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► **To cite this version:**

Johanne Morel, Stéphane Claverol, Sébastien Mongrand, Fabienne Furt, Jérôme Fromentin, et al..
Proteomics of plant detergent resistant membranes. *Molecular and Cellular Proteomics*, 2006, 5 (8),
pp.1396-1411. 10.1074/mcp.M600044-MCP200 . hal-00068719

HAL Id: hal-00068719

<https://hal.science/hal-00068719v1>

Submitted on 10 Dec 2024

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Proteomics of Plant Detergent-resistant Membranes*[§]

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A large body of evidence from the past decade supports the existence, in membrane from animal and yeast cells, of functional microdomains that play important roles in protein sorting, signal transduction, or infection by pathogens. Recent reports demonstrated the presence, in plants, of detergent-resistant fractions isolated from plasma membrane. Analysis of the lipidic composition of this fraction revealed its enrichment in sphingolipids and sterols and depletion in phospho- and glycerolipids as previously observed for animal microdomains. One-dimensional gel electrophoresis experiments indicated that these detergent-resistant fractions are able to recruit a specific set of plasma membrane proteins and exclude others. In the present study, we used mass spectrometry to give an extensive description of a tobacco plasma membrane fraction resistant to solubilization with Triton X-100. This led to the identification of 145 proteins whose functional and physicochemical characteristics were analyzed *in silico*. Parameters such as isoelectric point, molecular weight, number and length of transmembrane segments, or global hydrophobicity were analyzed and compared with the data available concerning plant plasma membrane proteins. Post-translational modifications, such as myristoylation, palmitoylation, or presence of a glycosylphosphatidylinositol anchor, were examined in relation to the presence of the corresponding proteins in these microdomains. From a functional point of view, this analysis indicated that if a primary function of the plasma membrane, such as transport, seems under-represented in the detergent-resistant fraction, others undergo a significant increase of their relative importance. Among these are signaling and response to biotic and abiotic stress, cellular trafficking, and cell wall metabolism. This suggests that these domains are likely to constitute, as in animal cells, signaling platforms involved in these physiological functions. *Molecular & Cellular Proteomics* 5:1396–1411, 2006.

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Received, February 3, 2006, and in revised form, March 31, 2006
Published, MCP Papers in Press, April 28, 2006, DOI 10.1074/mcp.M600044-MCP200

The plasma membrane of eukaryotes delineates the interface between the cell and the environment. It thus plays a crucial role in many essential functions such as cell nutrition (involving transport of solutes in and out of the cell), endocytosis, or response to environmental modifications (including defense against pathogens). Although this renders the elucidation of the proteic composition of this membrane a hot topic for a better understanding of these cellular processes, only a minority of integral membrane proteins have been experimentally identified in plants. Indeed membrane proteins are widely known as an unsuitable material for classical proteomic analysis using two-dimensional gel electrophoresis separation followed by the identification of each spot by mass spectrometry: many hydrophobic proteins are not solubilized in the isoelectric focusing sample buffer and precipitate at their isoelectric point (1). Previous two-dimensional gel electrophoresis of tobacco leaf plasma membrane led to an estimation of 500 polypeptides (2) but with a clearly established under-representation of intrinsic membrane proteins. Two more recent studies performed on *Arabidopsis thaliana* plasma membrane using mass spectrometry directly after SDS-PAGE separation identified, respectively, 97 hydrophobic plasma membrane proteins (3) and 238 putative plasma membrane proteins (4). An *in silico* analysis deduced from the genome of the model plant *A. thaliana* has been undertaken to identify putative membrane proteins (5). However, the diversity of the types of proteins associated to the plasma membrane (inferred from studies already performed) and the lack of signal peptide or specific signature indicating the targeting to this plasma membrane make the results very uncertain.

A new aspect of the plasma membrane organization has arisen from biophysical and biochemical studies performed with animal cells for several years. Evidence has been given that the various types of lipids forming this membrane are not uniformly distributed inside the bilayer but rather spatially organized (6). This leads in particular to the formation of specialized phase domains, also called lipid rafts (7, 8). These domains, enriched in sterols and sphingolipids, form a liquid ordered phase inside the membrane. This structural characteristic renders these domains resistant to solubilization by non-ionic detergents, and this property has been widely used to isolate lipid rafts for further analysis (6). The most important hypothesis to explain the function of these domains is that they provide for lateral compartmentalization of membrane proteins and thereby create a dynamic scaffold to organize certain cellular processes. This ability to temporally and spatially organize protein complexes, while excluding others, conceivably allows for synchronization efficiency and speci-

ficity of cellular responses (9). In yeasts and animal cells, the association of particular proteins with these specialized microdomains has emerged as an important regulator of crucial physiological processes such as signal transduction, polarized secretion, cytoskeletal organization, generation of cell polarity, and entry of infectious organisms in living cells (10).

In plants, the investigations about the presence of such microdomains is very recent and limited to a small number of publications (for a review, see Ref. 11). A few years ago, Peskan *et al.* (12) reported for the first time the isolation of Triton X-100-insoluble fractions from tobacco plasma membrane. Mongrand *et al.* (13) provided a detailed analysis of the lipidic composition of such a detergent-resistant fraction indicating that it is highly enriched in a particular species of sphingolipid (glycosylceramide) and in several phytosterols (stigmasterol, sitosterol, 24-methylcholesterol, and cholesterol) compared with the whole plasma membrane from which it originates. Similar results were then obtained with detergent-resistant membranes prepared from *A. thaliana* callus membranes (14). So the presence in plant plasma membrane of domains that share with animal rafts a particular lipidic composition now seems established. The above cited groups have also shown, using one- and two-dimensional gel electrophoresis, that the global proteic composition of microdomains seems quite different from that of the whole plasma membrane. However, the question of the physiological processes involving these domains is still wide open. To investigate in that direction, an inventory of the proteins associated to these plant detergent-resistant domains seems essential. Preliminary studies have indicated the presence in these domains of typical plant plasma membrane proteins such as different isoforms of H⁺-ATPase and the water channels, aquaporins. They also suggested a possible enrichment in signaling components such as leucine-rich repeat receptor kinases or small GTP-binding proteins. The limits of these few tentative studies (summarized in Ref. 11) are that each of them was incomplete and that they were performed on various plant materials (detergent-resistant membranes extracted from plasma membranes of tobacco leaves, mustard cotyledons, or *Arabidopsis* cotyledons or from microsomal membranes of *Arabidopsis* callus).

The purpose of the present work is to give, on a unique material, an extensive description of the proteic composition of plant detergent-resistant membranes (DRMs).¹ Tobacco Bright Yellow 2 (BY-2) suspension cells were chosen for several reasons related either to the type of material or to the plant species. First, the plasma membrane is probably the most diverse membrane of the cell with a proteic composition

that varies with cell type, developmental stage, and environment. In this respect, using an undifferentiated cell suspension as a starting material for such proteomic analyses should be an advantage. Moreover the cell suspension is an appropriate starting material to perform subcellular fractionation and get significant amounts of material (*i.e.* plasma membranes and DRMs) compatible with biochemical studies. Concerning the particular choice of the BY-2 cell suspension, lipid content and ultrastructure of BY-2 cell plasma membrane DRMs have been thoroughly characterized previously (13). Furthermore tobacco has long been an essential model plant for physiological studies and particularly plant defense, and BY-2 cells represent a widely used material for both signal transduction and *in vivo* imaging studies. Such approaches will constitute further development of work that should lead to future investigations concerning the role of these microdomains in plant physiology.

In this study, we first prepared a highly purified plasma membrane fraction, which constitutes a first step of subcellular fractionation. This plasma membrane was further submitted to a treatment by the non-ionic detergent Triton X-100 to isolate the "detergent-resistant membranes"; this should be considered as a further step of subfractionation. As discussed above, membrane proteins are poorly soluble in the detergents used for two-dimensional (2D) electrophoresis; this limitation is all the more marked for proteins selected on the basis of their insolubility in non-ionic detergent, the criteria for isolation of DRMs. We therefore developed a buffer consisting of both ionic/non-ionic detergents and chaotropic agents to thoroughly solubilize DRMs. The subsequent problem was to detect low abundance proteins inside this complex mixture. To this respect, we tested three different methods for protein identification: namely the classic SDS-PAGE followed by one-capillary HPLC-MS/MS, 2D LC-MS/MS, and gas phase fractionation (GPF) MS/MS. This led to the identification, in BY-2 cell plasma membrane DRMs, of 145 proteins whose functional and physicochemical characteristics are further discussed.

EXPERIMENTAL PROCEDURES

Materials—BY-2 cells (*Nicotiana tabacum* cv. Bright Yellow 2) were grown in Murashige and Skoog medium, pH 5.6, containing Murashige and Skoog salt (15), 1 mg/liter thiamine HCl, 0.2 mg/liter 2,4-dichlorophenylacetic acid, 100 mg/liter *myo*-inositol, 30 g/liter sucrose, 200 mg/liter KH₂PO₄, and 2 g/liter MES. Cells were maintained by weekly dilution (2:80) into fresh medium.

Preparation and Purity of Tobacco Plasma Membrane—All steps were performed at 4 °C. Cells were collected by filtration, frozen in liquid N₂, and homogenized with a Waring Blendor in grinding medium (50 mM Tris-MES, pH 8.0, 500 mM sucrose, 20 mM EDTA, 10 mM DTT, and 1 mM PMSF). The homogenate was centrifuged at 16,000 × *g* for 20 min. After centrifugation, supernatants were collected, filtered through two successive screens (63 and 38 μm), and centrifuged at 96,000 × *g* for 35 min. This microsomal fraction was purified by partitioning in an aqueous two-phase system (polyethylene glycol 3350/dextran T-500, 6.6% each) to obtain the plasma membrane fraction (16). Marker activities used to evaluate the contamination of the plasma membrane fraction were as follows: azide-sensitive ATPase activity at pH 9 for mitochondria, nitrate-sensitive ATPase

¹ The abbreviations used are: DRM, detergent-resistant membrane; PM, plasma membrane; BY-2, Bright Yellow 2; ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; GPI-AP, glycosylphosphatidylinositol-anchored protein; PIP, plasma membrane intrinsic protein; 2D, two-dimensional; GPF, gas phase fractionation; SCX, strong cation exchange; TM, transmembrane; ABC, ATP-binding cassette; RLK, receptor-like kinase; LRR, leucine-rich repeat; NSF, *N*-ethylmaleimide-sensitive factor; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; SYP, syntaxin of plants.

activity at pH 6 for tonoplast, antimycin-insensitive NADH cytochrome c reductase for endoplasmic reticulum, and analysis of lipid monogalactosyldiacylglycerol contents for chloroplasts.

