Identification and characterization of anion channel genes in plants represent a goal for a better understanding of their central role in cell signaling, osmoregulation, nutrition, and metabolism. Though channel activities have been well characterized in plasma membrane by electrophysiology, the corresponding molecular entities are little documented. Indeed, the hydrophobic protein equipment of plant plasma membrane still remains largely unknown, though several proteomic approaches have been reported. To identify new putative transport systems, we developed a new proteomic strategy based on mass spectrometry analyses of a plasma membrane fraction enriched in hydrophobic proteins. We produced from Arabidopsis cell suspensions a highly purified plasma membrane fraction and characterized it in detail by immunological and enzymatic tests. Using complementary methods for the extraction of hydrophobic proteins and mass spectrometry analyses on mono-dimensional gels, about 100 proteins have been identified, 95% of which had never been found in previous proteomic studies. The inventory of the plasma membrane proteome generated by this approach contains numerous plasma membrane integral proteins, one-third displaying at least four transmembrane segments. The plasma membrane localization was confirmed for several proteins, therefore validating such proteomic strategy. An in silico analysis shows a correlation between the putative functions of the identified proteins and the expected roles for plasma membrane in transport, signaling, cellular traffic, and metabolism. This analysis also reveals 10 proteins that display structural properties compatible with transport functions and will constitute interesting targets for further functional studies. Molecular & Cellular Proteomics 3: 675–691, 2004.

The plasma membrane (PM) is an organized system serving as a structural and communication interface with the extracellular environment for exchanges of information and substances. In animal cells, PM proteins represent a point for potential therapeutic intervention, making the PM a source of drug targets, for instance in cancer research (1). In plant cells too, as signaling processes controlling responses to biotic and abiotic factors occur in PM, a better knowledge of the PM proteome would help developing defense strategies. Indeed, in plant cells as well as in animal cells, the PM is controlling many primary cellular functions, such as metabolite and ion transport, endocytosis, cell differentiation and proliferation, etc. All these processes involve a large array of proteins with highly diverse structure and function. In addition, the strength of their association to the membrane varies, some being well embedded in the membrane lipid core while others are more peripheral proteins, sometimes reversibly associated with the membrane. However, due to their poor solubility, only a minority of integral membrane proteins have been identified, and most of them came out from in silico analyses since the genome of the model plant Arabidopsis thaliana was completed (2). The Arabidopsis Membrane Protein Library (AMPL; www.biosci.cbs.umn.edu/Arabidopsis/) was established by clustering the predicted membrane proteins based on sequence and sorted into families of known, predicted, or unknown functions (3). In ARAMEMNON, dedicated to Arabidopsis integral membrane proteins, averaging the predictions from seven publicly available programs led to the identification of ~6,500 proteins displaying at least one transmembrane domain (TM) of the 25,500 predicted protein sequences (aranemnon.botanik.uni-koeln.de) (4). Some 1,800 of these proteins contain four TM or more and are possibly linked to transport functions. Among those, it is not possible to identify PM proteins because no signal peptide or specific signature specifying the targeting to PM have been identified so far. On

1 The abbreviations used are: PM, plasma membrane; Cyt b5, cytochrome b5; 2-D, two-dimensional; GFP, green fluorescent protein; GRAYV, grand average of hydrophathy; pl, isoelectric point; TM, transmembrane segment; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; LC, liquid chromatography; PEG, polyethylene glycol; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; C/M, chloroform/methanol; TIP, tonoplast intrinsic protein; PIP, PM intrinsic proteins; VDAC, voltage-dependent anion channel.
the basis of two-dimensional (2-D) gel electrophoresis, Masson and Rossignol (5) estimated that 500 polypeptides were present in the PM, corresponding to about 3% of total cellular proteins identified at that time. The total number of ~750 PM proteins can now be inferred from the entire Arabidopsis proteome (2).

Nowadays, the real challenge is to find the way of extracting and identifying the whole set of PM proteins, including especially the integral proteins. Several methods have been developed on various animal and plant biological systems and already allowed identification of plant integral or PM-associated proteins. First, immunoscreening of a cDNA expression library with an antiserum raised against PM proteins was used to identify genes encoding PM proteins in soybean (6) and A. thaliana (7). Then, the sequence trap technique was designed to clone, in mammalian COS cells, cDNAs encoding secreted or membrane-associated proteins (8). However, the most commonly used technique for all organisms to identify new membrane proteins has been the solubilization of proteins from membrane-enriched fractions with detergents and their separation by 2-D gel electrophoresis (9–12). Though numerous PM-specific proteins were identified by this method, most of these studies highlighted the fact that 2-D gel separation was not appropriate for a comprehensive mapping of membrane proteins. The first major problem is the chemical heterogeneity of proteins (isoelectric point (pI), molecular mass, and solubility). New 2-D PAGE procedures were developed to overcome this problem (13, 14). The second major limitation of this technique concerns protein solubility and/or hydrophobicity leading to an under-representation of the most hydrophobic proteins in 2-D gels (15, 16). Indeed, such proteins often precipitate or aggregate during the isoelectric focusing electrophoresis. The solubility of membrane proteins in 2-D gels has been improved with the use of new zwitterionic detergents during sample preparation (17), and combined with the development of mass spectrometry analysis, many new proteins were identified in PM from bacteria (18), human cells (1), and Arabidopsis plants (19). However, the majority of proteins identified by this technique remains mainly peripheral proteins (20). The last major problem is linked to the dynamics of protein expression in the cell. Low-abundance proteins, including regulatory proteins and rare membrane proteins, are out of the scope of standard proteomic techniques. One way to bring low-abundance proteins into view is to analyze subproteomes based on subcellular compartmentation (21). For instance, mammalian phagolysosome (22) and nuclear pore complex (23), PM in plant cells (9, 24) or animal cells (1), mitochondria from mammals (25) and plants (26), and different chloroplast membranes (27–29). Complexity of the membrane fractions to be analyzed can be reduced using different strategies such as chloroform/methanol extraction (30), reverse-phase chromatography (31), or blue natives gels (32).

The aim of this work was to uncover new membrane proteins from Arabidopsis cell PM and especially new transport systems and ion channels. In addition, by focusing on proteins of the most intrinsic core of the membrane, which are difficult to pick up with classical proteomic approaches, we expected to complement previous studies of membrane proteins separated by 2-D PAGE. In this article, we report mass spectrometry analyses of proteins solubilized from PM fractions from Arabidopsis cell suspensions. As an informative subcellular proteomic approach requires highly purified subfractions to be obtained, the purity of PM prepared from Arabidopsis cell suspensions was systematically assessed. Then, proteins were extracted from PM using different procedures in order to retrieve proteins within a wide range of hydrophobicity, i.e. chloroform/methanol extraction and alkaline treatment. Mass spectrometry analyses (matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF), electrospray ionization (ESI) tandem mass spectrometry (MS/MS), and nano-liquid chromatography (LC)-MS/MS) led to the identification of 102 different proteins. Database annotations and predictions indicate that more than 50% of them are integral proteins with 1–12 TM domains. Most of these proteins had never been identified before in the course of 2-D gel analysis of Arabidopsis PM (33). Moreover, PM localization of five new proteins was demonstrated by confocal microscopy. As to our objective, 34 proteins with at least four TM domains were identified, among which 20 proteins had been already characterized as transporters, and nine proteins display characteristics compatible with a transport function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and PM Purification**

