Proteomics of the Chloroplast Envelope Membranes from *Arabidopsis thaliana**

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The development of chloroplasts and the integration of their function within a plant cell rely on the presence of a complex biochemical machinery located within their limiting envelope membranes. To provide the most exhaustive view of the protein repertoire of chloroplast envelope membranes, we analyzed this membrane system using proteomics. To this purpose, we first developed a procedure to prepare highly purified envelope membranes from *Arabidopsis* chloroplasts. We then extracted envelope proteins using different methods, *i.e.* chloroform/methanol extraction and alkaline or saline treatments, in order to retrieve as many proteins as possible, from the most to least hydrophobic ones. Liquid chromatography tandem mass spectrometry analyses were then performed on each envelope membrane subfraction, leading to the identification of more than 100 proteins. About 80% of the identified proteins are known to be, or are very likely, located in the chloroplast envelope. The validation of localization in the envelope of two phosphate transporters exemplifies the need for a combination of strategies to perform the most exhaustive identification of genuine chloroplast envelope proteins. Interestingly, some of the identified proteins are found to be N^{α}-acetylated, which indicates the accurate location of the N terminus of the corresponding mature protein. With regard to function, more than 50% of the identified proteins have functions known or very likely to be associated with the chloroplast envelope. These proteins are a) involved in ion and metabolite transport, b) components of the protein import machinery, and c) involved in chloroplast lipid metabolism. Some soluble proteins, like proteases, proteins involved in carbon metabolism, or proteins involved in responses to oxidative stress, were associated with envelope membranes. Almost one-third of the proteins we identified have no known function. The present work helps understanding chloroplast envelope metabolism at the molecular level and provides a new overview of the biochemical machinery of the chloroplast envelope membranes. ***Molecular & Cellular Proteomics 2:325–345, 2003.***

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Plastids are semiautonomous organelles that present a wide structural diversity and contain unique biosynthetic pathways. They are strongly dependent on proteins that are nuclear encoded, translated in the cytoplasm, and imported into this organelle. A pair of membranes called the envelope surrounds all plastids. As the vast majority of plastid proteins are nuclear encoded, the plastid envelope contains a protein import machinery. Translocation at the envelope membranes is directed by a general import machinery composed of the outer-membrane Toc complex and the inner-membrane Tic complex (for reviews, see Refs. 1–4).

Located at the interface between the stroma and the cytosol, the envelope is also the site of various transports and exchanges of ions and metabolites required for the integration of the plastid metabolism within the plant cell. Few envelope transporters have been identified and characterized at the molecular level: the triose-phosphate/phosphate translocator, an ADP/ATP translocator, several substrate-specific outer membrane channels, and two dicarboxylate translocators (for a review, see Ref. 5). Recently, a putative hexose transporter was also identified (6). More recently we described a proteomic approach that allowed the identification of several putative transporters of the chloroplast envelope (7).

A unique biochemical machinery is also present in envelope membranes. The chloroplast envelope is the site of specific biosynthetic functions *i.e.* synthesis of plastid membrane components (glycerolipids, pigments, prenylquinones), chlorophyll breakdown, synthesis of lipid-derived signaling molecules (fatty acid hydroperoxydes, growth regulators, or chlorophyll precursors), and participates in the coordination of the expression of nuclear and plastid genes (for a review, see Ref. 8). So far, and as for other plastid envelope components, few proteins catalyzing these biosynthetic functions have been identified and characterized at the molecular level.

Subcellular proteomic studies are essential to get access to protein location in relation with their function (for a review, see Ref. 9). Plant proteomics exemplifies perfectly this functional dimension with the recent explosion of proteomic initiatives, which are more and more focused on the analyses of subcellular compartments (for review, see Ref. 10). Plant mitochondria (11, 12), chloroplast (13), plasma membrane (14), peroxisome (15), endoplasmic reticulum (16), and the cell wall (17) have recently been studied with proteomic approaches. Subproteome sample complexity can also be reduced for a more

accurate protein location. For instance, the chloroplast can be subdivided into the envelope membranes, the stroma, and the thylakoids. Recent papers describe both a systematic proteomic and an *in silico* approaches aiming at the identification of the thylakoid luminal and peripheral proteins (18, 19). We recently reported a subcellular proteomic analysis aiming at identifying the hydrophobic core of the chloroplast envelope (7). Using spinach chloroplast envelope fractions, this approach allowed the identification of various previously uncharacterized proteins, most of them corresponding to components of the envelope transport systems.

The aim of the present work was to enhance our understanding of the biochemical machinery of plastid envelope membranes. We applied various extraction procedures (chloroform/methanol extraction and NaOH and NaCl treatments) to get a more exhaustive array of the chloroplast envelope membrane proteins, from the most to the least hydrophobic ones. For database searching purposes, the present proteomic approach was based on *Arabidopsis thaliana* samples, this organism being fully sequenced (20). However, in the context of plant subproteomic studies, *A. thaliana* is generally not the best biochemical model as far as getting highly pure fractions of an organelle is concerned. As an informative subcellular proteomic approach requires highly purified organelle subfractions to be obtained, the procedure of chloroplast envelope purification was optimized and adapted for *A. thaliana* samples. Using the present strategy, we identified more than 100 envelope components of various hydrophobicity such as ion and metabolite transporters, proteins involved in fatty acids, glycerolipids, vitamins, and pigments metabolism, components of the protein import machinery, proteases, as well as many proteins of unknown function and of previously unknown subcellular localization. The identification of these proteins is extensively discussed with respect to their chloroplastic location and their implications in the chloroplast envelope metabolism. New insights of the envelope chloroplast metabolism are presently suggested.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions—*A. thaliana* (ecotype Ws) seeds were grown in growth rooms at 23 °C (12-h light cycle) with a light intensity of 150 μmol·m^{-2} s^{-1}. Plants were grown 3–4 weeks before harvesting.

Purification of Arabidopsis Chloroplasts—Chloroplasts from *A. thaliana* were purified according to Kunst (21) with the following modifications: crude chloroplasts were obtained from 400 to 500 g *A. thaliana* leaves and purified by isopycnic centrifugation using 6 Percoll gradients. Percoll gradients were preformed by centrifugation at 38,700 × *g* for 55 min (Sorvall SS-34 rotor; Sorvall, Newtown, CT). Leaves were ground two times 2 s, and the filtrate was centrifuged at 2070 × *g* for 2 min (Sorvall GS 3 rotor). After resuspension, chloroplasts were loaded on the top of the preformed Percoll gradients, and the gradients were centrifuged at 13,300 × *g* for 10 min (Sorvall swinging HB-6 rotor). Intact chloroplasts were collected from the gradients, diluted three to four times, and centrifuged at 2070 × *g* for 2 min (Sorvall swinging HB-6 rotor). All operations were carried out at 0–5 °C.

Purification of Envelope Membranes from Arabidopsis Chloroplasts—Purified intact chloroplasts were lysed in hypotonic medium in the presence of protease inhibitors (10 mM 4-morpholinepropanesulfonic acid (MOPS)[1]-NaOH, pH 7.8, 4 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM ϵ-amino caproic acid). Envelope membranes were purified from the lysate by centrifugation at 70,000 × *g* for 1 h (Beckman SW41-Ti rotor; Beckman, Urbana, IL) on sucrose gradients (0.93 M, 0.6 M, 0.3 M sucrose). Envelope membranes were collected at the 0.93/0.6 M interface and concentrated (after dilution three to four times in 10 mM MOPS-NaOH, pH 7.8 buffer containing protease inhibitors) using a centrifugation at 110,000 × *g* for 1 h (Beckman SW 41 Ti rotor). Envelope membrane preparations were stored in liquid nitrogen in 10 mM MOPS-NaOH, pH 7.8 (in the presence of protease inhibitors).

Differential Extractions of Envelope Membrane Proteins—Protein contents of membrane fractions were estimated using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) (22). In order to remove most of the soluble stromal proteins contaminating the chloroplast envelope vesicles, envelope membrane preparations were first treated by sonication as previously described (23). 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 stromal contaminants) were discarded.

The more hydrophobic proteins of the chloroplast envelope were extracted from envelope preparations using a 5/4 (v/v) chloroform/methanol mixture as previously described (7, 24, 25). Envelope membranes (0.5-mg proteins in 0.1-ml storage buffer) were slowly diluted in 0.9 ml of cold chloroform/methanol (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 resuspended in 50 μl of SDS-PAGE buffer.

Chloroplast envelope proteins were also extracted using alkaline (NaOH, 0.5 M) or salt treatments (NaCl, 1 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 white pellets and resuspended in 50 μl of SDS-PAGE buffer.

SDS-PAGE and Western Blot Analyses—Proteins were loaded on 12% acrylamide gels for SDS-PAGE analyses (26). For the analyses of subplastidial fractions and for Western blot analyses, each fraction contained 15 μg of proteins. For tandem mass spectrometry (MS/MS) experiments, 30–50 μg of proteins (estimations from SDS-PAGE analyses) were loaded on 12% acrylamide gels (7-cm gels, Bio-Rad). The ceQORH protein was detected using the purified antibodies diluted 1:5000 and using alkaline phosphatase staining as previously described (23). The light harvesting complex proteins (LHCPs) were detected using rabbit polyclonal antibodies (a gift from Dr. Olivier Vallon; Institut de Biologie Physico-Chimique, Paris, France) diluted 1:10,000 and using alkaline phosphatase staining.

Mass Spectrometry and Protein Identification—After SDS-PAGE (migration was stopped just between the stacking and the separating gels so that proteins were concentrated on a very fine band for further

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; GFP, green fluorescent protein; MS/MS, tandem mass spectrometry; LHCP, light harvesting complex protein; LC, liquid chromatography; MS, mass spectrometry; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; LACS, long-chain acyl-CoA synthetases; ACCase, acetyl-CoA carboxylase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; P_i, inorganic phosphate.

analyses), a discrete band was excised from the Coomassie blue-stained gel. The in-gel digestion was carried out as previously described (25). 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, Sunnyvale, CA) nanoLC system and first preconcentrated on a 300 μm $\times$ 5 mm PepMap C18 precolumn. The peptides were then eluted onto a C18 column (75 μm $\times$ 150 mm). The chromatographic separation used a gradient from solution A (5% water, 95% acetonitrile, 0.1% formic acid) to solution B (5% acetonitrile, 95% water, 0.1% formic acid) over 60 min at a flow rate of 200 nl/min. The liquid chromatography (LC) system was directly coupled to quadrupole time-of-flight (QTOF) 1 or QTOF Ultima mass spectrometer (Waters, Milford, MA). MS and MS/MS data were acquired and processed automatically using MassLynx 3.5 software. Database searching was carried out using the MASCOT 1.7 program. Two protein databases were used; an updated compilation of SwissProt and Trembl (us.expasy.org/databases/sp_tr_nrdb/) and the *Arabidopsis* Genome Initiative protein database (ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/). Proteins that were identified with at least 2 peptides showing both a score higher than 40 were validated without any manual validation. For proteins identified by only 1 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 (prospector.ucsf.edu/) and Blast (www.ncbi.nlm.nih.gov/BLAST/) were used for database searching.

Prediction Methods—Predictions for chloroplast localization and membrane-spanning regions were achieved using the software programs ChloroP (27) and HMMTOP (28), respectively. Predictions of functions were carried out using BLAST (www.ch.embnet.org/software/BottomBLAST.html) and InterProScan (www.ebi.ac.uk/interpro/scan.html) tools.

Transient Expression in Arabidopsis and Tobacco Cell—The green fluorescent protein (GFP) reporter plasmid 35Ω-sGFP(S65T) and the 35Ω-sGFP(S65T)-derived plasmid containing the transit peptide (TP) sequence from RBCS fused to GFP [35Ω-TP-sGFP(S65T)] were described previously (29).