Isolation of Detergent-resistant Membranes—Plasma membranes were resuspended in a buffer A containing 50 mM Tris-HCl, pH 7.4, 3 mM EDTA, and 1 mM 1,4-dithiothreitol and treated with 1% Triton X-100 (w/v) for 30 min on ice with very gentle shaking every 10 min. Solubilized membranes were placed at the bottom of a centrifuge tube and mixed with 60% sucrose (w/w) to reach a final concentration of 48% (w/w) and overlaid with a discontinuous sucrose gradient (40, 35, 30, and 20%, w/w). After centrifugation for 20 h at $100,000 \times g$, a ring of Triton X-100-insoluble membranes was recovered at the 30–35% interface, diluted in buffer A, and centrifuged for 4 h at $100,000 \times g$. The pellet was resuspended in buffer A, and protein concentrations were determined using the Bradford reagent with BSA as the standard.

Protein Separation by SDS-PAGE—DRM proteins were solubilized in a buffer consisting of 6 M urea, 2.2 M thiourea, 5 mM EDTA, 0.1% SDS, 2% *N*-octyl glucoside, and 50 mM Tris-HCl. Samples were first incubated at room temperature for 15 min and then in a sonic bath for another 15 min. After a centrifugation at $16,000 \times g$ for 15 min, no pellet was observed. An aliquot of solubilized proteins (5–10 μ g) was added to Laemmli buffer and boiled for 2 min before being deposited on an 8% acrylamide gel and separated by SDS-PAGE. Proteins were visualized by Coomassie Blue staining. Each lane was systematically cut into 15 bands of equal volume for MS/MS protein identification. For GPF and 2D LC experiments, SDS-PAGE migration was stopped as soon as proteins entered the separation gel. A unique band was then cut from the gel and submitted to further trypsin digestion.

Protein Digestion—Each band was cut into 1×1 -mm cubes, and gel pieces were incubated in 25 mM ammonium bicarbonate and 50% ACN until destained. Spots were subsequently rinsed twice in ultra-pure water and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature, covered with the trypsin solution (10 ng/ μ l in 50 mM NH_4HCO_3), rehydrated at 4 °C for 10 min, and finally incubated overnight at 37 °C. Spots were then incubated for 15 min in 50 mM NH_4HCO_3 at room temperature with rotary shaking. The supernatant was collected, and an $\text{H}_2\text{O}/\text{ACN}/\text{HCOOH}$ (47.5:47.5:5) extraction solution was added onto gel slices for 15 min. The extraction step was repeated twice. Supernatants were pooled and concentrated in a vacuum centrifuge to a final volume of 25 μ l. Digests were finally acidified by addition of 1.5 μ l of acetic acid (5%, v/v) and stored at -20 °C.

On-line Capillary HPLC Nanospray Ion Trap MS/MS Analyses—Peptide mixture was analyzed by on-line nano HPLC (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LCQ Deca XP Plus ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Ten microliters of peptide digests were loaded in solvent A (0.1% formic acid in 5% ACN) onto a 300- μ m-inner diameter \times 5-mm C_{18} PepMap™ trap column (LC Packings) at a flow rate of 30 μ l/min. The peptides were eluted from the trap column onto an analytical 75- μ m-inner diameter \times 15-cm C_{18} PepMap column (LC Packings) with a 5–65% linear gradient of solvent B (0.1% formic acid in 80% ACN) in 30 min. The separation flow rate was set at 200 nl/min. The mass spectrometer operated in positive ion mode at a 2-kV needle voltage and a 38-V capillary voltage. Data were acquired in a data-dependent mode alternating an MS scan survey over the range m/z 300–2000 and three MS/MS scans in an exclusion dynamic mode. MS/MS spectra were acquired using a 2- m/z unit ion isolation window, a 35% relative collision energy, and a 0.5-min dynamic exclusion duration.

For GPF_{*m/z*} experiments, 12 sequential nano-LC-MS/MS analyses with slower gradient (5–65% of solvent B in 60 min) were registered with the ion trap mass spectrometer scanning over the following ranges: m/z 300–440, 440–570, 570–670, 670–760, 760–850, 850–

930, 930–1010, 1010–1100, 1100–1200, 1200–1340, 1340–1600, and 1600–2000. Ranges were chosen so that a similar number of MS/MS experiments were performed for each injection.

For 2D LC-MS/MS experiments, 20 μ l of DRM sample were loaded onto a 500- μ m-inner diameter \times 5-mm BioX-SCX trap column (LC Packings) at a flow rate of 30 μ l/min. Peptides were transferred from the cation exchange column onto the 300- μ m-inner diameter \times 5-mm C_{18} PepMap desalting cartridge and separated onto the 75- μ m-inner diameter \times 15-cm C_{18} PepMap column using a 5–55% linear gradient of solvent B in 100 min. Bound peptides were displaced from the SCX column onto the C_{18} cartridge by successive injections of increasing concentrations of ammonium acetate (*i.e.* 0 mM, 5 mM, 10 mM, 20 mM, 50 mM, 0.1 M, 0.2 M, 0.5 M, 1 M, and 2 M) in 0.1% formic acid. A set of 11 LC-MS/MS analyses was then collected.

Database Search—Data were searched by SEQUEST through Bioworks 3.1 interface (ThermoFinnigan) against a subset of the UniProt database (download June 3, 2005) consisting of plant species sequences (203,309 entries). DTA files were generated for MS/MS spectra that both reached a minimal intensity ($5 \cdot 10^4$) and a sufficient number of ions (>35). The DTA generation authorized the averaging of several MS/MS spectra corresponding to the same precursor ion with a tolerance of 1.4 Da. Spectra from peptides higher than 3500 Da or lower than 500 Da were rejected. The search parameters were as follows: mass accuracy of the monoisotopic peptide precursor and peptide fragments was set to 1.5 and 0.5 Da, respectively. Only b- and y-ions were considered for mass calculation. Oxidation of methionines (+16) was considered as differential modifications. Two missed trypsin cleavages were allowed. The results of the individual analyses (15 bands for SDS-PAGE, 12 m/z ranges for GPF, or 11 steps for 2D LC) were merged into a single assessment. Only peptides with Xcorr values higher than 1.5 (single charge), 2 (double charge), and 2.5 (triple charge) were retained. MS/MS spectra corresponding to proteins identified by less than four peptides were checked manually. The complete list and characteristics of peptides corresponding to proteins identified is presented in Supplemental Table S1. Most protein identifications were based on a minimum of two-peptide assignments (when indicated, unique peptide-based identifications were considered: in those cases the corresponding spectra are joined in Supplemental Table S2). When ortholog proteins were identified, the choice was made to select those from genus *Nicotiana*, when possible, or from nearest species. Importantly, peptides shared between several proteins were only counted for the protein that has over all the most matching peptides. The protein list is thus the smallest set of proteins explaining the identified peptide presence.

To evaluate the false positive rate in our protein listing, a more stringent filtering of the database search results was conducted based on retaining peptides with probability $p(\text{pep}) < 1\text{E}-3$ in the Bioworks 3.2 interface (ThermoFinnigan). The obtained results led to an identification confidence higher than 95%. Protein candidates considered as ambiguous were thus checked manually, and a specific MS/MS spectrum showing the presence of each protein is given in Supplemental Table S2.

Prediction Methods—Predictions for number and length of trans-membrane (TM) domains were achieved by using the HMMTOP server (www.enzim.hu/hmmtop). The GRAVY (17), theoretical pI, and theoretical molecular weight were obtained using the software program ProtParam tool (www.expasy.org/cgi-bin/protparam).

For post-translational modifications, glycosylphosphatidylinositol (GPI) modification sites were predicted by Big-PI Plant Predictor (mendel.imp.univie.ac.at/gpi/plant_server.html), and myristoylation sites were predicted by NMT-The MYR Predictor (mendel.imp.univie.ac.at/myristate/SUPLpredictor.htm). Putative palmitoylation

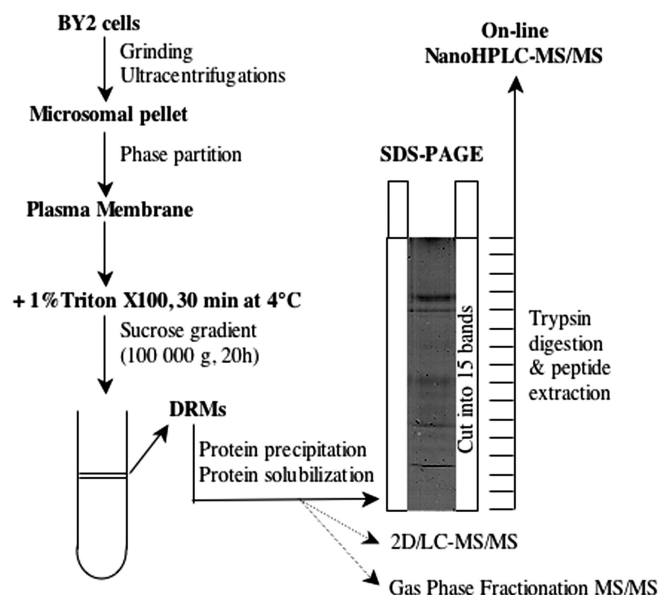


FIG. 1. Schematic representation of tobacco DRM isolation and analysis procedures.

sites were searched manually in amino acid sequences according to Ref. 18.

RESULTS

Tobacco Plasma Membrane Microdomains Extraction

The procedure of extraction of plasma membrane DRMs from cultured BY-2 tobacco cells is schematized in Fig. 1. We selected the conventional phase partition procedure to obtain a highly enriched plasma membrane (PM) fraction. Typical preparations were enriched by a factor of 7–8 in the PM marker, vanadate-sensitive ATPase activity, compared with the starting material consisting of a crude microsomal fraction. Biochemical characterization of this PM fraction revealed that it was virtually free of mitochondrial contamination. Endoplasmic reticulum marker enzyme was depleted by a factor of 8, and tonoplast marker was depleted by a factor of 20 between microsomes and PM. Finally the absence of monogalactosyldiacylglycerol indicated a lack of contamination by thylakoid envelope (data not shown). These results indicate that this PM fraction, contaminated very little by other endomembranes, constitutes a suitable starting material for extraction of DRMs (as described in Ref. 13) and for further proteomic analysis.