A. thaliana cells were cultured in complete medium (34) under controlled conditions, continuous light, 23 °C, and 150 rotations per minute. Cells were collected after 5 days of culture, during the exponential phase. A microsomal fraction was obtained after grinding the cells and applying a series of differential centrifugations (35). A PM-enriched fraction was purified from microsomes by the two-phase partitioning between polyethyleneglycol (PEG) and dextran (6.4% w/w) (36). In this condition, the PEG upper phase is enriched in PM vesicles though the dextran lower phase, named endomembrane fraction, contains all other membranes. To eliminate contamination of the PM fraction with PEG, the PM fraction was again partitioned in a two-phases partition system consisting of 0.7 M PEG/K-PO4, pH 7 (37). In this system, PM is recovered in the saline lower phase. After an ultracentrifugation (110,000 × g), PM is recovered in the pellet. Proteins were resuspended in 50 mM 4-morpholinepropanesulfonic acid (MOPS)/NaOH, pH 7.8, 1 mM dithiothreitol (DTT). Protein amounts were estimated using Bradford procedure (38), and the proteins were concentrated at 10 mg/ml. The sensitivity of the Mg2+-ATPase activity to vanadate and KNO3 was used as a marker of PM and tonoplast, respectively (39). Cytochrome c oxidase activity was used as a marker of mitochondria (39).

**Chloroform/Methanol (C/M) Extraction**

Hydrophobic proteins were extracted from the purified PM fraction using a C/M (v/v) treatment as described by Seigneurin-Berny et al. (27). For the standard conditions, 0.2 ml (2 mg) of PM fraction was slowly diluted in 1.8 ml of cold 5/4 C/M solution. The resulting mixture was stored for 15 min on ice before centrifugation (4 °C) for 20 min at
17,600 × g. Insoluble proteins in the organic phase were recovered as a white pellet at the bottom of the tube. The organic phase, which contains the proteins soluble in C/M solutions, was removed for further protein analyses. C/M-soluble proteins were dried under nitrogen, precipitated with acetone (80%), resuspended in 20 μl of SDS-PAGE buffer (4×), and stored at −80 °C. Before SDS-PAGE separation, the proteins were solubilized at 95 °C in Laemmli buffer.

To analyze the effect of pH on the extraction efficiency, a 20 mM NaPi, pH 6 buffer was used instead of the classical 50 mM MOPS/NaOH, pH 7.8. To increase the diversity of extracted proteins, in some experiments the PM fraction was pretreated by 0.5% Triton X-100. After centrifugation (18,000 × g, 4 °C), the pellet and the supernatant were resuspended independently in the same volume and in the same buffer, leading to two subfractions, soluble and insoluble Triton (ST and IT). In that case, both ST and IT were used for C/M extraction. Denaturation at 37 °C before the SDS-PAGE was also tested, some proteins being damaged at 95 °C.

**Treatment of PM Proteins with NaOH or Nonionic Detergents**

The PM proteins (0.2 mg) were diluted in 0.2 ml of solubilization solution (50 mM MOPS/NaOH, pH 7.8, 1 mM DTT) containing either 1% (v/v) Triton X-100 or 0.1 M NaOH. After 30 min incubation on ice, the mixture was centrifuged (17,600 × g, 15 min, 4 °C) to separate two fractions: the supernatant containing proteins solubilized by the treatment and the pellet containing the insoluble proteins. The pellet proteins were then suspended in 50 μl of the initial solubilization solution (50 mM MOPS/NaOH, pH 7.8, 1 mM DTT) and both fractions were analyzed by SDS-PAGE as described above for C/M extracts.

**SDS-PAGE and Western Blot Analyses**

The different samples were separated on 10 or 12% acrylamide gels for SDS-PAGE analyses. Both gels (stacking and separation) and migration buffers contained 0.1% SDS. For some analyses, protein migration was stopped just between the stacking and the separating gels so that proteins were concentrated on a very thin band for further nanoLC-MS/MS analyses (30). Western blot analyses were performed after SDS-PAGE analysis of cell subfractions (mesosome, endomembrane and PM fractions) as described by Maniatis et al. (40), each fraction contained 20 μg of proteins. Western blot experiments were performed with markers of different membrane compartments. The anti-H^+ -ATPase (P-type) antibody is raised against the PM H^+ -ATPase of Nicotiana plumbaginifolia (used at 1/250) (kindly provided by M. Brouy). The anti-E 37 antibody is raised against a tobacco tonoplast protein (used at 1/2,000) (kindly provided by M. Boutry). The anti-E 37 antibody is raised against a protein from the inner envelope membrane of spinach chloroplast (used at 1/20,000). The anti-tonoplast intrinsic protein (TIP) antibody is directed against a tobacco tonoplast protein (used at 1/2,000) (kindly provided by C. Maurel and P. Gerbeau). The anti-Nad 9 antibody is raised against an extrinsic protein of the wheat mitochondrial inner membrane (used at 1/2,000), and the anti-TOM 40 antibody recognizes an outer membrane protein of yeast mitochondria (used at 1/2,000) (both kindly provided by J. M. Grienenberger and G. Bonnard). These antibodies were detected using alkaline phosphatase staining. The corresponding pre-immune sera were tested and gave no signal on blots.

**Mass Spectrometry and Protein Identification**

The in-gel digestion was carried out as previously described (41). Briefly, after separation by SDS-PAGE, individual bands were excised from the Coomassie blue-stained gel and washed with 50% acetonitrile and 25 μM NH₄HCO₃. Gel pieces were dried in a vacuum centrifuge and reswollen in 20 μl of 25 μM NH₄HCO₃ containing 0.5 μg of trypsin (sequencing grade; Promega, Madison, WI). After 4-h incubation at 37 °C, a 0.5-μl aliquot was removed for MALDI-TOF analysis and spotted onto the MALDI sample probe on top of a dried 0.5-μl mixture of 4 volumes solution of saturated n-cyano-4-hydroxy-trans-cinnamic acid in acetonitrile and 3 volumes of nitrocellulose (10 mg/ml) dissolved in acetone/isopropanol 1/1 (v/v). Samples were rinsed by placing a 5-μl volume of 0.1% (v/v) trifluoroacetic acid on the matrix surface after the analyte solution had dried completely. After 2 min, the liquid was blown off by pressurized air. Tryptic digests were then subjected to MALDI-MS analysis on an Autoflex instrument (Bruker, Billerica, MA) in order to obtain peptide mass fingerprints. For protein identification purposes, mass spectrometric data were searched using the MS-Fit (prospector.ucsf.edu) or the Mascot softwares (www.matrixscience.com). Searching parameters were as follows: one missed cleavage, 100 ppm mass accuracy, six peptides allowed. For hits that did not fit with the tolerated mass accuracy and the occurrence of six peptides per protein, protein identification was achieved by MS/MS analysis.

For ESI-MS/MS analyses, after in-gel tryptic digestion the gel pieces were then extracted with 5% (v/v) formic acid solution and acetonitrile. The extracts were combined with the original digest, and the sample was evaporated to dryness in vacuum centrifuge. The residues were dissolved in 0.1% (v/v) formic acid and desalted by using C18 Zip Tips (Millipore, Bedford, MA). Elution of the peptides was performed with 5–10 μl of a 50:50:0.1 (v/v) acetonitrile/H₂O/formic acid solution. The peptide solution was introduced into a glass capillary (Protana, Odense, Denmark) for nanoESI. MS/MS experiments were carried out on a quadrupole TOF (QTOF) hybrid mass spectrometer (Waters, Micromass, Manchester, United Kingdom). Interpretation of MS/MS spectra was achieved manually and with the help of the PEPSEQ program (MassLynx software; Micromass). MS/MS sequence information was used for database searching by using either BLAST (www.ncbi.nlm.nih.gov/blast) or MS pattern (prospector.ucsf.edu) programs.

