002). Mitochondrial membranes (1.1 mg proteins in 0.1 ml 10 mM MOPS–NaOH, pH 7.8) were slowly diluted in 0.9 ml of cold C/M (2:1, v/v) solution. The resulting mixture was stored for 15 min on ice before centrifugation (4 °C, 20 min, 12,000*g*). Proteins insoluble in the organic phase were recovered as a white pellet and discarded. Proteins present in the organic phase were precipitated with cold acetone (−20 °C) and re-suspended in 50 μl of SDS–PAGE buffer (Chua, 1980).

Mitochondrial membrane proteins were also extracted using alkaline (0.8 mg proteins in 0.2 ml NaOH, 0.1 M) or salt treatments (0.8 mg proteins in 0.2 ml NaCl, 0.5 M). In order to solubilize membrane proteins present in both the outer and the inner surfaces of the vesicles, sonication of the membrane preparations was also performed during these two treatments. The resulting mixtures were stored for 15 min on ice before centrifugation (4 °C, 20 min, 12,000*g*). Insoluble proteins were recovered as pellets and re-suspended in 50 μl of SDS–PAGE buffer.

3.4. SDS–PAGE and Western blot analyses

Proteins were loaded on 12% acrylamide gels for SDS–PAGE analyses (Chua, 1980). For the analyses of mitochondrial fractions and for Western blot analyses, each fraction contained 20 μg of proteins. For MS/MS experiments, 30–50 μg of proteins (estimations from SDS–PAGE analyses) were loaded on 12% acrylamide gels (7 cm gels, Bio-Rad system). The T subunit of the glycine–decarboxylase complex (mitochondrial marker) was detected using the purified antibodies diluted 1:10,000 and using alkaline phosphatase staining as previously described (Vauclare et al., 1996). The LHCPs (plastidial marker) were detected using rabbit polyclonal

antibodies (a gift from Dr. Olivier Vallon; IBPC, Paris, France) diluted 1:10,000 and using alkaline phosphatase staining as previously described (Ferro et al., 2003).

3.5. Mass spectrometry and protein identification

After SDS–PAGE (migration was stopped just between the stacking and the separating gels so that proteins were concentrated in a very fine band for further analyses), a discrete band was excised from the Coomassie blue-stained gel. The in-gel digestion was carried out as previously described (Ferro et al., 2000). Gel pieces were then extracted with 5% [v/v] formic acid solution and acetonitrile. After drying, tryptic peptides were resuspended in 0.5% aqueous trifluoroacetic acid. The samples were injected into a LC-Packings (Dionex) nanoLC system and first preconcentrated on a 300 μm × 5 mm PepMap C18 precolumn. The peptides were then eluted onto a C18 column (75 μm × 150 mm). The chromatographic separation used a gradient from solution A (5% acetonitrile: 95% water: 0.1% formic acid) to solution B (95% acetonitrile: 5% water: 0.1% formic acid) over 60 min at a flow rate of 200 nL min^{-1}. The LC system was directly coupled to QTOF1 or QTOF Ultima mass spectrometer (Waters). MS and MS/MS data were acquired and processed automatically using MassLynx 3.5 software. Samples retrieved from each differential extraction (C/M extraction, NaOH and NaCl treatments) were individually analyzed by one LC-MS/MS, except from the NaCl treatment for which 2 LC-MS/MS experiments were performed. Besides, a sample retrieved from NaCl treatment was analyzed by two-dimensional chromatography coupled to mass spectrometry, referring to the principle described by Washburn et al. (2001). Separation by ion exchange chromatography was added upstream to reverse phase C18 separation. A four-step chromatographic run was carried out using 5 mM KH_2PO_4 with 0, 100, 200 and 400 mM KCl, respectively, for each of the four steps.

Database searching was carried out using the MASCOT 1.7 program and two protein databases: an updated compilation of SwissProt and Trembl (ftp://us.expasy.org/databases/sp_tr_nrdb/) and the AGI protein database (ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/). Proteins, which were identified with at least two peptides showing both a score higher than 40, were validated without any manual validation. For proteins identified by only one peptide having a score higher than 40, the peptide sequence was checked manually. Peptides, with scores higher than 20 and lower than 40, were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion. The remaining unassigned peptides were interpreted manually and internet MS-Pattern (http://prospector.ucsf.edu/) and Blast (http://www.ncbi.nlm.nih.gov/BLAST/) were used for database searching.

3.6. Prediction methods

Predictions for mitochondria localization and membrane-spanning regions were achieved using the software programs Predotar (version 0.5; http://www.inra.fr/predotar/), TargetP (Emanuelsson et al., 2000; http://www.cbs.dtu.dk/services/TargetP/) or Psort for plants (http://www.psort.org/, Nakai and Horton, 1999) respectively and HMMTOP (http://www.enzim.hu/~tusi/hmmtop/html/submit.html; Tusnády and Simon, 2001). Predictions of functions were carried out using BLAST (http://www.ch.embnet.org/software/BottomBLAST.html) and InterProScan (http://www.ebi.ac.uk/interpro/scan.html) tools. The GRAVY (Grand Average of Hydropathicity) index (Kyte and Doolittle, 1982) was calculated using the ProtParam tool available on the Expasy server (http://au.expasy.org/tools/protparam.html).

Acknowledgements

This work was supported by Génoplante (No. 2001027), CNRS and CEA research programs. Sabine Brugière was funded by Genome Express, 11 Chemin des Près, Meylan, France.

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