It is a well known feature that, in biological membranes, the formation of microdomains correlates with resistance to solubilization by non-ionic detergents, and this property has been widely used for biochemical characterization of these domains (6). Here plasma membranes were incubated at 4 °C with Triton X-100 (final concentration, 1%) at a detergent/protein ratio of 15, previously established as the most suitable for DRM extraction from this material (13). In these conditions, the amount of proteins recovered in the DRM fraction was around 5% (w/w) of the initial quantity present

in the plasma membrane fraction. The previous extensive analysis of the lipidic composition of this fraction revealed that it is highly enriched in sterols and sphingolipids compared with the PM (13). To get the most extensive description of its proteic content, this detergent-resistant membrane fraction, which constitutes a subfraction of purified plasma membrane, was used for proteomic analysis using three different approaches.

Analysis by Mass Spectrometry of the Proteic Composition of Tobacco Microdomains

Tobacco plasma membrane DRMs were found to be soluble in a buffer consisting of both non-ionic (*N*-octyl glucoside) and ionic (SDS) detergents and high concentration of chaotropic agents. Such a buffer would have resulted in an important decrease of trypsin efficiency and in incompatibility problems with the subsequent reverse phase separation. To circumvent these problems, it would have been necessary to dilute the sample before digestion and to include additional steps for detergent removal. Finally sample complexity rendered prefractionation necessary. We then chose to separate the protein mixture by SDS-PAGE. The complete electrophoresis lane was cut into 15 pieces, and digests obtained from each piece after trypsin addition were separately analyzed by nano-HPLC coupled to a nanospray ion trap mass spectrometer. The SDS-PAGE separation strategy thus allows reduction of sample complexity while making samples compatible with subsequent LC-MS/MS analysis. Alternative experiments consisting in short SDS-PAGE migration, unique band excision, digestion, and subsequent 2D LC-MS/MS or GPF_{m/z} were also conducted.

A total of 145 different proteins (Tables I–V) was identified using the SDS-PAGE/LC-MS/MS method, which proved to be far most efficient, in our hands, than the two other methods. Indeed 54 proteins were identified by 2D LC-MS/MS, 43 were identified by GPF, and all the proteins identified by any of those two methods were also present in the result of the SDS-PAGE/LC-MS/MS analysis. The results presented in Tables I–V were obtained from at least three experiments from independent suspension cells each time.

The disappointing results obtained with GPF and 2D LC could arise from the prominence, in the sample, of highly abundant proteins (*i.e.* ATPases) that would mask the signal of less abundant proteins. The SDS-PAGE separation would then be more efficient in the segregation of these major proteins. In addition, the use of step rather than gradient SCX separation would result in a loss of resolution in the first chromatographic dimension, which would also account for the contamination by major protein species.

Functional Survey of Identified DRM Proteins

Based on sequence homologies with known proteins, putative functional classes could be assigned for 140 proteins

among the 145 identified. These proteins can be divided in five groups, which are clearly in agreement with main physiological cellular processes involving the plant plasma membrane: transport (36 proteins, Table I), signaling and response to biotic or abiotic stress (55 proteins, Table II), cellular trafficking (17 proteins, Table III), cell wall synthesis and degradation (14 proteins, Table IV), and metabolism and other functions (18 proteins, Table V). For each of these groups, the relevance of the presence of some specific proteins (or family of proteins) in DRMs will be developed under the "Discussion."

Physical and Chemical Characteristics of Tobacco BY-2 Cell DRM Proteins

To examine putative characteristic features of DRMs associated proteins, the whole set of proteins listed in Tables I–V was analyzed with respect to physical parameters such as molecular weight, isoelectric point, number and length of transmembrane segments, or global hydrophobicity. For each parameter (except the length of transmembrane segment), these values were compared with results obtained with proteins identified in the whole plasma membrane from plant. As the whole plasma membrane proteome is not available in databases, we collected 330 sequences of proteins previously demonstrated as plant plasma membrane proteins in different studies (3, 4, 19–25) or referred to as plasma membrane proteins in databases. Proteins were retrieved from the National Center for Biotechnology Information (NCBI) database (version February 2006) using the following keywords: plasma membrane or plasmalemma and *Arabidopsis* (to eliminate redundancy due to ortholog proteins in different species). This set of proteins was used as a "plasma membrane protein database" for a comparison with DRM proteins.

Isoelectric Point—The theoretical isoelectric points of all the proteins identified range from 4 to 12 and are mainly represented in two groups: an acidic group (pH 5–7) and a basic group (pH 8–10), which represent, respectively, 46 and 38% of the DRM proteins identified (Fig. 2). This distribution is not radically different from the one obtained analyzing plasma membrane proteins from our database (Fig. 2). However, a detailed comparison indicated slight differences: if the proportion of "neutral proteins" ($6 < pI < 8$) is comparable in the two fractions, the relative amount of acidic proteins ($pI < 6$) seems higher in plasma membrane than in DRMs, and inversely, basic proteins ($pI > 8$) are more abundant in DRMs.

Molecular Weight—The molecular masses of proteins listed in Tables I–V are between 11 and 232 kDa with a majority of proteins (70%) exhibiting a molecular mass between 20 and 80 kDa (Fig. 3). This also seems to be a relatively classical feature of plant plasma membrane proteins (19). It appears, nevertheless, that proteins of low molecular mass (<20 kDa) seem rather excluded from DRMs, whereas proteins of high

molecular mass (>180 kDa) are more represented in this fraction than in plasma membrane (Fig. 3).

Number of Transmembrane Helices and Length of Transmembrane Segments—The *in silico* analysis of all the proteins listed in Tables I–V indicated that 59% comprised at least one putative transmembrane helix (Fig. 4). The result is quite similar (61%) for proteins of the whole plasma membrane (Fig. 4). The same analysis performed on plasma membrane from *A. thaliana* previously enriched in hydrophobic proteins (3) led to 51% of proteins exhibiting at least one transmembrane helix. Among the DRM proteins supposed to contain at least one transmembrane α -helix, 45% exhibited one to three membrane-spanning segments, 15% exhibited four to six, 32% exhibited seven to 12, and 7.5% exhibited more than 12 (Fig. 4). These data are quite comparable to the data obtained with the plasma membrane proteins from our database (Fig. 4). However, the proportion of transmembrane proteins containing more than seven spanning helices in this study (40%) is significantly higher than in a previous study on *A. thaliana* plasma membrane proteome (21%) (4) and even than in a study focused on intrinsic plasma membrane proteome of the same plant (30%) (3).

Using x-ray diffraction analysis, it has been shown that DRMs are 9 Å thicker than detergent-soluble membranes for a bilayer system containing an equimolar mixture of sphingomyelin, cholesterol, and an unsaturated phospholipid (26). This difference in thickness of rafts and non-raft membranes has been proposed to be involved in the targeting of proteins to microdomains from the Golgi apparatus: proteins with relatively short transmembrane domains would tend to be localized in thin non-raft membranes, whereas certain proteins with longer transmembrane domains would be segregated to thicker microdomains enriched in sphingolipids and sterols that form transport vesicles destined to the plasma membrane (27). The basis of transmembrane α -helix prediction is that a stretch of 15–30 hydrophobic amino acids is likely to be an α -helix in a membrane bilayer. All the helices identified in DRM proteins by the prediction programs were examined with regard to the number of amino acids. The results of this analysis indicate a broad distribution from 17 to 25 amino acids per helix with a maximum representation for helices comprising 20 or 21 amino acids (Fig. 5). This length is similar to the average length of transmembrane helices in a database generated from the Swiss-Prot database and containing a total of 3596 different putative membrane proteins (28).

Global Hydrophobicity—Another parameter commonly used to describe the hydrophobicity of proteins is the GRAVY index, which takes into account the size and charge of the whole protein. It ranges classically from -2 to $+2$, and positive values refer to hydrophobic proteins (17). In this study most of the proteins (77%) have a GRAVY index between -0.4 and $+0.4$, which could not really discriminate their hy-

TABLE I
Transport proteins

Sp., plant species abbreviated as follows: *A.t.*, *A. thaliana*; *L.e.*, *Lycopersicon esculentum*; *M.c.*, *Mesembryanthemum crystallinum*; *N.e.*, *Nicotiana excelsior*; *N.g.*, *Nicotiana glauca*; *N.p.*, *Nicotiana plumbaginifolia*; *N.t.*, *N. tabacum*; *O.s.*, *Oryza sativa*; *S.t.*, *Solanum tuberosum*; Acc. no., accession number in UniProtKB database; Pep., number of different sequences from the corresponding protein; %cov., percentage of coverage of the identified proteins; TM, putative TM domains predicted by HMMTOP; TML, length of the predicted TM domains; GRAVY, hydrophobicity value; pl, theoretical pl; MW, theoretical molecular weight; Loc., protein location (according to literature or UniProtKB database information), in italics when putative; Int., integral proteins; Peri., peripheral proteins; ER, endoplasmic reticulum; Tono., tonoplast; MDR, multidrug resistance; CaMB, calmodulin binding.