For the LC-MS/MS, after the in-gel digestion 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) or a CapLC (Waters) nanoLC system and first preconcentrated on a 300 μM × 5 mm PepMap C18 precolumn. The peptides were then eluted onto a C18 column (75 μm × 150 mm). The chromatographic separation used a gradient of solution A (5% acetonitrile:95% water:0.1% formic acid) to solution B (95% acetonitrile:5% water:0.1% formic acid) over 60 min at a flow rate of 200 nl/min. The LC system was directly coupled to QTOF1 or QTOF Ultima mass spectrometer (Waters), MS and MS/MS data were acquired and processed automatically using MassLynx 3.5 software. Database searching was carried out using the MASCOT 1.7 program available via internet. The parameters for Mascot searches were as follows: two missed cleavages; 2 and 0.4 Da mass accuracy allowed for parent and the fragment ions, respectively, carbamidomethyl as fixed modification, oxidized methionine, acetylation of the peptide or for parent and the fragment ions, respectively, carbamidomethyl as fixed modification, oxidized methionine, acetylation of the peptide or for parent and the fragment ions, respectively, carbamidomethyl as fixed modification, oxidized methionine, acetylation of the peptide or...
A. thaliana database (NCBI A. thaliana specified or TAIR (ftp.arabidopsis.org/home/tair/) databases).

### Prediction Methods

Predictions for membrane-spanning regions were achieved by using the ARAMEMNON database (aramemnon.botanik.uni-koeln.de) (4). The GRAVY (grand average of hydropathicity) was obtained using the software program ProtParam tool (42). The same software program was used to analyze the other parameters (pl and molecular mass) (us.expasy.org/tools/protparam.html). The presence of conserved domain in all proteins was searched on the NCBI conserved domain database (CCD; www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). CCD currently contains domains derived from two commonly used collections, Smart and Pfam, plus contributions from colleagues at NCBI, such as COG.

### Transient Expression of Protein Fusions in Onion Epidermal Cells or in Arabidopsis Protoplasts

The AtNRAMP3 and AtNRAMP4 plasmids were kindly provided by S. Thomine. cDNAs encoding translational fusions between P31 or P24 and the green fluorescent protein (GFP), RS(35S-GFP), (43, 44), were constructed as follows. To remove the stop codon and introduce BamHI and XbaI restriction sites, P31 cDNA was amplified with the oligonucleotides Xba-P31 (5'-GGT TAG AAT GGG GAA TTA TCT CAG GCC C-3') and Bam-P31 (5'-GGA TCG AGG CAT TGT TGG CCT G-3'). P24 cDNA was amplified with the oligonucleotides Xba-P24 (5'-GGT TAG AAT GGG TTA CTG GAA TCA CAA G-3') and Bam-P24 (5'-GGA TCG TCT GGT GTG CAG CC-3'). P31 and P24 were subsequently cloned in the BamHI and XbaI sites in the plant transient expression vector CD3-327 (weedsworld.arabidopsis.org.uk/vol3ii/sol-modGFP.html) to generate a fusion with the GFP as an additional C-terminal domain, under the control of the CaMV 35S promoter. To construct a cDNA encoding a translational fusion between POR2 and the DsRed2, Cyt b5 and DsRed2 were isolated from protoplasts by PEG-mediated transformation as described in Thomine et al. (45). Briefly, Arabidopsis suspension cells were digested in Gamborg's B5 medium supplemented with 0.17 mM glucose, 0.17 mM mannitol, 1% cell wall-degrading enzyme. The protoplasts were isolated from Arabidopsis suspension cells and used as a control for the CaMV 35S promoter.

### Enzymatic characterization of the PM fraction

| Activity                                      | Value       | n |
|-----------------------------------------------|-------------|---|
| Vanadate-sensitive ATPase activity            | 3.7 ± 0.3a  | 9 |
| Nitrate-sensitive ATPase activity             | 0.9 ± 0.1a  | 9 |
| Cyt. c oxidase activity                       | 2.9 × 10^3a | 6 |
| Vanadate-sensitive ATPase activity (PM)       | 91.6%       | 7 |
| Total ATPase activity (PM)                    |             |   |

*: Ratio between the activity of the plasma membrane fraction and the activity of the microsomal fraction. Cyt. c, cytochrome c. n = number of independent experiments.

### Fluorescence Visualization

Fluorescent cells were imaged by confocal microscopy (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany) upon excitation by an argon laser at 488 nm, the fluorescence emission signal of GFP was recovered between 495 and 530 nm. Upon excitation by a helium-neon laser at 543 nm, the emission signal was recovered between 555 and 620 nm for DsRed2. In co-localization experiments, the wavelength windows for collecting the two fluorescence signals at the same time were narrowed, in order to avoid any emission of one fluorescent protein into the observation range of the other one.

### RESULTS AND DISCUSSION

**Extraction and Analysis of Hydrophilic Proteins from Arabidopsis PM**

**Protein Extraction—**PM fractions were isolated from Arabidopsis cell suspensions using the two-phase partitioning method (36). The purity of membrane preparations was estimated from enzymatic assays (Table I). The enrichment in PM was estimated at 3.5–4 times compared with the microsomal fraction. A slight decrease in the ratio of NO_3^- sensitive ATPase activities in both fractions revealed a depletion of tonoplast in the PM fraction. A major part (92%) of the total ATPase activity associated with the PM fraction was vana-date-sensitive. This suggests that the PM contamination by tonoplast and other membranes displaying ATPase activities (endoplasmic reticulum, mitochondria, chloroplasts) was at most 8%, a likely overestimation considering the relative lack of specificity of the inhibitors used in the enzymatic assays.
Cytochrome c oxidase activity, an enzymatic marker of mitochondria, was almost undetectable in the PM fraction (Table I). This set of results was completed by immunological tests using antibodies against antigens specifically associated with various membrane systems. Western blot profiles (Fig. 1) show that antigens from the vacuolar membrane (γ-TIP), mitochondrial membranes (Nad9 and TOM 40 residing in the inner and outer membranes, respectively), or chloroplast membranes (E37 from the inner envelope) are present in microsomal fractions but cannot be detected in PM fractions. The anti-H⁺-ATPase IgG preparation does recognize this P-type enzyme mostly in the PM fraction, further illustrating the enrichment detected by the enzymatic test. These data confirm the high degree of enrichment in the PM fraction and its low level of contamination by organelles and endomembranes.

A survey of the literature shows that hydrophilic proteins are routinely extracted by nonionic detergents like Triton X-100 or by alkaline treatments such as NaOH leading to the recovery, after centrifugation, of hydrophobic proteins in the pellet (11, 46). Organic solvents have been used successfully for solubilizing highly hydrophobic proteins from membranes of the chloroplast envelope (27) and thylakoids (41). Preliminary experiments showed that it was also efficient on PM proteins. Several extraction conditions were compared: various C/M ratios (3/6, 5/4, 7/2), different extraction pH (6.0, 7.8) and temperatures for protein solubilization in Laemmli buffer (37 °C, 95 °C). Besides the solubilization in organic solvents, a Triton X-100 treatment or an alkaline wash (NaOH) of the membrane samples was performed to increase the diversity of recovered proteins. Whatever the method used, proteins were then submitted to one-dimensional SDS-PAGE analyses. The polypeptide profiles of the different fractions (soluble and insoluble) obtained after C/M extraction (C/M ratio 5/4, pH 7.8, and solubilization temperature 95 °C) and NaOH treatment are shown in Figure 2, A and B, respectively. When varying the C/M extraction conditions, almost similar profiles were obtained, except a few new bands appearing in the extreme molecular masses. Triton X-100 extraction led to polypeptide profiles that were similar for soluble and insoluble fractions and was not used in further experiments (data not shown). The most hydrophobic proteins contained in the soluble C/M fractions and in the NaOH pellet were analyzed. Discrete bands were excised from the gels and digested by trypsin prior to mass spectrometry analyses using MALDI-TOF and ESI-MS/MS techniques. Moreover, to detect the low-abundance proteins, new C/M and NaOH fractions were prepared and analyzed by nanoLC-MS/MS.