Construction of the plasmids for expression of P56-2 or P56-4 *Arabidopsis* proteins fused to GFP was performed as follows. The coding region of the *Arabidopsis* P56-2 protein was PCR-amplified using flanking primers *Xho*I-N-ter (ATCCTCGAGATGAACGCGAGAGCTCTTCTTTGC) and *Bsp*HI-C-ter (GAATGGTCATGACGATTATCTTCTCTCCGGTTG). The coding region of the *Arabidopsis* P56-4 protein was PCR-amplified using flanking primers *Xho*I-N-ter (AGACTCGAGATGGCCCTCGGTGGCTTGATTTC) and *Afl*III-C-ter (GGTACATGTCGAGAATTTTTTCTCCGGTTGCG). Both PCR products were cloned into the pBluescript SK$^-$ Vector. The *Xho*I-*Bsp*HI (P56-2) or *Xho*I-*Afl*III (P56-4) fragments cleaved from these plasmids were inserted into the *Sal*I-*Nco*I-digested GFP reporter plasmid 35Ω-sGFP(S65T) to create the 35Ω-P56-2-sGFP(S65T) and 35Ω-P56-4-sGFP(S65T) vectors, respectively. Correct orientation and sequence of the inserted fragments were controlled. The plasmids used for tissue bombardment were prepared using the QIAfilter Plasmid Midi Kit (Qiagen Laboratories, Hiden, Germany).

For transient expression of the proteins, plasmids of appropriate constructions (1 μg) were introduced into *Arabidopsis* and BY2 cells using a pneumatic particle gun (PDS-1000/He; Bio-Rad). Growth of *Arabidopsis* or BY2 cells, conditions of cell bombardment, and fluorescence microscopy were as previously described (23).

RESULTS AND DISCUSSION

Purification of Arabidopsis Chloroplasts and Chloroplast Subfractions—Chloroplasts from *A. thaliana* were purified according to Kunst (21) with some modifications of the previously described protocol in order to optimize yield and purity. Using this protocol, an average of 54 mg chloroplast proteins was purified from 500 g of 3-wk-old *Arabidopsis* leaves. Excellent purity of these Percoll-purified *Arabidopsis* chloroplasts was confirmed through proteomic analysis. Only 5% (6 out of 112) of the identified *Arabidopsis* proteins may correspond to nonplastid proteins (Table I). Among them, one protein appeared to correspond to a previously characterized major plasma membrane component; four proteins may indicate contamination by major tonoplast proteins and one from a glyoxysome (specialized peroxisomes) compartment. Considering the high sensitivity of present mass spectrometers, it was not surprising to detect minute amounts of these few extra-plastidial contaminants, which are major proteins in their respective subcellular compartment. However, because the subcellular localization of some of these proteins remains to be unambiguously determined, the contamination of the Percoll-purified *Arabidopsis* chloroplasts may be overestimated. Indeed, we were recently able to demonstrate the unique plastid localization of a phosphate transporter (7) that was previously suspected to be associated to the plasma membrane (30). It is also important to notice that none of the 112 identified proteins appears to derive from mitochondria, an organelle known to represent a classical contaminant of purified plastids.

In order to purify plastid subfractions (envelope membranes, stroma, and thylakoid), Percoll-purified intact chloroplasts were lyzed in hypotonic medium and the three fractions were separated on sucrose gradients. From these plastid preparations, an average of 31 mg proteins from the stroma, 22.5 mg thylakoid proteins, and 300 μg envelope proteins could be purified. Purified envelope, stroma, and thylakoid proteins were further analyzed by SDS-PAGE, and these three fractions were shown to be poorly cross-contaminated. Indeed, Fig. 1 shows that the RuBisCo, the major stroma protein, was hardly detected in other subplastidial fractions. It is also noticeable that the LHCPs, which are major thylakoid proteins, were highly enriched in the thylakoid fraction and hardly visible in other subplastidial fractions. Finally, the phosphate/triose phosphate translocator, known as the major envelope component, was exclusively detected in the envelope fraction. This enrichment of envelope proteins in the purified fraction was further confirmed using Western blot experiments (Fig. 1) demonstrating enrichment of ceQORH, which is exclusively located in the plastid envelope (23).

Being the most likely source of membrane contamination of the purified envelope fraction, thylakoid cross-contamination was precisely assessed. Chlorophyll being the most conspicuous thylakoid component, we first analyzed the presence of this pigment in envelope membrane preparations. Indeed, the

TABLE I

List and general characteristics of the proteins identified in chloroplast envelope membranes (classification according to protein function)

Da, molecular mass of the predicted protein precursor. Acc nb, accession number in SwissProt, TrEMBL, or NCBI. AGI acc nb, corresponding AGI accession number. NbP, number of unique peptides that allowed identification of the corresponding protein. The functions were assumed according to the BLAST, Blocks, and Profile Scan investigations. cTp, plastid transit peptide and putative maturation site predicted by ChloroP. TM, putative transmembrane domains predicted by HMMTOP. R/TM, R is the number of AA residues of predicted protein precursor. Loc, indicates the subplastidial location: IM, inner envelope membrane; OM, outer envelope membrane; E, envelope; S, stroma; T, thylakoids; To, tonoplast; PM, plasma membrane; EB or TB, referenced interaction with envelope components or thylakoid membranes.

Da	Acc nb	Protein names and AtDB annotations	cTP	TM	R/TM	pI	Loc	AGI Acc nb	NbP
Ions and metabolite transporters									
59,213	Q9LXV3	IEP45 = 2-oxoglutarate/malate translocator	+ (69)	14	40	9.75	IM	At5g12860	4
64,986	Q9LQ76	HP64 (putative K Efflux antiporter KEA1)	-	13	48	6.10	E?	At1g01790	3
58,634	Q9LF13	IEP62 (putative sugar transporter)	+ (80)	12	47	9.14	IM	At5g16150	2
59,991	Q9FMF7	HPSOT (2-oxoglut/malate translocator homolog)	+ (68)	12	47	9.34	IM	At5g64290	2
59,829	Q9FIF2	HP59 (putative sugar transporter)	+ (31)	12	47	9.02	IM?	At5g59250	1
46,536	Q9FNL1	IEP30 = Phosphate/triose-phosphate translocator	-	11	39	9.70	IM	At5g46110	4
47,645	Q9LTG3	HP47 (low homology C4-carboxylate tp)	+ (73)	10	46	9.96	IM?	At5g52540	2
36,066	O81017	HP36 (putative Na^+/taurocholate transporter)	-	7	48	8.87	IM?	At2g26900	1
44,224	P92991	IEP33 = Phosphate/phosphoenolpyruvate translocator	+ (30)	7	58	10.16	IM	At5g33320	1
77,926	Q8LPQ6	HP77 (ABC transporter)	+ (24)	7	102	9.13	IM?	At4g25450	4
88,188	Q9M3H5	AHM1 (potential cadmium/zinc-transporting ATPase HMA1)	+ (60)	7	117	8.06	IM?	At4g37270	1
90,626	Q8VZP7	HP90 (putative mitochondrial carrier protein)	-	5	165	8.96	E?	At2g35800	1
15,482	Q9ZV24	HP15 (putative membrane channel protein = amino acid-selective channel?)	-	4	37	9.16	OM	At2g28900	4
26,992	Q9SJH9	HP25 (similar to 22 kDa perox membrane protein)	+ (46)	4	61	9.7	IM ?	At2g42770	2
30,663	Q8L9G1	HP30c (peroxisomal membrane protein PMP22 homolog)	+ (74)	4	72	10.17	IM ?	At5g19750	2
45,090	Q9M024	HP45b (putative perox Ca^{2+}-dependent solute carrier)	+ (61)	4	104	9.82	IM?	At5g01500	1
35,663	Q9SVB2	HP35 (putative mitochondrial carrier-like protein)	+ (23)	3	110	9.6	E ?	At4g39460	2
Protein import components									
22,899	O82251	IEP16 (low homology TIC20)	+ (49)	4	52	10.28	IM	At2g47840	5
22,379	Q9FJ60	HP22 (low homology TIM17/TIM22)	-	4	53	9.08	E?	At5g55510	1
27,772	Q9FLT9	HP30-2 (low homology TIM17/TIM22)	-	4	65	9.60	E?	At5g24650	1
27,982	Q9SCK3	HP30 (low homology TIM17/TIM22)	-	4	65	9.49	E?	At3g49560	4
60,607	Q9SK50	TIC55 (putative Rieske iron-sulfur protein)	+ (48)	3	180	8.94	IM	At2g24820	6
62,604	Q9SZZ3	HP62 (Rieske [2Fe-2S] containing-domain protein; low homology TIC55)	+ (55)	2	274	8.86	E?	At4g25650	2
64,025	Q9LVH5	HP64b (TOC64 homolog)	-	2	295	8.62	OM	At3g17960	1
112,120	Q8LPR9	HP112 (IAP100, putative chloroplast inner envelope protein, component of the chloroplastic protein import machinery)	+ (50)	2	508	5.73	IM	At1g06950	11
26,599	O64701	HP26c (low homology TIC62)	-	1	246	6.45	E?	At2g34460	1
34,723	Q9GDD2	TOC34 (protein import apparatus component)	-	1	313	9.42	OM	At5g05000	1
48,903	Q9FMD5	TIC40 (translocon Tic40-like protein)	+ (42)	1	447	5.38	IM	At5g16620	4
89,188	Q9STE8	OEP75 (TOC75) = Chloroplast import-associated channel homolog	+ (79)	1	818	8.37	OM	At3g46740	6
94,388	O22774	OEP86 (TOC159) (putative chloroplast outer envelope 86-like protein)	-	1	865	5.83	OM	At4g02510	3
32,939	Q94B42	TOC33 (similar to GTP-binding protein TOC34)	-	0	-	9.11	OM?	At1g02280	2
65,160	Q9FLP1	HP65b (Trigger factor-like protein; Synecocystis homolog involved in protein export)	+ (27)	0	-	5.22	E?	At5g55220	1
Fatty acid and glycerolipids metabolism									
25,219	Q9M2W3	HP25b (phosphatidylglycerophosphate synthase-like)	+(78)	6	39	9.61	IM?	At3g55030	1
32,158	O80952	HP32c (Putative CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase)	+ (39)	6	49	10.31	IM?	At2g39290	1
23,505	Q9M0A2	LPAAT (ACT2) = plastidial lysophosphatidic acid acyltransferase	-	3	71	9.76	IM	At4g30580	1