Protein name (number of identified proteins)	Sp.	Acc. no.	Pep.	%cov.	TM	TML	GRAVY	pl	MW	Loc.
Transport (36)										
PM ATPases (6)										
Plasma membrane proton ATPase PMA2	<i>N.p.</i>	Q42932	34	40.8	10	21	0.113	6.31	105	PM
Plasma membrane ATPase PMA3	<i>N.p.</i>	Q08436	32	36.1	10	21	0.129	6.43	105	PM
Plasma membrane ATPase PMA4	<i>N.p.</i>	Q03194	49	43.7	10	21	0.048	6.52	105	PM
Plasma membrane proton ATPase PMA5	<i>N.p.</i>	Q5U9D4	7	9.1	10	21	0.031	5.56	102	PM
Plasma membrane proton ATPase PMA6	<i>N.p.</i>	Q9SWH2	9	11.4	10	21	0.115	6.48	105	PM
Plasma membrane proton ATPase PMA9	<i>N.p.</i>	Q9SWH0	6	4.8	10	20	0.114	5.79	105	PM
Calcium-transporting ATPase (1)										
Potential calcium-transporting ATPase 9	<i>A.t.</i>	Q9LU41	2	2.3	10	22	0.059	6.31	117	PM
Vacuolar H ⁺ -ATPases (7)										
Vacuolar H ⁺ -ATPase A1 subunit isoform	<i>L.e.</i>	Q84XW6	18	38.5	0	^a	-0.164	5.20	69	Peri. <i>PM</i>
Vacuolar H ⁺ -ATPase A2 subunit isoform	<i>L.e.</i>	Q84XV7	20	38.5	0	^a	-0.185	5.30	69	Peri. <i>PM</i>
Vacuolar H ⁺ -ATPase B subunit	<i>N.t.</i>	Q9M5Z8	15	38.5	0	^a	-0.268	5.11	54	Peri. <i>PM</i>
Vacuolar ATP synthase subunit D	<i>A.t.</i>	Q9XGM1	3	11.5	0	^a	-0.243	9.52	29	Peri. <i>PM</i>
Putative vacuolar H ⁺ -ATPase subunit E (fragment)	<i>L.e.</i>	Q9LKG0	4	20.3	^a	^a	^a	^a	^a	Peri. <i>PM</i>
Vacuolar ATP synthase subunit G1	<i>N.t.</i>	O82702	3	30.9	0	^a	-0.775	5.94	13	Tono. ER <i>PM</i>
Putative vacuolar ATPase subunit H	<i>M.c.</i>	Q8GUB3	3	7.7	0	^a	-0.125	6.73	53	Peri. <i>PM</i>
ABC transporters (4)										
MDR-like ABC transporter mdr8	<i>O.s.</i>	Q7FMW3	3	3.3	12	23	0.077	6.82	138	Int.
P-glycoprotein-like protein pgp3	<i>A.t.</i>	Q9SY12	5	6.2	12	23	0.227	8.23	133	Int.
Putative ABC transporter	<i>A.t.</i>	O80725	8	8.2	12	23	0.107	6.16	139	Int.
Putative ABC transporter	<i>A.t.</i>	Q9FWX8	6	5.9	12	23	0.207	8.33	132	Int.
Water channels, aquaporins (7)										
Water channel protein	<i>N.e.</i>	O09224	7	21.3	6	20	0.390	8.61	31	Int.
Water channel protein	<i>N.e.</i>	O09222	2	12.6	6	20	0.375	8.81	30	Int.
Aquaporin NT2	<i>N.t.</i>	Q40595	6	13.7	6	20	0.399	9.08	31	Int.
Aquaporin NtPIP2;1	<i>N.t.</i>	Q8W506	3	13.0	6	20	0.467	9.05	30	PM
Aquaporin 1 NtAQP1	<i>N.t.</i>	O24662	5	20.6	6	20	0.414	8.29	31	PM
Putative PIP MIP5 (fragment)	<i>N.g.</i>	Q9FPZ6	4	26.3	6	^a	^a	^a	^a	<i>PM</i>
Putative PIP2 MIP4	<i>N.g.</i>	Q9FPZ7	2	8.1	6	20	0.556	9.02	30	<i>PM</i>
Cyclic nucleotide-gated ion channels (2)										
Cyclic nucleotide-gated calmodulin-binding ion channel CBP4	<i>N.t.</i>	Q9LM04	1	1.8	6	21	-0.109	8.86	81	PM
Probable cyclic nucleotide-gated ion channel CNGC 6 (or 9 ^b)	<i>A.t.</i>	O82226	2	3.9	6	21	-0.167	9.38	85	<i>PM</i>
Other transporters (9)										
Inorganic phosphate transporter NtPT1	<i>N.t.</i>	Q9LLS5	12	24.8	12	21	0.291	8.96	59	Int.
Phosphate transporter	<i>N.t.</i>	Q9ST22	10	21.8	12	21	0.341	8.77	59	Int.
Phosphate transporter NtPT4	<i>N.t.</i>	Q9AYT1	5	11.7	12	21	0.371	8.84	57	Int.
Transmembrane protein PT3	<i>S.t.</i>	Q8W4W9	2	3.7	12	22	0.373	8.78	59	Int.
H ⁺ /monosaccharide co-transporter MST1	<i>N.t.</i>	Q06312	5	10.3	12	23	0.562	9.04	58	Int.
High affinity nitrate transporter protein	<i>N.t.</i>	Q84MZ8	1	3.4	12	21	0.351	9.19	58	Int.
Ammonium transporter 1 AMT1	<i>L.e.</i>	P58905	1	2.9	11	20	0.436	6.62	52	Int.
CaMB-channel protein	<i>N.t.</i>	O04182	3	13.3	0	^a	-0.400	9.47	27	Unknown
Lysine- and histidine-specific transporter LHT1	<i>A.t.</i>	O24405	1	2.7	8	24	0.398	9.25	51	Int.

^a No data.

^b Unable to distinguish between these proteins.

drophobicity (Fig. 6). Only 25% of the proteins have a strictly positive GRAVY among which 5% exhibit a GRAVY above 0.4. Interestingly the same analysis performed on the proteins of

the plasma membrane database indicated a similar repartition but with a significantly higher proportion (14%) of clearly hydrophobic proteins (GRAVY > +0.4) (Fig. 6).

TABLE II
Signaling and biotic/abiotic stress-related proteins

Sp., plant species abbreviated as follows: *A.m.*, *Ammopiptanthus mongolicus*; *A.t.*, *A. thaliana*; *C.an.*, *Capsicum annuum*; *C.ar.*, *Cicer arietinum*; *C.s.*, *Cucumis sativa*; *D.c.*, *Daucus carota*; *G.h.*, *Gossypium hirsutum*; *G.m.*, *Glycine max*; *H.v.*, *Hordeum vulgare*; *L.e.*, *L. esculentum*; *L.j.*, *Lotus japonicus*; *M.d.*, *Malus domestica*; *M.i.*, *Mangifera indica*; *N.at.*, *Nicotiana attenuata*; *N.b.*, *Nicotiana benthamiana*; *N.p.*, *N. plumbaginifolia*; *N.t.*, *N. tabacum*; *O.s.*, *O. sativa*; *S.d.*, *Solanum demissum*; *S.t.*, *S. tuberosum*; Acc. no., accession number in UniProtKB database; Pep., number of different sequences from the corresponding protein; %cov., percentage of coverage of the identified proteins; TM, putative TM domains predicted by HMMTOP; TML, length of the predicted putative TM domains; GRAVY, hydrophobicity value; pl, theoretical pl; MW, theoretical molecular weight; Loc., protein location (according to literature or UniProtKB database information), in italics when putative; Cyto., cytoplasm; Endo., endosomes; Int., integral proteins; Mb., membrane proteins; Nuc., nucleus; PTM, post-translational modifications; 16:0, palmitoylation; 14:0, myristoylation; PDR, pleiotropic drug resistance.

Protein name (number of identified proteins)	Sp.	Acc. no.	Pep.	%cov.	TM	TML	GRAVY	pl	MW	Loc.	PTM
Signaling (34)											
Putative receptor protein kinases (10)											
LRR (9)											
Leucine-rich repeat receptor-like protein kinase NtTMK1	<i>N.t.</i>	Q9M7A8	2	3.2	1	25	-0.161	6.64	102	Int.	
BRI1-associated receptor kinase 1 BAK1	<i>A.t.</i>	Q94F62	1	1.6	1	24	-0.187	5.62	68	PM/Endo.	
Putative receptor-like kinase	<i>O.s.</i>	Q9LDG0	2	3.4	1	24	-0.004	8.62	68		
Putative leucine-rich repeat transmembrane	<i>O.s.</i>	Q8H811	2	2.2	1	24	-0.026	6.59	71	Int.	
Receptor-like protein kinase RKL1	<i>A.t.</i>	Q9ZT08	4	6.5	1	24	-0.198	8.30	70		
Receptor kinase, putative	<i>A.t.</i>	Q8LD58	3	4.1	1	24	-0.084	6.28	70		
Putative receptor-like protein kinase	<i>O.s.</i>	Q7XHW7	2	4.8	1	25	-0.133	7.62	70		
Receptor-like protein kinase 1-like	<i>O.s.</i>	Q5ZBN0	2	2.8	2	20	0.135	7.18	71		
Putative leucine-rich repeat receptor kinase	<i>A.t.</i>	Q9SUQ3	1	2.2	1	25	-0.134	6.37	71	Int.	
Non-LRR (1)											
Receptor protein kinase-like protein	<i>C.an.</i>	Q8VZH5	2	4.2	0	^a	-0.231	6.21	71		
Calcium-dependent protein kinase (7)											
Calcium-dependent protein kinase 1 NtCDPK1	<i>N.t.</i>	O81390	9	24.4	0	^a	-0.664	6.57	62	Nuc.; PM	14:0, 16:0
Calcium-dependent protein kinase 8	<i>N.p.</i>	Q6KC54	4	7.4	1	20	-0.468	6.43	59		14:0, 16:0
Calcium-dependent protein kinase MdCPK1	<i>M.d.</i>	Q6EE26	4	9.9	1	20	-0.537	6.19	61		14:0, 16:0
Calcium-dependent protein kinase 4	<i>C.an.</i>	Q5EDD1	3	7.4	1	20	-0.516	5.87	59		14:0, 16:0
Calcium-dependent protein kinase	<i>D.c.</i>	P28582	2	5.5	1	20	-0.506	6.13	61		14:0, 16:0
Calcium-dependent calmodulin-independent protein kinase	<i>C.s.</i>	Q9ARI5	2	5.4	1	22	-0.377	5.55	58		
Calcium-dependent protein kinase CDPK2	<i>C.ar.</i>	Q7XZK4	2	6.1	1	20	-0.562	6.20	61		14:0, 16:0
Calcium/calmodulin-dependent protein kinase (3)											
Calcium/calmodulin-dependent protein kinase CaMK1	<i>N.t.</i>	Q8W561	3	7.2	0	^a	-0.389	8.98	67		16:0
Putative calcium/calmodulin-dependent protein	<i>O.s.</i>	Q8GSB1	2	5.1	1	19	-0.202	8.88	65		14:0, 16:0
Calmodulin-like domain protein kinase isoenzyme γ	<i>G.m.</i>	Q24431	4	10.4	0	^a	-0.395	6.05	60		16:0
Other kinases (5)											
Serine/threonine protein kinase	<i>L.j.</i>	Q53VM2	2	6.7	0	^a	-0.248	7.62	51		
Protein kinase-like	<i>A.t.</i>	Q9FHD7	4	8.6	0	^a	-0.409	5.92	55		14:0, 16:0
Protein kinase-like protein	<i>O.s.</i>	Q9FWF0	3	7.5	1	18	-0.448	7.91	58		14:0, 16:0
F2K11.13 protein kinase-like	<i>A.t.</i>	Q9SH35	4	7.5	3	24	-0.211	6.87	70		
OSJNBa0088H09.6 (serine/threonine protein kinase)	<i>O.s.</i>	Q7XPV0	5	12.2	0	^a	-0.381	5.63	55		14:0, 16:0
Other signaling proteins (9)											
NtRac2/NTGP3	<i>N.t.</i>	Q9ZRD2	1	7.1	1	17	-0.137	9.39	22		
NtRac4	<i>N.t.</i>	Q8GT44	2	12.9	0	^a	-0.125	9.29	23		
Ras-related protein RAB8-3	<i>N.t.</i>	Q8W3J3	2	13.0	2	18	-0.328	7.65	24		
Ras-related protein RAB8-5	<i>N.t.</i>	Q8W3J2	2	13.0	2	18	-0.340	7.65	24		
ARF1-like GTP-binding protein	<i>C.an.</i>	Q9M7P4	5	32.0	1	18	-0.220	6.43	21		14:0