**In Silico Characterization of the Hydrophobic Proteins Analyzed by Mass Spectrometry**—A total of 468 different peptides were sequenced and matched with 102 different proteins. Because we were using *Arabidopsis*, which has been completely sequenced, the analysis of a single peptide containing about 10 amino acids is usually sufficient for identifying the protein, though some sequenced peptides belong to several proteins of a same family, which might led to at most 125 identified proteins. Among the identified proteins, 59 were solubilized in the C/M mixture and 60 were recovered in the insoluble NaOH pellet, including 17 proteins that are common to both extraction conditions. Among the NaOH-insoluble phase, we identified five ribosomal proteins, showing that alkaline treatment does not totally eliminate major soluble proteins. These ribosomal proteins were considered as contaminants and were excluded from further analyses. In silico analyses of the whole set of 97 proteins were performed combining the use of several programs for physico-chemical parameters and structure predictions (see Tables II to V).
The molecular masses of the identified proteins ranged from 8 to 112 kDa, with 67% of them ranging from 20 to 40 kDa. The values of their pI are also largely scattered, between 4 and 11. By averaging the data obtained from seven different prediction programs (ARAMEMNON, Ref. 4) based on the detection of hydrophobic or amphiphilic \( \alpha \)-helices, 51% of the proteins were classified as TM candidate proteins, displaying at least one predicted TM span. Among them, 16% exhibited one to three TM, 20% showed four to six TM, and 15% had seven to 12 TM (Fig. 3A). This classification, based on the presence of putative \( \alpha \)-helices, shows its limit in the identification of membrane proteins, because the proteins of the

The following table summarizes the membrane transporters identified:

| AGI Acc. no. | kDa   | pI  | TM/M | GRAVY | AtDB annotations | Conserved domains | T | NbP | C/M | NaOH |
|--------------|-------|-----|------|-------|------------------|------------------|---|-----|-----|-------|
| ATPases      |       |     |      |       |                  |                  |   |     |     |       |
| At2g18960    | 104.2 | 6.25| 10   | 0.077 | Plasma membrane proton ATPase type 1 (PMA1) | MgtA, ZntA, KdpB, E1–E2-ATPase, Hydrolase, ZntA, KdpB, Cation-ATPase | L* | 2   | X   | X    |
| At4g30190    | 16.6  | 8.62| 4    | 0.954 | H\(^+\)-transporting ATPase 16K chain, vacuolar (ava-p1) |                  | L  | 2   |     |      |
| Transporters |       |     |      |       |                  |                  |   |     |     |       |
| At2g38940    | 58.6  | 8.35| 11   | 0.323 | Phosphate transporter (ATP2) | Sugar-tr | E* | 8   | X   | X    |
| At5g43360    | 57.3  | 9.11| 12   | 0.388 | Inorganic phosphate transporter (PHT3) | Sugar-tr, MelB, UhpC, | L  | 2   | X   |      |
| Transporters |       |     |      |       |                  |                  |   |     |     |       |
| At4g27720    | 50.9  | 6.82| 9    | 0.510 | Putative transporter |                       | L  | 1   | X   |      |
| At5g95920    | 38.3  | 5.99| 8    | 0.529 | Zinc transporter ZIP2 | Zip, Predicted divalent heavy-metal cations transporter | L  | 1   | X   |      |
| Channels     |       |     |      |       |                  |                  |   |     |     |       |
| At4g1430     | 30.7  | 9.14| 6    | 0.367 | PIP1;1 | MIP | L  | 5   | X   | X    |
| At2g59600    | 30.6  | 9.16| 6    | 0.405 | PIP1;2 | MIP | L* | 4   | X   | X    |
| At4g23400    | 30.6  | 9.02| 6    | 0.381 | PIP1;3 | MIP | L* | 4   | X   |      |
| At4g00430    | 31.6  | 9.00| 6    | 0.405 | Probable PIP1;4 | MIP | L  | 2   | X   |      |
| At3g34200    | 30.5  | 8.89| 6    | 0.401 | Probable PIP1;5 | MIP | L* | 2   | X   | X    |
| At3g17180    | 30.4  | 7.69| 6    | 0.506 | PIP2;1 | MIP | L* | 6   | X   | X    |
| At2g37170    | 29.6  | 8.82| 6    | 0.486 | PIP2;7 | MIP, GlpF, GlpF | L* | 6   | X   | X    |
| At4g35100    | 25.8  | 4.92| 7    | 0.791 | TIP1;2 (\( \gamma \)) | MIP | L  | 1   | X   |      |
| At4g26240    | 25.0  | 5.30| 6    | 0.974 | TIP2;1 (\( \delta \)) | MIP | E* | 1   | X   | X    |
| At4g18910    | 31.3  | 8.63| 6    | 0.446 | NIP2;2 (NOD26-like) | MIP | L  | 1   | X   |      |
| At5g15090    | 29.2  | 7.84| 0    | −0.183 | Putative porin (POR2) | Euk-porin | M* | 22  | X    |      |
| At3g01280    | 29.4  | 8.77| 0    | −0.114 | Putative porin (POR1) | Euk-porin | L  | 1   | X    |      |
| At4g57490    | 30.9  | 7.93| 0    | −0.166 | Putative porin | Euk-porin | L  | 1   | X    |      |