TABLE I— *continued*

51,174	P46310	FD3C = omega-3 fatty acid desaturase	+ (81)	3	149	8.15	IM	At3g11170	2
51,225	Q9M094	FD6C = omega-6 fatty acid desaturase	+ (78)	3	149	9.01	IM	At4g30950	3
81,465	Q8W471	HP81 (A6 anther-specific protein, AMP-binding protein, long chain acyl-CoA synthetase)	+ (65)	3	242	8.87	E?	At4g14070	2
20,779	Q9LJ85	HP20b (putative plant lipid transfer)	+ (73)	2	102	6.10	E?	At3g22620	1
88,516	Q9LD43	HP88b (alpha subunit of ACCase)	+ (54)	2	398	5.78	E?	At2g38040	1
55,609	P56765	ACCD = Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	-	1	488	5.90	S/EB	Plastid gene	1
76,175	Q9CAP8	HP76 (putative acyl-CoA synthetase)	-	0	-	6.54	E?	At1g77590	2
58,196	Q96242	CP74 Allene oxide synthase, chloroplast precursor(Cytochrome P450 74A)	+ (32)	0	-	8.75	E?	At5g42650	5
Vitamin and pigment metabolism									
48,844	Q9LHM7	HP43 (low homology UBIA prenyltransferase)	+ (91)	8	55	4.62	IM?	At3g11950	1
37,926	Q9LY74	IEP37 = Putative chloroplast inner envelope protein (SAM-dependent methyl transferase)	+ (51)	2	169	9.19	IM	At3g63410	5
43,359	P21218	PORB = NADPH-protochlorophyllide oxidoreductase B	+ (43)	1	401	9.23	OM+ TB	At4g27440	7
Proteases and chaperones									
87,837	Q9FIM2	HP87 (metalloprotease-like, FtsH)	+ (62)	3	269	7.66	E?	At3g47060	1
32,356	Q9S834	NCLPP1 (put. ATP-dependent CLP protease)	+ (62)	2	149	8.35	S / EB	At1g02560	2
103,455	O48931	ClpC = ATP-dependent Clp protease (ATP-binding subunit)	+ (26)	1	928	6.36	S/EB	At5g50920	8
Response to oxidative stress									
26,507	P21276	SODF = superoxide dismutase [Fe], chloroplast precursor	-	2	117	6.87	E?	At4g25100	1
31,572	Q42564	AP = Ascorbate peroxidase	-	1	287	6.47	S / TB	At4g35000	2
26,016	P52032	GSHY (PHGP) = Phospholipid hydroperoxide glutathione peroxidase	+ (72)	0	-	9.42	S	AT2G25080	1
19,664	O48737	THM1 = thioredoxin M-type 1	+ (48)	0	-	9.13	E?	At2g28900	1
Carbon metabolism									
28,344	Q9FDZ0	CAHC = Carbonic anhydrase	-	1	259	5.36	S/EB	At3g01500	2
42,405	Q9SN86 O81844	MDH (putative chloroplast malate dehydrogenase)	+ (80)	1	403	8.66	S	At3g47520	6
44,015	O64688	HP44b (putative pyruvate dehydrogenase complex E1 beta subunit)	+ (70)	1	406	5.53	S	At2g34590 (At1g30120)	1
20,202	P10795	RBS1 = Ribulose biphosphate carboxylase small chain 1A	+ (54)	0	-	7.59	S/EB	At1g67090	7
20,286	P10796	RBS2 = Ribulose biphosphate carboxylase small chain 1B	+ (54)	0	-	7.58	S/EB	At5g38430	1
42,489	P25856	G3PA = Glyceraldehyde 3-phosphate dehydrogenase A	+ (45)	0	-	7.62	S/EB	At3g26650 At1g12900	2
47,659	P25857	G3PB = Glyceraldehyde 3-phosphate dehydrogenase B	+ (45)	0	-	6.33	S/EB	At1g42970	1
51,981	P10896	RCA = Ribulose bisphosphate carboxylase/oxygenase activase	+ (58)	0	-	5.87	S	At2g39730	2
52,955	O03042	RBL = RuBisCO large subunit	-	0	-	5.88	S/EB	Plastid gene	7
63,746	P21240	RUBB = RuBisCO subunit binding-protein beta subunit, chloroplast precursor (60 kDa chaperonin beta subunit) (CPN-60 beta)	+ (53)	0	-	6.01	S	At1g55490	1
Unclassified other functions									
70,778	***Q93Y07***	HP70 (putative ß-glucosidase)	-	4	155	8.80	E?	At3g06510	2
88,157	Q9LYZ1	HP88 (putative protein kinase)	-	3	259	6.61	E?	At5g02940	3
52,258	Q9SF45	HP52b (Putative oxidoreductase)	+ (34)	1	477	7.03	E?	At3g09580	2
Unknown function									
54,017	Q9FVQ4	HP45 unknown function	+ (13)	12	43	9.63	IM	At1g32080	5
45,294	Q9SPJ2	RCP1 (root cap1)	+ (47)	10	41	9.38	IM?	At5g17520	1
28,838	Q9T0H9	HP28b unknown function	+ (72)	6	46	7.68	IM?	At4g13590	1
36,687	NP_1916 18	HP36b unknown function	+ (61)	6	55	9.22	IM?	At3g60590	1
25,788	Q9M2L9	HP26b (putative inner envelope protein)	+ (33)	5	48	9.16	IM?	At3g57280	4
41,323	Q94CJ5	HP40 unknown function	+ (61)	5	77	7.01	IM?	At5g12470	7
46,703	Q9FGP9	HP46 unknwn function	+ (25)	5	86	5.14	IM?	At5g22790	1
22,867	Q9FZH4	HP23 unknown function	-	4	51	9.45	E?	At1g67080	1
25,827	Q9FM22 +Q9FM2 1	IEP18 unknown function	+ (65)	4	61	10.14	IM	At5g62720	2
27,986 31,536	Q9SD32 Q94C78	HP28 unknown protein	+ (35)	4 4	62 70	10.20 10.24	IM?	At3g51140	1

TABLE I—*continued*

36,701	Q9ZVH7	HP36c (putative non-green plastid inner envelope membrane protein)	+ (72)	4	84	7.77	IM?	At2g38550	2
56,812	Q93W02	HP56b unknown function	+ (41)	4	130	9.83	IM?	At5g24690	7
23,704	Q9SUE0	HP24 unknown function	+ (83)	3	73	11.28	E?	At4g27990	1
28,911	Q9FNA1	HP29c unknown function	+ (60)	3	87	9.03	E?	At5g1237201	2
35,252	Q9C9Z2	HP35b unknown function	+ (59)	2	169	9.01	E?	At3g028640	2
48,258	Q9SJX7	HP48 unknown function	-	2	216	7.71	E?	At2g311190	1
53,076	Q9FNN4	HP53 unknown function	-	2	235	8.73	E?	At5g018540	2
7,256	Q9M1X3	OEP6 unknown function	-	1	69	9.05	OM	At3g63160	2
27,432	Q9LHC4	HP27b unknown function	+ (61)	1	250	9.61	E?	At3g32930	1
40,982	Q9LM68	HP40b unknown function	+ (67)	1	364	9.08	E?	At1g20830	1
41,630	Q9LTR2	HP41b unknown function	+ (45)	1	381	8.90	E?	At3g20320	1
57,645	Q9LQ77	HP57 unknown function	+ (49)	1	526	4.94	E?	At4g00640	3
73,946	Q9XIK2	HP73 unknown function	+ (2)	1	681	7.93	E?	At1g10510	4
103,926	Q9FF91	HP103 unknown function	+ (47)	1	946	4.62	E?	At5g23890	1
26,482	Q941D3	HP26d unknown function	+ (50)	0	-	9.49	E?	At5g19940	1
35,630	Q9LS48	HP35c unknown function	+ (39)	0	-	5.24	E?	AT3g18420	1
36,392	O80796	IM30 (Putative lipid transfer protein)	+ (64)	0	-	9.18	E+T?	At1g65260	1
49,830	O80503	HP50 unknown function	-	0	-	8.95	E?	At2g44640	7
64,952	Q9M016	HP65 unknown function	+ (48)	0	-	5.38	E?	At5g01590	1
Stromal translation proteins									
12,060	P56807	RR18 = Chloroplast 30S ribosomal protein S18	-	0	-	12.16	S	Plastid gene	1
51,630	P17745	EFTU = Elongation factor Tu, chloroplast precursor	+ (67)	0	-	5.84	S	At4g20360	1
Thylakoid electron tranfer chain									
25,881	Q01667	CAB-6A = chlorophyll A-B binding protein of LHCI type	+ (35)	3	80	6.21	T	At3g54890	2
29,103	Q43381	LHC3 = PSI type III chlorophyll a/b-binding protein	+ (48)	3	91	8.61	T	At1g61520	5
28,170	Q39142	CB21 (putative photosystem II type I chlorophyll a b binding protein)	+ (23)	3	89	5.15	T	At2g34430	3
23,051	Q9SUI4	PSAL = PSI reaction center subunit XI	+ (50)	2	109	9.85	T	At4g12800	1
27,522	Q9XF90	Lhcb6	+ (46)	2	129	6.75	T	At1g15820	1
28,803	Q9XF87	LHCB2 = Light harvesting chlorophyll A/B-binding protein	+ (28)	2	133	5.62	T	At3g27690	1
17,085	Q9S7N7	PSAG = Photosystem I reaction center subunit V	+ (59)	1	160	9.57	T	At1g55670	1
24,366	Q9ZR03	UCRI (cytochrome B6-F complex iron sulfur)	+ (50)	1	229	8.80	T	At4g03280	1
27,733	P27521	CB24 = chlorophyll A-B binding protein 4	+ (49)	1	251	6.22	T	At3g47470	4
Contaminants from other cell compartments									
25.619	P25818	TIGP = tonoplast intrinsic protein, gamma	-	7	36	6.02	To	At2g36830	1
25,0227	Q43352	HP25c (delta tonoplast integral protein, putative membrane channel protein root specific)	-	7	36	5.30	To?	At3g16240	1
371,369	Q9ZP05	MDHG = malate dehydrogenase, glyoxysomal precursor	-	1	354	8.14	G	At5g09660	1
54,304	Q9SZN1	HP54 (Probable H+-transporting ATPase)	-	0	-	5.03	To?	At4g38510	1
68,812	O23654	VATA (putative vacuolar ATP synthase catalytic subunit A)	-	0	-	5.11	To?	AT1G78900	2
30,688	P43285	WC1A = Plasma membrane intrinsic protein 1A (Aquaporin 1) (Plasma membrane aquaporin 1)	-	6	48	9.14	PM?	At3g61430	1

yellow color of the purified envelope fractions first indicates that purified envelope vesicles contain very few thylakoids (Fig. 2*B*). On a protein basis, the ratio of chlorophyll content detected in the purified envelope and thylakoid membranes, respectively, was 1/40. Western blot analyses were further performed with LHCP polyclonal antibodies as markers of thylakoid contamination. As presented in Fig. 2*A*, the proteins present in several independent envelope preparations appeared to contain between 1 and 3% thylakoid protein.

Toward the Exhaustive Identification of the A. thaliana Chloroplast Envelope Proteins—We recently demonstrated that proteins extracted by chloroform/methanol mixtures are the most hydrophobic proteins of a membrane system (24, 25). Thus the hydrophobic core of the spinach chloroplast envelope was analyzed and new envelope transporters were identified (7). The present study shows the importance of using complementary methods aiming at the most exhaustive identification of envelope proteins. Using three different extraction procedures, *i.e.* chloroform/methanol extraction, alkaline treatment, and saline treatment, more than 100 proteins could be identified from highly purified fractions of the *A. thaliana* chloroplast envelope (Table I). These three complementary

methods were used in order to retrieve and identify as many envelope proteins as possible; from the most to the least hydrophobic ones (Fig. 3). Indeed, about two-third (69/112) of identified proteins were found from only one extraction method and not from any of the two other ones (Fig. 4). This provides additional evidence to demonstrate the interest in using a wide set of methods to get the most exhaustive repertoire of a complex protein mixture. 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 allowed the highest percentage of highly hydrophobic proteins to be retrieved, as already shown in previous works (7, 24, 25). This extraction method allowed the identification of putative transporters (*e.g.* HP22, HP23, HP24, HP25, HP36b, HP47, IEP18, and RCP1; Table I), which were not detected using either alkaline (NaOH) or saline (NaCl) treatment and which are likely to be low abundant transporters. Indeed, transporters are often minor components of a membrane system and most of them are highly hydrophobic. By concentrating highly hydrophobic proteins, chloroform/methanol extraction makes some minor transporters amenable to mass spectrometric analysis and further identification. However, such an extraction procedure requires enough material as the yield of protein recovery in this fraction is about 5–10% of the total amount of proteins present in the native membrane. Although being less stringent with respect to the hydrophobicity and providing the use of highly purified fractions, alkaline and saline treatments allowed the identification of some highly hydrophobic proteins altogether with more hydrophilic ones. As expected, the more stringent the extraction method, the less the number of proteins identified. Chloroform/methanol is the most stringent method and alkaline treatment is more stringent than saline treatment with regard to the recovery of hydrophobic proteins. Indeed, chloroform/methanol extraction, alkaline, and saline treatments allowed the identification of 37, 51, and 74 proteins, respectively. The specificity toward hydrophobicity of each extraction method was exemplified by the identification of hydrophobic contaminants. The identification of only 9 thylakoid proteins in the overall analyses of the chloroplast envelope confirms the low thylakoid contamination level, considering that, in chloroplasts, the overall thylakoid membrane protein is about 40 times more abundant than envelope proteins (Table I). The percentage of thylakoid contaminants was found different according to the extraction method used. Indeed, the alkaline (NaOH) or saline (NaCl) treatments, which do not solubilize integral membrane proteins, provided a fair evaluation of the percentage of thylakoid contamination (2 and 4%, respectively, as estimated from the number of detected thylakoid proteins in the analyzed envelope sample).