TABLE II—continued

Protein name (number of identified proteins)	Sp.	Acc. no.	Pep.	%cov.	TM	TML	GRAVY	pI	MW	Loc.	PTM
Calcineurin B-like protein CBL1	A.m.	Q5EE13	2	10.3	0	^a	-0.084	4.60	24		14:0, 16:0
Calmodulin CaM-2 CaM-1 (fragment)	N.p.	O82773	3	37.7	0	^a	^a	^a	^a		
Calmodulin	N.at.	Q5QJ50	3	30.9	0	^a	-0.619	4.10	17		
Phospholipase D δ isoform 1b	G.h.	Q8H1U0	3	3.7	0	^a	-0.412	6.79	96	PM	
Biotic/abiotic stress (21)											
NADPH oxidase NtrbohD	N.t.	Q8RVJ9	2	3.1	6	20	-0.344	8.93	106	PM	
Respiratory burst oxidase homolog StrbohB	S.t.	Q948T9	2	2.9	6	20	-0.291	9.21	100	PM	
Remorin 1	L.e.	Q9XEX8	3	16.8	0	^a	-0.802	5.64	22	PM	
Harpin-inducing protein 1-like 9	N.t.	Q6L7J8	7	27.9	2	19	-0.310	9.06	26	PM	
Harpin-inducing protein 1-like 18	N.t.	Q6L7J7	6	24.9	2	19	-0.308	9.29	26	PM	
Hypersensitive-induced reaction protein	C.an.	Q5GI04	14	48.8	0	^a	-0.121	6.02	31		16:0
Hypersensitive-induced response protein	A.t.	Q9FM19	14	49.7	0	^a	-0.101	5.29	31		16:0
Putative hypersensitive-induced response protein	O.s.	Q6L4S3	11	35.1	0	^a	-0.135	6.01	31		16:0
Hypersensitive-induced response protein Lj-HIR1	L.j.	Q5KSB6	8	35.3	0	^a	-0.174	5.22	31		14:0, 16:0
Putative hypersensitive-induced response protein	O.s.	Q8H9I8	6	17.8	0	^a	-0.136	5.81	32		16:0
Hypersensitive-induced reaction 2 HIR2	H.v.	Q8H1V5	5	19.0	0	^a	-0.096	5.57	31		
Putative hypersensitive-induced reaction protein	S.d.	Q6L4B0	4	12.0	0	^a	-0.206	5.40	33		16:0
Putative Pto-like serine/threonine kinase (fragment)	M.i.	Q64GC6	2	15.3	^a	^a	^a	^a	^a		
Pto kinase interactor 1 Pti1	L.e.	Q41328	4	13.3	0	^a	-0.264	7.16	39		16:0
PDR-type ABC transporter 1 NtPDR1	N.t.	Q76CU2	3	3.1	12	21	0.028	6.46	162	PM	
PERK1-like protein kinase (fragment)	N.t.	Q76BK8	2	10.5	^a	^a	^a	^a	^a	PM	
Calcium-dependent protein kinase CDPK1	L.e.	Q94KH6	7	20.7	1	20	-0.390	6.62	58		14:0, 16:0
Rop subfamily GTPase rop1 (or Rac5 ^b)	N.t.	O24142	3	23.3	0	^a	-0.097	9.30	21	PM	
Ascorbate peroxidase	C.an.	Q8W4V7	2	9.4	1	22	-0.370	7.74	32		
Elicitor-inducible protein (EIG-J7)	N.t.	Q9FXT1	2	11.9	1	20	-0.148	5.41	20	Mb.	
Molecular chaperone Hsp90-2	N.b.	Q6UJX5	5	9.2	1	25	-0.583	4.98	80	Cyto.	

^a No data.^b Unable to distinguish between these proteins.

Post-translational Modifications

Specific post-translational modifications have been reported to be correlated with the presence in rafts of specific subsets of proteins. GPI anchoring was the first and best characterized of these signals (29), but N-terminal double acylation of membrane proteins has also been reported (18, 30).

Addition of a GPI anchor is a post-translational modification occurring in endoplasmic reticulum. Such proteins are then characteristically embedded with the lipid portion of the GPI anchor into the exoplasmic leaflet of the plasma membrane (29). In the present study, four GPI-anchored proteins were identified: a pectinesterase-like protein, two monocopper oxidases, and a fasciclin-like arabinogalactan protein. Two of these proteins (the two monocopper oxidases) have also been identified (with four other GPI-anchored proteins (GPI-APs)) in DRMs from *Arabidopsis* callus (14).

Another example of lipid modification previously observed in some proteins associated with animal lipid rafts is palmitoylation (addition of a 16-carbon saturated fatty acid) or myristoylation (addition of a 14-carbon saturated fatty acid). In this case the acyl chain inserts into the cytoplasmic face of

the plasma membrane. Although the mechanism responsible for accumulation of dually acylated proteins to microdomains has not yet been elucidated, one could speculate that insertion of fatty acyl anchors into a liquid ordered domain would be energetically favorable (31). Both myristoylation and palmitoylation can be dynamically regulated: the myristate moiety can be sequestered through the use of myristoyl switches, whereas palmitate can be removed by protein palmitoyl thioesterases (18). In our study, 22 proteins appear to undergo this kind of post-translational modification, and among those proteins 13 possess both myristoylation and palmitoylation sites (Tables I–V).

DISCUSSION

How Many Proteins Are in Tobacco DRMs?

Previous studies performed on plant DRMs (summarized in Ref. 11) led to the identification of 11 proteins enriched in DRMs from tobacco BY-2 cell plasma membrane, 35 proteins present in DRMs obtained from microsomes of *A. thaliana* callus, and 34 proteins present in DRMs of *A. thaliana* cotyledons plasma membrane (32). The analysis presented here

TABLE III
Intracellular trafficking-related proteins

Sp., plant species abbreviated as follows: *A.t.*, *A. thaliana*; *G.m.*, *G. max*; *N.t.*, *N. tabacum*; *O.s.*, *O. sativa*; *S.t.*, *S. tuberosum*; Acc. no., accession number in UniProtKB database; Pep., number of different sequences from the corresponding protein; %cov., percentage of coverage of the identified proteins; TM, putative TM domains predicted by HMMTOP; TML, length of the predicted putative TM domains; GRAVY, hydrophobicity value; pl, theoretical pl; MW, theoretical molecular weight; Loc., protein location (according to literature or UniProtKB database information); Cell pl., cell plate; Mb., membrane proteins.

Protein name (number of identified proteins)	Sp.	Acc. no.	Pep.	%cov.	TM	TML	GRAVY	pl	MW	Loc.	
Intracellular trafficking (17)											
Clathrin heavy chain	<i>G.m.</i>	Q39834	5	4.4	0	^a	-0.168	5.37	193	PM	
Syntaxin 71 AtSYP71	<i>A.t.</i>	Q9SF29	3	12.0	1	18	-0.515	5.09	30	Mb.	
Putative syntaxin 131 AtSYP131	<i>A.t.</i>	Q9SRV7	1	4.2	1	21	-0.617	8.67	35	Mb.	
Syntaxin-related protein Nt-syr1	<i>N.t.</i>	Q9ZSD5	1	4.0	1	20	-0.551	7.76	34	Mb.	
<i>N</i> -Ethylmaleimide-sensitive fusion protein NtNSF-1	<i>N.t.</i>	Q40495	3	6.2	0	^a	-0.134	5.81	81		
Putative NPSN12	<i>O.s.</i>	Q5Z9Q0	1	6.3	0	^a	-0.721	5.06	23		
α -Soluble NSF attachment protein α -SNAP	<i>S.t.</i>	Q9M5P8	2	9.7	0	^a	-0.470	5.11	32		
β -Tubulin 1 B1	<i>G.m.</i>	P12459	8	20.7	0	^a	-0.318	4.88	50		
α -Tubulin A3	<i>N.t.</i>	Q8VXC9	3	11.8	0	^a	-0.208	4.86	50		
Actin 54 (fragment)	<i>N.t.</i>	P93373	5	17.4	^a	^a	^a	^a	^a		
Centrin CEN1	<i>N.t.</i>	Q9SQI5	4	29.9	0	^a	-0.901	4.68	20	Mb.	
Centrin CEN2	<i>N.t.</i>	Q9SQI4	6	47.5	0	^a	-0.922	4.72	20	Mb.	
Dynammin-related protein 1C (DRP1C)	<i>A.t.</i>	Q8LF21	2	6.0	0	^a	-0.360	7.22	69		
Dynammin-related protein 1D (DRP1D)	<i>A.t.</i>	Q8S3C9	3	5.2	0	^a	-0.198	8.08	68		
Dynammin-related protein 2B (DRP2B)	<i>A.t.</i>	Q9LQ55	3	4.0	0	^a	-0.557	9.10	100		
SDL5A (phragmoplastin)	<i>G.m.</i>	Q39828	6	11.5	0	^a	-0.293	8.04	68	Cell pl.	
Phragmoplastin	<i>N.t.</i>	Q9SMB6	10	19.2	1	20	-0.291	7.61	68	Cell pl.	

^a No data.