The functions and conserved domains were assumed according to the BLAST and BLAST smart domains as sources. T, mass spectrometry techniques permitting the protein identification; M, MALDI-TOF; E, ESI-MS/MS; L, LC-MS/MS. Asterisks indicate protein found at least twice in two different gels. NbP, number of different sequences from the corresponding protein. C/M, protein extracted by C/M. NaOH, Proteins identified in the membrane after NaOH 0.1 M washing.
| AGI Acc. no. | kDa   | pl   | TM/M | GRAVY | AtDB annotations | Conserved domains | T | NbP | C/M | NaOH |
|-------------|-------|------|------|-------|------------------|------------------|---|-----|-----|------|
| **Receptors** |       |      |      |       |                  |                  |   |     |     |      |
| At2g01210   | 78.3  | 5.75 | 1    | −0.118| TM protein kinase putative | Pkinase, S-TKc, TyrKc, SPS1, LRR | L | 1   | X   |      |
| At3g02880   | 67.7  | 8.60 | 2    | −0.091| TM protein kinase putative | Pkinase, S-TKc, TyrKc, SPS1, LRR | L*| 2   | X   |      |
| At4g02600   | 59.1  | 8.71 | 7    | 0.192 | AtMlo-h1-like protein | Mlo              | L | 1   | X   |      |
| **GTP binding and related proteins** |       |      |      |       |                  |                  |   |     |     |      |
| At4g02080   | 22.0  | 6.97 | 0    | −0.170| SAR1/GTP-binding secretory factor | SAR, ARF, GTPase, SAR1 | L | 1   | X   |      |
| At3g62560   | 23.0  | 5.21 | 0/pr | −0.386| GTP-binding protein RAB7D putative | RAB, RAS, SAR1, RHO, RAN, ARF | L | 1   | X   |      |
| At1g56330   | 22.6  | 5.02 | 0/pr | −0.374| GTP-binding protein ara-5 | RAS, GTPase, SAR1 | L | 2   | X   |      |
| At3g7410    | 24.3  | 4.74 | 0/pr | −0.364| GTP-binding protein putative | RAS, GTPase, SAR1 | L | 1   | X   |      |
| At5g7520    | 24.4  | 5.98 | 0/pr | −0.323| GTP-binding protein putative | RAS, GTPase, SAR1 | L | 1   | X   |      |
| At1g16920   | 24.0  | 5.59 | 0/pr | −0.341| GTP-binding protein Rab11 | RAS, GTPase, SAR1 | L | 1   | X   |      |
| At4g18800   | 30.2  | 7.74 | 5    | 0.367 | Putative protein | Yip1, Rab GTPase-interacting factor | L | 1   | X   |      |
| At5g7200    | 22.3  | 5.27 | 0/pr | −0.290| Ras-related small GTP-binding protein like | RAS, GTPase, SAR1 | L | 3   | X   |      |
| At4g17530   | 22.3  | 5.27 | 0/pr | −0.273| Ras-related small GTP-binding protein RAB1c | RAS, GTPase, SAR1 | L | 4   | X   |      |
| At3g53610   | 23.9  | 8.36 | 0/pr | −0.346| Ras-related small GTP-binding protein putative | RAS, GTPase, SAR1, RHO | L | 3   | X   |      |
| At5g59840   | 23.8  | 8.36 | 0/pr | −0.298| Putative GTP-binding protein | RAS, RAB, GTPase, SAR1 | L | 5   | X   |      |
| At3g60600   | 23.8  | 7.65 | 0/pr | −0.296| GTP-binding protein ara-3 | RAS, RAB, GTPase, SAR1 | L | 5   | X   |      |
| At1g07940   | 49.5  | 9.19 | 0    | −0.325| Elongation factor 1 α (EF-1α) | GTP-EFTU-D2, D3, ATP-binding | L*| 2   | X   |      |
| At5g60390   | 21.2  | 6.91 | 0/my | −0.182| ADP-ribosylation factor putative | ARF, SAR, GTPase, SAR1, RAS, RAB | L | 1   | X   |      |
| At1g10630   | 20.4  | 7.64 | 0/my | −0.029| ADP-ribosylation-like protein | ARF, SAR, GTPase, SAR1, RAS, RAB | L | 1   | X   |      |
| **Cellular traffic proteins** |       |      |      |       |                  |                  |   |     |     |      |
| At1g04920   | 49.5  | 4.93 | 0    | −0.195| Tubulin α2/α4 chain | Tubulin, tubulin C | L | 1   | X   |      |
| At3g97470   | 30.0  | 5.09 | 1    | −0.515| Syntaxin SYP71 | t-SNARE | L | 3   | X   |      |
| At3g11820   | 38.0  | 9.06 | 1    | −0.573| Syntaxin SYP121(At-SYR1) | SynN, t-SNARE, Syntaxin, Synaptobrevin, SNC1 | L | 1   | X   |      |
| At2g33120   | 24.9  | 9.07 | 1    | −0.122| Synaptobrevin protein (AtVAMP722) | Synaptobrevin | L | 3   | X   |      |
| At3g17440   | 30.4  | 6.86 | 1    | −0.548| Novel plant SNARE 13 (AtNPSN13) | t-SNARE | L | 2   | X   |      |
| At1g84240   | 71.7  | 6.11 | 6    | −0.095| Unknown protein | PuO, Type II secretory pathway | L | 1   | X   |      |
| **Stress induced proteins** |       |      |      |       |                  |                  |   |     |     |      |
| At3g01290   | 31.3  | 5.67 | 0/my | −0.069| Expressed protein (P31) | Band-7, HflC Membrane protease subunits, PHB | M*| 15  | X   | X    |
| At5g62740   | 31.4  | 5.29 | 0/my | −0.101| Hypersensitive-induced response protein | Band-7, HflC Membrane protease subunits, PHB | L | 7   | X   |      |
### TABLE III—continued

| AGI Acc. no. | kDa | pl  | TM/M | GRAVY | AtDB annotations | Conserved domains | T | NbP | C/M | NaOH |
|-------------|-----|-----|------|-------|------------------|------------------|---|-----|-----|------|
| At3g63080  | 19.3| 9.28| 0/my | −0.343| Prohibitin-related | Band-7, HflC Membrane protase subunits, PHB | L* | 2   | X   |      |
| At4g11600  | 18.6| 6.59| 0    | −0.253|                   |                   | L* | 7   | X   |      |
| At5g53560  | 15.0| 5.11| 1    | −0.342|                   |                   | E* | 1   | X   |      |
| At4g03280  | 24.4| 8.80| 1    | −0.087|                   |                   | E* | 5   | X   |      |
| At4g27270  | 22.3| 6.30| 1    | −0.084|                   |                   | M* | 4   | X   |      |
| At4g27890  | 21.5| 6.84| 0    | −0.028|                   |                   | M* | 4   | X   |      |
| At5g54500  | 30.4| 6.99| 1/my | −0.108|                   |                   | E* | 5   | X   |      |
| At4g93750  | 28.7| 5.17| 0    | −0.210|                   |                   | E* | 5   | X   |      |
| At1g13440  | 36.9| 6.67| 0    | −0.137|                   |                   | L* | 2   | X   |      |
| At5g10730  | 32.6| 9.34| 0    | 0.056 | Expressed protein  |                   | L  | 1   | X   |      |
| At2g02400  | 34.8| 5.82| 0    | 0.049 |                   |                   | E  | 1   | X   |      |
| At3g02600  | 40.8| 6.23| 5    | 0.079 |                   |                   | L  | 1   | X   |      |
| At1g80050  | 21.0| 5.69| 1    | 0.229 |                   |                   | L  | 1   | X   |      |
| At4g25100  | 23.8| 6.06| 0    | 0.291 |                   |                   | M* | 6   | X   |      |
| At1g63290  | 24.1| 5.72| 0    | 0.082 |                   |                   | E* | 3   | X   |      |

### TABLE IV

**Metabolism**

| AGI Acc. no. | kDa | pl  | TM/M | GRAVY | AtDB annotations | Conserved domains | T | NbP | C/M | NaOH |
|-------------|-----|-----|------|-------|------------------|------------------|---|-----|-----|------|

**Stress regulated proteins**

| AGI Acc. no. | kDa | pl  | TM/M | GRAVY | AtDB annotations | Conserved domains | T | NbP | C/M | NaOH |
|-------------|-----|-----|------|-------|------------------|------------------|---|-----|-----|------|
| At3g61260  | 23.1| 5.55| 0    | −0.765| Putative DNA binding protein | Remorin-N, remorin-C | M* | 8   | X   |      |
| At2g55820  | 21.0| 8.63| 0    | −0.814| Remorin | Remorin-N, remorin-C | L  | 1   | X   |      |
| AT4g12420  | 65.6| 9.16| 4    | −0.236| Pollen-specific protein (SKU5), predicted GPI-anchored protein | Cu-oxidase, SufI | M* | 14  | X   |      |

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**Plant Plasma Membrane Proteomic**

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porin-type, known to form a β-barrel embedded in the membrane lipid bilayer, have no α-helices and are therefore not predicted as TM proteins (Table II). Some of the proteins lacking putative α-helices can be predicted to be anchored to the membrane owing to hydrophobic tails: eight proteins have putative myristoylation sites and 10 proteins have putative prenylation sites (Table III).