In contrast, traces of some of the major thylakoid proteins in envelope subfractions appear to be concentrated during the extraction of hydrophobic proteins using chloroform/methanol (see supplemental data). Indeed, extraction in organic solvent allows the recovery of more than 30% of the thylakoid membrane proteins but only 5% of the envelope proteins (as estimated from the ratio of soluble and insoluble proteins

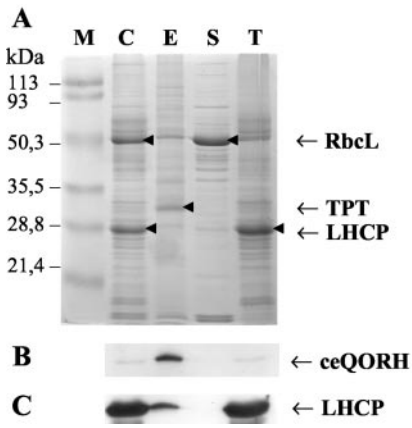

FIG. 1. **Characterization of Percoll-purified *Arabidopsis* chloroplast and subplastidial fractions.** *C*, crude chloroplast proteins; *E*, envelope membrane proteins; *S*, stroma proteins; *T*, thylakoid proteins. Each fraction contained 15 μg proteins. *A*, Fractions were analyzed on a 12% SDS-PAGE. *B* and *C*, Western blots were performed with polyclonal antibodies raised against ceQORH (envelope marker) or LHCP (thylakoid marker) respectively.

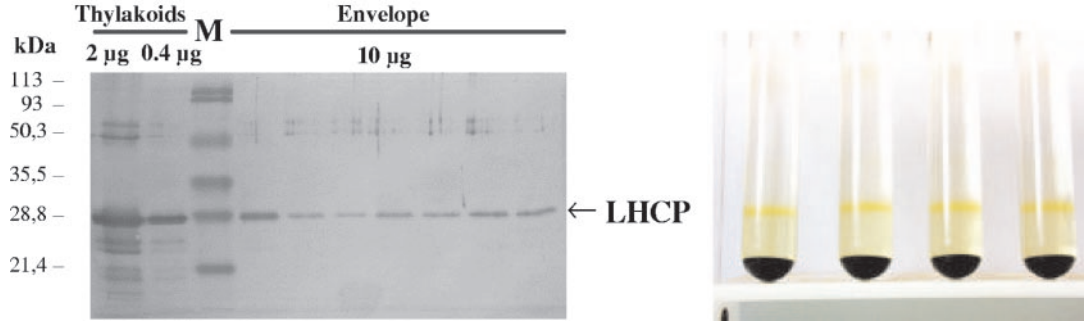

FIG. 2. **Estimation of thylakoid membrane cross-contamination in envelope preparations from *Arabidopsis* chloroplasts.** *Left*, Western blots performed with polyclonal antibodies raised against the LHCP (thylakoid marker). Note the low (< 2%) cross-contamination of envelope preparations. *Right*, Sucrose gradients performed to purify the envelope fraction from *Arabidopsis* chloroplasts. The yellow color of the envelope fraction validates the poor cross-contamination with thylakoid membranes.

using SDS-PAGE analyses of dilutions obtained from both fractions; see Ref. 25). Consequently, extractions of envelope fractions using organic solvents resulted in the enrichment of major thylakoid integral membrane proteins (19% thylakoid proteins), even if original thylakoid contamination was shown to be very low (about 2% in average).

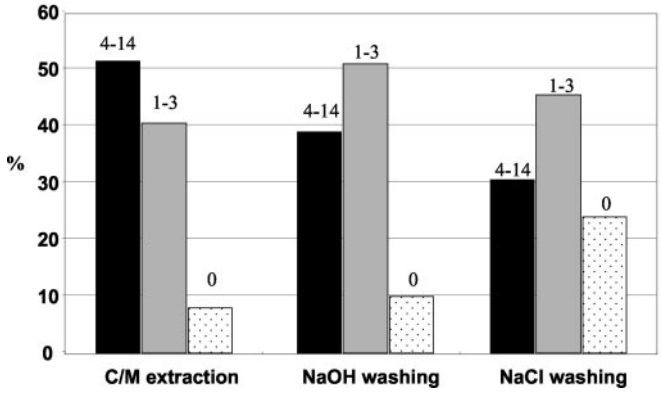

FIG. 3. **Percentage of proteins identified in envelope membrane extracts according to the purification method and the number of transmembrane domains.** Ranges of number of transmembrane domains (as predicted by HMMTOP): 0 (*spotted column*); 1–3 (*gray column*); 4–14 (*black column*).

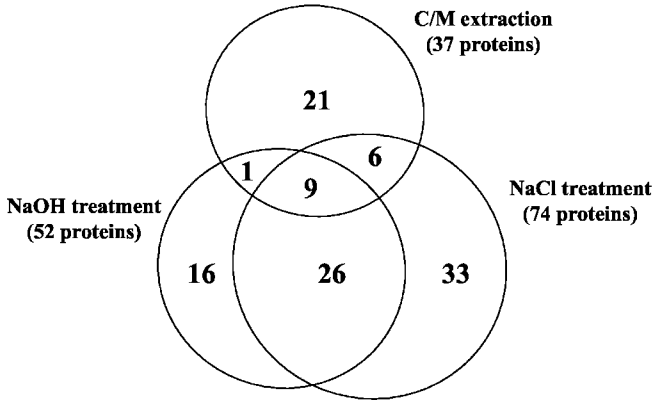

FIG. 4. **Number of proteins identified in envelope membrane extracts according to the extraction method.**

The aim of a targeted proteomic study, such as the present one, is to provide relevant identifications for subsequent functional study. Our aim being the identification of envelope proteins, the identified proteins were classified according to their known (*IM*, *OM*, *S*, *T*, *S/EB*, *S/TB*, *To*) or putative (*IM?*, *OM?*, *E?*, *PM?*, *G?*, *To?*) localization (see Table I). Known localizations were essentially retrieved from previous studies. Although being arbitrary, putative localizations were assessed using rigorous criteria. Putative localization in the inner membrane (referred to *IM?* in Table I) was suggested with respect to the strong hydrophobicity and the presence of a predicted chloroplastic transit peptide (7). Moreover, as discussed further, some of these proteins have putative functions that are compatible with their localization in the chloroplast inner envelope membrane.

Putative localization in the outer membrane refers to high homology with previously characterized outer envelope proteins (orthologous proteins) from other plant species (referred to *OM?* in Table I). Putative localization in the envelope (referred to *E?* in Table I) was suggested because of the very low contamination level, keeping in mind that all identified contaminants are major and characterized proteins in their respective subcellular compartments. Some of these proteins (HP90, HP22, HP30-2, HP30, and HP35), likely to be located in the envelope, show different levels of homology with proteins, previously classified as members of mitochondrial transporter families. As no known protein from mitochondria was detected in the present proteomic approach, these putative (but uncharacterized) transporters are rather unlikely to be actually located in mitochondria. Other putative locations (referred to *To?*, *PM?*, and *G?* in Table I) were deduced from the strong homology with proteins of known location. According to the classification described above, about 80% (89/112)

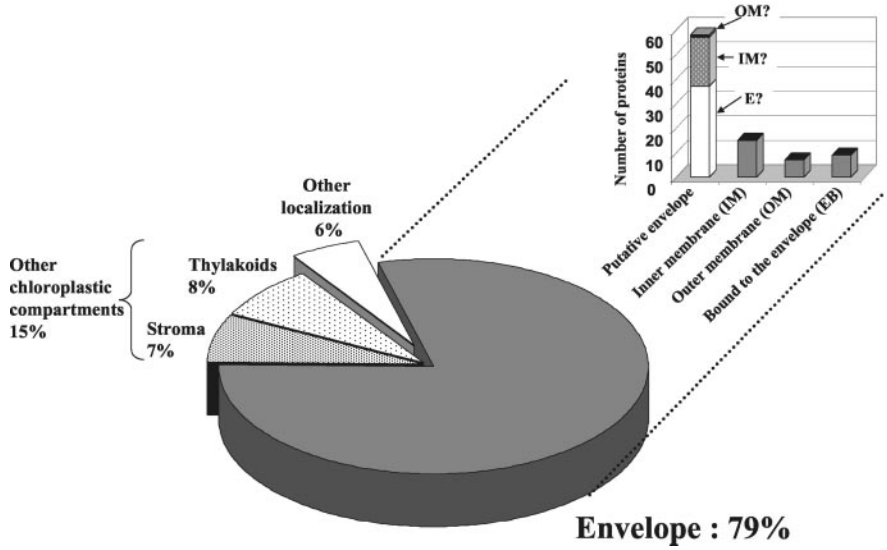

FIG. 5. **Localization of the identified proteins.** *OM*, outer envelope membrane; *IM*, inner envelope membrane; *EB*, bound to the envelope; *OM?*, putative localization in the outer envelope membrane; *IM?*, putative localization in the inner envelope membrane; *E?*, putative localization in the envelope (see Table I).

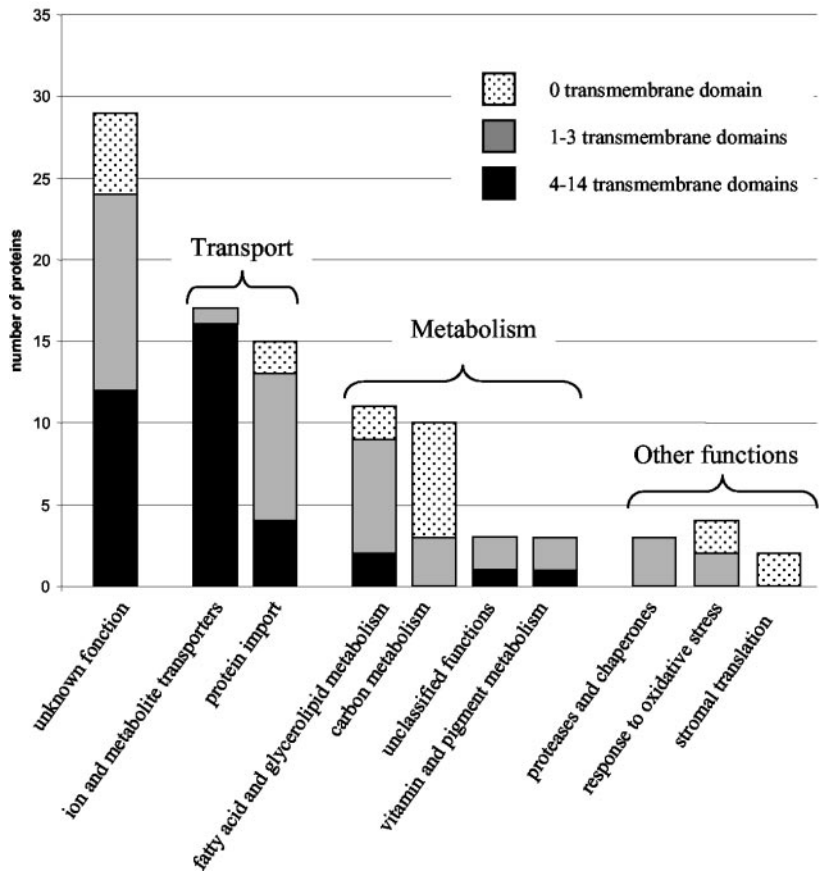

FIG. 6. **Survey of the functions of the proteins identified in chloroplast envelope membranes from *A. thaliana* (according to Table I).** Contaminants from thylakoids and other subcompartments than chloroplast are not listed. Ranges of number of transmembrane domains (as predicted by HMMTOP): 0 (*spotted column*); 1–3 (*gray column*); 4–14 (*black column*).

of the identified proteins can be considered as genuine envelope proteins (Fig. 5), only 9/89 being peripheral envelope proteins (referred to *S/EB* in Table I) and the 80/89 remaining ones being either genuine outer or inner envelope membrane proteins. However, one cannot exclude that some of the stromal proteins could actually be bound to the envelope, as suggested, for example, for the multienzyme complexes with Benson-Calvin cycle activities (31) or for the acetyl-CoA carboxylase (32). Finally, 15% are proteins from other chloroplast subcompartments (stroma and thylakoids), and only 6% of the identified proteins are not chloroplastic proteins.