TABLE IV
Cell wall-related proteins

Sp., plant species abbreviated as follows: *A.t.*, *A. thaliana*; *G.h.*, *G. hirsutum*; *L.e.*, *L. esculentum*; *N.al.*, *Nicotiana glauca*; *O.s.*, *O. sativa*; Acc. no., accession number in UniProtKB database; Pep., number of different sequences from the corresponding protein; %cov., percentage of coverage of the identified proteins; TM, putative TM domains predicted by HMMTOP; TML, length of the predicted putative TM domains; GRAVY, hydrophobicity value; pl, theoretical pl; MW, theoretical molecular weight; Loc., protein location (according to literature or UniProtKB database information); CW, cell wall; GA, Golgi apparatus; Mb., membrane; PTM, post-translational modifications; GPI, GPI-anchored proteins.

Protein name (number of identified proteins)	Sp.	Acc. no.	Pep.	%cov.	TM	TML	GRAVY	pl	MW	Loc.	PTM
Cell wall-related (14)											
Putative callose synthase	<i>O.s.</i>	Q8H046	9	4.7	9	22	-0.005	8.30	232	Mb.	
Putative callose synthase CFL1	<i>G.h.</i>	Q9XEG1	9	4.8	17	20	-0.037	8.42	219	Mb.	
Callose synthase catalytic subunit-like protein	<i>A.t.</i>	Q9LXT9	5	3.5	11	20	-0.182	8.40	226	Mb.	
Callose synthase catalytic subunit-like	<i>A.t.</i>	Q9LTG5	5	3.9	12	22	0.104	8.44	155	Mb.	
Putative 1,3- β -glucan synthase	<i>O.s.</i>	Q5VS25	12	7.8	15	22	-0.014	8.64	202	Mb.	
Putative glucan synthase	<i>A.t.</i>	Q9SJM0	7	9.8	8	19	0.042	8.64	89	Mb.	
Putative glucan synthase component	<i>A.t.</i>	Q9ZT82	8	5.6	16	21	-0.034	9.02	207	Mb.	
Putative 1,3- β -glucan synthase (fragment)	<i>N.al.</i>	Q93XQ2	11	46.7	^a	^a	^a	^a	^a	Mb.	
Putative glucan synthase	<i>A.t.</i>	Q9SFU6	8	4.7	16	20	-0.013	8.63	223	Mb.	
Putative 1,3- β -D-glucan synthase	<i>A.t.</i>	Q8S8D4	3	4.2	8	19	-0.028	9.01	101	Mb.	
Pectinesterase-like	<i>A.t.</i>	Q9FHN6	3	6.1	2	18	-0.205	5.87	66	Mb.	GPI
Endo-1,4- β -glucanase Cel3	<i>L.e.</i>	O04890	1	1.9	3	19	-0.378	8.94	69	PM/GA	
Putative monocopper oxidase SKU5 (precursor)	<i>A.t.</i>	Q9SU40	2	4.9	0	^a	^a	^a	^a	CW	GPI
Fasciclin-like arabinogalactan protein 8 (precursor)	<i>A.t.</i>	O22126	2	3.6	1	23	^a	^a	^a	Mb.	GPI

^a No data.

led to the identification of 145 proteins. A first reference is to compare this result with the total number of proteins present on the plasma membrane. However, this is not a trivial purpose. The experimental data concerning the number of proteins present on plant plasma membrane are very fragmentary due to the technical difficulties encountered with this material.

Previous studies either based on two-dimensional gel electrophoresis or using mass spectrometry directly after SDS-PAGE separation led, respectively, to an estimation of 500 polypeptides, but with a clearly established under-representation of intrinsic membrane proteins (2), or to the identification of 97 (3) and 238 (4) putative plasma membrane proteins.

TABLE V
Metabolic enzymes and hypothetical and other proteins

Sp., plant species abbreviated as follows: *A.t.*, *A. thaliana*; *C.an.*, *C. annuum*, *L.e.*, *L. esculentum*, *N.p.*, *N. plumbaginifolia*, *N.t.*, *N. tabacum*, *O.s.*, *O. sativa*, *S.m.*, *Solanum melongena*, *S.t.*, *S. tuberosum*; Acc. no., accession number in UniProtKB database; Pep., number of different sequences from the corresponding protein; %cov., percentage of coverage of the identified proteins; TM, putative TM domains predicted by HMMTOP; TML, length of the predicted putative TM domains; GRAVY, hydrophobicity value; pI, theoretical pI; MW, theoretical molecular weight; Loc., protein location (according to literature or UniProtKB database information); Cyto., cytoplasm; Mb., membrane; Mito., mitochondria; Nuc., nucleus; Tono., tonoplast; PTM, post-translational modifications; GPI, GPI-anchored proteins, Pr., isoprenylation.

Protein name (number of identified proteins)	Sp.	Acc. no.	Pep.	%cov.	TM	TML	GRAVY	pI	MW	Loc.	PTM
Metabolism (8)											
Monocopper oxidase-like protein SKS1 (precursor)	<i>A.t.</i>	Q8VXX5	2	3.9	0	^a	^a	^a	^a	Mb.	GPI
Aldehyde oxidase AAO1	<i>A.t.</i>	Q7G193	2	1.8	0	^a	-0.113	6.02	150		
1,4-Benzoquinone reductase-like	<i>A.t.</i>	Q9LSQ5	5	29.9	0	^a	-0.108	5.96	22		
Glyceraldehyde-3-phosphate dehydrogenase (fragment)	<i>N.t.</i>	P09094	2	8.6	0	^a	^a	^a	^a	Cyto.	
Phosphoglycerate kinase	<i>N.t.</i>	Q42962	2	8.5	0	^a	0.103	5.69	42	Cyto.	
Inorganic pyrophosphatase	<i>N.t.</i>	Q43801	4	7.1	16	23	0.589	5.11	80	Tono.	
ATP synthase α chain	<i>N.p.</i>	P05495	2	5.9	0	^a	-0.059	5.84	55	Mito.	
S-Adenosyl-L-methionine synthetase	<i>N.t.</i>	Q6SYB9	1	3.8	0	^a	-0.282	5.74	43		
Other functions (10)											
Elongation factor 1- α	<i>N.t.</i>	P43643	7	18.1	0	^a	-0.325	9.20	49		
NTPF2 (DNAJ protein) (fragment)	<i>N.t.</i>	Q9ZRE0	1	10.2	^a	^a	^a	^a	^a		Pr.
Heat shock cognate 70-kDa protein 1	<i>L.e.</i>	P24629	2	5.1	0	^a	-0.389	5.15	71		
DREPP2 protein	<i>N.t.</i>	Q9M3U5	1	11.2	0	^a	-0.368	4.98	23	PM	
60 S ribosomal protein L9	<i>O.s.</i>	P49210	2	16.8	0	^a	-0.219	9.62	21	Cyto.	
Small subunit ribosomal protein S16 SRP2 (fragment)	<i>C.an.</i>	Q6RK71	2	22.0	^a	^a	^a	^a	^a	Cyto.	
Eukaryotic initiation factor 4A-9	<i>N.t.</i>	Q40471	10	29.8	0	^a	-0.246	5.54	47	Cyto.	
Eukaryotic translation initiation factor 3 eIF-3	<i>N.t.</i>	Q40554	2	1.8	1	18	-0.710	9.40	112	Cyto.	
Histone H4-like protein	<i>S.m.</i>	Q76N07	2	21.3	0	^a	-0.524	11.48	11	Nuc.	
34-kDa outer mitochondrial membrane protein porin	<i>S.t.</i>	P42055	2	9.4	0	^a	-0.205	8.69	29	Mito.	
Hypothetical/unknown (5)											
Hypothetical protein F18O21_60	<i>A.t.</i>	Q9LYN1	2	2.8	3	21	-0.013	7.93	77		
Hypothetical protein F2G19.25	<i>A.t.</i>	Q9C636	2	6.1	1	23	-0.352	9.90	37		
F1E22.10 hypothetical protein At1g65730	<i>A.t.</i>	Q9SHY2	3	4.5	14	19	0.473	9.17	76		
Hypothetical protein	<i>O.s.</i>	Q94EF4	3	5.1	3	18	-0.109	5.88	96		
Putative membrane protein	<i>S.t.</i>	Q9AR55	1	5.8	5	19	0.008	9.60	44	Mb.	

^a No data.

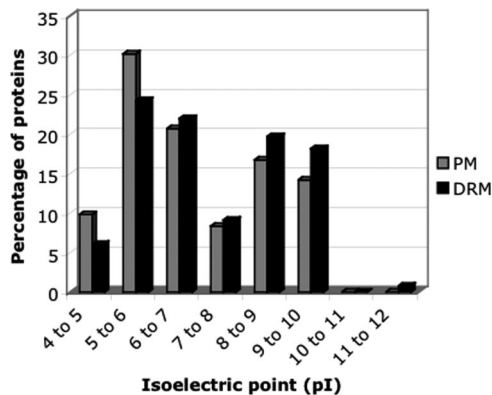


FIG. 2. Classification of DRM and PM proteins according to their pI. pI values were calculated using the ProtParam tool on the ExpASy server.

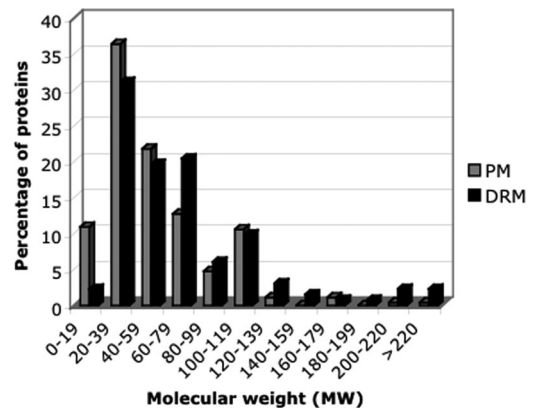


FIG. 3. Classification of DRM and PM proteins according to their molecular weight. Molecular mass values (in kilodaltons) were calculated using the ProtParam tool on the ExpASy server.

The question is also very complex when tackled with genomic tools. The Institute for Genomic Research (TIGR) now has annotated about 30,000 proteins among which 25% are predicted to be integral membrane proteins (5). However it is not possible to identify PM proteins from these sequences because no signal peptide or specific signature specifying the

targeting to the PM have been identified so far. Furthermore the plasma membrane is probably the most diverse membrane of the cell with a proteic composition that varies with cell type, developmental stage, and environment; therefore the proteins present at a precise time on the PM of a particular

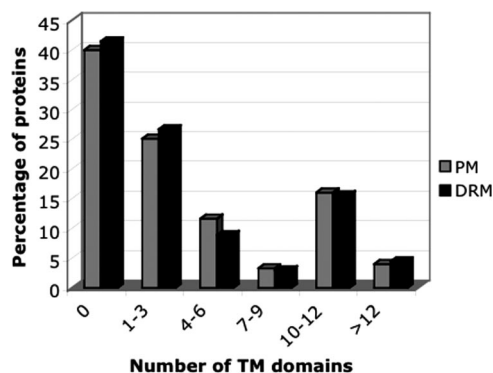


FIG. 4. Classification of DRM and PM proteins according to their number of TM domains. TM domains were predicted by HMMTOP when it was not already known in the literature.