In the literature, the GRAVY index is also commonly used to describe the hydrophobicity properties of proteins. The GRAVY score (42) takes into account the size and the charge of the whole protein and ranges for instance from –2 to +2 in the whole proteome predicted from the Escherichia coli genome sequence (16), positive values referring to hydrophobic proteins. The GRAVY of the Arabidopsis plasma membrane proteins analyzed ranges from –1.6 to +1 (Fig. 3B). Only 20% of the proteins display a GRAVY value higher than +0.4 and can thus be considered as highly hydrophobic, such as the glucose transporter STP1 (+0.482, Table II). The majority of the analyzed proteins has a GRAVY between +0.4 and –0.4, which could not discriminate their hydrophobic or hydrophilic nature (11, 20). For instance, on one hand, the P-

| AGI Acc. no. | kDa | pI | TM/M | GRAVY | AtDB annotations | Conserved domains | T | NbP | C/M | NaOH |
|-------------|-----|----|------|-------|------------------|------------------|---|-----|-----|------|
| At1g32050   | 30.0| 7.66| 4    | 0.233 | Secretory carrier membrane protein putative | SCAMP | L  | 1   | X   |
| At1g61250   | 32.6| 8.87| 4    | 0.185 | Putative secretory carrier membrane protein | SCAMP | L  | 1   | X   |
| At3g45600   | 31.9| 8.93| 4    | 0.246 | Putative protein senescence-associated transmembrane 4 | Transmembrane 4 | L  | 2   | X   |
| At2g23810   | 30.6| 8.86| 4    | 0.230 | Hypothetical protein senescence-associated transmembrane 4 | Transmembrane 4 | L  | 1   | X   |
| At1g73650   | 33.0| 9.14| 7    | 0.516 | Expressed protein | Predicted membrane protein | L  | 2   | X   |
| At1g18180   | 33.6| 9.22| 7    | 0.466 | Hypothetical protein | Predicted membrane protein | L  | 1   | X   |
| At3g61560   | 29.8| 6.36| 3    | -0.010 | Putative protein | Reticulon | L  | 1   | X   |
| At4g23630   | 30.5| 8.32| 3    | -0.068 | Putative protein | Reticulon | L  | 1   | X   |
| At1g75500   | 42.6| 9.16| 9    | 0.566 | Nodulin-like protein | DUF6, DUF6 RhaT | L  | 1   | X   |
| At4g15630   | 20.1| 9.90| 4    | 0.658 | Expressed protein | DUF588 | L  | 2   | X   |
| At5g24670   | 27.8| 9.60| 3    | -0.125 | Unknown protein | Tim17, lipoprotein lipid attachment site | L  | 1   | X   |
| At4g38840   | 11.1| 6.56| 0    | -0.115 | Auxin-induced protein-like | Auxin-inducible | L  | 1   | X   |
| At2g37970   | 23.7| 7.74| 0/amy| -0.407 | Expressed protein | SOUL heme-binding protein | L  | 1   | X   |
| At1g18380   | 25.7| 5.55| 2    | -0.076 | Unknown protein | | L* | 2   | X   |
| At4g20260   | 24.6| 4.99| 0    | -0.706 | Endomembrane-associated protein (P24) | | M* | 5   | X   | X   |
| At1g13930   | 16.1| 4.82| 0    | -0.883 | Unknown protein | | E  | 2   | X   |
| At2g30930   | 16.9| 4.92| 0    | -0.251 | Expressed protein | E* | 4   | X   |
| At5g11680   | 23.1| 6.35| 0    | -0.199 | Putative protein | | L  | 2   | X   |
| At5g39730   | 20.0| 5.01| 0    | -0.703 | Avirulence-induced gene (AIG) like | | L  | 1   | X   |
| At5g44610   | 18.5| 4.92| 0    | -0.886 | Expressed protein | | L  | 1   | X   |
| At1g81080   | 15.1| 11.07| 0 | -1.006 | Expressed protein | | L  | 1   | X   |
| At5g53880   | 7.8 | 9.19| 0    | -1.564 | Expressed protein | | L  | 1   | X   |
| At2g1420    | 10.7| 4.31| 0    | -0.946 | Unknown protein, proline-rich protein family | | L  | 1   | X   |
| At3g22231   | 8.5 | 4.39| 0    | -0.130 | Expressed protein | | L  | 1   | X   |
| At5g12010   | 56.8| 5.68| 0    | -0.504 | Putative protein | | L  | 1   | X   |

**Fig. 3.** Classification of PM proteins according to physicochemical properties. A, classification according to the number of TM segments. TM were predicted using ARAEMMON database accessible at aramemnon.botanik.uni-koeln.de (4). B, protein distribution according to the GRAVY indexes (42). GRAVY values were automatically calculated using the ProtParam tool on the ExPasy server at expasy.hcuge.ch/sprot/protparam.html.
On the other hand, the D-ribulose-5-phosphate 3-epimerase, without predicted TM domain, has a GRAVY of only -0.082 (Table IV). To better define subclasses in the population, the GRAVY index was plotted versus pI (Fig. 4). Basically, three groups of proteins can be distinguished, those with relatively low pI (between 4 and 7) and mainly negative GRAVY (class I), those with high pI (from 7 to 10) and negative GRAVY (class II), and the third class (class III) displaying high pI (pI > 8.8) and high hydrophobicity expressed as the ratio of the number of amino acid residues per number of predicted TM (Res/TM < 100). Our analysis of the PM hydrophobic proteome did not reveal any particular characteristic trait specifying PM proteins, as pI values were spread over 7 pH units and Res/TM ratios were often higher than 100. The difference between these two hydrophobic proteomes might be related to the lipid composition of these membranes, as well as to the structure of PM proteins, which often exhibit longer extracellular and cytosolic loops and longer N- and C-terminal extensions when compared with envelope proteins. In other respects, none of the different parameters used in the present study (number of TM, GRAVY index) was sufficient on its own to characterize the hydrophobicity of the PM proteins. It thus appeared necessary to validate experimentally their association with the PM.

**Confirmation of the PM Localization of Newly Identified PM Proteins**

The quality of the purified PM fraction, as assessed by enzymatic and immunological assays, was the first criterion for establishing the PM localization of the proteins identified in the course of this study. Indeed, mass spectrometry analyses.
did not reveal the presence of major proteins from other organelles, such as the phosphate/triose-phosphate translocator or the RuBisCo for the chloroplasts, or of elements of the NADH dehydrogenase, the HSP60 complex, the F0F1-ATP synthase, or the cytochrome c reductase, the four dominant protein complexes of mitochondria. Moreover, several proteins identified (H+\text{-}ATPase, PM intrinsic proteins (PIPs)) were already well-known PM proteins. Other proteins, already described as PM resident proteins but in other plants, are present in the \textit{Arabidopsis} PM fraction, thus strengthening their PM localization. This is the case for remorins, close to potato remorins (53), the phosphate transporters AtPT1 and AtPT2 homologous to LePT1 identified in tomato (54), AtSYR1 similar to the tobacco syntaxin Nt-Syr1 (55), and the metal transporter ZIP2 (56).