The possible function of the identified proteins was also assessed using bioinformatic tools such as Blast or InterProScan (Fig. 6; Table I). Many of the 89 identified chloroplast envelope proteins are proteins involved in actual or putative transport functions (32%). Another 30% of these chloroplast proteins have unknown function, and none of the bioinformatic tools we used were able to suggest any function. This opens up real new and challenging investigation areas to find new and unsuspected functions for these new chloroplast envelope proteins. Moreover, some of them being highly hydrophobic, new families of transport systems might have been pointed out. Indeed, it is suspected that plant cells might have evolved unique transport properties that have no homolog in prokaryotic or other eukaryotic cells (33). The implication of identified proteins with characterized or putative function for the chloroplast metabolism is discussed further.

Proteomics for Genome Annotation: Accurate Location of the N Terminus of Mature Proteins—The present study gave clues for the accurate location of the N terminus of some proteins. Except for most proteins from the outer envelope membrane (for a recent review, see Ref. 4) and for the recently described ceQORH (23), all chloroplastic proteins bear a cleavable N-terminal transit peptide. Although efficient bioinformatic tools, like TargetP or ChloroP, are able to predict such transit peptides, these tools are not 100% reliable. Fig. 7 shows the analyzed peptides that are very likely to correspond to the N terminus of some identified mature proteins. This assumption was based i) on the location of these peptides at the very N terminus end of the predicted protein sequence and/or ii) on their location near a predicted chloroplastic transit peptide maturation site and/or iii) on the N-acetylation of their N terminus.

HP15 was found to be a putative membrane channel protein, very likely to be located in the outer membrane. In agreement with this location, no transit peptide signal was predicted for this protein. The present proteomic approach confirmed this prediction as the N terminus of this protein could be analyzed by mass spectrometry. Like for many proteins, the methionine at the N terminus was removed (Fig. 7).

FIG. 7. **Predictions of transit peptide signal *versus* proteomics data.** The *bold* and *grayed* sequences represent analyzed peptides that correspond to the probable N terminus. *Asterisks* indicate N-acetylation of the following amino acid. The *bold underlined* amino acid corresponds to the end of the chloroplastic transit peptide predicted by ChloroP, if one was predicted (*cTP+*). At the end of each definition line (>) *At* refers to identification from the present *A. thaliana* study and *So* to a previous work based on *Spinacia oleracea* samples (7). The species of the identified proteins is the one of the starting material (*A. thaliana* or *S. oleracea*). If not, the species of the homologous protein is specified (*e.g.* IEP33 and HP36).

In the case of CP74 and HP26b proteins, the N-terminal peptides were found downstream of the predicted transit peptide but very close to the predicted maturation site (Fig. 7). Interestingly, in the case of HP35 a MS/MS analyzed peptide was found upstream of the predicted transit peptide, indicating that the putative location of the maturation site is not accurate (Fig. 7). The N-terminal peptides analyzed for HP35, CP74, and HP26b did not result from a specific trypsin cleavage. Although unspecific enzymatic cleavages are known to occur, the location of these nontryptic peptides very close to the predicted transit peptide signal strongly suggests that these peptides represent the N terminus of the processed proteins.

Other peptides located at the N terminus of some *Arabidopsis* proteins were found N-acetylated. With respect to this observation, previously identified spinach envelope protein-derived tryptic peptides (7) were checked for the presence of such posttranslational modifications. N^{α}-acetylation is acknowledged to be a common post-translational modification

of eukaryotic protein N termini. Indeed it was estimated that 70% of eukaryotic soluble proteins were N-acetylated (34, 35). Some proteins, HP22, OMP24, and OEP6, were not predicted for having a chloroplastic transit peptide. Moreover, two of them, OMP24 and OEP6, are known to be located in the outer envelope membrane, which excludes the requirement for a transit chloroplastic peptide. In agreement with these predictions, N^{α}-acetylation occurred at the very N-terminal end of the protein and concerned the N-terminal methionine (OEP6) or the second amino acid residue (OMP24, HP22).

Other proteins were predicted for having a chloroplastic transit peptide. For proteins HP30c, HP27b, IEP18 (At), IEP18 (So), IEP62, FD6C, and IEP33 (So), N-acetylated peptides were found very close to the predicted maturation site. For proteins HP45 and IEP33 (At), N-acetylated peptides were found about 60 amino acids downstream of the predicted maturation site, thus suggesting that the prediction is not correct for these two proteins. Two other cases were not in agreement with ChloroP predictions: HP36 and HP60. Indeed, no maturation site was predicted for these proteins, while an N-acetylated peptide was found close to the N terminus of these proteins. Nevertheless, the relative connection between predictions and proteomic results shows that the maturation site prediction of plastid envelope proteins using the ChloroP program is rather accurate.

Many post-translational modifications have been described for plant and more precisely for chloroplast proteins. Indeed, methylation and carbamylation of proteins of the RuBisCo complex (36, 37), glycosylation of a chloroplastic coupling factor (38), palmitoylation of chloroplastic herbicide-binding protein (39), and phosphorylation of a thylakoid protein (40) have been described. N-acetylation of thylakoid proteins has emerged as an intriguing feature of the photosystem II. In addition to being found in four LHC II molecules, it is also found in three out of four phosphoproteins of the photosystem II core (41–43). The three identified N-acetylated peptides correspond to the N termini of DI, D2, and CPa-2 (PsbA, PsbC, and PsbD) proteins and each begins with *N*-acetyl-0-phosphothreonine. In a paper dealing with thylakoid proteins, Peltier *et al.* (18) quoted that among 55 proteins analyzed by Edman degradation none were likely to be blocked at the N terminus. The authors concluded that the N termini of most mature chloroplastic proteins are unlikely to be further modified (18). Indeed, all nuclear-encoded chloroplastic proteins, which bear a transit peptide, are processed at the N terminus after import to remove this peptide. Thus, possible N^{α}-terminal modifications of the protein precursors that occurred in the cytosol are likely to be removed for proteins targeted to the chloroplast *via* a cleavable transit peptide. Conversely, N^{α}-acetylation of processed proteins that are known to be targeted to the inner envelope membrane, *e.g.* IEP18 (24), HP45 and IEP60 (7), and FD6C (44), would imply an N^{α}-acetylation process in the course or after transit peptide cleavage. The question arising from these N-acetylations is the following: do they result from an *in vivo* process or are they an experimental artifact? For instance, it is acknowledged that carbamylation can occur in the presence of urea (45) and thus can be artifact. In the present study, N-acetylation was only observed for peptides likely to correspond to the N terminus of the chloroplast envelope proteins. Therefore, if chemical N^{α}-acetylation occurred, it would have been before tryptic cleavage. The procedure used from chloroplast fractionation to SDS-PAGE analysis and before trypsin digestion is not in favor of a chemical N^{α}-acetylation. Consequently, *in vivo* N^{α}-acetylation of chloroplast envelope proteins is very likely to occur in the course or after transit peptide cleavage. As described in the literature (34), N^{α}-acetyltransferases have specific substrates, either glycine, alanine, serine, or threonine residues (GAST substrates) or methionine residue (M substrate). In good agreement, among the 14 N^{α}-acetylations described in Fig. 7, 12 correspond to GAST substrates and 1 to an M substrate. Furthermore, in the case of the M substrate, N^{α}-acetylation occurred because the adjacent amino acid residue is a glutamic acid, in agreement with the literature (34). Therefore, and as previously observed for thylakoid proteins (41–43), we can conclude that N^{α}-acetylation actually occurs for chloroplast envelope proteins. Consequently, when such N^{α}-acetylated tryptic peptides are identified, this suggests that these peptides might be the potential N termini of the corresponding mature proteins.

The question of the exact location of the N terminus of these chloroplast envelope proteins could be addressed by intact protein mass measurements. This strategy has been advocated for chloroplast thylakoid proteomics (43) and developed into a viable proteomics strategy with LC-MS analyses (46). The technology to perform intact protein mass measurements on integral membrane proteins, including transporters with up to 15 transmembrane helices, using electrospray ionization has been developed (47–50) and would be of great help to answer this question.

A Combination of Strategies and Plant Models Is Required to Perform the Exhaustive Identification of the Chloroplast Envelope Proteins—The present proteomic study, performed on subfractions deriving from *Arabidopsis* chloroplast envelope membranes, allowed identifying more than 100 plastid proteins, most of them being genuine envelope components. Because the *Arabidopsis* genome has been fully sequenced, this plant is the model of choice for proteomic analyses as far as protein identification is concerned. It appears, however, that a combination of strategies (proteomic and *in silico* approaches) and plant models is required to perform the exhaustive identification of the chloroplast envelope proteins. When focusing on the same chloroform/methanol extraction of envelope purified from both *Arabidopsis* (this work) and spinach (7) chloroplasts, it appears that 15 proteins were exclusively found in *Arabidopsis* while more than 20 were exclusively found in the spinach samples (see supporting data, "Extraction Methods"). This suggests that several plant models may be required

TABLE II

A combination of strategies is required to perform the exhaustive identification of the chloroplast envelope proteins

ChloroP (27). Nt, not tested.

Protein names (AGI accession numbers)	Plastid localization using ChloroP prediction	Spinach envelope	*Arabidopsis* envelope (this work)	Transient expression in plant	Reference for plastid envelope localization
P56-4 (At4g00370)	none (*Arabidopsis*)	**Yes**	Not found	**plastid**	this work
P56-2 (At2g29650)	**plastid (*Arabidopsis*)**	Not found	Not found	**plastid**	this work
Pht2;1 (At3g26570)	**plastid (*Arabidopsis*)** none (spinach)	**Yes**	Not found	**plastid**	7, 115
ceQORH (At4g13010)	none (*Arabidopsis*) none (spinach)	**Yes**	Not found	**plastid**	23
TPT (IEP30) (At5g46110)	none (*Arabidopsis*) **plastid (spinach)**	**Yes**	**Yes**	nt	116, 7, this work
HP36 (At2g26900)	none (*Arabidopsis*)	**Yes**	**Yes**	nt	7, this work
OEP21 (At1g76405)	none (pea) none (*Arabidopsis*)	**Yes**	**Yes**	nt	53, 7, this work
Toc159 (At4g02510)	none (pea) none (*Arabidopsis*)	Not found	**Yes**	nt	51, 52, this work
Tic55 (At2g24820)	**plastid (*Arabidopsis*)** **plastid (Pea)**	Not found	**Yes**	nt	54, this work

to identify chloroplast envelope proteins.

All known inner envelope proteins contain a classical N-terminal plastid transit peptide and thus should be predicted as localized in plastids using *in silico* approaches. However, while localized in the inner membrane of the chloroplast envelope, the ceQORH identified in the plastid envelope could not be predicted to be localized in plastids (Table II), because it lacks a classical N-terminal and cleavable plastid transit peptide (23). Likewise, the HP36 protein could not be predicted to be plastid localized (Table II) because of an error in the prediction of the 5′ region in the *Arabidopsis* open reading frame during the *Arabidopsis* genome annotation (7). Toc159 (51, 52) or OEP21 (53), some major outer envelope proteins, could not be predicted to be localized in plastids (Table II) because they lack a classical N-terminal chloroplast transit peptide, like most outer envelope membrane proteins. Toc159 and Tic55 were initially identified in pea chloroplast envelope membranes (54) as components of the chloroplast protein import machinery. These two proteins were identified in *Arabidopsis* but not detected in spinach (Table II).