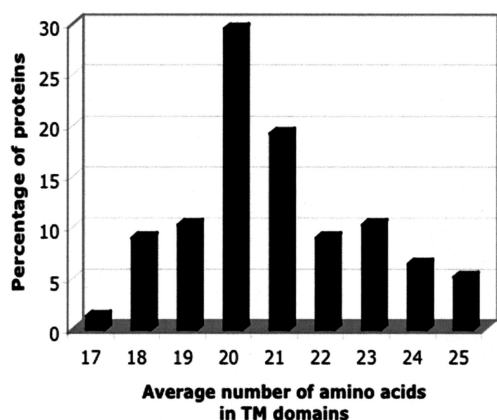


FIG. 5. Classification of detergent-resistant membrane proteins according to the length of their TM domains. The average number of amino acids in the TM domains was calculated on the basis of HMMTOP predictions.

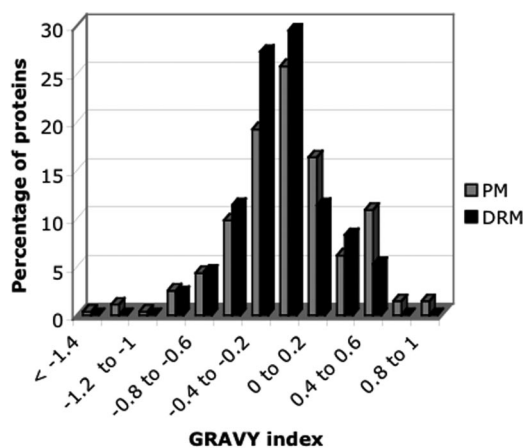


FIG. 6. Classification of DRM and PM proteins according to the GRAVY index. GRAVY values were calculated using the ProtParam tool on the ExPASy server.

type of cell are probably not representative of the genomic data corresponding to the whole set of proteins putatively addressed to this membrane.

In summary, no precise data are available concerning the

total number of plant plasma membrane proteins. However, taking into account all the elements discussed above, one may reasonably hypothesize the presence of about a thousand proteins on this membrane. In this context, the number of DRM proteins identified here (145) would represent about 15% of this total. This is not inconsistent with our experimental results indicating that 5% (w/w) of the total amount of proteins present in a PM fraction are recovered in the corresponding DRM fraction (i.e. 50 μ g of DRM proteins from 1 mg of PM proteins) and assuming that low abundance proteins are enriched in DRMs. Similar proteomic analyses in animal cells indicate a number of plasma membrane proteins between 900 and 1400 (33, 34) and a number of DRM-associated proteins between 200 and 350 according to the cell type (35).

What Kind of Proteins Are in Tobacco DRMs?

The putative functions of the proteins identified in tobacco DRMs are clearly in agreement with the main physiological cellular processes involving the plant plasma membrane. For each of these families, the relevance of the presence of some specific proteins in DRMs is discussed below.

Membrane Transporters (Table I)—Six isoforms of the most representative integral protein of the plant plasma membrane, the P-type H⁺-ATPase, were identified in tobacco DRMs (Table I). This result is in agreement with previous analysis indicating an enrichment of this protein in tobacco DRMs (13, 14). Interestingly the yeast counterpart of this protein has been demonstrated to be enriched in lipid rafts (36). Furthermore its immunolocalization in minor veins of *Vicia faba* showing a non-homogeneous distribution in transfer cells is consistent with its localization in specialized microdomains (37).

Different proteins initially identified as subunits of a vacuolar H⁺-ATPase are also present in DRMs (Table I). This result is supported by the identification of such proteins in two proteomic studies performed on *Arabidopsis* plasma membrane (3, 4) and by previous reports indicating the presence of different isoforms of this protein on the plasmalemma of tobacco (38) or *A. thaliana* (39). Furthermore these proteins have already been identified in DRMs from *Arabidopsis* (14).

Several members of the aquaporin family (plasma membrane intrinsic proteins (PIPs)) are also present in DRMs. Thirteen PIPs have been identified in *Arabidopsis*, and two recent studies detected eight isoforms on plasma membrane from cell culture (3) or green leaves (4) of this plant. A recent work also demonstrated the location of an aquaporin in caveolin-rich membrane microdomains of mouse keratinocytes (40).

Four proteins belonging to the ABC transporters family appear in Table I. Most ABC transporters characterized in plants have long been localized in the vacuolar membrane, but recent evidence indicated that members of this family could also be located on the plasma membrane (4).

Signaling and Response to Biotic or Abiotic Stress (Table II)—The plasma membrane, as the interface between the cell and the exterior, is the primary target for various signals supposed to trigger cell responses required for adaptation to environmental changes. To this respect, the presence on plant plasma membrane of proteins involved in signaling processes is relevant. In tobacco DRMs, these proteins represent 38% of the total proteins identified, and this proportion is significantly higher than in the whole plasma membrane (see below). In agreement with our results, a quantitative proteomic study of lipid rafts from HeLa cells demonstrated a significant enrichment of signaling proteins such as kinases and small GTP-binding proteins in this fraction compared with the plasma membrane (41). Among these proteins, receptor kinases (most of them exhibiting leucine-rich repeats), calcium-dependent protein kinases, calcium/calmodulin-dependent protein kinases, and GTP-binding proteins have been identified. All these families of proteins have been identified previously on plant plasma membrane, and their presence has also been observed in DRMs extracted from *Arabidopsis* callus (14) or cotyledons (32). Moreover, evidences of the involvement of animal lipid rafts in signaling processes come from studies performed with lymphocytes that demonstrated in different signaling processes the presence of particular receptors and kinases in these domains (10).

Proteins of the receptor-like kinase (RLK) family are well represented in DRMs as evidenced in this study (Table II) and other recent work (32). These proteins are defined by an extracellular domain, a single pass transmembrane domain, and a cytoplasmic serine/threonine protein kinase domain (42). An extensive phylogenetic analysis of the RLKs in *A. thaliana* has revealed that more than 400 genes encode putative RLK proteins, which can be classified on the basis of their extracellular domain (42). Among these proteins, leucine-rich repeat (LRR)-containing receptor kinases represent the largest group (42). The phenotypes associated with mutations in various LRR RLKs show that they play roles in diverse processes such as growth and development, setting up of symbiosis, or plant pathogen recognition (43). This particular point illustrates the general difficulty related to classifying proteins according to physiological functions because some LRRs that appear in Table II under “Signaling” should probably appear under “Defense” for example. Phospholipase D, isoform δ , a lipid-utilizing enzyme whose function is clearly established in plant signaling (44), was also found in DRMs (Table II).

In addition to signaling proteins or proteins whose precise functions are still unknown, we also listed in Table II proteins that have all been proved to be involved in plant responses to stress. Two of them, the NADPH oxidase NtrbohD and its regulator, the small G protein Rac5, involved in the signal transduction triggered by the fungal elicitor cryptogein, have been previously immunologically detected in tobacco DRMs (13). Two other proteins present in DRMs, HIN9 and HIN18,

have been proved to be induced during hypersensitive response due to tobacco mosaic virus infection (45), and seven others are the products of a plant defense gene family named HIR (hypersensitive-induced reaction) associated with pathogen resistance (46).

Pti1 is a serine/threonine kinase that is phosphorylated by the product of the *Pto* resistance gene of tomato that functions in a gene-for-gene interaction with the *avrPto* avirulence gene of the pathogen *Pseudomonas syringae* (47). Pti1 is believed to be involved in downstream signaling leading to the hypersensitive response (47). Interestingly both Pti1 and a Pto-like serine/threonine kinase were identified in tobacco DRMs (Table II).

Our previous work indicated that the protein remorin, which was identified in this study, is particularly well represented in tobacco DRMs as attested by one- and two-dimensional electrophoresis experiments (13, 32). Remorin is the putative receptor of oligogalacturonic acids, components of the extracellular pectic matrix that regulate the expression of a variety of defense genes (48). Taken altogether, these results indicating the presence in DRMs of various proteins involved in plant defense suggest, as already established in animal cells, a crucial role of these microdomains in the signaling linked to host-pathogen interactions.

Proteins Involved in Intracellular Trafficking (Table III)—The establishment and maintenance of intracellular organelles are dependent upon the proper trafficking of macromolecules and membrane fusion. The plant endomembrane system, which is critical for biosynthetic and endocytic trafficking to the plasma membrane and vacuole, is comprised of the endoplasmic reticulum, Golgi apparatus, and intermediate organelles such as prevacuolar compartment (49). Several proteins known to be involved in membrane trafficking were identified in this study (Table III).

SNAREs (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) are a family of membrane-tethered coiled-coil proteins that regulate fusion reactions and play a crucial role in the correct targeting of proteins to the different organelles of the endomembrane system (50). Syntaxins are a subset of SNARE proteins originally isolated from the presynaptic plasma membrane of neuronal and secretory cells (50). Two syntaxins of the SYP (*synt*axin of *pl*ants) subfamily (SYP 131 and SYP 71), which is the group most closely related to the plasma membrane-associated syntaxins in yeasts and mammals, and the tobacco SNARE NtSyr1 were identified in this study (Table III). The role of NtSyr1 in the regulation of guard cells responses to abscisic acid has been demonstrated (51). This result is in agreement with various experimental data suggesting a possible regulation of stress responses by intracellular vesicle trafficking (52). The soluble *N*-ethylmaleimide-sensitive factor attachment proteins (SNAPs) are eukaryotic soluble proteins required for membrane fusion. SNAPs act as adapters between NSF, a hexameric ATPase, and membrane SNARE proteins (SNAP re-

ceptors). Within the NSF-SNAP-SNARE complex, SNAPs contribute to the catalysis of an ATP-driven conformational change in the SNAREs, resulting in dissociation of the complex (53). NSF and SNAPs have also been identified in tobacco DRMs, indicating that all the partners necessary to the trafficking machinery are present in these domains.