Despite these observations, it remains an uncertainty in the subcellular localization of previously unknown gene products. Moreover, some gene products (such as porins, TIP, or Rieske protein) were previously described in membrane compartments other than PM. Even if no major proteins from other organelles were identified in the PM fraction, it might contain minute amounts of contaminating proteins from other cell compartments, and some proteins may have a dual localization. Therefore, we decided to investigate the subcellular compartmentation of several of the identified proteins with uncertain localization to validate our PM proteomic strategy. The first chosen proteins were unknown abundant proteins, identified from the first MALDI-TOF analyses, exhibiting negative GRAVY values and belonging to class I: a protein with prohibitin motives (P31; Table III), a protein previously described as endomembrane-associated (P24; Table V), and Cyt \textit{b5} (Table IV), which is often described in internal membrane compartments (57, 58). Transient expression of fusion proteins with fluorescent protein reporters, such as GFP or DsRed2, was performed in protoplasts isolated from \textit{Arabidopsis} cell suspensions and followed by confocal microscopy observations (Fig. 5). Green fluorescence associated with the GFP marker clearly delineated the PM upon expression of p24::GFP or p31::GFP fusions. The Cyt\textit{b5}::DsRed2 fusion was co-expressed with GFP fused to AtNRAMP3, a metal transporter targeted to the vacuolar membrane (45). Red and green fluorescence signals appear very close to each other, but a close-up view of the nucleus area reveals distinct green labeling of the tonoplast and red labeling of the PM, thus confirming the PM localization of Cyt \textit{b5} (Fig. 5). The expression of another GFP fusion with a protein from the quinone reductase family (P22; Table IV) led to a strong labeling of the PM together with a fluorescence background in the cytosol (data not shown). In other respects, a porin-like protein (POR2; Table II) was identified in the PM fraction, though members of this family are commonly described as mitochondrial components (59, 60). In our classification, POR2 belongs to class II with regards to its properties, no predicted TM, negative GRAVY, and high pI. As the overexpression of POR2::GFP was lethal for \textit{Arabidopsis} protoplasts, onion epidermal cells were used as an alternative transient expression system. Co-expression of a POR2::DsRed2 fusion with a tonoplast metal transporter, AtNRAMP4::GFP (S. Thomine, personal communication), was performed (Fig. 6). The merge of red and green fluorescence signals clearly showed a PM localization for the POR2 protein in reference to the tonoplast targeting of the AtNRAMP4 protein. PM localization could thus be demonstrated by confocal microscopy for the five proteins chosen for further investigation, even when \textit{in silico} analyses did not predict a TM structure. A significant proportion of the proteins identified in the hydrophobic PM fraction are in fact peripheral proteins, most likely because they are more abundant than integral proteins, as previously sug-

![PM targeting of P24, P31 and Cyt b5 in Arabidopsis protoplasts.](image)
The Rieske protein (At4g03280) (31, 67, 68), has been found to be a component of the chloroplastic cytochrome complex, although its role is still under investigation. Robinson et al. (66) also observed an im-munological labeling of TIP proteins associated with the PM. The latter hypothesis, Robinson et al. (66) also observed an immunological labeling of TIP proteins associated with the PM. Concerning putative protein contaminants of the PM fraction, members from several protein families usually described as residing in other membrane compartments have been identified in the hydrophobic PM proteome. Proteins such as the V-ATPase and the porins are classically accepted to reside in the tonoplast (61) and in the mitochondrial membrane (60), respectively. However, they were found here in the PM fraction. It is surprising to find this protein in the present PM proteome because no abundant chloroplastic protein was present.

Functional Survey of the Analyzed PM Proteins

Among the 100 proteins identified in the course of this study, only 23% were present in protein databases. All others were only previously described at the level of either cDNA (67%) or even only from genomic sequence (10%). Sequence analyses using programs for domain predictions were used to classify the protein population. The percentage of known proteins (or paralogues of known proteins) is 49%, whereas putative (proteins with a low homology with known proteins) and unknown proteins represent 19 and 32%, respectively.

Based on sequence homologies with known proteins and on identified functional domains, putative functional classes could be assigned for ~75% of the proteins. The whole set of proteins can be divided mainly in three large functional groups (Tables II–IV). In each group, several proteins share common putative function or at least functional domains, thus defining families. The unknown proteins are grouped in a fourth class (Table V).

Membrane Transporters (Table II)—Several transporters, pumps, and channels were identified in the hydrophobic PM fraction. Among them, it is not surprising to find the P-type H\(^+\)-ATPase (PMA1 and/or PMA2 forms), the most representative integral protein of the PM (49). In contrast, we identified the two subunits 16K and G of the V-type H\(^+\)-ATPase in the PM fraction, although they have been described as associated with the tonoplast (61). Such a surprising localization is in fact supported by previous observations: Rouquié et al. (69) identified the G subunit in tobacco PM, whereas Robinson et al. (66) identified by immuno-localization studies the 16K subunit in pea PM, in agreement with the localization observed in many other eukaryotic cells (62). In addition to such components of the proton pump, the PM fraction contained the phosphate transporters AtPT1 and AtPT2 homologous to LePT1 identified in tomato. LePT1 was previously shown to be localized in PM by Western blot experiments (54). Furthermore, glucose transporters of the STP family, STP1 and STP4, are known to function as monosaccharide/H\(^+\) symporters through the PM (47, 70). Very recently, MtZIP2, the orthologue of the metal transporter AtZIP2, has been localized in PM (56). Interestingly, the family of PIPs is well represented in the population of hydrophobic proteins extracted from PM as at least eight different PIPs are present. The physiological role of PIPs is well documented (71, 72), and evidence for their localization in the PM were brought for PIP2;1 (51), PIP2;3 (73), and more recently for several isoforms of PIP1 and PIP2 (24). Our study provides further evidence for the plasma membrane association of a large number of PIP proteins. The presence of two TIPs in the population, δ-TIP found in each analyzed fraction and γ-TIP found only once, could result from...
the extraction of small specialized vacuoles together with PM vesicles, to which they are associated, storage vacuoles in the case of δ-TIP and lytic vacuoles for γ-TIP (65).

Porins from the voltage-dependent anion channel (VDAC) family have also been identified in the PM hydrophobic fraction. These proteins are peculiar as they show no predicted TM domain, though they are integral membrane proteins characterized by the presence of 12 β-sheets forming a TM β-barrel structure within the membrane. VDAC proteins have been found in most of the organisms and are often described as membrane proteins forming a pore in the outer membrane of mitochondria and playing an essential role in apoptosis (59, 60). However, several studies based on electrophysiology, immunological techniques (74, 75), and, more recently, on the use of chimeric proteins VDAC::GFP in mammalian cells (64, 76, 77) demonstrated the presence of VDAC proteins at the cell surface in the PM. By confocal microscopy experiments, we confirm here for the first time that one VDAC, POR2, identified by MS in the PM fraction, is indeed targeted to the PM in plant cells (Fig. 6).

**Signaling and Cellular Traffic Components (Table III)**—We have identified three TM receptors, among which are two protein kinases, sharing 35% identity at the protein level and belonging to the large family of the leucine-rich repeats (LRR) receptor kinases. These proteins were described only as mRNA in databases, and this work provides evidence for the existence of the two proteins and their presence at the PM. A Mlo-h1-like protein, another type of receptor characterized by seven TM domains, is present in the PM fraction. Mlo-h1 is a genetic locus for resistance to mildew in barley (78), and the protein was identified by Western blot in PM vesicles from *A. thaliana* (79).