The case of phosphate transporters further highlights the requirement for using different biological models and strategies. As presented in Table II, the Pht2;1 phosphate transporter that was localized in the spinach chloroplast envelope (7) was not identified during the study performed on *Arabidopsis*. With an *in silico* analysis, using ChloroP, a correct plastid localization was predicted for the *Arabidopsis* Pht2;1 (At3g2657), but not for the spinach protein (7). Conversely, the same prediction tool predicts the plastid localization of the spinach triose-phosphate/phosphate translocator but does not predict that of the *Arabidopsis* protein (At5g46110) (Table II). Using recent information deriving from full-length sequencing of *Arabidopsis* cDNAs, we could identify some proteins corresponding to previously unassigned spinach peptides (7). Among these proteins is the HP56-4 protein (At4g00370), a putative phosphate transporter. A close homologue of this protein, HP56-2 (At2g29650), was also identified in *Arabidopsis* Genome Initiative predictions using a BLAST-based method. Only HP56-2 *Arabidopsis* protein is predicted (*in silico*) to be localized in plastids (Table II). However, and in contrast with the HP56-4 homologue, we have not detected the HP56-2 protein by proteomics. Using transient expression of GFP fusions in *Arabidopsis* cells (Fig. 8), we demonstrated that both HP56-2 and HP56-4 proteins were targeted to plastids, thus strengthening the HP56-4 envelope localization and supporting the hypothesis that HP56-2 is indeed a plastid protein. This latter protein may be i) too poorly represented in chloroplasts purified from *Arabidopsis* or spinach leaves, ii) only expressed in nongreen tissues of the plant, or iii) only expressed at a given time of plant development, thus strengthening the interest of an *in silico* approach based on the prediction of protein structure and subcellular localization. Databases, like ARAMEMNON (55), that use a set of different prediction programs to provide a statistical view of the possible subcellular localization, are therefore very interesting. Alternatively, subcellular-specific proteomic approaches have to be performed on other plant tissues that are compatible with the preparation of highly purified plastids (proplastids from cauliflower inflorescence, amyloplasts from sycamore cells, etc.).

A Functional Survey of Envelope Proteins Identified by Proteomics

Transport Systems in Chloroplast Envelope Membranes—Several proteins were identified that correspond to validated

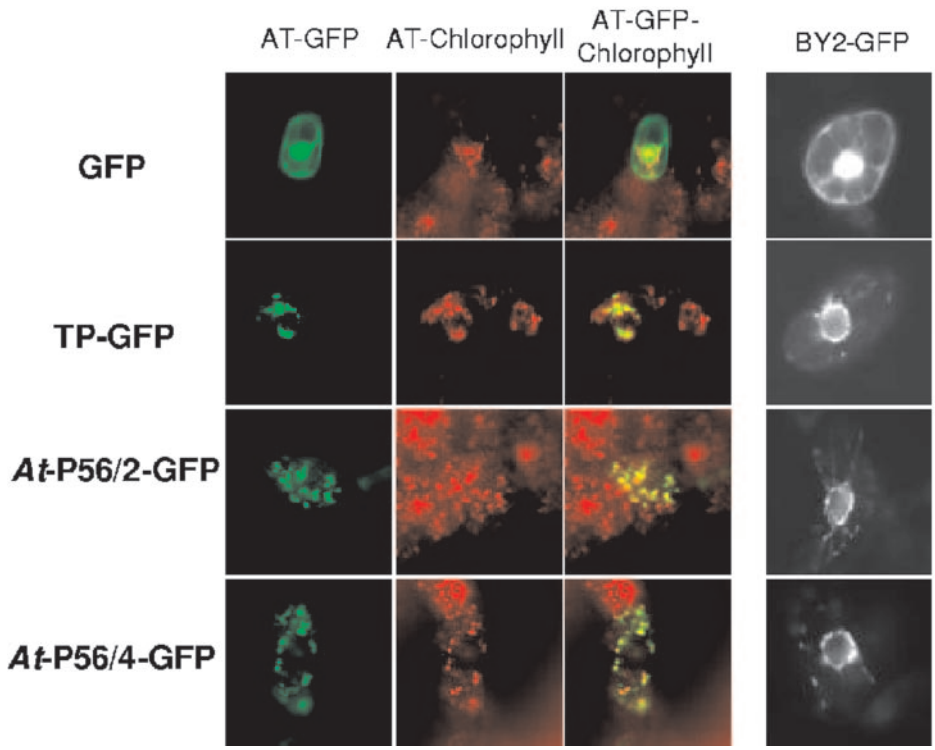

FIG. 8. **Transient expression of *Arabidopsis* phosphate transporter homologues P56/2 and P56/4 fused to GFP-tag in *Arabidopsis* and BY2 tobacco cells.** 35Ω-sGFP(S65T) plasmid (*GFP*) and the 35Ω-TP-sGFP(S65T) plasmid (*TP-GFP*) containing the transit peptide sequence from RbcS fused to GFP were used as controls. Colocalization of GFP and chlorophyll fluorescence was observed for both the plastidial control TP-GFP and the two putative *Arabidopsis* phosphate transporters P56/2 (*At-P56/2*) and P56/4 (*At-P56/4*).

or putative ion and metabolite transporters. They represented a high proportion of the proteins present in the chloroform/methanol extract. Nine proteins correspond to previously identified chloroplast envelope components (7), and eight are previously unidentified envelope components that may catalyze transport activities across the chloroplast envelope (Table I). IEP45 (At5g12860) and HPSOT (At5g64290) are identified members of the 2-oxoglutarate/malate translocator family, and IEP30 (At5g46110) and IEP33 (At5g33320) are known members of the phosphate translocator family. IEP62 (At5g16150) and HP59 (At5g59250) are two putative sugar transporters, HP35 (At4g39460) is a putative mitochondrial carrier-like protein, and HP36 (At2g26900) is the previously identified member of the Na^+/taurocholate transporter family. The implication of the association of these proteins in the envelope was previously discussed (7). HP15 (At2g28900) is the *Arabidopsis* homologue of the previously identified OEP16 pea protein (amino acid-selective outer membrane channel; Ref. 56). The previously identified HP25 (At2g42770) and the new protein HP30c (At5g19750) are two members of the PMP22-like family that share homology with major integral peroxisomal membrane proteins suspected to act as solute transporters. HP45b (At5g01500) is a new member of the putative peroxisomal calcium-dependent solute carrier family but is distinct from the member previously identified in spinach. HP90 (At2g35800) is a new member of the calcium-dependent mitochondrial solute carrier family distinct from the member previously identified in spinach. Members of this last family transport C5-C7 oxodicarboxylates across the inner membranes of mitochondria. They can transport 2-oxoadipate, 2-oxoglutarate, adipate, glutarate, and, to a lesser extent, pimelate, 2-oxopimelate, 2-aminoadipate, oxaloacetate, and citrate (57). HP77 (At4g25450) is a member of the nonintrinsic ABC transporter family (NAP8) and could be a subunit of multimeric quarter-molecule ABC transporters that, when subjected to phylogenetic analyses, group with the MDR/TAP/ATM family (58). HP64 (At1g01790) is referred to as the putative K^+ efflux antiporter KEA1 and shares more than 40% identity with the KEFC homologue from *Escherichia coli*, a transport system that facilitates potassium efflux, possibly by proton antiport (59). This HP64 protein may thus participate in the modulation of K^+/H^+ counterfluxes across the envelope (60). AHM1 (At4g37270) is referred as a potential cadmium/zinc-transporting ATPase (HMA1) and belongs to the cation transport ATPases family (E1-E2 ATPases, subfamily IB). Its sequence is distinct from that of the previously described chloroplast envelope Ca^{2+}-ATPase-like protein (61). Finally, HP47 (At5g52540) is a protein containing 10 potential transmembrane helices sharing low homology with C4-carboxylate transporters. Other identified proteins of unknown function but containing several potential transmembrane helices may correspond to new chloroplast envelope transporters (Table I). Indeed, 7 proteins among the 29 unknown proteins found in *Arabidopsis* envelope membranes combine all the characteristics for putative chloroplast envelope transporters, *i.e.* i) a chloroplast transit peptide, ii) a ratio of number of amino acid residues to putative transmembrane domains (Res/TM) below 100, iii) at least four transmembrane domains, and iv) a pI value above 8.8 (7).

The Protein Import Machinery of Chloroplast Envelope Membranes—Import of nuclear-encoded precursor proteins into the chloroplast occurs through translocon complexes at the outer (Toc complex) and inner (Tic complex) envelope membranes. Numerous biochemical studies resulted in the characterization of components of the chloroplast import machinery (for reviews, see Refs. 3 and 4). We identified a series of proteins previously known to be part of the Toc or the Tic complexes. Some of them have a high homology with pea (*Pisum sativum*) proteins (from which most of the import proteins were identified). Table I demonstrates the identification of the three main components of the Toc complex: Toc34, Toc75 (OEP75), Toc159 (OEP86). We also identified Toc33, a protein similar to Toc34, and a Toc64-like (HP64b) protein. Toc34 and Toc159 are GTP-binding proteins involved in precursor recognition, and Toc75 is forming an aqueous protein-conducting channel (for review, see Ref. 3). Toc64 is expected to function as a docking protein for cytosolic cofactors of the protein import into chloroplasts (62). Concerning components of the Tic complex, we identified a series of proteins such as Tic40, Tic55, a Tic55-like (HP62) protein, and proteins with homology with Tic20 (IEP16) and Tic62 (HP26c). Tic22 was the only lacking component of the previously identified members of the Tic complex. This is not surprising because Tic22 is highly hydrophilic, contains no apparent membrane spanning domains, and is localized in the intermembrane space of the envelope (for review, see Refs. 1–4). Therefore, Tic22 is likely to be excluded from the samples we analyzed because it was extracted from the envelope by all the different treatments performed in this study. In contrast, Tic20 is a hydrophobic, integral membrane protein playing a role in preprotein conductance at the inner envelope membrane (63, 64). Tic40 was proposed to have a putative Hsp70 chaperone-interacting function (65). Tic55 contains a predicted Rieske-type iron-sulfur cluster and was shown to be part of the core of the Tic complex (54). The N terminus of Tic62 shows strong homologies to NAD(H) dehydrogenases in eukaryotes and to Ycf39-like proteins present in cyanobacteria and nongreen algae (66). We also identified several chaperones involved in protein import, HP112 (IAP100) and ClpC. IAP100 was proposed to serve in recruiting chaperonin for folding of newly imported proteins (67). ClpC, a soluble Hsp100 chaperone that appears to interact directly with Tic110, is thought to provide the driving force for chloroplast protein import (54, 68). Although identified as a stroma protein, ClpC is clearly functionally associated with the envelope membrane.

In addition, we found three proteins (HP22, HP30, HP30-2) with some homology to components of the mitochondrial import machinery (Tim17/Tim22). This confirms our previous findings on spinach, where we identified another homolog of the Tim complex, namely HP20 (7). Because we can rule out a specific contamination of chloroplast envelope membranes by Tim components (see above), our analyses demonstrate the unexpected presence in chloroplast envelope membranes of a series of proteins having homologies with components of the mitochondrial protein import machinery. In mitochondria, Tim17 (together with Tim23) constitute the import channel for preproteins containing amino-terminal hydrophilic presequences (69). The Tim17, Tim22, and Tim23 proteins have in common a similar topology in the membrane and a homologous amino acid sequence. Interestingly, they show a sequence similarity to OEP16, a channel-forming amino acid transporter in the outer envelope of chloroplasts, and to LivH, a component of a prokaryotic amino acid permease (69). One can question whether these proteins are true components of the Tic/Toc complexes or whether another import system is present in chloroplast envelope membranes. A similar question raised by the identification of an inner envelope protein containing internal targeting information (23) is whether it is imported by distinct import mechanism, like in mitochondria (70, 71), or by the normal Tic/Toc complexes used by precursor proteins containing cleavable N-terminal transit sequences.