Clathrin is a protein involved in endocytosis via the formation of clathrin-coated pits that eventually form vesicles by budding from the membrane. Clathrin heavy chain, listed in Table III, has already been identified in proteomic analysis of the plasma membrane from *Arabidopsis* (4) and in DRMs from various animal cell types (41, 54). In mammalian cells, the final scission of the clathrin-coated vesicles from the plasma membrane relies on dynamins through the action of their pleckstrin homology domain that interacts with lipids (55). Among the three large GTPase dynamins identified in this study, DRP2B contains this type of domain in its sequence and may be involved in endocytosis and membrane recycling via clathrin-coated vesicles (55).

Actin and microtubules are the main components of plant cytoskeleton. Cellular responses to various endogenous or environmental factors involve the reorganization of this cytoskeleton, which provides tracks for the delivery of molecules to their sites of action (56). Actin and α - and β -chains of tubulin were identified in tobacco DRMs (Table III). Studies performed on animal cells already demonstrated the presence of these proteins in DRMs (41) and suggested a possible role for actin in the stabilization of the microdomains (57).

The presence in tobacco DRMs of various elements of the cell trafficking machinery is in agreement with data provided by studies performed on animal cells. It has been shown that a subset of plasma membrane skeleton proteins from bovine neutrophils co-isolates with DRMs (54). Numerous experimental evidences indicate the involvement of lipid rafts in the internalization of various proteins, suggesting the existence of several distinct raft-mediated endocytotic pathways. Finally rafts play an important role in polarized membrane trafficking (10).

Cell Wall-related Proteins (Table IV)—The crucial role of cytoskeletal elements in the establishment of cellular polarity involves their interaction with membranous and extracellular matrix (ECM) molecules. A reciprocal relationship exists, in particular in plants, between cytoskeleton and ECM: cytoskeleton determines the orientation of cell wall cellulosic components, and plant ECM can affect the orientation of microtubules with the cell cortex (58). The presence in tobacco DRMs of a set of cell wall proteins is thus relevant. The identification, in particular, of several isoforms of callose synthase, which acts as one class of cytoskeleton-cell wall linkers of plant cells (58), seems quite interesting.

Metabolism Proteins and Other Proteins (Table V)—As discussed above, only a few enzymes (about 6% of the total proteins identified) involved in metabolism were detected in DRMs when compared with the whole PM (3). Among these

are probably some remnant contaminants such as the mitochondrial ATP synthase α -chain and the tonoplasmic inorganic pyrophosphatase or some ribosomal proteins. This result is not surprising, even if our starting material is a highly enriched plasma membrane fraction, because the presence of a variable amount of contamination by other endomembranes is unavoidable.

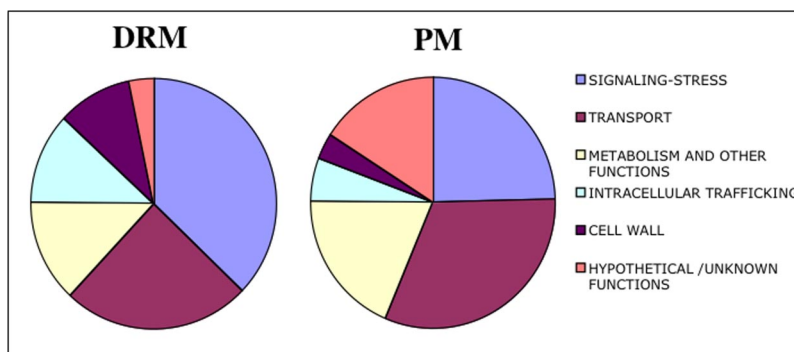
How Are These Proteins Associated to Tobacco DRMs?

Slight differences were observed between DRMs and plasma membrane proteins: a few more basic or heavy proteins and fewer highly hydrophobic proteins were found in DRMs, and a few more acidic or light proteins were found in the plasma membrane. To our knowledge, this work constitutes the first large scale comparison of the characteristics of proteins identified in a particular membrane (here the tobacco plasma membrane) and in DRMs isolated from this membrane. Moreover, studies concerning the proteic composition of animal DRMs have not been performed considering physicochemical but rather functional characteristics of the proteins identified, and mostly, with a particular signaling pathway in prospect. The interpretation of the differences evidenced in this work is made difficult by this lack of data. Taken as a whole, the physicochemical characteristics of the proteins listed above do not seem to constitute discriminant characters potentially responsible for the targeting to DRMs. However, according to the raft model developed from animal cells studies, an important determinant for the association of a protein to rafts seems to be the presence of a lipid involving post-translational modification (9).

Experimental evidence for the association of GPI-APs with lipid rafts from animal cells is based on the observation that these proteins are found in DRMs and suggests that the GPI anchor might be a signal to target proteins to these microdomains. At least two explanations could support this hypothesis. Experiments carried out in artificial membranes suggested that the proper lipid environment for insertion of a GPI-AP is provided by cholesterol, which specifically interacts with the acyl chain of the GPI anchor (29). Another possibility is that GPI-APs may interact with glycosphingolipids through their GPI saccharide or inositol residues (59). GPI-APs are widely distributed in eukaryotic organisms and have been found in almost all tissues and cells examined (29). Mining of the *A. thaliana* genome sequences has led to the prediction of 248 putative GPI-APs, and a combination of biochemical methods and mass spectrometry led to the identification of 44 GPI-APs in a plasma membrane fraction from *A. thaliana* (60).

A total of eight GPI-APs have been identified so far in plant DRMs (Ref. 14 and this work). This may appear too low to be a decisive criterion for raft targeting. Furthermore in the same study that led to the identification of 44 GPI-APs in a plasma membrane fraction from *A. thaliana* (60), only six GPI-APs were found in a human lipid raft-enriched membrane fraction.

FIG. 7. **Functional grouping of DRM and PM proteins.** Grouping was assumed according to the putative functions given in the databases (UniProt, www.expasy.org/uniprot; and NCBI, www.ncbi.nlm.nih.gov/entrez).



Similarly a proteomic study of lipid rafts from human HeLa cells led to the identification of 241 proteins among which only eight exhibited a GPI anchor (41). So if enrichment of GPI proteins in animal lipid rafts compared with the whole plasma membrane has been observed, their relative abundance among all the proteins of these domains is not documented.

Under our experimental conditions, myristoylated and palmitoylated proteins were quantitatively far more represented than GPI-AP in plant DRMs because 22 proteins identified in tobacco DRMs exhibit putative fatty acid modification sites. This represents about 16% of the total proteins identified and 26% (22/83) of the proteins exhibiting no or one transmembrane segment. This proportion is far from being insignificant. However, there is no possible comparison with other models because this type of modification has not been examined previously in others studies concerning plant microdomains or whole plasma membrane. Concerning animal lipid rafts, myristoylation and palmitoylation have been demonstrated for some proteins such as Src kinases, G proteins, or endothelial nitric-oxide synthase (18), and the importance of such post-translational modifications for targeting to microdomains has been demonstrated (61). However, to our knowledge, no quantitative data are available about the relative amount of raft-associated proteins undergoing such modifications. It should be noted that, among the 22 proteins identified in this study as palmitoylated or myristoylated, 13 displayed no predicted transmembrane domain, and nine exhibited only one. This relatively high proportion of DRM proteins associated to the membrane only by a post-translational modification is probably related to the lower values of GRAVY index observed in DRM proteins compared with plasma membrane proteins (Fig. 6). As for the proteins with several membrane-spanning segments, this criterion is probably not relevant. For such proteins, the association with microdomains could require electrostatic interactions between charged amino acids of the protein and oppositely charged head groups of sphingolipids. In this respect, characterization of structural motifs involved in the interaction of proteins with sphingolipids and cholesterol is of primary importance.

It is particularly interesting to note that these different post-translational modifications are not randomly distributed among the different physiological functions represented in

these DRMs: sites for fatty acylation are present exclusively in proteins involved in signaling or response to stress, whereas GPI anchors correspond to cell wall-related proteins (Table IV). This is all the more relevant given that GPI-APs are characteristically embedded with the lipid portion of the GPI anchor into the exoplasmic leaflet of the plasma membrane and that the reversibility of the palmitoylation/myristoylation process is quite suitable for dynamic signal transduction processes.

From DRMs to Rafts: Toward Physiological Functions of Plant DRMs

A very interesting point would be to compare the relative importance of these functional classes in DRMs and in the whole plasma membrane to determine a possible specialization of these domains. Such an analysis revealed very relevant features (Fig. 7). If primary functions of the plasma membrane, such as transport and metabolism, seem under-represented in DRMs, others undergo a significant increase of their relative importance in DRMs compared with plasma membrane. Among these are signaling and response to biotic and abiotic stress, cellular trafficking, and cell wall metabolism. This observation is all the more relevant given that these functions, except the plant-specific cell wall metabolism, have been identified as the main physiological processes involving animal lipid rafts (9, 10, 62, 63). This could suggest that beyond the similarity of their lipidic composition and biophysical properties, the plant DRMs could constitute the functional equivalent of animal lipid rafts.

Conclusion

This “large scale,” although certainly not exhaustive, proteomic analysis of plant DRMs from a single material (tobacco cell plasma membrane) proved to be very instructive. The essential point is probably that it provides a physical support where several physiological realities or processes, so far linked functionally two by two, can converge: for instance cytoskeleton and ECM, defense and ECM, or cytoskeleton and defense. Moreover the gathering in DRMs of components associated with these physiological functions is in agreement with the “patchy” localization around the cell previously ob-

served in confocal microscopy for some of these components (37, 64).

The enrichment of DRMs, compared with the whole plasma membrane, in signaling proteins contributing to the essential physiological processes listed above suggests (i) that we could probably consider these DRMs as the biochemical equivalent of microdomains present on plant plasma membrane *in vivo* and (ii) that these domains are likely to constitute, as in animal cells, signaling platforms potentially involved in these physiological functions.

The prospects offered by the demonstration of the presence in plants of such microdomains and the inventory of their proteic composition are numerous. The next challenge will be, for scientists involved in the study of a particular signaling pathway related to the physiological processes discussed here, to take hold of this microdomain concept and determine how it can contribute to the development of their research. A crucial point is probably to connect unambiguously the biochemical data presented here to a reality occurring in living cells as nicely undertaken by Bhat and Panstruga (11) with the visualization of the recruitment of plant penetration resistance components in a plasma membrane microdomain during the penetration of powdery mildew in plants.

* This work was supported in part by the Agence Nationale de la Recherche (ANR). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at <http://www.mcponline.org>) contains supplemental material.

§ Supported by ALTADIS and the Conseil Régional de Bourgogne.

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