The great majority of proteins in Table III belongs to the superfAMILY of small GTP-binding proteins and related proteins (ADP-ribosylation factors), which play key roles in signaling pathways, and in vesicular traffic and targeting to the PM (80). This classification is based on the identification in the sequences of conserved domains like RAB, RAS, ARF, or SAR domains. Membrane anchoring of these proteins is a prerequisite to their activity and/or their interaction with effector proteins. Their association to membranes is mediated by post-translational modifications such as prenylation, palmitoylation, or myristoylation (81, 82). All the GTP-binding proteins found here show a putative site of prenylation in their C-terminus, and the two ADP-ribosylation factors are predicted to be myristoylated (83, 84). Their presence in the PM is well documented; it relies on immunological evidence (85, 86) and, more recently, on subcellular localization studies using GFP fusions (87, 88). Several other proteins, displaying a t-SNARE domain, are also associated with the PM fraction as expected from the role of that membrane as a target for vesicular traffic. Moreover, the tobacco syntaxin Nt-Sy1, highly similar to the *Arabidopsis* SYP121 (72% identity), has been localized by immunogold labeling in PM (55).

Members from the PID (proliferation, ion, and death) superfamily, as defined by Nadimpalli *et al.* (89) in a study performed on maize, are also present in our hydrophobic fraction. They include two prohibitins usually involved in cell proliferation and three HIR (hypersensitive-induced reaction) proteins, associated with HR (hypersensitive response), cell death, and resistance to pathogens. These proteins are described in the literature as containing a short TM helix in their N-terminal region (90), and, in animal cells, Terashima *et al.* (91) have already shown the PM localization of two prohibitins from B lymphocytes. One of the *Arabidopsis* HIR proteins identified here is predicted to possess one TM using the ARAMEMNON database, and the four others have a putative site of myristoylation. One of them (At5g62740), corresponding to P31, has been localized during the present work in the PM by expression of a GFP fusion. Other proteins involved in defense responses, remorins, are recovered in the PM fraction, in good agreement with the PM localization of potato remorin reported by Raymond *et al.* (53). Finally, we also found SKU5 in the group of signaling proteins. This PM protein acts on cell wall components in a signaling pathway affecting directed cell expansion (50).

**Metabolism (Table IV)**—Most of the enzymatic proteins found in the hydrophobic PM fraction are biotic and abiotic stress-regulated enzymes. They are responsible for electron transfer to a large variety of membrane-associated substrates, leading to membrane antioxidant protection. This is the case for phospholipid hyperoxide glutathione peroxidases (PHGPX), Cyt b5 protein, and FMN-reductases. PHGPX proteins are membrane-bound as previously shown in rat (92), and the plastid targeting of PHGPX isoforms indicates the existence of membrane-associated activity in plants too (93). The Cyt b5 protein has been described in microsomal membranes from developing safflower cotyledons where it functions as an intermediate electron donor in fatty acid desaturation (94). The Cyt b5 isoform found in this study, among at least six different isoforms encoded by the *Arabidopsis* genome, was localized in the PM (this work), whereas the other isoforms are found in different cell compartments (57, 58). In agreement with our data, FMN-reductases, known to participate in cell detoxication during oxidative stress, have been shown to be strongly bound to the PM (95, 96).

Cinnamoyl-CoA reductase (CCR), displaying a 3β-HSD domain, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are also stress-regulated proteins. In particular, CCR genes are differentially expressed in response to infection by pathogenic bacteria (97). These proteins have no predicted TM domains. However, a PM localization, at least transient, would be in agreement with their physiological role. The GAPDH, already identified in a PM proteome of *Arabidopsis* (9), is known, in mammal cells to have a NO-dependent membrane association catalyzing membrane fusion (98, 99).

Superoxide dismutase (SOD) and one enzyme from the pentose phosphate pathway, α-ribulose 5-phosphate 3-epi-
Phosphoribosyltransferases (APRT) and phosphatidate phosphatases (LPP) are both involved in second messenger synthesis, but their localization is still controversial. Investigating the respective contributions of three APRT isoforms of Arabidopsis to adenine and cytokinin metabolism, Allen et al. (102) provided evidence for the cytosolic localization of APRT1 and APRT3 proteins but could not conclude on the localization of APRT2. We propose that the isoform we identified, APRT2, might be PM-associated. The LPP (PAP2) identified here corresponds to AtLPP3p (103), thus suggesting the presence of this protein in the PM. This is in agreement with the localization attributed to the animal phosphatidate phosphohydrolase PAP type 2 from porcine thymus (104) or LPP1/1a from rat lung (105).

Unknown Proteins (Table V)—Database analyses reveal that more than 30% of the proteins identified in this work are considered as unknown proteins, which have no match with proteins of already known functions in other organisms. About 5%, though unknown, possess functional domains allowing their classification in the categories described above. This is the case for “expressed proteins” displaying t-SNARE domains and likely participating in the secretory pathway (Table III). The 25% unknown proteins left are gathered in Table V. Among these, P24, initially referred to as EMAP (endomembrane-associated protein), is the only gene product that had been previously identified as a protein and localized at the PM (9), like its tobacco orthologue (106). Moreover, its localization to the PM was further confirmed by our confocal microscopy experiments with EMAP::GFP fusion protein (Fig. 5).

Proteins belonging to four families identified on the basis of the presence of conserved domains (“SCAMP,” “transmembrane 4,” “predicted membrane protein,” and “reticulon”) were present in our fraction. This suggests that these families might be specific for the PM, although their functions still remain unknown.

CONCLUDING REMARKS

In this article, we report an inventory of the hydrophobic proteins of a highly purified PM fraction isolated from Arabidopsis cell suspensions. Using complementary methods for the extraction of hydrophobic proteins and mass spectrometry analyses, about 100 proteins could be identified; 95% of them represent newly identified PM proteins.

The proteins of the hydrophobic PM proteome have been grouped in three main functional classes: transport systems, proteins involved in signaling and cellular traffic, and different enzymes from metabolism (Fig. 7). Like for chloroplast envelope proteins (30), the PM proteome reveals a correlation between the putative functions of the identified proteins and the expected roles for the PM. Part of the proteins identified as putative PM proteins have no TM, but are anchored, likely transiently, to the PM through myristoylation or prenylation (GTP-binding proteins) or other uncharacterized process (P24, for instance). The presence of peripheral proteins, already localized in PM by Santoni et al. (9), is in agreement with one of the main PM functions, cellular signaling. It is noteworthy that the hydrophobic PM proteome contains a large part of stress-regulated proteins, illustrating the role of PM as a barrier to environment. The high number of unknown proteins reflects the poor knowledge of the PM due to difficulties in extracting and separating hydrophobic proteins for mass spectrometry identification. Although the inventory remains incomplete, this proteomic approach leads to the identification of a significant number of new PM proteins and brings new insights for developing functional studies on plant PM.

Our objective was aiming at the identification of new transport systems and ion channels. Indeed, our study allowed us to identify, for the first time, numerous TM proteins, 34 of them containing at least four TM. Among them, nine proteins are good candidates as new putative transporters of PM on the basis of their physico-chemical properties (Tables II and V). In addition, we have also localized an anion channel, POR2, at the PM. In this particular case, we confirmed our proteomic analyses by confocal microscopy analysis of the subcellular localization of POR2::DsRed2 fusion proteins. This is the first time that a channel belonging to the large VDAC family is identified in the PM of plant cells, in agreement with previous reports in mammalian cells (76). The expression of the gene encoding this protein was shown to be induced during the HR triggered by the infection of Arabidopsis cells by pathogenic bacteria (107), pointing to a role of PM in the control of cell death mechanisms. These new putative transport systems are now available for functional studies combining several complementary approaches like electrophysiol-
ology, yeast mutant complementation, subcellular localization, and plant mutant characterization.

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[5] The on-line version of this manuscript (available at http://www.mcponline.org) contains supplemental material.

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