Finally, our results provide further evidence for the use of cyanobacterial ancestor genes to build up the chloroplast envelope import machinery. Several examples have been already described. For instance, Toc75 is rather close, in sequence as well as in topography, to a cyanobacterial channel-forming protein (51, 72). We identified HP65b, a protein having homology with the *Synechocystis* protein Q55511. This cyanobacterial protein is likely to be involved in protein export and could act as a chaperonin by maintaining the newly synthesized protein in an open conformation. A similar function of the related chloroplast protein is therefore rather possible.

Envelope Membranes and Enzymes of the Lipid Metabolism—Through our proteomics studies on spinach (7) and *Arabidopsis* chloroplast envelope membranes (this work, Table I), we have identified several proteins involved in lipid metabolism (for a survey of the genes encoding these enzymes, see Ref. 73). The first step of chloroplast membrane lipid biosynthesis is the acylation of glycerol 3-phosphate to form phosphatidic acid. This is catalyzed by two acyltransferases: the first one, responsible for lysophosphatidic acid biosynthesis, is a stromal enzyme active in the vicinity of the inner envelope membrane (74), whereas the second one, responsible for phosphatidic acid biosynthesis, resides to the inner membrane (for review, see Ref. 75). Indeed, we identified 2-lysophosphatidate acyltransferase (76), an enzyme catalyzing the transfer of 16:0 (from 16:0-ACP) to the sn-2 position of lysophosphatidic acid, leading to the synthesis of phosphatidic acid, the precursor for chloroplast glycerolipids. MGD1, one of the three *Arabidopsis* monogalactosyldiacylglycerol (MGDG) synthases, the last committed step in MGDG biosynthesis, was identified in our previous study on spinach (7). This protein is a very minor envelope protein (77), thus providing some reason for its absence in the list of *Arabidopsis* proteins. Concerning the formation of other chloroplast-specific glycerolipids, we identified proteins that could participate to phosphatidylglycerol (PG) synthesis. The protein

HP32c is a phosphatidylglycerophosphate synthase (or CDP-diacylglycerol:glycerol-3-phosphate phosphatidyltransferase); an *Arabidopsis* mutant, impaired in the corresponding gene (*pgp1*), has an overall PG content reduced by 30% and shows an 80% reduction in plastidial enzyme activity (78). Envelope membranes also contain a protein (HP25b) that is a phosphatidylglycerophosphate synthase-like protein (for review, see Ref. 79). Interestingly, *in silico* analyses (7) suggested that a putative CDP-diacylglycerol synthetase (At4g26770) could be present in envelope membranes. This enzyme could be the first step committed to chloroplast PG synthesis.

Chloroplast glycerolipids synthesized through the envelope membranes contain saturated (16:0) and monounsaturated fatty acids (18:1) and are therefore substrates for fatty acid desaturases that catalyze the formation of the polyunsaturated molecular species characteristic of plastid glycerolipids. Although genetic approaches shed new light on chloroplast membrane desaturases with the characterization of *Arabidopsis* mutants (for review, see Ref. 80), there was only little evidence for such enzymes to be present in chloroplast envelopes (81). Indeed, we identified in envelope membranes two desaturases, namely FD3C and FD6C, corresponding respectively to omega-3 and omega-6 fatty acid desaturases. Genetic analyses on *fad6* (FD6C) and *fad7* (FD3C) mutants demonstrated that they are both chloroplast enzymes active on glycerolipids, and especially on galactolipids (for review, see Ref. 80). FD6C (oleate desaturase) catalyzes the formation of C18:2 from monounsaturated fatty acids, whereas FD3C (plastidial linoleate desaturase) introduces the third double bond leading to the formation of linolenate. Fatty acid desaturation is a complex process that requires an electron transfer chain. Using electron paramagnetic resonance spectroscopy, Jäger-Vottero *et al.* (82) characterized in the spinach chloroplast envelope electron paramagnetic resonance signals corresponding to putative components of an electron transfer chain. None of the envelope components responsible for such signal have been identified to date, but the identification of a putative quinone oxidoreductase (IEP41; Ref. 23) in our spinach preparation may provide a first clue toward characterization of members of an envelope electron transfer chain. Interestingly, we also identified a putative flavin-containing oxidoreductase (HP52b).

In plant cells, most saturated and monounsaturated fatty acids are synthesized within the plastid stroma, but they are also used in the endoplasmic reticulum for the biosynthesis of phospholipid (phosphatidylcholine, phosphatidylethanolamine, etc.) and therefore have to be exported to the cytosol. An hypothesis is that the acyl-CoA synthetase (which is located on the outer envelope membrane, Ref. 83) could be involved in fatty acids export from chloroplasts. We identified in *Arabidopsis* envelope membranes two proteins (HP76 and HP81) that could correspond to acyl-CoA synthetases. They were both among the 11 putative acyl-CoA synthetases identified by Shokey *et al.* (84) in their survey of the *Arabidopsis* genome. These authors demonstrated that only nine of these genes actually encoded long-chain acyl-CoA synthetases (LACS). Although containing a putative AMP-binding site, HP81 is actually not a LACS because i) it was unable to complement yeast mutants and ii) the overexpressed protein was unable to synthesize acyl-CoAs. The exact function of this envelope protein therefore remains to be identified. In contrast, HP76 corresponds to one of the LACS proteins, namely LACS9. This protein was demonstrated by Schnurr *et al.* (85) to complement yeast mutants and to catalyze the synthesis of acyl-CoAs. Most interestingly, they found that this protein is the major plastid acyl-CoA synthetase, and, by *in vitro* protein import and GFP fusion experiments, they demonstrated that it was targeted to the chloroplast envelope. Interestingly, LACS9 could not be extracted by a NaOH washing of chloroplast envelope membranes, in good agreement with our findings. This protein does not have any transit peptide and resides at the outer envelope membrane, as shown by previous biochemical studies (83).

Oxylipins are metabolites produced by the oxidative transformation of unsaturated fatty acids via a series of diverging metabolic pathways. Blée and Joyard (86) demonstrated that chloroplast envelope membranes can synthesize oxylipins, owing to enzymes like allene oxide synthase and hydroperoxyde lyase. Indeed, Froehlich *et al.* (87) demonstrated that these two proteins are respectively targeted to the inner and outer envelope membrane. We identified one of these two proteins in *Arabidopsis* envelope membranes, namely the allene oxide synthase (CP74). We also identified a phospholipid hydroperoxide glutathione peroxidase. This enzyme, which converts fatty acid hydroperoxides into alcohols, can be part of an ascorbate-glutathione cycle (see below). Altogether, these data suggest a role for envelope membranes in plant defense-signaling pathways, because such processes involve oxylipins.

Among the proteins involved in fatty acid metabolism, we identified two proteins, HP88b and ACCD, corresponding to two subunits of the acetyl-CoA carboxylase (ACCase) complex, respectively the α and β subunits. In general, the ACCase complex is considered as soluble and is indeed easily extracted from the chloroplast stroma. However, our results strongly support a series of observations (88–90), suggesting that IEP96 (corresponding to HP88b) could be the α subunit of the ACCase. More recently, Thelen and Ohlrogge (32) demonstrated the presence of α and β subunits of ACCase in envelope preparations by Western blot experiments. They proposed that the binding of ACCase to the chloroplast envelope could occur through nonionic interactions to the carboxyltransferase subunits. Our results provide further support to such observations: these proteins should be present at the stroma side of the inner envelope membrane, anchoring the ACCase complex to the membrane. Interestingly the β subunit of ACCase is chloroplast encoded.

We also identified IM30, a protein that could be involved in lipid transfer between the inner envelope membrane and thy-

lakoids. This function was postulated because of its dual localization; immunocytochemical localization of this protein revealed that the protein occurred in clusters in the vicinity of both the envelope and the thylakoid (91). Another protein, namely HP20b, was shown to contain domains homologous to domains present in lipid transfer proteins. Such proteins are essential in chloroplasts because the envelope is the site of membrane lipid biosynthesis, whereas thylakoids are the site for their accumulation.

Envelope Membranes and the Biosynthesis of Terpenoid Compounds—Chloroplast membranes contain a series of compounds deriving (at least in part) from isopentenyl pyrophosphate: carotenoids, prenylquinones, and chlorophyll precursors (for reviews, see Refs. 75 and 92). These compounds are synthesized in envelope membranes (92), but some controversy still remains (especially for prenylquinone biosynthesis). Concerning the biosynthesis of chlorophyll precursors, protochlorophyllide oxidoreductase was characterized in spinach as well as in *Arabidopsis*. The presence of this protein in envelope membranes was first demonstrated functionally (93), then with antibodies (94), thus suggesting that envelope membranes could play a role in the biosynthesis of chlorophyll precursors (94). Indeed, several other enzymes involved in the biosynthesis of protochlorophyllide were demonstrated to be present in purified envelope membranes (see for instance Refs. 95–97). To date, none of these enzymes could be detected by proteomics.

In chloroplasts, the inner envelope membrane was shown to be the site of α-tocopherol and plastoquinone-9 synthesis (98). In contrast, Swiezewska *et al.* (99) proposed that plastoquinone and ubiquinone biosynthesis was in fact localized in Golgi membranes and that a specific transport system was required for plastoquinone and ubiquinone transfer, respectively, to chloroplasts and mitochondria (see also Ref. 100). Due to the difficulty in handling such membrane-associated enzymes using classical biochemical approaches, alternative approaches to clone the corresponding genes were developed. To date, only a nuclear-encoded methyltransferase (AtCOQ3) catalyzing the last step in ubiquinone biosynthesis and localized in the inner membrane from plant mitochondria was characterized (101). We identified in envelope membranes two proteins that are candidates for a role in chloroplast prenylquinone biosynthesis. IEP37, one of the major inner envelope membrane protein, is a *S*-adenosyl-L-methionine-dependent methyltransferase (102) having homology with UbiE/COQ5 methyltransferase, whereas HP43 presents a low homology with UBIA prenyltransferase. Interestingly, a mutant containing a transposon within the gene encoding IEP37 was shown to have a much lower content of chloroplast prenylquinones, thus suggesting that IEP37 is a methyltransferase committed to the biosynthesis of plastid prenylquinones.[2] One intriguing question is why this protein is present in such large amounts in the inner envelope membrane (IEP37 is one of the major inner envelope membrane protein). Altogether, these observations are strong arguments in favor of a major role of envelope membranes in the biosynthesis of plastid prenylquinones.

Finally, major terpenoid compounds in envelope membranes are carotenoids (75). Although enzymes involved in carotenoid biosynthesis and metabolism (abscisic acid biosynthesis) have not been yet detected by proteomics in spinach or *Arabidopsis* chloroplast envelope membranes, *in silico* analyses demonstrated that β-carotene hydroxylase, an enzyme of the zeaxanthin pathway, could be localized in envelope membranes (7).

Oxyradical Scavenging and Antioxidant Capacities of Envelope Membranes—Plants are submitted to a wide variety of environmental stresses, like light, drought, nutrient, and temperature, that can induce oxidative stress through the formation of reactive oxygen species. This lead to major damages within membrane constituents, like lipids and proteins. For instance, large amounts of fatty acid hydroperoxides can be formed within envelope membranes. They can be metabolized through the ascorbate-glutathione cycle (for review, see Ref. 103). Indeed, we identified in *Arabidospis* envelope membranes several proteins that could be involved in oxidative stress responses: namely a phospholipid hydroperoxide glutathione peroxidase (PHGPx), an ascorbate peroxydase, and a superoxide dismutase. Superoxide dismutase is a soluble protein that produces hydrogen peroxide. Ascorbate peroxydase is also a soluble protein. It mediates hydrogen peroxide detoxification in a series of reactions coupled to the functioning of PHGPx. PHGPx selectively acts on hydroperoxides, converting them to alcohols and therefore preventing lipid peroxidation. It is not very clear whether PHGPx is soluble or membrane bound, but it should be active in the vicinity of the membrane. Little is known on a possible role of chloroplast PHGPx in envelope membranes. But the demonstration that mitochondrial PHGPx is involved in the protection from inactivation of the adenine nucleotide translocator during hypoglycemia-induced apoptosis (104) provides some clues toward the characterization of such a role in envelope membranes. We also identified an m-type thioredioxin (type 1), an enzyme that can induce hydrogen peroxide tolerance in chloroplasts (105). Unfortunately, we do not know the actual target of this key enzyme in chloroplast redox network.

Ascorbate also serves as an antioxidant in many other detoxification reactions, such as the scavenging of hydroxyl radicals and the reduction of tocopheryl radicals produced by α-tocopherol, by quenching autocatalytic lipid peroxidation. Because α-tocopherol is the major prenylquinone in envelope membranes (for review, see Ref. 75) and because hydroxyl radicals can be formed by various reactions in this membrane system, the association of the ascorbate-glutathione cycle to the envelope membranes (rather likely to the inner membrane) is probably a physiological requirement for protecting the membrane against harmful reactive oxygen species responsible for lipid peroxidation. Furthermore, the possible pres-

[2] K. Shinozaki, personal communication.

ence of a β-carotene hydroxylase in envelope membranes (7) is also in favor of such a role. As a matter of fact, Davidson *et al.* (106) demonstrated that *A. thaliana* plants in which the *chyB* gene that encodes β-carotene hydroxylase was overexpressed were more tolerant to conditions of high light and high temperature, as shown by reduced lipid peroxidation.

Finally, the damages caused to membrane proteins submitted to an oxidative stress requires the presence of active repair mechanisms. We identified several proteases in *Arabidopsis* envelope membranes, namely two members of the ATP-dependent Clp family and one of the GTP-dependent FtsH family. Indeed, a possible role for these proteases could be to remove the damaged protein components from the envelope membrane.

Altogether, our observations demonstrate that in addition to antioxidant molecules, like α-tocopherol, a whole set of enzymes involved in prevention of oxidative stress (responses and repair mechanisms) are present in chloroplast envelope membranes. Because the thylakoid lumen also contains enzymes active in membrane protection against oxidative stress (18), these data together with ours demonstrate that both chloroplast membrane systems (envelope membranes and thylakoids) contain complex enzymatic and nonenzymatic equipment to protect the chloroplast against oxidative stress.

CONCLUDING REMARKS

The functional integration of plastids within the plant cell involves a series of unique envelope proteins like metabolite transporters, ion channels, pumps, permeases, pore proteins, etc. For instance, the envelope phosphate/triose phosphate translocator regulates photosynthesis (for review, see Ref. 107), whereas the dicarboxylate transporters are essential to control the interaction between carbon and nitrogen metabolism (108). The identification, in chloroplast envelope membranes, of these well-characterized transporters further validates the whole proteomic strategy we have developed (this work and Ref. 7). In addition, we identified several proteins with multiple α-helical transmembrane regions corresponding to putative transport proteins of predictable function (for instance sugar, phosphate, amino acid, or taurocholate transporters, etc.) as well as a series of proteins with unknown function, many of them bearing features (such as a strong hydrophobicity and an alkaline isoelectric point, Ref. 7) that make them good candidates for being new transport systems of the inner membrane of the chloroplast envelope. One of the major findings of these proteomic analyses (this work and Ref. 7) is the identification of several proteins that could be involved in phosphate transport across the envelope (Table II). Interestingly, whereas some transporters, like the members of the triose-P/inorganic phosphate (P_i), PEP/P_i, or Glucose-6P/P_i translocators, catalyze an equimolar exchange of P_i, others, like the putative H^+/P_i transporter, could catalyze a net import of P_i in the chloroplast. Such transporters are likely to be essential for controlling the stromal phosphate level and homeostasis required to initiate the Calvin cycle. Identification of these new phosphate transport systems in chloroplasts is expected to lead to a better understanding of their role in cell metabolism. Altogether, the identification of these new putative transport systems paves the way for extensive functional analyses of chloroplast envelope membranes.

The development of fully functional plastids relies on a complex set of envelope enzymes for the biosynthesis of specific lipid constituents of plastid membranes, such as glycerolipids (galactolipids, sulfolipid, phosphatidylglycerol), pigments (carotenoids and chlorophyll), as well as prenylquinones (α-tocopherol, plastoquinone-9, etc.). Altogether, our proteomic analyses of spinach and *Arabidopsis* envelope membranes (this work and Ref. 7) are consistent with a key role of chloroplast envelope membranes in lipid metabolism. They first confirm the presence in envelope membranes of enzymes involved in chloroplast membrane lipid biosynthesis (MGDG and PG). A second major observation is the unambiguous identification of two chloroplast desaturases in *Arabidopsis* envelope membranes. Together with some elements of a putative electron transfer chain, our results provide experimental evidence for desaturation of fatty acids to occur in chloroplast envelope membranes. Furthermore, our results also provide clear evidence for a more general participation of chloroplast envelope membranes in fatty acid metabolism. Obviously, fatty acid biosynthesis probably occurs in the close vicinity of the inner envelope membrane because our results provide support to a series of converging evidence suggesting that acetyl-CoA carboxylase could be attached to the stromal face of this membrane. The newly synthesized fatty acids can therefore be used either directly by the enzymes of the Kornberg-Pricer pathway for glycerolipid biosynthesis or exported outside the chloroplasts owing to a series of proteins like the acyl-CoA synthetase. Our data also confirm that polyunsaturated fatty acids can be metabolized into oxylipins, providing further support for a role of envelope membranes in the synthesis of lipid-derived plant growth regulators and defense compounds in response to extracellular stimuli. Furthermore, fatty acid hydroperoxides whose formation can be induced by various reactive oxygen species can also be metabolized through the ascorbate-glutathione cycle that seems to be associated to envelope membranes (probably the inner) through key enzymes of the pathway.

We found in envelope membrane preparations several proteins that are expected to reside in the chloroplast stroma. The use of antibodies raised against stromal proteins demonstrated unambiguously that purified envelope membranes contains several stroma enzymes, the most conspicuous being RuBisCo (109). An obvious explanation to this observation is that some stroma is trapped inside envelope vesicles during the osmotic shock used to prepare the envelope. However, a second possibility, namely the functional association of envelope proteins with soluble enzymes, cannot be entirely ruled out because we analyzed highly purified envelope subfrac-

tions washed with either NaOH or NaCl and that should therefore be mostly devoid of soluble contaminants (especially from the stroma). A functional relationship with the inner membrane is possible for the enzymes of the ascorbate-glutathione cycle we identified in envelope subfractions (see above). This can also be the case for the carbonic anhydrase we identified, in good agreement with previous observations of Villarejo *et al.* (110) on *Chlamydomonas* envelope membranes and with the function of ycf10, an envelope protein involved in the transport of inorganic carbon through the envelope (111). The Clp proteins we identified are possibly linked to their importance in the functioning of the protein import machinery of the envelope membranes (for reviews, see Refs. 4 and 112). As already mentioned, envelope membranes contain some RuBisCo. This protein is the most abundant stromal contaminant in envelope preparation, but functional association of the most abundant protein in the biosphere with the inner envelope membrane cannot be completely ruled out. For instance, Babadzhanova *et al.* (31) characterized free and membrane-bound forms of Calvin-cycle multienzyme complexes in cotton chloroplasts. The activities in the membrane-bound complex were significantly higher than the activities of the free form. Interestingly, RuBisCo, phosphoglycerate kinase, and glyceraldehyde phosphate dehydrogenase, which were among the proteins present in such complexes, are also present in our survey of the proteins in envelope membranes subfractions.

Despite the considerable progress in analyzing chloroplast envelope membranes, we do not have yet a complete picture of the chloroplast envelope protein equipment and therefore functions. For instance, many proteins we expect to be present in our preparations from our knowledge of chloroplast envelope metabolism are still missing. This is the case for several enzymes of membrane lipid synthesis or fatty acid metabolism. The same is true for enzymes of pigment or prenylquinone biosynthesis. Several transport systems expected to be present in chloroplast envelope membranes are missing. There are at least two main reasons for this. First, envelope membranes have a wide variety of functions and therefore contain a much larger set of protein that many other membranes. Such proteins are likely to be present only in minor amounts. The peptides deriving from such minor proteins are therefore difficult to identify among hundreds of peptides deriving from the major proteins. Second, many of these proteins have not yet been characterized and therefore are absent from the databases. One can hypothesize that several of the expected proteins are among the unknown proteins we have identified. Because chloroplast proteins have little chance to be found in nonplant organisms, there is little possibility for identifying homologs in nonplant protein databases. Functional analyses of such unknown proteins represent a true challenge for the future.

Another problem is the unambiguous identification of outer envelope proteins. With the increasing number of envelope proteins being characterized, it now appears rather clearly that most outer envelope proteins are synthesized without a chloroplast targeting sequence. Only few exceptions to this rule have been found (OEP75, for instance). Furthermore, extensive genome analyses and comparison with increasing expression data (expressed sequence tag or proteins) demonstrate that correct genome annotation is difficult to achieve at the 5′ end of the putative genes, *i.e.* where most of the targeting information is expected to be located. In addition, predictions for identifying putative targeting sequences are still not completely efficient and sometimes they propose that the sequence under evaluation has no targeting sequence despite of the contrary. Therefore, it is difficult to identify among the hypothetical proteins expected to be synthesized without chloroplast transit sequences those that are genuine outer envelope membranes. In addition, the situation is even more complex because precursors from some inner membrane proteins also appear to be devoid of a cleavable targeting peptide (23). Specific methods should therefore be developed to unambiguously identify proteins residing at the outer envelope membrane. The same is true for proteins of the intermembrane space of the envelope. Because they are soluble, such proteins are expected to be present in the fraction obtained from NaCl washing of the membranes. Unfortunately, this fraction contains mostly stromal contaminants. Stromal proteins represent about half of the chloroplast proteins, whereas one cannot expect envelope proteins of the intermembrane space to represent no more than a few 1‰ of the total chloroplast proteins. The situation is therefore much more complex than for another chloroplast intermembrane space, *i.e.* the thylakoid lumen (18). Despite the fact that thylakoid peripheral proteins are also present in the extract, proteomic analyses of the thylakoid lumen seems to be rather reliable because i) protein targeted to the lumen have transit peptides sharing common features, which can be used to predict localization (27, 113), and ii) the integrity of thylakoids can be preserved during their preparation and purification, in contrast to envelope membranes.

Finally, chloroplasts are only the most characterized type of plastid, a unique feature of photosynthetic tissues. Plastids are present in almost every plant cell, and their structural and functional diversity reflects their role in different cell types. According to their developmental stage, juvenile (proplastids), differentiating, mature, and senescent plastids can be distinguished. The metabolism of these various types of plastids is linked to the function of the tissue in which they are found. For instance, whereas the chief function of chloroplasts is photosynthesis, root plastids are mainly involved in the assimilation of inorganic nitrogen. Amyloplasts contain starch grains and behave as storage reservoirs in stems, roots, and tubers. Chromoplasts synthesize carotenoids and are present in petals, fruits, and even roots. Interconversions between these different plastids are accompanied by dramatic changes including the development or the regression of specific enzy-

matic equipment reflecting specialized metabolism. A more general view of the envelope proteome through various plastid types within tissues having specialized functions is needed. However, one should keep in mind that extensive purification and correct fractionation of plastid other than chloroplasts is a goal difficult to achieve. Therefore, one should first develop classical biochemical approaches to achieve this goal on the best plant model for the type of plastid to be analyzed. Then bioinformatics is necessary to make the link between proteomic data and genome information to identify the protein. To date, only preliminary work has been done in this direction. Obviously, *in silico* strategies (see for instance Refs. 7 and 114) combined with comparison of gene expression databases (expressed sequence tags) may help identifying envelope proteins expressed in a given type of tissue and therefore a given type of plastid. Again, such strategies will be limited by the present stage of the genome annotation and of the programs aiming to identify organelle targeting sequences. In addition, outer envelope proteins will remain very difficult to identify, if not impossible.

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